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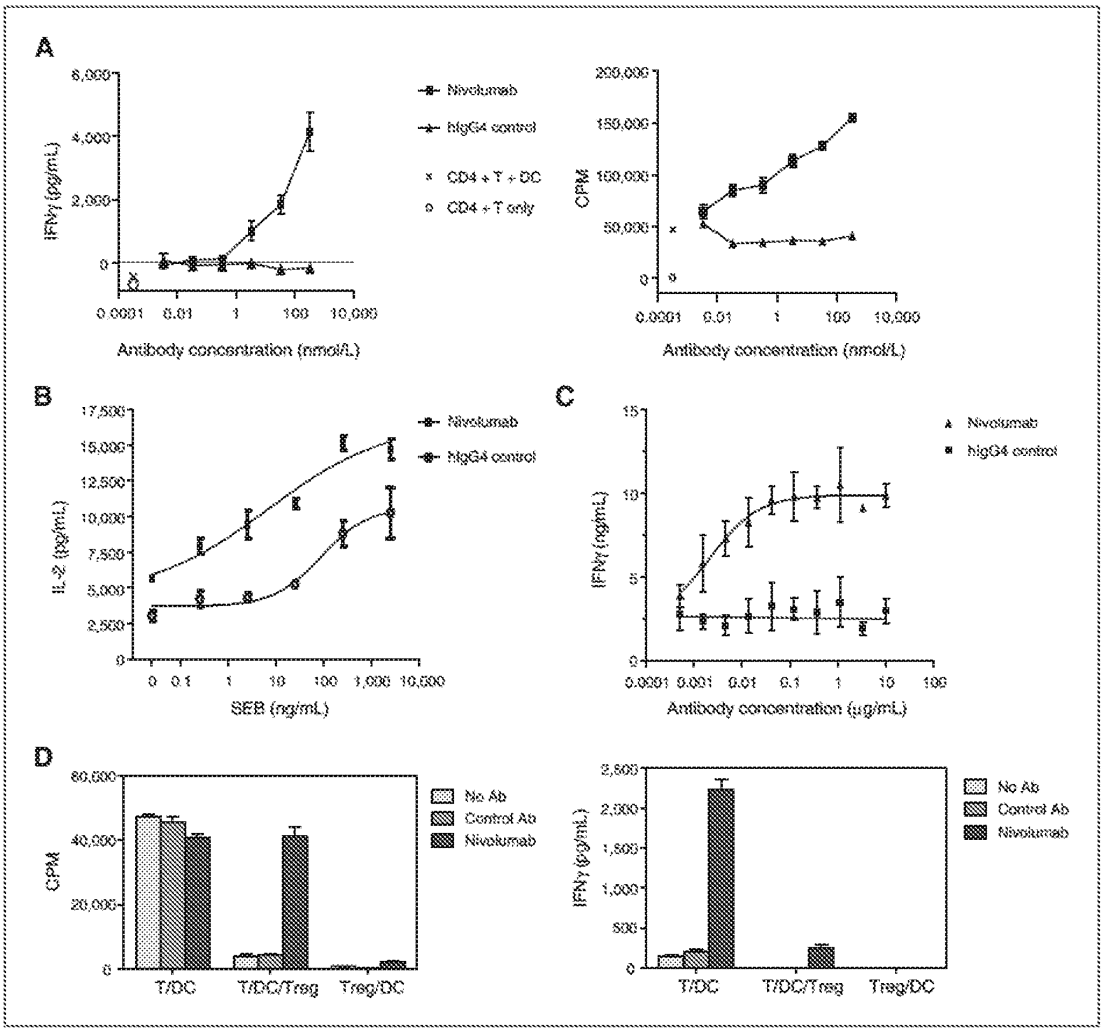



Figure 2. PD-1 blockade enhances T-cell function. A, $10^5$ purified $CD4^+$ T cells were cocultured with $10^4$ allogeneic monocyte-derived DCs in the presence of a titration of nivolumab or isotype control antibody in triplicates for 6 days. Supernatants were collected at day 5 and measured for IFNγ production by ELISA. The cultured cells were labeled with 1 μCi $^3$H-thymidine for another 18 hours before being analyzed for proliferation. Representative data from multiple donor DC/T-cell pairs are shown. CPM, counts per minute. B, $10^5$ PBMCs were stimulated with serial dilutions of SEB in the presence of a fixed amount of nivolumab or isotype control antibody in solution (20 μg/mL). Supernatants were collected after 3 days for measurement of IL-2 by ELISA. Representative data from multiple healthy donors ($n = 18$) are shown. C, $2 \times 10^5$ PBMCs from a CMV-positive donor were stimulated with lysate from CMV-infected cells in the presence of nivolumab or isotype control. Supernatants were collected after 4 days and assayed for IFNγ secretion by ELISA. D, $5 \times 10^4$ $CD4^+CD25^+$ Tregs were cocultured with $10^5$ $CD4^+CD25^-$ responder T cells and $2 \times 10^4$ DCs in the presence of 20 μg/mL of nivolumab or isotype control antibody in an allogeneic MLR for 6 days. IFNγ was analyzed from the supernatants collected at day 5 and proliferation was measured at day 6 after 18 hours of $^3$H-thymidine labeling.

enhancement of IFNγ release, and in some donor T-cell/DC pairs, enhanced T-cell proliferation was observed (Fig. 2A). Nivolumab also enhanced IL-2 secretion over isotype control in response to SEB using PBMCs (Fig. 2B). Addition of nivolumab increased IL-2 secretion by a mean of 97% to 139% over control (Supplementary Table S1). Using a CMV-restimulation assay, nivolumab, compared with an isotype control, resulted in a concentration-dependent augmentation of IFNγ secretion from CMV-responsive donors (Fig. 2C). Although PD-1 expression can be observed in T cells before stimulation by allogeneic DCs, SEB, or CMV antigen, PD-1 expression is upregulated after T-cell activation in all of these assays (Supplementary Fig. S4A and S4B and data not shown). In addition, PD-L1 expression and upregulation can be observed in multiple cell subsets in these assays (Supplementary Fig. S4B and data not shown).



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As Tregs also express PD-1, nivolumab was assessed in an allogeneic MLR in which Tregs suppressed the proliferation and cytokine secretion of purified $CD4^+CD25^+$ responder T cells, which were stimulated by allogeneic DCs. In this assay, nivolumab completely restored proliferation and partially restored IFNγ release by the alloreactive T cells (Fig. 2D).

Taken together, these data show that nivolumab can, at very low concentrations (~1.5 ng/mL), enhance T-cell reactivity in the presence of a T-cell receptor stimulus. However, nivolumab had no stimulatory effect in the absence of antigen or T-cell receptor stimulus. Specifically, there was no significant release of inflammatory cytokines, including IFNγ, TNFα, IL-2, IL-4, IL-6, or IL-10, from unstimulated whole blood after coincubation with nivolumab. In contrast, positive control anti-CD3 antibody potently increased cytokine release (Supplementary Table S2). These results demonstrate that nivolumab does not cause nonspecific lymphocyte activation.

Finally, the ability of nivolumab (tested from 0.003 to 50 μg/mL) to mediate ADCC activity *in vitro* was tested. Using IL-2-activated PBMCs as a source of natural killer (NK) cells and activated human $CD4^+$ T cells expressing high levels of cell-surface PD-1 as target cells, nivolumab [IgG4 (S228P)] did not mediate ADCC (Fig. 3). Limited ADCC activity was observed at high concentrations with the parental form of nivolumab, an IgG1 antibody purified from hybridoma supernatant, whereas positive control anti-MHC class I antibody was able to mediate ADCC of T cells at low antibody concentrations. In addition, nivolumab did not mediate complement-mediated cytotoxicity (CDC) of activated human $CD4^+$ T cells in the presence of human complement (data not shown).

### Pharmacokinetics, immunogenicity, and toxicity of nivolumab in cynomolgus monkeys

Single, i.v. administration of nivolumab to cynomolgus monkeys at 1 and 10 mg/kg was well tolerated with no effects on body weight or clinical observations. Mean concentration–time profiles for serum nivolumab were qualitatively similar for males and females at 1 mg/kg and for males at 10 mg/kg. Mean concentrations declined in a multi-phasic manner from $C_{max}$, observed within 0.5 hour at both doses. Serum PK parameter estimates are shown in Table 1. Mean apparent terminal elimination half-life estimates for males and females at 1 mg/kg were similar (124 and 139 hours, respectively), and the mean half-life estimate for males at 10 mg/kg was 261 hours. Anti-nivolumab antibodies were detected on day 28, but seemed to have no substantial impact on PK assessment [i.e., mean retention time (MRT), total clearance (CLT), and steady-state volume of distribution ($V_{ss}$)]. In general, serum nivolumab had a relatively slow clearance with limited extra vascular distribution, as demonstrated by a $V_{ss}$ value consistent with plasma volume. Five of the 6 animals in group 1 (1 mg/kg nivolumab), and 2 of 3 in group 2 (10 mg/kg nivolumab) were positive for an anti-nivolumab antibody response (including neutralizing antibodies) at day 28 (data not shown), but with no observable adverse effects.

In a 3-month toxicity study in cynomolgus monkeys, twice weekly i.v. administration of nivolumab at doses of 10 mg/kg and 50 mg/kg was also well tolerated, with no effect on body weight, and no other clinical findings. Serum chemistry changes were limited to a reversible 28% decrease in T3 at week 13 in females treated with 50 mg/kg. T4 and TSH levels were unchanged. In males treated with 50 mg/kg, there were no changes in T3, T4, or TSH levels. Nivolumab exposure increased in an approximately dose-proportional manner between 10 and 50 mg/kg, with no substantial sex differences noted (data not shown). Anti-nivolumab antibodies were detected in only 1 of 24 animals, although high nivolumab concentrations could have interfered with the assay. The highest well-tolerated dose in this study, 50 mg/kg twice weekly, is at least 20 times greater than doses reported to demonstrate antitumor activity in humans (≤10 mg/kg, every other week; ref. 26).

In phenotypic analyses of PBMCs 1 day after the last dose, no significant difference in total T- and B-cell numbers was observed between groups (data not shown). There were significantly more $CD8^+$ effector memory T cells in the 50 mg/kg group than in the 10 mg/kg and untreated groups (Supplementary Fig. S5A), and a nonsignificant trend toward more $CD8^+$ central memory T cells in the nivolumab group, especially in the 50 mg/kg group. Naïve T-cell populations were decreased in the 50 mg/kg group, suggesting that PD-1 blockade may facilitate activation and differentiation of naïve T cells. Finally, there were more $CD11c^+$ DCs in the 50 mg/kg group than in the untreated group ($P = 0.014$; Supplementary Fig. S5B), suggesting a possible role for PD-1 blockade in promoting DC differentiation.

### Immune responses in cynomolgus monkeys to cellular and particulate virus-like particle vaccines coadministered with nivolumab or ipilimumab

Enhancement of vaccine responses can demonstrate the activity or potency of immunomodulatory antibodies in non-human primates. Previous studies demonstrated that CTLA-4 blockade potentiated immune responses to an HBsAg vaccine or SK-MEL-3 melanoma cell vaccine (37). A similar experiment was conducted to examine the ability of PD-1 blockade to potentiate vaccine responses. As previously observed, ipilimumab strongly enhanced humoral immune responses to HBsAg as compared with control, whereas nivolumab showed no effect on HBsAg titers over control treatment (Fig. 4A). All groups had normal anamnestic responses with measurable antibody titers following the second vaccine dose, which peaked after the third dose and declined thereafter.

Responses to the SK-MEL-3 vaccine were elevated in both nivolumab- and ipilimumab-treated groups compared with control (Fig. 4B). There was a modest increase in the humoral vaccine response in the nivolumab group, with a greater increase in the ipilimumab group. Titers increased markedly following the second vaccine dose, but did not change substantially after the third dose, followed by a rapid decline in all groups. Antibody titers to HLA-A2404 (an allele of HLA expressed by SK-MEL-3 cells) were also increased in animals treated with ipilimumab (4.3-fold) or nivolumab (2.4-fold) on day 71 (Fig. 4C).

## Discussion

Nivolumab is a fully human IgG4 PD-1 antibody that binds to human and cynomolgus PD-1 with high affinity and blocks the



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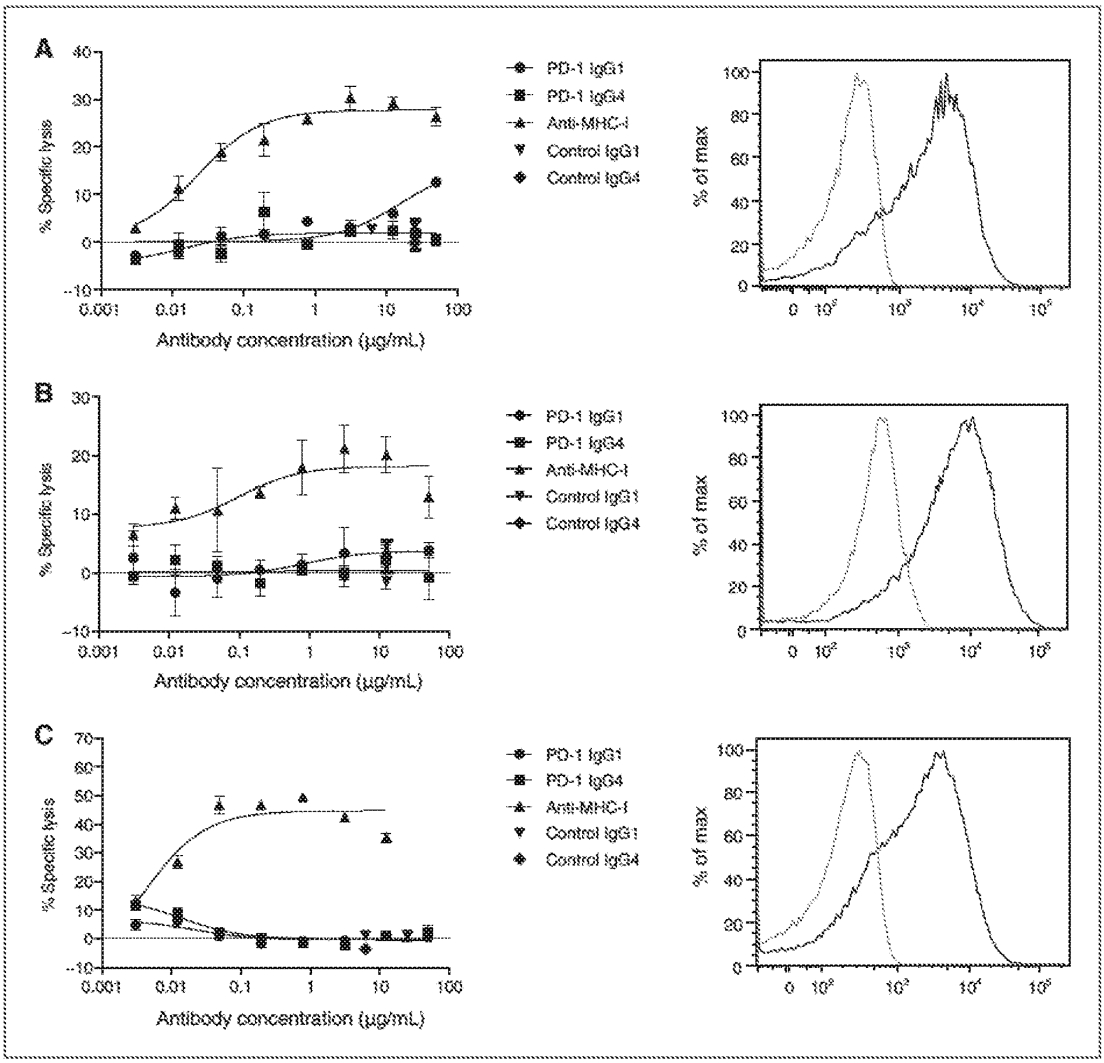



Figure 3. The absence of ADCC by nivolumab *in vitro*. IL-2–activated human PBMCs (effector cells) were incubated with activated human $CD4^+$ T cells (target cells) in an effector-to-target cell ratio of 50:1 in the presence of serial dilutions of nivolumab or a positive control anti-MHC class I antibody for 3 hour at 37°C. A–C, data from three individual ADCC assays using cells from different donors are shown. Purified $CD4^+$ T cells were activated by coated anti-CD3 antibody (4 μg/mL) plus soluble anti-CD28 antibody (1 μg/mL) and IL-2 (100 U/mL) for 3 days. PD-1 expression on activated $CD4^+$ T cells in each of the ADCC assays is shown in the right (solid line for PD-1, gray line for isotype control).

interaction of PD-1 with both PD-L1 and PD-L2 ligands. In functional *in vitro* assays, nivolumab enhanced cytokine production in human T-cell/DC MLR, SEB, and CMV recall response assays. In addition, antigen-specific $CD8^+$ T-cell responses from patients with melanoma increased after incubation with nivolumab and peptide antigen, and not by stimulation with an irrelevant peptide (30). Importantly, while anti-PD-1 antibody enhanced antigen-specific T-cell responses, it did not stimulate nonspecific responses by human blood cells, as determined by cytokine release upon incubation with antibody alone. In a Treg suppression assay, nivolumab completely restored $CD4^+$ T-responder cell proliferation and partially restored IFNγ production. Although it is unclear whether nivolumab acts directly on $CD4^+$ T-responder or Tregs, previous data have demonstrated that nivolumab could overcome Treg suppression of $CD8^+$ T cells by increasing resistance to Treg suppression, and also by directly limiting Treg-suppressive capacity (38).

The heavy chain constant region of nivolumab is a human IgG4 isotype with an S228P mutation, which replaces a serine



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*Table 1. Mean and SD for serum PK parameter estimates for nivolumab following single i.v. administration to monkeys*

| Gender | Dose, mg/kg | $C_{max}$, μg/mL | $T_{max}$, h | $AUC_{(0-T)}$, μg × h/mL | $AUC_{(INF)}$, μg × h/mL | $T_{1/2}$, h | MRT, h | CLTs, mL/h/kg | $V_{ss}$, L/kg |
|---|---|---|---|---|---|---|---|---|---|
| Male | 1 | 33.8 ± 2.36 | 0.25–0.5 | 4,910 ± 645[a] | 4,470 ± 423 | 124 ± 20.3 | 200 ± 5.2 | 0.224 ± 0.021 | 0.046 ± 0.005 |
| Female | 1 | 26.6 ± 0.661 | 0.25–0.25 | 3,570 ± 573[a] | 4,060 ± 616 | 139 ± 12.7 | 210 ± 27 | 0.250 ± 0.039 | 0.052 ± 0.002 |
| Male | 10 | 330 ± 34.2 | 0.25–0.5 | 47,100 ± 12,400[b] | 64,200 ± 27,400 | 261 ± 226 | 400 ± 250 | 0.172 ± 0.059 | 0.060 ± 0.016 |

NOTE: Values are mean ± SD except for $T_{max}$, which is the range.

Abbreviations: CLT, total clearance; MRT, mean retention time; $V_{ss}$, steady state volume of distribution.

[a]T = 384 hours.

[b]T = 648 hours.

residue in the hinge region with the proline residue found at the corresponding position in IgG1 isotype antibodies. This mutation prevents Fab arm exchange with endogenous IgG4 antibodies, while retaining the low affinity for activating Fc receptors associated with wild-type IgG4 antibodies. Engagement of activating Fc receptors by a PD-1-blocking antibody could conceivably deplete antitumor effector T cells. However, no *in vitro* ADCC or CDC activity was observed with nivolumab in assays using PD-1-expressing activated T cells as target cells, suggesting that nivolumab is unlikely to deplete PD-1-positive cells. Lack of nivolumab-mediated ADCC or CDC activity is consistent with the expected lack of effector function of IgG4 Fc region, as observed by others (39, 40). Moreover, an IgG1 isotype of nivolumab resulted in limited ADCC activity at high antibody concentrations, indicating that the epitope recognized by nivolumab may not lead to potent ADCC activity. Although IgG4 isotype antibodies show low affinity for FcγRI *in vitro* as compared with IgG1 (41), it is unclear whether this translates into ADCC or phagocytic activity by FcγRI-expressing monocytes or macrophages *in vivo*. Normal levels of human immunoglobulin in sera have been reported to strongly inhibit IgG1-mediated ADCC (42). Although phase I trial data showed a transient decrease of peripheral blood T cells after nivolumab treatment, this was probably related to T-cell extravasation (25). In other clinical studies, nivolumab monotherapy did not change the median absolute lymphocyte count (ALC), or the number of activated $CD4^+$, $CD8^+$, or Tregs, suggesting that nivolumab does not mediate overt changes in T-cell percentages (24).

Immunohistochemical studies evaluated nivolumab reactivity to lymphoid cells and assessed nontarget tissue binding. Reactivity of nivolumab to lymphocytes in various tissues was observed as expected; however, there was unexpected moderate-to-strong cytoplasmic staining of rare-to-occasional endocrine cells in the adenohypophysis. This was considered to be low-affinity binding, as staining occurred only at higher antibody concentrations, and is unlikely to have physiologic consequences because of limited accessibility to cytoplasmic compartments *in vivo*. Cynomolgus tissue staining showed similar lymphocyte-binding patterns, as well as cytoplasmic staining of endocrine cells in the adenohypophysis (data not shown). Potential adverse effects of this binding were not borne out in cynomolgus toxicity studies or human clinical trials (25, 26).

Vaccination studies in cynomolgus monkeys have been used as surrogates for evaluating activity of T-cell inhibitory or costimulatory molecules in the absence of tumor models (37, 43). Although anti-CTLA-4 antibody promoted significantly increased humoral responses in HBsAg-vaccinated monkeys, nivolumab did not, despite its high affinity for cynomolgus PD-1. However, slightly higher antibody titers to a cellular vaccine directed, at least in part, against the HLA-A2404 antigen expressed on SK-MEL3 cells, were detected in monkeys treated with 10 mg/kg of nivolumab. In mice, anti-PD-1 antibody can promote T-cell responses to a GVAX cellular vaccine (44) and to peptide-loaded DC vaccination (K. Bahjat et al.; unpublished data). Although not a vaccine study, it is noteworthy that simian immunodeficiency virus (SIV)–infected monkeys



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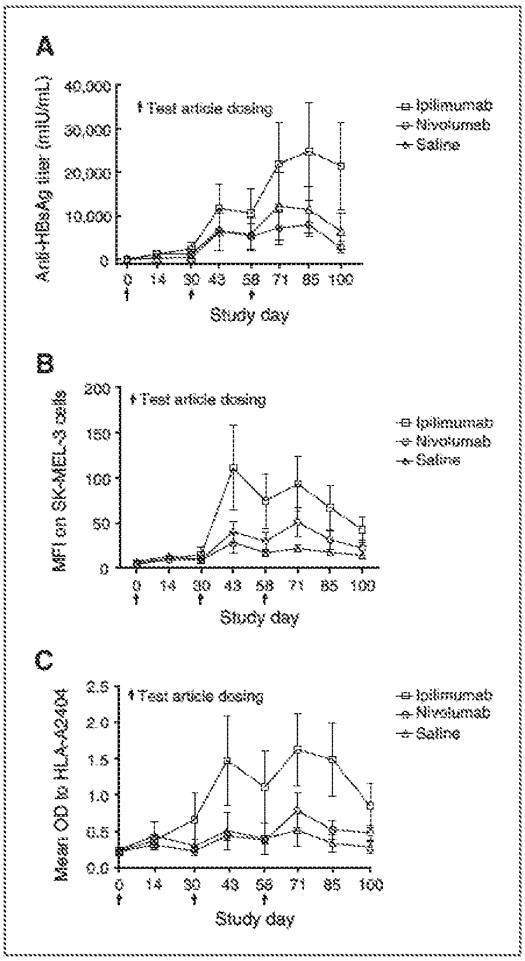



Figure 4. Effect of nivolumab or ipilimumab on immune responses to vaccination in cynomolgus monkeys. A, humoral immune responses to a particulate HBsAg vaccine by ELISA. Plasma samples obtained at the indicated times were analyzed for anti-HBsAg Abs. B, antibody responses to an SK-MEL-3 vaccine as assessed by flow cytometry. Vaccine-specific antibody responses were measured by incubation of SK-MEL-3 cells with plasma collected at 2-week intervals. Data points represent the mean ± SEM of the mean fluorescence intensity (MFI) values in each treatment group at each collection time point. C, antibody responses to HLA-A2404 were determined from plasma by ELISA. Data points represent the mean ± SEM of the mean OD values in each treatment group. All samples were analyzed at least two times with similar results.

treated with an anti-PD-1 antibody showed increased humoral responses to SIV antigens (45).

Nivolumab was well tolerated when administered to cynomolgus monkeys as twice-weekly i.v. injections for 3 months at doses up to 50 mg/kg, with no adverse effects on any parameters. Although there was a low incidence of anti-nivolumab antibodies, they were not associated with any adverse effects (i.e., hypersensitivity reactions), and had no substantial impact on PK parameters. Furthermore, anti-drug antibody responses in animals are not considered predictive of responses in humans (46). Thus, the results of the nonclinical studies in monkeys suggested a favorable risk:benefit ratio to support initial clinical trials with nivolumab. Although nivolumab seems to lack toxicity in monkeys, toxicities have been observed in human clinical trials. In a phase I trial, nivolumab had a favorable safety profile (26). Adverse events were generally similar to those observed with ipilimumab, although with lower incidence and of less severity, and comprised gastrointestinal, endocrine, and skin toxicities, and pulmonary inflammation. Interestingly, pneumonitis has been observed in PD-1–deficient mice bred onto the MRL genetic background (8), but not in PD-1–deficient mice with other genetic backgrounds (6, 7). In cynomolgus toxicity studies with anti-CTLA-4 (ipilimumab), no or only rare toxicities were observed, although they were evident in human studies. These observations highlight the difficulty of predicting toxicities in humans with antibodies mediating checkpoint blockade, such as anti-PD-1 or anti-CTLA-4 antibodies, from results in mice and nonhuman primates.

Increased numbers of $CD8^+$ T-effector memory cells were detected in cynomolgus monkey peripheral blood after repeated treatment at the highest dose of nivolumab for 3 months. Although recent data (24) suggest that activated T cells are not potential pharmacodynamic markers of nivolumab treatment, it remains to be determined whether nivolumab treatment increases $CD8^+$ effector memory cells in humans with cancer. A marked accumulation of $CD8^+$ effector memory cells in lymphoid organs and tissues of PD-1–deficient mice has been described previously (47). Increases in $CD11c^+$ DCs were also observed in the cynomolgus monkey safety study, although the underlying mechanism is unclear.

In early clinical trials, nivolumab produced durable responses and stable disease, and an encouraging survival profile, in patients with advanced melanoma, lung, and renal cancers. In some patients, tumor regression persisted after discontinuation of nivolumab (26, 27, 48). Nivolumab was generally well tolerated, even with prolonged dosing (26, 27, 48). Another PD-1–blocking antibody, pembrolizumab, has shown similar activity and safety in metastatic melanoma (49). These studies further validate the concept of modulating immune responses with checkpoint blockade for cancer immunotherapy, as first demonstrated in clinical trials with ipilimumab (1, 2).

Exploration of nivolumab combinations with other immuno-oncology approaches, as well as standard of care therapies, is warranted. PD-1 pathway blockade combined with anti-CTLA-4 or anti-LAG-3 antibody showed synergistic antitumor activity superior to the single agents in murine tumor models (15–17). Preliminary clinical data in patients with melanoma receiving nivolumab plus ipilimumab showed rapid and durable responses: 31% of responders had tumor regression of 80% or more by week 12, a superior profile to monotherapies (50). The combination is currently being clinically evaluated in multiple tumor types.



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## Disclosure of Potential Conflicts of Interest

C. Wang, M.J. Selby, X.-T. Wang, and A.J. Korman have ownership interest (including patents) in Bristol-Myers Squibb stock. No potential conflicts of interest were disclosed by the other authors.

## Disclaimer

All pivotal toxicology studies were conducted in compliance with the Good Laboratory Practice Regulations for nonclinical Laboratory Studies of the U.S. Food and Drug Administration (21 CFR Part 58) and were approved by the laboratory's Institutional Animal Care and Use Committee.

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Cancer Immunology Research

# *In Vitro* Characterization of the Anti-PD-1 Antibody Nivolumab, BMS-936558, and *In Vivo* Toxicology in Non-Human Primates

Changyu Wang, Kent B. Thudium, Minhua Han, et al.

*Cancer Immunol Res* 2014;2:846-856. Published OnlineFirst May 28, 2014.

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Review

Clinical Cancer Research

# Microsatellite Instability as a Biomarker for PD-1 Blockade

Jonathan C. Dudley[1], Ming-Tseh Lin[2], Dung T. Le[3], and James R. Eshleman[2,3]

## Abstract

Initial results by Le and colleagues, which were published in the June 25, 2015 issue of the *New England Journal of Medicine*, report significant responses of cancers with microsatellite instability (MSI) to anti–PD-1 inhibitors in patients who failed conventional therapy. This finding fits into a broader body of research associating somatic hypermutation and neoepitope formation with response to immunotherapy, with the added benefit of relying on a simple, widely used diagnostic test. This review surveys the pathogenesis and prognostic value of MSI, diagnostic guidelines for detecting it, and the frequency of MSI across tumors, with the goal of providing a reference for its use as a biomarker for PD-1 blockade. MSI usually arises from either germline mutations in components of the mismatch repair (MMR) machinery (MSH2, MSH6, MLH1, PMS2) in patients with Lynch syndrome or somatic hypermethylation of the *MLH1* promoter. The result is a cancer with a 10- to 100-fold increase in mutations, associated in the colon with poor differentiation, an intense lymphocytic infiltrate, and a superior prognosis. Diagnostic approaches have evolved since the early 1990s, from relying exclusively on clinical criteria to incorporating pathologic features, PCR-based MSI testing, and immunohistochemistry for loss of MMR component expression. Tumor types can be grouped into categories based on the frequency of MSI, from colorectal (20%) and endometrial (22%–33%) to cervical (8%) and esophageal (7%) to skin and breast cancers (0%–2%). If initial results are validated, MSI testing could have an expanded role as a tool in the armamentarium of precision medicine. *Clin Cancer Res; 22(4); 813–20. ©2016 AACR.*

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The members of the planning committee have no real or apparent conflicts of interest to disclose.

### Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the pathogenesis and clinicopathologic associations of microsatellite instability, approaches to diagnosing it, its frequency across tumor types, and its potential value as a biomarker for PD-1 blockade immunotherapy.

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## Introduction

Recent work has highlighted the importance of the immunologic response to cancer for therapy. Interaction of the PD-1 T-cell coreceptor and its ligand B7-H1/PD-L1 promotes an immunosuppressive tumor microenvironment, and antibodies to either molecule have shown promise for inducing durable tumor responses, even in late-stage patients who have failed multiple previous lines of therapy (1–3). Despite this success, only a minority of patients respond, and it has been unclear which patients and which tumors are the best candidates for this therapy.

At the same time, immunologic factors have shown promise as prognostic tools. Characterizing the lymphocytic response to tumors can be more prognostically valuable than traditional histopathologic parameters. In colorectal cancer, Galon and colleagues showed that tumors with a high infiltrate of $CD8^+$ CTL and T helper 1 (Th1)-type cells had a superior prognosis to those with a low infiltrate, with the degree of infiltrate surpassing

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tumor–node–metastasis staging in prognostic value (4, 5). Although there is evidence that such infiltrate suppresses tumor growth and metastasis (5), it has been unclear why some tumors elicit it and others do not and how tumors with such a response are able to survive it.

Recent publications have clarified the answers to these questions. By analyzing the genetics and immune microenvironment of colorectal cancer specimens in parallel, Llosa and colleagues demonstrated, consistent with previous studies, that tumors with a high Th1/CTL infiltrate had defects in mismatch repair (MMR), resulting in microsatellite instability (MSI; ref. 6). Following previous studies (7), the authors suggested that the increased mutational burden in such tumors created neoepitopes responsible for the immune response. Most significantly, they found that tumors with MSI had significant upregulation of immune checkpoint proteins, including PD-1 and PD-L1, enabling them to survive. In MSI colorectal cancer, the PD-L1 expression appears not to be on tumor cells, but rather on tumor-infiltrating lymphocytes and/or myeloid cells. This model is presented in Fig. 1.

A follow-up clinical trial demonstrated the utility of MSI status as a predictive marker for response to PD-1 blockade in stage IV cancer patients. The article reported on 11 patients with MMR-deficient (dMMR) colorectal cancer, 21 with MMR-proficient colorectal cancer, and 9 with dMMR noncolorectal cancer (4 ampullary or cholangiocarcinomas, 2 endometrial carcinomas, 2 small bowel carcinomas, and 1 gastric carcinoma). Patients were stage IV and had failed multiple other chemotherapies. They were treated with pembrolizumab, an anti–PD-1 antibody. MSI was a significant predictor of the immune-related objective response rate (40% in dMMR colorectal cancer, 71% in dMMR noncolorectal cancer, 0% in MMR-proficient colorectal cancer) and also the immune-related progression-free survival rate (78%, 67%, and 11%, respectively). Whole-exome sequencing of tumor tissue revealed an average of 1,782 somatic mutations in cancers with MSI (578 were predicted to result in neoantigens) versus 73 somatic mutations in cancers without MSI (21 of which were predicted to result in neoantigens; ref. 8).

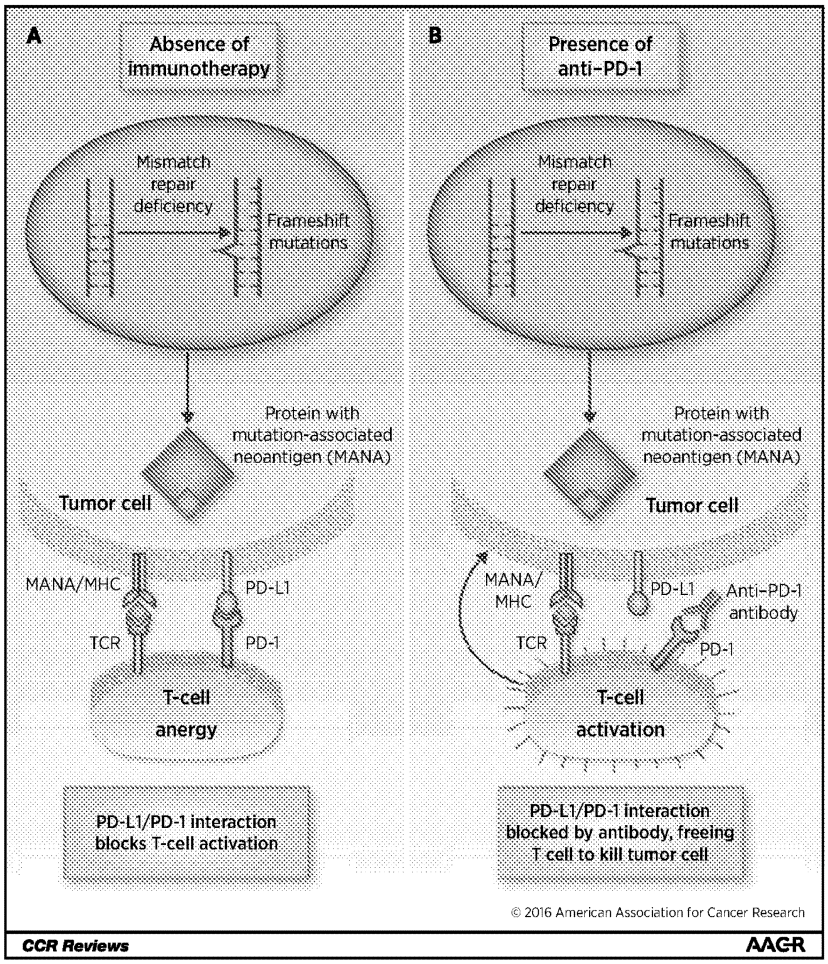



**Figure 1.**
Proposed relationship between MSI status and immunologic response. Tumor cell (top) and T cell (bottom) in the absence (A) or presence (B) of immunotherapy. For simplicity, the tumor cell is drawn as the source for the PD-L1; however, in some tumors, such as MSI colorectal cancer, the dominant source may be macrophages or other tumor-infiltrating lymphocytes and myeloid cells (6). In the absence of functional MMR, nascent strand slippage (green base) goes unrepaired, producing frameshift mutations (red bases) and resulting in proteins (blue diamond) containing a mutation-associated neoantigen (MANA, red square). MANA-MHC complexes are presented to T cells. MANAs can also arise from missense mutations (not shown). MHC, major histocompatibility complex; TCR, T-cell receptor.



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These results have the potential to significantly improve therapeutic options. Immune checkpoint blockade inhibition is less toxic than chemotherapeutic regimens and has potential for durable responses in advanced cancer patients who would otherwise live a few months. About 4% of advanced colorectal, 18% of advanced endometrial, and 11% of advanced ovarian cancers have been estimated to harbor MMR deficiency (9). Additional studies are under way to test PD-1 inhibition in early-stage and chemotherapy-naive patients. Positive results in these groups could potentially spare these patients' chemotherapy and expand the number of patients who could benefit. As noted below, MSI is a common phenomenon across a significant fraction of tumor types, reaching 10% to 30% in frequency.

Many causes of somatic hypermutation exist other than MSI: mutations in the DNA polymerases encoded by *POLE* or *POLD1*, exposure to external mutagens (cigarette smoke, UV radiation), and endogenous mutagens (reactive oxygen species; refs. 10, 11). It can be predicted that tumors with hypermutation caused by these alternative mechanisms would also have enhanced sensitivity to checkpoint blockade. Indeed, Rizvi and colleagues demonstrated a strong correlation between response to PD-1 blockade and burden of nonsynonymous mutations in non–small cell lung cancer (12). Snyder and colleagues examined cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade in melanoma, finding a correlation between mutational load and clinical benefit. More significantly, they identified a set of putative neoepitopes able to predict clinical benefit in an independent patient cohort (13). These approaches, and others not yet utilized in clinical settings (14), have significant promise, but they rely on whole-exome sequencing of tumor samples followed by extensive bioinformatic analysis, an approach not yet practical for routine diagnostic use.

MSI testing, by contrast, is routinely performed in most diagnostic laboratories, increasing its potential as an immediately useful approach to predict immunotherapeutic response in patients who have failed conventional therapy. The purpose of this review is to examine current knowledge of MSI and the clinical assays used to detect it, with a focus on its potential role as a predictive biomarker for immune checkpoint blockade.

## Pathogenesis of MSI in Colorectal Cancers

Microsatellites or "short-tandem repeats" are defined as repeats (usually 10–60 times) of the same base or sequence of bases, with a unit length ranging from one to six bases. They are scattered throughout coding and noncoding regions of the genome. DNA polymerases are more prone to make mistakes in these regions, by either inserting additional bases when slippage occurs on the synthesized strand or removing them when slippage occurs on the template strand, leading to mismatched DNA strands. MSI cancers are associated with 100- to 1,000-fold increased mutation rates of frameshift and missense mutations (experimentally measured; refs. 15–17), which result from defects in the DNA MMR system (18–23). Following DNA replication, the MMR machinery slides along the DNA and targets mismatches for correction when it encounters them. Its core components are two heterodimers consisting of MSH2/MSH6 and MLH1/PMS2 (MSH2 may also form a heterodimer with MSH3). Patients with a defect in any one of these components, or, in a gene upstream of *MSH2* that encodes EPCAM that results in loss of MSH2 expression when mutated (24), develop frameshift mutations leading to truncated protein products. Genes most commonly affected include *TGFBRII*, *ACVR2A*, *TCF4*, *IGF2R*, *BAX*, *MSH6*, and *MSH3* (25–28). About 15% of all colorectal cancers have MSI, with about 2.5% resulting from genetic inheritance and the remaining 12.5% being sporadic (29).

Patients with MSI due to germline mutations in one of the MMR genes are defined as having Lynch syndrome. Lynch syndrome is an autosomal dominant condition characterized by an elevated risk for cancers of the ovaries, kidneys, bladder, stomach, small bowel, bile ducts, and brain, with the biggest increase in risk for endometrial cancer (60% of females) and colorectal cancer (80% of patients; ref. 30). Sporadic MSI usually arises from epigenetic silencing of the *MLH1* promoter, often from a global increase in CpG island methylation, and is associated with a somatic *BRAF* p.V600E mutation (29, 31–33). Less commonly, it may arise from biallelic somatic inactivation of the genes encoding a MMR component (34). The manifestation of Lynch syndrome reflects the specific mutation and genetic background of the patient. Compared with patients with an *MLH1* mutation, for example, those with an *MSH2* mutation have a higher risk of a noncolorectal malignancy and those with an *MSH6* mutation have a higher risk of endometrial cancer (35, 36). Patients with an *MLH1* mutation, in turn, are more likely to develop colorectal cancer at a young age in the presence of specific risk alleles (37).

Whether hereditary or sporadic, MSI colorectal cancers have unique clinicopathologic associations. Whereas microsatellite stable (MSS) cancers tend to arise from adenomatous polyps, distributed anywhere in the colon, MSI malignancies tend to arise from sessile serrated adenomas in the proximal colon (38). MSS cancers are associated with chromosomal instability, whereas MSI cancers are typically diploid, with preserved chromosomal architecture (39). Under the microscope, MSS colorectal cancers typically show infiltrating glands with dirty necrosis, whereas MSI tumors classically show tumor-infiltrating lymphocytes and the Crohn-like lymphocytic reaction, poor differentiation with pushing margins, and mucinous differentiation (40).

## Prognostication and Prediction

Even apart from its potential value as a biomarker for PD-1 blockade response, MSI status has been used to predict outcomes. MSI colorectal cancer is associated with a lower stage at diagnosis and improved stage-specific prognosis (though conflicting results have been observed in stage IV patients; refs. 41, 42), the likely result of a significant immunologic response elicited by neoepitopes. Assessing for somatic mutations in *BRAF* in conjunction with MSI status is also prognostically valuable (43, 44). The p.V600E mutation renders BRAF constitutively active, resulting in a worse prognosis. A recent study stratified colorectal cancer patients based on MSI and *BRAF* status into three prognostic groups: MSI/*BRAF*-wild type or mutant (best prognosis), MSS/*BRAF*-wild type (intermediate prognosis), and MSS/*BRAF* mutant (worst prognosis; ref. 43), though other studies have reached conflicting results (45), and no consensus exists to date on the best prognostic subgroupings.

The role of MSI as a predictive marker for therapy has been controversial. A 2003 study in stage II or III colorectal cancer patients examined the relationship between MSI status and response to 5-fluorouracil (5-FU)–based adjuvant chemotherapy. Although MSS patients showed a benefit from 5-FU treatment, MSI patients did not (46), a finding confirmed in smaller follow-up



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studies (47, 48). Laboratory-based investigations have suggested that components of the MMR machinery bind to 5-FU incorporated DNA, raising the possibility that they mediate the observed cytotoxic response (49, 50). Further clinical studies have generally substantiated the finding that MSI patients do not benefit from 5-FU therapy, with some exceptions (51). A 2011 study of 2,141 colorectal cancer patients suggested the benefits of 5-FU therapy were restricted to stage III MSI patients with germline (versus sporadic) mutations (52). More recently, a 2015 meta-analysis of 14 studies concluded that there is a trend for lack of benefit of 5-FU in MSI cancers ($P = 0.11$), which did not reach statistical significance (53). Further studies are needed to reconcile these findings.

## Diagnosis

Diagnostic guidelines relating to MSI status have evolved over the past two-and-a-half decades, from using clinical criteria to identify colorectal cancer patients with Lynch syndrome to using clinical, histologic, and genetic criteria to identify all patients with Lynch syndrome (including those with noncolorectal tumors) and distinguish sporadic and inherited cases of MSI.

The Amsterdam criteria were set forth in 1991, before the discovery of the genes responsible for Lynch syndrome. At least three relatives had to be diagnosed with histologically confirmed colorectal cancer, with the following additional criteria met: One patient had to be a first-degree relative of the other two; one relative had to have been diagnosed with colorectal cancer before the age of 50 years; and the colorectal cancer had to involve at least two generations (54).

The Bethesda guidelines, first proposed in 1997 and then revised in 2003, expanded the scope of testing, adding criteria for testing when patients do not fulfill the Amsterdam requirements (55, 56). These guidelines are especially applicable when there is no known family history of cancer. Only one of the following criteria need to be met in the revised guidelines: colorectal cancer in a patient under the age of 50 years, presence of other Lynch syndrome–related tumors (including synchronous or metachronous colorectal cancers), typical MSI histology in a patient under the age of 60 years, a colorectal cancer patient with a first-degree relative under the age of 50 years with a Lynch syndrome–related cancer, or a colorectal cancer patient with at least two first- or second-degree relatives with Lynch syndrome–related cancers (56). Patients who fulfill the Amsterdam criteria are also recommended to undergo further testing.

The National Comprehensive Cancer Network (NCCN) has issued the most recent guidance. The 2015 NCCN guidelines provide criteria for testing based on clinical or pathologic parameters. The clinical testing criteria recommend further evaluation of patients who meet the Amsterdam criteria or revised Bethesda guidelines, in addition to patients under the age of 50 years with endometrial cancer, patients with known Lynch syndrome in the family, and patients with a 5% or higher likelihood of having a germline mutation (as assessed by risk-prediction modeling). The pathologic testing criteria focus on patients with colorectal cancer, recommending that all patients be tested for MSI regardless of whether or not they meet other criteria or, at a minimum, testing all colorectal cancer patients under the age of 70 years and those over the age of 70 years who meet the revised Bethesda guidelines. A 2012 study of the performance characteristics of different screening approaches for detecting Lynch syndrome in 10,206 colorectal cancer patients found the revised Bethesda guidelines to have a sensitivity of 87.8% and specificity of 97.5%, the strategy endorsed by the NCCN a sensitivity of 95.1% and specificity of 95.5%, and universal tumor testing a sensitivity of 100% and specificity of 93% (57).

Although the criteria for deciding which patients to test have developed, the methods of testing have also evolved. As the human genome has several hundred thousand microsatellites, it is only practical to examine representatives. To ensure reproducibility and standardization, the 1997 Bethesda guidelines recommended a reference panel of five microsatellites (the "Bethesda panel") for testing: two mononucleotide loci (BAT-25 and BAT-26) and three dinucleotide loci (D2S123, D5S346, and D17S250). These regions are amplified within both tumor and normal tissue via fluorescent multiplex PCR and their size assessed by capillary electrophoresis (58). A shift (usually downward) in the size of at least two of the five microsatellite loci in tumor relative to normal is defined as the MSI-high (MSI-H) phenotype, or of 30% or more loci when larger panels are tested. A shift in the size of one locus constitutes the MSI-low (MSI-L) phenotype, which behaves in a similar manner to MSS tumors (55). The Promega Corporation has developed a widely used alternative to the Bethesda panel, called the MSI Analysis System, replacing the dinucleotide markers with mononucleotide markers (NR-21, NR-24, and MONO-27; refs. 56, 59, 60). The resulting panel has several practical advantages, though recent work has suggested that a panel targeting longer loci may have improved sensitivity, especially for endometrial cancer (61). Genome- or exome-wide sequencing of matched tumor and normal tissue has also been used to characterize MSI (12, 13, 62). This approach is useful in a research setting to identify affected genes, but it is expensive and technically challenging due to the repetitive nature of microsatellites (62).

Immunohistochemistry for MMR proteins has comparable performance characteristics to MSI testing and a high concordance rate (29). MLH1 and MSH2 are stable without their dimer partners (PMS2 and MSH6, respectively), but the reverse is generally not true. As a result, tumors with an *MLH1* mutation will show loss of both MLH1 and PMS2 on immunohistochemistry and tumors with an *MSH2* mutation will show loss of MSH2 and MSH6. Mutations in *PMS2* or *MSH6*, by contrast, lead to loss of only the affected protein (an exception is that MSH2 may be lost when *both* of its binding partners, MSH6 and MSH3, are lost). These features allow one to determine which of the MMR genes is likely mutated (or methylated) by immunohistochemistry, an advantage not present with MSI testing. An additional advantage of immunohistochemistry is its ability to work with paucicellular specimens. However, about 5% to 11% of MSI cases will not show MMR protein loss, likely due to retained antigenicity in an otherwise nonfunctional protein. Given its focus on representative microsatellites, MSI panel testing itself is an imperfect marker for Lynch syndrome, missing an estimated 0.3% to 10% of cases (29). The sensitivity of both tests varies based on which MMR gene is mutated (63).

These limitations have led the Association of Molecular Pathology to recommend all new colorectal cancer cases to be subjected to concurrent MSI analysis, immunohistochemistry for MMR proteins, and *BRAF* mutation screening (Fig. 2; refs. 29). This approach would allow almost all cases to be categorized and managed appropriately: Lynch syndrome patients would need screening for other Lynch syndrome–associated malignancies and counseling of family members; patients with sporadic MSI would



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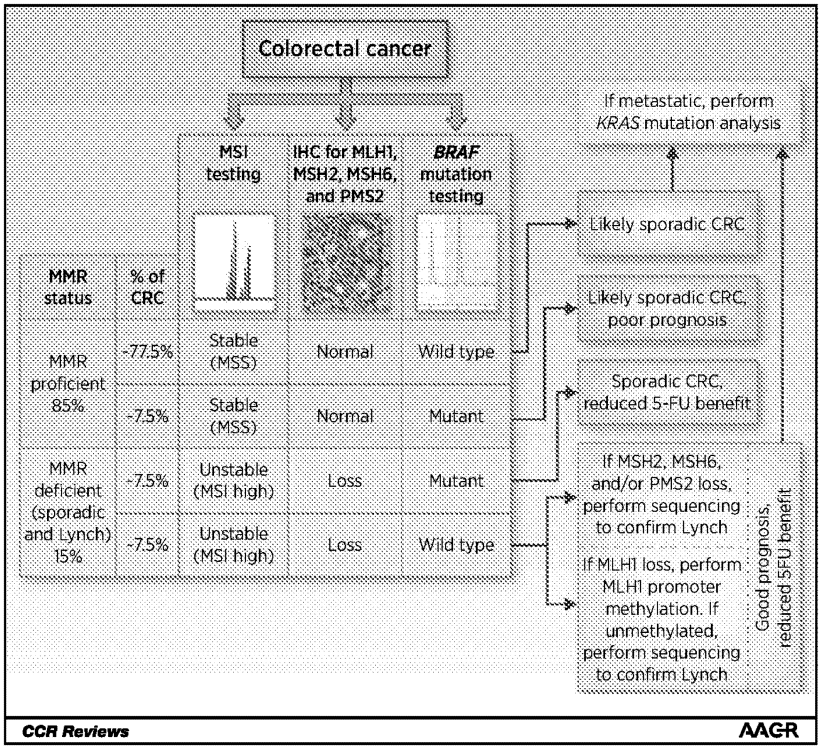



**Figure 2.**
MSI testing algorithm proposed in 2012 by the Association for Molecular Pathology. Reprinted with permission from ref. 29. This figure was published in *The Journal of Molecular Diagnostics*, Vol. 14, Funkhouser WK, Jr, Lubin IM, Monzon FA, Zehnbauer BA, Evans JP, Ogino S, et al. Relevance, pathogenesis, and testing algorithm for mismatch repair-defective colorectal carcinomas: a report of the Association for Molecular Pathology, 91–103. Copyright Elsevier 2012.

be apprised of their superior prognosis and potential lack of response to 5-FU chemotherapy; and MSS cases could plan for a poorer prognosis but could also potentially take advantage of 5-FU (29). Whether this is the most cost-effective approach however, is debatable. Two cost-effectiveness analyses evaluating money spent per life-year gained have favored screening patients with immunohistochemistry (64, 65). A more recent cost-effectiveness study favored PCR-based MSI testing as an initial measure (66). In either case, the biggest impact on cost saved per life-year gained came from identifying and properly managing affected relatives.

## Frequency of MSI Across Human Cancers

The inference that a hypermutation syndrome such as MSI may serve as a biomarker for checkpoint blockade response across tumor types is supported by the efficacy of such blockade in other tumors with high mutational burdens. Further, of the noncolorectal tumors for which the information is available, endometrial, gastric, esophageal, and sebaceous tumors with MSI have been found to have an elevated rate of lymphocytic infiltration relative to those without MSI (67–69). Further investigation will benefit from understanding the frequency of MSI in different tumors so they can be appropriately tested.

Tables 1–3 compare the frequency of MSI-H across tumor types. MSI-H is here defined, unless otherwise noted, as size variation in at least 2/5 or 30% of tested microsatellite loci. In constructing the tables, preference was given to studies published after the revised Bethesda guidelines were released, studies with larger cohorts, and studies with unselected or minimally selected cohorts. Because the frequencies given here

**Table 1.** Cancers with an MSI-H frequency greater than 10%

| Tumor type | Frequency, % (*n*) | Study |
|---|---|---|
| Colorectal cancer | 13% (1066) | Hampel et al. (72) |
| Endometrial | 22% (543), 33% (446) | Zighelboim et al. (73), Hampel et al. (74) |
| Gastric | 22% (295) | TCGA (75) |
| Hepatocellular carcinoma | 16% (37)[a] | Chiappini et al. (76) |
| Ampullary carcinoma | 10% (144) | Ruemmele et al. (77) |
| Thyroid | 63% (30)[a] | Mitmaker et al. (78) |
| Skin (sebaceous tumors) | 35% (20)[a], 60% (25)[a] | Cesinaro et al. (79), Kruse et al (80) |
| Skin (melanoma) | 11% (56)[a] | Palmieri et al. (81) |

[a]Studies of less than 100 patients.

**Table 2.** Cancers with an MSI-H frequency between 2% and 10%

| Tumor type | Frequency, % (*n*) | Study |
|---|---|---|
| Ovarian | 10% (1234) | Murphy and Wentzensen (82) |
| Cervical | 8% (344)[a] | Lazo (83) |
| Esophageal adenocarcinoma | 7% (76) | Farris et al. (84) |
| Soft-tissue sarcoma | 5% (40) | Kawaguchi et al. (85) |
| Head and neck SCC | 3% (153)[b] | Glavac et al. (86) |
| Renal cell carcinoma | 2% (152) | Hammerschmied et al. (87) |
| Ewing sarcoma | 2% (55) | Alldinger et al. (88) |

Abbreviation: SCC, squamous cell carcinoma.
[a]This number represents an aggregate of studies with different definitions of MSI-H, not all of which meet the Bethesda guidelines.
[b]MSI-H was defined as positivity in at least 2/8 markers.



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**Table 3.** Cancers with an MSI-H frequency less than 2%

| Tumor type | Frequency, % (*n*) | Study |
|---|---|---|
| Skin (squamous cell) | 0% (30), 0% (56) | Reuschenbach et al. (89)[a] |
| Skin (basal cell) | 0% (53), 2% (104) | Reuschenbach et al. (89)[a] |
| Prostate | 1% (79) | Burger et al. (90) |
| Lung | 0% (80), 2% (55) | Okuda et al. (91), Ninomiya et al. (92) |
| Osteosarcoma | 0% (68) | Entz-Werle et al. (93) |
| Glioblastoma | 0% (109) | Martinez et al. (94) |
| Pancreatic ductal adenocarcinoma | 0%–2% (338) | Laghi et al (95) |
| Breast | 0% (267), 0% (34), 0% (52), 1% (100) | Anbazhagan et al. (96), Adem et al. (97), Kuligina et al. (98), Toyama et al. (99) |
| Bladder | 1% (84) | Catto et al. (100) |
| Testicular germ cell | 0% (100) | Mayer et al. (70) |

[a]The first percentage given resulted from looking at a series of cases with three mononucleotide markers (BAT25, BAT26, and CAT25).The second number was derived by the authors by summing the results of several other studies, not all of which defined MSI-H in a manner consistent with the Bethesda guidelines.

are for unselected groups, they will be underestimates for the frequency of MSI in tumors of patients already known to have Lynch syndrome.

Several points regarding Tables 1, 2, and 3 deserve mentioning. Despite selecting the largest studies found for each tumor category, many of these studies still included relatively few patients, highlighting the need for further investigation to better characterize the frequency and characteristics of MSI in many tumor types. Cancers that have a very low proportion in unselected groups, potentially making standard MSI testing impractical, may have a significantly higher proportion in specific subgroups. A study of testicular germ cell tumors, for example, found no cases of MSI in an unselected series of 100 tumors but 5 cases in a series of 11 cisplatin-resistant tumors ($P = 0.001$; ref. 70). By contrast, a study of hepatocellular carcinoma in 55 Japanese patients failed to reveal a single case of MSI, despite its relatively high frequency in other cohorts (71). These examples illustrate that the optimal approach to MSI testing will be based not only on tumor type but also on specific clinicopathologic characteristics.

## Conclusions

Further studies are needed to assess the potential for MSI status to guide immunotherapy across tumor types. If initial results are validated, however, it would represent a significant advance in precision medicine. If the mechanism proposed for the efficacy of MSI-guided immunotherapy is correct (Fig. 1), the ultimate biomarker for immunotherapeutic response is not MSI or even the mutational burden but the presence of immunogenic neoepitopes. This could potentially be assessed more directly with future assays, leading to more precise guidance of immunotherapy, but at present, MSI status is a practical surrogate.

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Clinical Cancer Research

# Microsatellite Instability as a Biomarker for PD-1 Blockade

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# Dual Blockade of PD-1 and CTLA-4 Combined with Tumor Vaccine Effectively Restores T Cell Rejection Function in Tumors

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## Abstract

Tumor progression is facilitated by regulatory T cells (Tregs) and restricted by effector T cells. In this study, we document parallel regulation of $CD8^+$ T cells and $Foxp3^+$ Tregs by programmed death-1 (PD-1, PDCD1). In addition, we identify an additional role of cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitory receptor in further promoting dysfunction of $CD8^+$ T effector cells in tumor models (CT26 colon carcinoma and ID8-VEGF ovarian carcinoma). Two thirds of $CD8^+$ tumor-infiltrating lymphocytes (TIL) expressed PD-1, while one third to half of $CD8^+$ TIL co-expressed PD-1 and CTLA-4. Double-positive ($PD\text{-}1^+CTLA\text{-}4^+$) $CD8^+$ TIL had characteristics of more severe dysfunction than single-positive ($PD\text{-}1^+$ or $CTLA\text{-}4^+$) TIL, including an inability to proliferate and secrete effector cytokines. Blockade of both PD-1 and CTLA-4 resulted in reversal of $CD8^+$ TIL dysfunction and led to tumor rejection in two-thirds of mice. Double blockade was associated with increased proliferation of antigen-specific effector $CD8^+$ and $CD4^+$ T cells, antigen-specific cytokine release, inhibition of suppressive functions of Tregs, and upregulation of key signaling molecules critical for T cell function. When used in combination with GVAX vaccination (consisting of GM-CSF-expressing irradiated tumor cells), inhibitory pathway blockade induced rejection of CT26 tumors in 100% of mice and ID8-VEGF tumors in 75% of mice. Our study indicates that PD-1 signaling in tumors is required for both suppressing effector T cells and maintaining tumor Tregs, and that PD-1/PD-L1 pathway (CD274) blockade augments tumor inhibition by increasing effector T cell activity, while attenuating Treg cell suppression.

## Introduction

An effective immune response that leads to meaningful anti-tumor effects requires not only an increase in immune activation but also reduction of suppressive or inhibitory elements of the immune system (1–3). Mechanisms regulating immune activation in cancer progression have been extensively investigated (4–16). There are multiple inhibitory mechanisms that

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G.F. draws royalties from patents regarding PD-1.

**Contributions**
J.D. and G.C. designed the experiments; G.F. provided the reagents; J.D. performed the experiments, analyzed the data and wrote the manuscript; G.C, G.F, and K.K. edited the manuscript.

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suppress immune responses, including inhibitory receptors, secreted soluble inhibitors (IL-10 and TGF-β), and regulatory T cells (Tregs) (3, 16–17, 19–22). However, how these factors interact with each other in the tumor environment needs further study.

It is well established that tumors employ PD-1 and CTLA-4 inhibitory pathways to silence the immune system. Both these pathways are critical for physiological homeostasis. While PD-1 is broadly expressed on activated T cells and other hematopoietic cells (23–25), CTLA-4 is expressed on activated T cells including regulatory T cells (26). PD-1 binds two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). Upregulation of PD-L1 occurs on a wide variety of human tumors suggesting that cancer cells coopt the PD-1/PD-L1 inhibitory pathway to evade the host immune response (4–16). PD-L1 can also interact with B7.1, resulting in inhibition of T cell activation (24–25). Despite these similarities as terminators of T-cell activation, the difference in regulatory roles of the PD-1 and CTLA-4 pathways has been recognized (27–29). $CD4^+CD25^+Foxp3^+$ T cells (Tregs) play critical roles in the control of anti-tumor immune responses (21–22, 30–38). Indeed, recent data have shown that blocking of PD-1 and CTLA-4 can modulate Treg functions and enhance antitumor responses (2, 35). However, the direct role and function of PD-1 in Tregs in the cancer environment remains unknown.

Immunoregulatory pathways involving PD-1, CTLA-4, and their ligands are highly complex. Co-expression of more than one receptor by $CD8^+$ T cells has been correlated with a more severe exhausted phenotype (39–42). Antibody blockade of either PD-1 or CTLA-4 can enhance effector T-cell responses and induce T cell-mediated tumor rejection in mouse models (2, 4–15). However, the intricacies of each pathway and cross-talk between them make it unlikely that blockade of PD-1 or CTLA-4 would have identical effects. Hence, to optimally design the next generation of immunotherapeutic strategies, careful delineation of the individual contributions of PD-L1/PD-1, PD-L2/PD-1, CTLA-4/B7-1, and PD-L1/B7-1 interactions ***in vivo*** will be crucial.

In this study, we provide evidence that reversal of T cell dysfunction can be achieved by simultaneously targeting effector T cells and Tregs. First, we show that CTLA-4 is preferentially expressed by $PD\text{-}1^+$ $CD8^+$ T cells, and co-expression of both PD-1 and CTLA-4 is associated with marked dysfunction of antigen-specific T cells. Second, blockade of PD-1 and CTLA-4 pathways reversed T cell dysfunction. Blockade therapy with GVAX vaccination further enhanced tumor rejection in mice. Third, adoptive transfer of $CD8^+CTLA\text{-}4^+PD\text{-}1^+$ TILs that had been pre-treated ***in vitro*** with αPD-1 and αCTLA-4 antibodies eliminated tumors ***in vivo***. Fourth, blockade of PD-1/PD-L1 pathway in Tregs attenuates their suppressive function. Taken together, our study describes an approach involving vaccination and inhibitory receptor blockade to reverse T cell exhaustion, which we envision to be a promising anticancer immunotherapy.

## Methods

### Mice and cell lines

Six to 8 week-old female BALB/c or C57BL/6 mice (Jackson Laboratory) were used. CT26 colon carcinoma cell line was purchased from American Type Culture Collection (43). ID8-VEGF ovarian carcinoma cell line was developed previously from a mouse ovarian epithelial papillary serous adenocarcinoma cell line (ID8) obtained from Dr. Paul F. Terranova, University of Kansas, and used as described (44–46).

pMG-Lyt2 vector expressing murine GM-CSF was kindly provided by Dr. J.P. Allison, M.D. Anderson Cancer Center, Houston, TX (2). Development of CT26 or ID8-VEGF cells expressing murine GM-CSF (CT26/GVAX or ID8-VEGF/GVAX) was based on the

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methods described previously (2, 47). CT26/GVAX and ID8-VEGF/GVAX cells showed secretion of murine GM-CSF 365 ng/$10^6$ cells/48 h (95% confidence interval, 231–499) and 397 ng/$10^6$ cells/48 h (95% confidence interval, 275–519), respectively, by ELISA (R&D Systems, Minneapolis).

### Tumor experiments and *in vivo* blockade

BALB/c or C57BL/6 mice were implanted subcutaneously (s.c.) on the right flank with either $5\times10^5$ CT26 or $5\times10^6$ ID8-VEGF tumor cells, respectively. Two hundred μg of rat α-mouse PD-1 (29F.1A12), PD-L1 (10F.9G2), PD-L2 (3.2) (24), or 100 μg of α-CTLA-4 (clone 9D9) were administered intraperitoneally (i.p.), either 3, 6, and 9 days following CT26 inoculation, or 10, 13, and 16 days following ID8-VEGF inoculation. In vaccination experiments, $10^6$ irradiated (150Gy) CT26-GVAX or ID8-VEGF-GVAX were given on the contralateral flank intradermally (i.d.) once either day 3, or day 10 after CT26 or ID8-VEGF inoculations, respectively. $CD8^+$ T cells and $CD4^+CD25^+$ T cells from tumor leucocytes were purified either on a MACS column or FACSAria.

### Adoptive transfer experiments

$CD8^+CTLA\text{-}4^+PD\text{-}1^+$ TILs from CT26 tumor (25 gms pooled from 80 mice) were sorted to more than 95% purity by FACS. Sorted TILs were treated ***in vitro*** with 10 μg/ml of α-PD-1 and α-CTLA-4 blocking antibodies and cultured in the presence of AH-1 peptide-loaded APCs for 7 days. The cells expanded an average of 10-fold ***in vitro*** in the week of culture. Following expansion, $5\times10^6$ purified $CD8^+$ T cells per mouse were injected intratumorally (i.t) 5 times on alternate days starting from day 10 following s.c. CT26 tumor inoculation.

### *in vitro* proliferation assay

$PD\text{-}1^+CTLA\text{-}4^+$ and $PD1^+CTLA\text{-}4^-$ $CD8^+$ TIL were sorted to more than 95% purity by FACS and co-cultured with splenocytes from naive $CD45.1^+$ C57BL/6 mice in the presence of AH-1 (CT-26) or FR-α (ID8-VEGF) peptides for 3 days (48–49). Thymidine incorporation was measured after pulsing with 0.5 μCi/well [$^3$H]-thymidine during the last 8 h.

### *in vitro* Treg suppression and cytokine analysis

CFSE-labeled $CD8^+$ T cells ($5 \times 10^4$) were stimulated with $1.5 \times 10^5$ irradiated splenic $CD45.1^-$ APCs in the presence of αCD3. $CD4^+CD25^+$ Tregs ($10^5$) from CT26 TILs, along with αPD-L1, αCTLA-4, or control rat IgG were added to the culture as indicated. Four days later, ***in vitro*** Treg suppression was determined based on CFSE dilution by flowcytometry.

The whole tumor TILs were isolated and adjusted to $2 \times 10^6$/ml in 24-well plates and cultured with AH-1 peptide ± 10 μg/ml αPD-1, αPD-L1, αCTLA-4, or control IgG. After 3 days, supernatants were analyzed for secretion of TGF-β, IL-10 and IFN-γ by ELISA and cytokine bead array.

## Results

### PD-1, CTLA-4, and their ligands are highly expressed in tumor models

To examine the interplay between the PD-1 and CTLA-4 in cancer, we used the murine CT26 colon carcinoma and ID8-VEGF ovarian carcinoma models. In the $CD8^+$ TIL population, two thirds expressed PD-1, and the majority of these (over two thirds) were $PD\text{-}1^{hi}$ (Fig. 1A and B). We also found expression of CTLA-4 on TIL, however CTLA-4 was expressed primarily in a subset of $PD\text{-}1^+CD8^+$ TIL, while $PD\text{-}1^-CD8^+$ cells were

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mostly CTLA-4$^-$. About half of PD-1$^+$CD8$^+$ CT26 TIL and one third of PD-1$^+$CD8$^+$ ID8-VEGF TIL were CTLA-4$^+$, while the remainder of PD-1$^+$CD8$^+$ TIL were CTLA-4$^-$. CTLA-4 was expressed on PD-1$^{hi}$ as well as PD-1$^{int}$ cells in CT26 TILs, whereas CTLA-4 was expressed on PD-1$^+$ cells in ID8-VEGF TILs. Taken together, among CD8$^+$ TIL, one third co-expressed PD-1 and CTLA-4, one third expressed PD-1 only, and the remainder were negative for either receptor (Fig. 1A and B). Among the CD4$^+$ TIL, only a third of the population expressed either of the receptors and approximately 10% were double-positive (Fig. 1A and B), while a quarter of splenic CD4$^+$ T cells expressed CTLA-4, yet there were very few PD-1$^+$ cells (Supplementary Fig. 1). We also found that CT26 and ID8-VEGF tumors upregulate PD-1 and CTLA-4 ligands on tumor and myeloid cells (Supplementary Fig. 2). Thus, CT26 and ID8-VEGF models are suitable for studying PD-1 and CTLA-4.

To begin defining the functional status of TIL, we examined their memory phenotype. Among CD8$^+$ T cells, effector memory ($T_{EM}$) cells were most prevalent in the double positive (PD-1$^{hi}$CTLA-4$^+$) population (approximately 90% of CT26 and 75% of ID8-VEGF cells), but also accounted for approximately 60% (CT26) and 40% (ID8-VEGF) of the PD-1$^{hi}$CTLA-4$^-$ population and half of the rare PD-1$^-$CTLA-4$^{Int}$ population (CT26). One third of the PD-1$^{Int}$ CT26 CD8$^+$ T cells were $T_{EM}$ independently of their CTLA-4 status. Both single- and double-positive CT26 CD4$^+$ T cells had a $T_{EM}$ phenotype, whereas only PD-1$^+$CTLA-4$^+$ ID8-VEGF CD4$^+$ T cells had a $T_{EM}$ phenotype (Fig. 1A and C). Thus, a significant fraction of TILs upregulated PD-1, and many of these cells were $T_{EM}$. A proportion of PD-1$^{hi}$ TILs also upregulated CTLA-4, and these double-positive cells were mostly $T_{EM}$.

## Co-expression of PD-1 and CTLA-4 correlates with more severe dysfunction of tumor-specific CD8$^+$ T cells

Given the strong association of PD-1 expression and $T_{EM}$ phenotype, we hypothesized that most tumor-reactive TIL are present in the PD-1$^+$ population. To address this, we used AH-1 ($gp70_{423–431}$) and H2-K$^b$ folate receptor ($FR_{161–169}$), CD8$^+$ T cell specific epitopes expressed by CT26 and ID8-VEGF tumor cells, respectively (45–46). We hypothesized that the PD-1$^+$CTLA-4$^+$ cells exhibit greater dysfunction in response to cognate antigens than the single-positive (PD-1$^+$) counterparts. To address this, we sorted PD-1$^+$CTLA-4$^+$ and PD-1$^+$CTLA-4$^-$ CD8$^+$ TILs, labeled them with CFSE, and co-cultured for 3 days with peptide-pulsed APCs. CFSE dilution was observed only in the PD-1$^+$CTLA-4$^-$ population but not in the PD-1$^+$CTLA-4$^+$ population (11.39% vs. 2.45% and 32.3% vs. 4.4% CT26 or ID8-VEGF CFSE$^{lo}$ cells, respectively), indicating a profound suppressed state of the double-positive cells (Fig. 2A).

Next, we directly compared the functional properties of sorted PD-1$^+$CTLA-4$^-$ or PD1$^+$CTLA-4$^+$ CD8 TILs by measuring cytokine secretion in response to peptide stimulation. The frequency of IFN-$\gamma^+$, TNF-$\alpha^+$, IL-2$^+$ or CD107a/b$^+$ cells was approximately 2-fold higher in the PD-1$^+$CTLA-4$^-$ subset compared with the PD1$^+$CTLA-4$^+$ subset (Fig. 2B; $p<0.05$). The PD1$^+$CTLA-4$^+$ CD8 T cells displayed higher levels of other inhibitory receptors such as 2B4, LAG-3, and TIM-3, along with lower levels of CD62L, and CD127 (Fig. 2B; $p<0.01$). These results therefore indicate that dysfunction of CD8$^+$ T cells in tumor was associated with coexpression of PD-1 and CTLA-4.

Next, we tested whether checkpoint blockade could rescue the proliferation of double-positive cells and/or enhance the proliferation of single-positive cells. We isolated PD-1$^+$CTLA-4$^+$ and PD-1$^+$CTLA-4$^-$ CD8$^+$ CT26 and ID8-VEGF TIL subsets and co-cultured them with AH-1 or FR peptide pulsed splenocytes (APCs) ± $\alpha$PD-1 and/or $\alpha$CTLA-4 antibodies. Again, in the absence of any neutralizing antibodies we observed modest proliferation of the PD-1$^+$CTLA-4$^-$ CD8$^+$ TIL and no proliferation of the

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PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL (Fig. 2C; $p$=0.019 and $p$=0.015 respectively). PD-1 blockade alone was able to enhance the proliferation of both PD-1$^+$CTLA-4$^-$ and PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL, although it was unable to completely restore the proliferation of double-positive cells. CTLA-4 blockade alone was able to enhance the proliferation only of PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL, and had no effect on CTLA-4$^-$ cells. However, dual blockade fully restored the proliferation of PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL (Fig. 2C; $p$=0.038 and $p$=0.004, respectively). Thus, the double-positive population represents cells with severely dysfunctional status, but their function can be rescued by double blockade.

## Combination therapy promotes CT26 and ID8-VEGF tumor rejection

Given that a significant proportion of tumor-reactive CD8$^+$ TILs were found to be PD-1$^+$CTLA-4$^+$, and given that their proliferation in response to cognate tumor antigen was restored by double blockade *in vitro*, we tested whether simultaneous blockade of PD-1 and CTLA-4 could promote tumor rejection in mice. We found that treatment with αPD-1 or αPD-L1 showed CT26 tumor regression in 25% and 33% of the mice, respectively (Fig. 3A; Table 1). CTLA-4 blockade alone led to tumor regression in 50% of the mice. Further, we noticed that combination of either αPD-1 or αPD-L1 with αCTLA-4 induced tumor regression in 75% of mice ($p$<0.01). Similarly, we found that treatment with αPD-1, αPD-L1 or αCTLA-4 showed ID8-VEGF tumor regression in 25%, 37.5%, and 25% of the mice, respectively (Fig. 3B; Table 1). Combined blockade induced tumor regression in 50% of mice. In contrast, αPD-L2 blockade did not induce tumor regression in both models. Further, *in vivo* CD4$^+$ and CD8$^+$ depletion completely abolished tumor regression (Supplementary Fig. 3).

Hypothesizing that the effects of inhibitory receptor blockade could be enhanced by increasing the frequency of tumor-reactive T cells, we combined our treatment with GM-CSF transduced whole tumor cells (GVAX) (50). Remarkably, 100% of CT26 bearing mice and 75% of ID8-VEGF bearing mice in the triple combination groups (GVAX/αPD-1/αCTLA-4) rejected their tumors (Fig. 3A & B; Table. 1). Thus, double checkpoint blockade has the potential to induce significant tumor rejection, which could be maximized by boosting antitumor immune response with vaccine.

## Adoptive transfer of *in vitro* pre-treated CD8$^+$CTLA-4$^+$PD-1$^+$ TILs with αPD-1 and αCTLA-4 antibodies cause regression of CT26 tumor in mice

Next we tested whether CD8$^+$CTLA-4$^+$PD-1$^+$ TILs treated *in vitro* with αPD-1 and αCTLA-4 would kill the tumor cells *in vivo*. We sorted CD8$^+$CTLA-4$^+$PD-1$^+$ TILs from CT26 tumor and cultured in the presence of α-PD-1 and α-CTLA-4 blocking antibodies and AH-1 peptide for 7 days. We found that *in vitro* treated cells showed profound increase in IFN-γ cytokine production and Ki-67 expression (Fig. 4B; left panel). These *in vitro* treated cells were adoptively transferred into CT26 tumor bearing mice as described in the Methods. We found that adoptive therapy resulted in tumor regression in 75% of mice (Fig. 4A). In addition, we found that the adoptively transferred TILs were still functional (IFN-γ$^+$Ki67$^+$) in the regressing tumors a week after the treatment (Fig. 4B; right panel).

## Combination blockade increases TIL activation and antigen-specific inflammatory cytokine production

Next, we tested whether tumor regression following *in vivo* blockade was associated with activation of TIL. We resected and analyzed tumors (n=8) that showed evidence of rejection from 13–15 day onwards. As expected, Ki-67 expression on TIL was markedly increased following PD-1, PD-L1, or CTLA-4 monotherapy ($p$=0.001). Confirming our observation *in vitro* (Fig. 2) combination of αPD-1 with αCTLA-4 doubled the frequency of Ki-67$^+$CD8$^+$ T cells (Fig. 5A; $p$=0.002), while αPD-L1 and αCTLA-4 combination increased the

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frequency of Ki-67$^+$CD8$^+$ T cells by 50% ($p$=0.012) compared with single antibody blockade (Fig. 5A). Combined blockade also increased granzyme B expression, suggesting an enhancement in cytolytic potential of effector T cells (Fig. 5B; $p$=0.01).

We corroborated the above findings by testing the phosphorylation status of key transcription factors by flowcytometry (51–52). The ribosomal S6 kinase (S6K) is implicated in IL-2 induced T cell proliferation (53). Validating the increased expression of Ki-67 by TIL, we observed an increase in the expression of phosphorylated (p)-S6K in CD8$^+$ T cells following single antibody (αPD-1) blockade, and combined PD-1 and CTLA-4 blockade further enhanced pS6K levels ($p$=0.01; Fig. 5C). T-bet and Eomes are two T-box transcription factors regulating Th1 and cytolytic function of CD8$^+$ cells (54–56). αPD-L1 antibody alone induced high levels of pT-bet, whereas αPD-1 or αCTLA-4 alone or combined showed moderate increases in pT-bet levels (Fig. 5C). Validating the enhanced granzyme B expression, blocking antibodies increased pEomes expression levels (Fig. 5C). Thus, checkpoint blockade induced TIL activation, and combined αPD-1 and αCTLA-4 therapy significantly increased proliferation and cytolytic function of TIL relative to monotherapy.

We next tested whether the functional capacity of tumor-reactive CD8$^+$ TIL increased following single versus double blockade. CT26 tumors from the various treatment groups were dissociated, and mixed cells from these tumors were seeded in primary co-cultures with AH-1 peptide. Cultures derived from single antibody (PD-1, PD-L1, or CTLA-4) treated mice showed modestly increased IFN-γ$^+$TNF-α$^+$ or IFN-γ$^+$IL-2$^+$ CD8$^+$ TILs (Fig. 5D; $p$=0.05). Cultures from combined αPD-1 and αCTLA-4 treatment, exhibited increased numbers of IFN-γ$^+$TNF-α$^+$ (Fig. 5D; $p$=0.05), but not IFN-γ$^+$IL-2$^+$ CD8$^+$ TILs (Fig. 5D;$p$=0.12). These data suggest that combination blockade induces polyfunctional activation of tumor antigen-specific CD8$^+$ T cells. As expected, combining GVAX with αPD-1, αPD-L1, or αCTLA-4 inhibitory blockade further increased the frequency of CT26-specific IFN-γ$^+$TNF-α$^+$ CD8$^+$ TIL (Fig. 5E; $p$=0.009).

### *in vivo* PD-1 and CTLA-4 blockade enhances infiltration of T cells and reduces Tregs in tumors

As expected based on the above experiments, combined blockade significantly increased total inflammatory infiltration and CD8$^+$ cells in tumors, but also CD4$^+$ cell infiltration (Fig. 6A; $p$=0.002, $p$=0.025, and $p$=0.001 respectively). Then we tested whether the effects of inhibitory receptor blockade could be further enhanced by depleting Tregs. Interestingly, we could not find any additional benefit by combining αCD25 antibody with blocking antibodies (Supplementary Fig. 4). This suggested that blocking antibodies could independently affect Treg accumulation in tumors as well as tumor draining lymph nodes (Supplementary Fig. 5).

To test this hypothesis, we analyzed the frequency of Tregs in tumors of mice following single or double blockade. PD-1 or PD-L1 blockade alone produced a moderate reduction in Treg levels in the tumor (Fig. 6B and 6C; $p$=0.024), while CTLA-4 blockade did not affect Treg numbers. However, combined blockade profoundly reduced the frequency of Tregs (from 9.06% to 3.24% of CD4$^+$; Fig. 6B and 6C; $p$=0.004). As a result, the CD8/Treg and CD4/Treg ratios increased markedly, especially after combined αPD-L1 and αCTLA-4 treatment.

We asked whether the reduction in Treg frequency was due to apoptosis. None of the blocking antibodies affected the apoptosis rate in Tregs (based on Annexin-V and 7-AAD staining - data not shown). Importantly, combined αCTLA-4 and αPD-1 reduced GITR (a functional marker of activated Tregs) expression from 66.7% to 45.8% of Tregs (Fig. 6B;

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$p<0.001$), indicating that double blockade could affect not only the number but also the function of tumor-infiltrating Tregs.

### Co-expression of PD-1 and CTLA-4 by Tregs is associated with heightened T cell dysfunction in tumor

Since we found approximately one-fourth of the $CD4^+$ TIL expressing PD-1 and/or CTLA-4 (Fig. 1), we first examined whether these $CD4^+$ TIL are effector cells or Tregs. We found that Tregs were primarily comprised in the CTLA-$4^+$ $CD4^+$ population. Importantly, among the CTLA-$4^+$ cells, most were $Foxp3^+CD25^+$ cells, representing activated effector Tregs (30, 35, 37), and were PD-$1^+$CTLA-$4^+$ double-positive cells (Fig. 7A). Notably, splenic Tregs expressed much lower levels of PD-1 and CTLA-4 (Fig. 7A). We also observed not only increased expression of CTLA-4, but also of inducible T cell co-stimulator (ICOS) and folate receptor 4 (FR-4) expression on PD-$1^{hi}$ Treg compared to PD-$1^{lo}$ Treg (Fig. 7B; $p=0.001$, $p=0.001$ and $p=0.034$, respectively), indicating an activated functional status. Thus, tumor Treg were mostly CTLA-$4^+$ and among them, the PD-$1^+$ Tregs appeared to be activated Tregs.

To understand the specific contribution of PD-$1^+$ Treg to the suppressive program, $CD4^+CD25^+$CTLA-$4^+$ Tregs were preincubated with PD-1 blocking antibody or control IgG. Control Tregs or PD-1-neutralized Tregs were then incubated with $CD8^+$ T cells stimulated by immobilized αCD3 antibody (responder cells) in the presence of splenic APCs ($CD11c^+$ cells) or PD-L1 neutralized tumor-derived APCs. Responder T cell proliferation was assessed by CFSE dilution. Whereas control Tregs were able to suppress responder cell proliferation, PD-1 neutralized Tregs as well as PD-L1-neutralized tumor-derived APCs were unable to suppress $CD8^+$ T cell proliferation (Fig. 7C, $p=0.0014$). Thus, the suppressive function of tumor-derived Tregs is critically dependent on PD-1 signaling, compatible with the notion that the PD-$1^+$CTLA-$4^+$ Tregs are responsible for the suppressor function.

Next, we tested whether PD-1 blockade could attenuate the suppressive phenotype of Tregs and their regulatory cytokine programming (Fig. 7E–G). The whole tumor digests from various *in vivo* treatment groups were co-cultured with AH-1 peptide for 5 hours, and we measured TGF-β, IL-10 and IFN-γ in the supernatants. We detected significant amounts of TGF-β and IL-10 and minimal IFN-γ in leukocyte co-cultures form untreated mice, indicating ongoing activity of Tregs and no reactivity of antitumor effector cells. TGF-β secretion was suppressed *ex vivo* following αPD-1 treatment, and more effectively after αCTLA-4 treatment *in vivo* (Fig. 7E; $p=0.017$). In contrast, IL-10 secretion was decreased *ex vivo* following αPD-1 treatment, but not after αCTLA-4 treatment *in vivo* (Fig. 7F; $p=0.024$). Furthermore, dual αPD-1 and αCTLA-4 blockade elevated AH-1-specific IFN-γ induction (Fig. 7G; $p=0.03$). Thus, checkpoint blockade reduces the ability of Tregs to secrete immunoregulatory cytokines.

## Discussion

In this study, we demonstrated a non-redundant regulation of $CD8^+$ T cell exhaustion by PD-1 and CTLA-4 in CT26 colon carcinoma and ID8-VEGF ovarian carcinoma models. About one fourth of $CD8^+$ TIL expressed both PD-1 and CTLA-4, and this was associated with more severe $CD8^+$ T cell exhaustion. Blockade of either PD-1 or CTLA-4 pathways enhanced effector T cell infiltration into the tumor. However, simultaneous blockade of both pathways had synergistic effects in activation of tumor antigen specific $CD8^+$ and $CD4^+$ effector T cells as well as affecting Tregs, ultimately improving long-term survival rates. Adoptive transfer of $CD8^+$CTLA-$4^+$PD-$1^+$ TILs that had been pre-treated *in vitro* with

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αPD-1 and αCTLA-4 antibodies eliminated CT26 tumors *in vivo*, further demonstrating the role of the PD-1 and CTLA-4 pathways in the tumor.

These effects were further augmented through combination with a GVAX vaccine. Combining blockade therapy with a single dose of GVAX vaccination resulted in enhanced intratumoral $CD45^+$ and $CD8^+$ T cells and as a result, the CD8/Treg ratios increased markedly. Moreover, mice treated with αPD-1/CTLA-4 combined with GVAX further increased IFN-$\gamma^+$ TNF-$\alpha^+$ $CD8^+$ TILs (Fig. 5E) resulting in enhanced tumor rejection. Hence our results suggest that the mechanisms underlying the synergistic effects of vaccine could possibly be due to the capacity of vaccine to increase the frequency of tumor-reactive $CD8^+$ TILs, which could be activated by CTLA-4 and PD-1 blockade. The levels of GM-CSF produced by CT26/GVAX and ID8-VEGF/GVAX vaccines are comparable to the previously published preclinical studies using B16/GVAX and Glioma/GVAX (2, 57). The lack of *in vivo* efficacy of PD-1/PD-L1 blockade with GVAX in B16 melanoma seen by Curran et al. (2) may be due to the fact that the B16 tumor line does not express PD-L1, whereas CT26 and ID8-VEGF cells express high levels. Our results are also in line with previous observations that higher dose of GVAX recruited MDSCs into the tumor (2, 57).

The PD-1/PD-L1 pathway mediates T cell exhaustion by antagonizing activation signaling pathways (28–29, 58–62). We investigated our therapy's ability to counteract these effects by comparing the levels of key signaling molecules in different treatment groups. First we looked at T-bet, which is expressed preferentially by type 1 effector T cells, and Eomes, which is expressed in memory T cells (61–62). We also monitored the S6K which, when phosphorylated, acts downstream of mTOR to activate cell proliferation, protein translation, and survival (53). In untreated mice, we found that tumor infiltrating T cells showed no or very low expression of pT-bet, pEomes, or pS6K. However, upon single αPD-L1 antibody treatment we observed a marked increase in pT-bet expression. αPD-L1 combined with αCTLA-4 antibody further increased pEomes, and αPD-1 combined with αCTLA-4 blockade further increased pS6K. That our treatments led to increased levels of these phosphorylated signaling molecules supports the contention that we were activating classical anti-tumor effector T cells. However, the differential effects of combining αCTLA-4 with αPD-1 versus αPD-L1 could be because αPD-1 blocks the inhibitory contributions of PD-1 binding to PD-L1 and PD-L2 but leaves the PD-L1/B7-1 inhibitory interaction unaffected (24–25). In contrast, αPD-L1 blocks both the PD-L1/PD-1 and the PD-L1/B7-1 inhibitory pathways but leaves the PD-1/PD-L2 inhibitory interaction unaffected (24–25).

Our experiments with sorted PD-$1^+$CTLA-$4^-$ and PD-$1^+$CTLA-$4^+$ cells showed that CT26 or ID8-VEGF antigen-specific $CD8^+$ T cells were exclusively located in the double-positive population and were highly dysfunctional, highlighting the relevance of these receptors in escape from the anti-tumor T cell response. Studies report that PD-1 mediates inhibition by blocking PI3K activation, whereas CTLA-4 functions through binding to the phosphatase PP2A, leading to inhibition of Akt phosphorylation (29, 59, 63–64). Our study proves that double blockade targets both pathways resulting in an additive response by targeting both PD-1 and CTLA-4 signaling molecules allowing more effective T-cell activation, proliferation and function of TILs, and further augmentation of therapeutic effect. Taken together, our results indicate that PD-1 and CTLA-4 pathways act non-redundantly to inhibit TIL function. This is an important lesson to bring to the clinic, highlighting why blockade of both pathways may be essential to completely regain T cell function.

Our therapy's ability to limit the Treg population *in vivo* is highly desirable. Here we found simultaneous expression of PD-1 and CTLA-4 on Tregs and distinct effects of blocking each pathway. The importance of the PD-1 pathway for Tregs, however, is not well characterized, although one recent study described PD-L1 expression by Tregs (36). While PD-1 enhanced

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*in vivo* Treg function, CTLA-4 blockade did not impact the suppressive function of Tregs in CT26 *in vivo* (Figs. 6). However, our findings indicate that complete restoration of $CD8^+$ T cell proliferation required combined αPD-1 and αCTLA-4 blockade both *in vivo* as well as *in vitro* (Figs. 6 and 7). In our models, PD-L1 on Tregs might bind directly to PD-1 on $CD8^+$ T cells (cell-autonomous), or it may indirectly activate nearby DCs by reversing suppression through PD-1, allowing the induction of a stronger adaptive immune response (non-autonomous). We investigated which of these mechanisms was occurring. We found that blocking either PD-1 or PD-L1 on Tregs led to an increase in $CD8^+$ T cell proliferation (Fig. 7C). Also, in the absence of PD-L1 depleted APCs, the Tregs were not able to suppress $CD8^+$ T cell proliferation (Fig. 7C). This demonstrates that PD-1 expression by effector T cells and Tregs, as well as PD-L1 expression by APCs, are all involved in the suppression of $CD8^+$ TIL responses. We also found that dual blockade reduces production of other immunoinhibitory cytokines including TGF-β and IL-10. This provides further novel insights into the mechanisms of dual blockade.

αCTLA-4 has already been approved by FDA for treatment of melanoma. The antitumor activity of αPD-1 and αPD-L1 are evident in animal models, and αPD-1 and αPD-L1 antibodies are now under intense clinical evaluation (65–67). It will be critical to understand the direct effects of blockade on effector T cells as well as Tregs. Our work suggests that administration of αPD-1 antagonists may harness the therapeutic potential of effector T cells *in vivo* while concomitantly suppressing the functions of activated Tregs. Our study provides the scientific basis for a clinical trial that would involve combination of tumor-cell vaccination and simultaneous PD-1 and CTLA-4 blockade for sustained tumor control in cancer patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **PD-1** | programmed death-1 |
| **PD-L** | PD-1 ligand |
| **CTLA-4** | cytotoxic T-lymphocyte antigen-4 |
| **CT26** | colon carcinoma |
| **ID8** | ovarian carcinoma |
| **Tregs** | regulatory $CD4^+$ T cells |
| **GVAX** | Tumor cells transduced to express GM-CSF |

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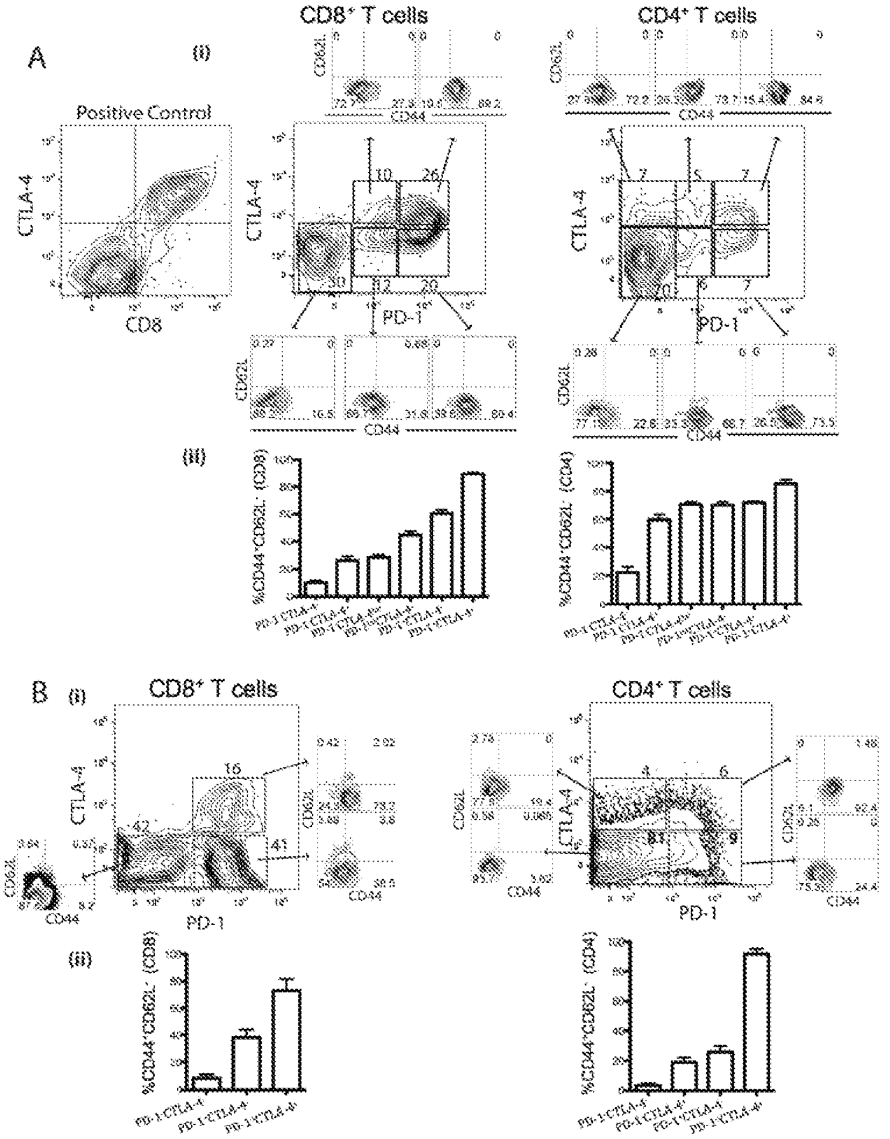



**Figure 1. Phenotypic analysis of CT26 and ID8-VEGF TIL**

TIL isolated from CT26 and ID8-VEGF tumors were stained with antibodies to CD45, CD8, CD4, PD-1, CTLA-4, CD44, and CD62L as well as live/dead stain. Representative CT26 (A) and ID8-VEGF (B) tumor samples showing expression of PD-1 and CTLA-4 on $CD8^+CD45^+$ and $CD4^+CD45^+$ TIL with distinct cell populations, which are further analyzed for CD44 and CD62L. Summary data of CTLA-4 and PD-1 expression on $T_{EM}$ ($CD44^{hi}CD62L^{lo}$) $CD8^+$ and $CD4^+$ TIL. Data represent mean ± SD of n=6 mice per group and are representative of 4 independent analyses.

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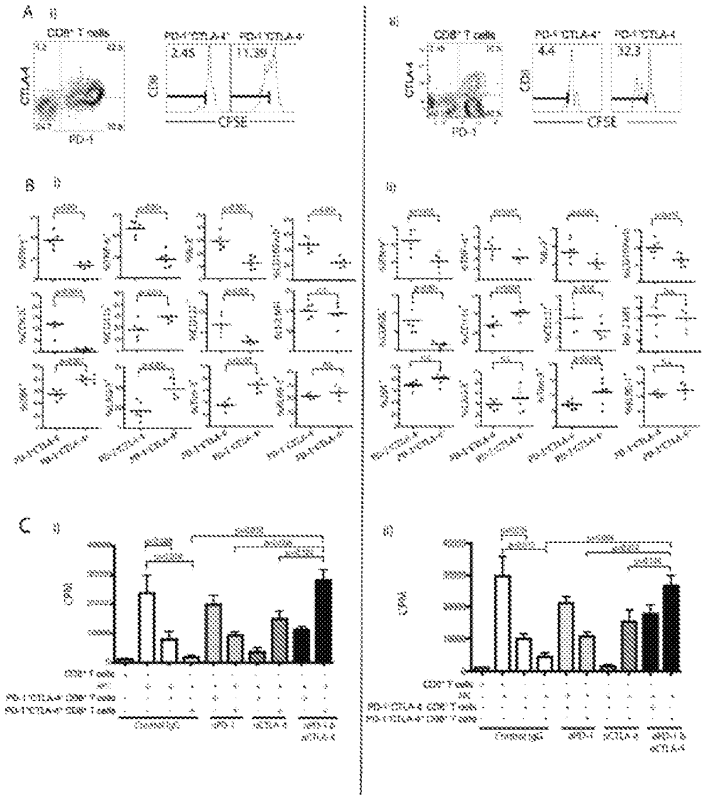


**Figure 2. Co-expression of PD-1 and CTLA-4 correlates with more severe dysfunction of CT26 and ID8-VEGF antigen-specific CD8$^+$ T cells**

A) PD-1$^+$CTLA-4$^+$ and PD-1$^+$CTLA-4$^-$ CD8$^+$ TIL populations from either CT26 (i) or ID8-VEGF (ii) tumors were sorted, labeled with CFSE, and stimulated with AH-1 or FR peptide, respectively for 3 days in the presence of irradiated splenic CD45.1$^-$ APCs. CD8$^+$ T cell proliferation was determined by dilution of CFSE; numbers indicate the percentage of CFSE$^{lo}$ (AH-1 or FR reactive cells). B) Frequency of CT26 (i) or ID8-VEGF (ii)-specific PD-1$^+$CTLA-4$^-$ and PD1$^+$CTLA-4$^+$ CD8$^+$ T cells showing cytokine and degranulation after peptide stimulation (n=6). PD-1$^+$CTLA-4$^-$ and PD1$^+$CTLA-4$^+$ CD8$^+$ TILs cells were further analyzed for differential phenotype and expression of other inhibitory receptors. C) PD-1$^+$CTLA-4$^+$ or PD-1$^+$CTLA-4$^-$ CD8$^+$ TILs from CT26 (i) or ID8-VEGF (ii) were stimulated with AH-1 or FR peptide, respectively and cultured with blocking antibodies for 3 days. Proliferation was determined by $^3$[H]-thymidine incorporation. Statistical significance was determined by Student's t-test.

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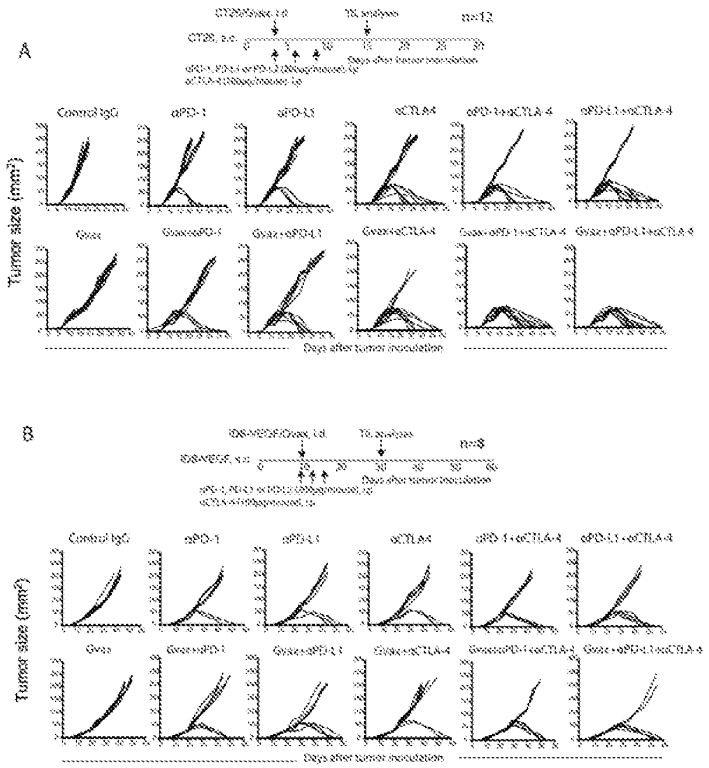



**Figure 3.** ***In vivo*** **PD-1 and CTLA-4 blockade promotes CT26 and ID8-VEGF tumor rejection in mice**

Regression and survival (<200 $mm^2$) of CT26 (A) and ID8-VEGF (B) inoculated mice following treatment as described in the Methods section. Figures represent one of the 5 (CT26) and 3 (ID8-VEGF) independent experiments.

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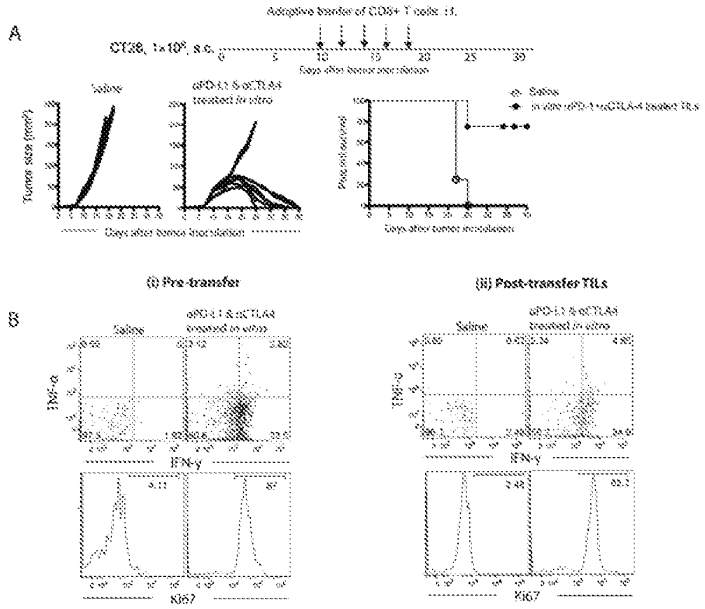



**Figure 4. Therapeutic adoptive transfer of *in vitro* α-PD-1 and α-CTLA-4 pre-treated TILs cause regression of CT26 tumors in mice**

A) Tumor regression in mice transferred with ***in vitro*** expanded CT26 antigen-specific $CD8^+CTLA\text{-}4^+PD\text{-}1^+$ CT26 TILs. (B) The percent of IFN-$\gamma^+$ and $Ki67^+$ of ***in vitro*** pre-treated $CD8^+$ T cells just before adoptive transfer (left panel) and the TILs recovered from tumor one week after the final transfer (right panel) are shown.

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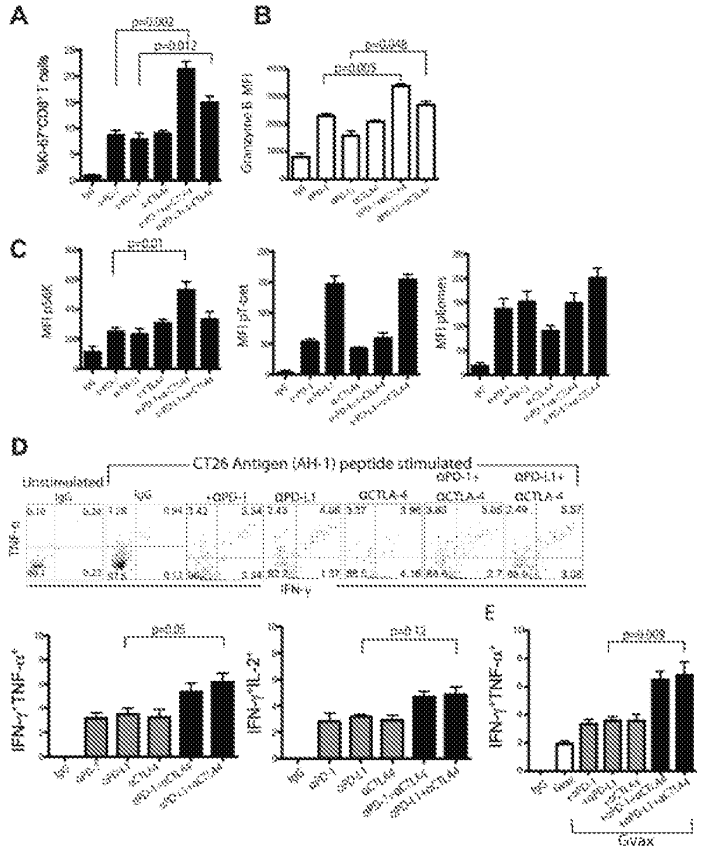



**Figure 5.** ***In vivo*** **PD-1 and CTLA-4 blockade increases TIL activation and CT26 antigen-specific inflammatory cytokine production**

Summary data showing percentage of Ki-67$^+$ CD8$^+$ TILs (A) and mean fluorescence intensity (MFI) of granzyme B$^+$ CD8$^+$ TILs (B) from mice treated with various antibodies, as indicated. C) Flow cytometry analysis showing MFI of pT-bet, pEomes and pS6K expression by CD8$^+$ TIL from treated mice. Representative and summary data showing IFN-γ and TNF-α production by CD8$^+$CD45$^+$ TIL from antibody (D) as well as GVAX ± antibody (E) treated mice 13–15 days following tumor inoculation. Statistical significance was determined by Student's *t*-test.

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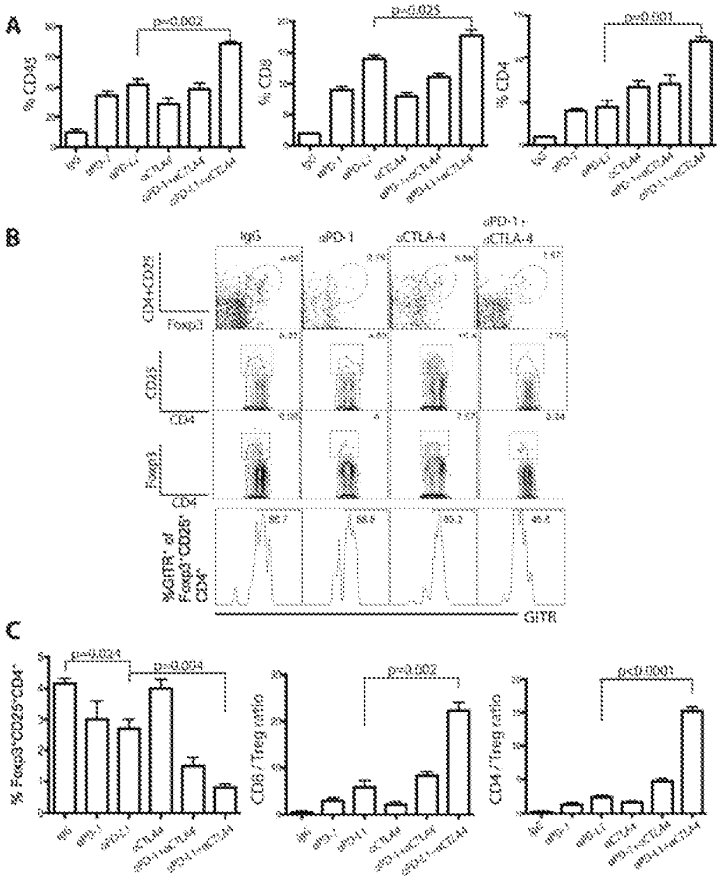



**Figure 6. PD-1 and CTLA-4 blockade enhance lymphocyte infiltration and reduce the frequency of Tregs in CT26 tumors**

A) $CD45^+$ leukocyte infiltration, and $CD8^+$ and $CD4^+$ TIL as percent of total $CD45^+$ cells 13–15 days after tumor inoculation. B) Representative data showing the percentage of Tregs and expression of GITR among Tregs. C) Percentage of $CD45^+$ TILs of Tregs, and the ratios of $CD8^+$ or $CD4^+$ effector T cells to Tregs in the tumors of treated mice with indicated blocking antibodies. Values are shown for mice analyzed independently and the sum of 3 independent experiments with 6–12 mice per group. Statistical significance was determined by Student's *t*-test.

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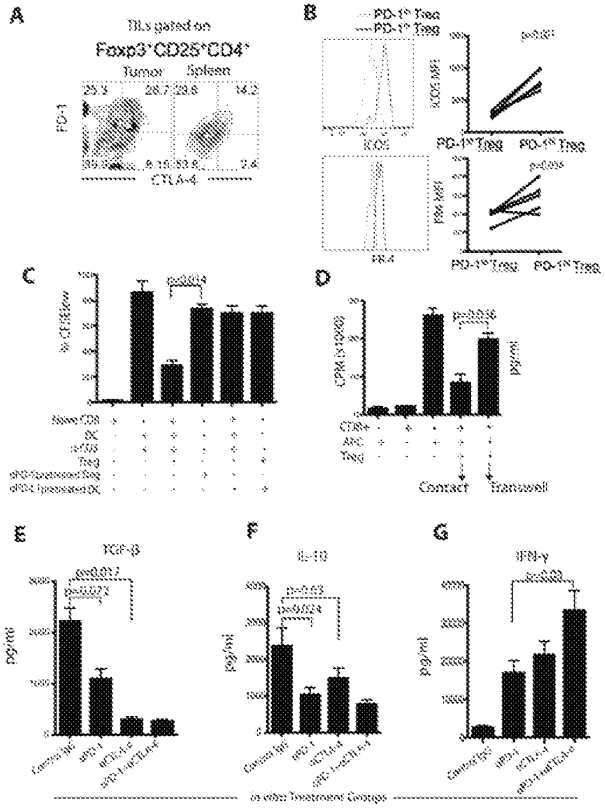



**Figure 7. PD-1 and CTLA-4 pathway blockade reduces Treg-mediated suppression of CD8$^+$ T cells *in vitro* and secretion of regulatory cytokines TGF-β and IL-10**

A) Representative expression of PD-1 and CTLA-4 by intra-tumoral and splenic Tregs in the same mouse. B) ICOS and folate receptor 4 (FR4) expression and MFI in PD-1$^{hi}$ and PD-1$^{lo}$ Tregs. C) TIL Tregs (± αPD-1) were co-cultured with CFSE-labeled CD8$^+$ T cells and DCs (pretreated) in the presence of αCD3. Dividing cells were quantified after 4-days of culture by gating on the CFSE$^{lo}$ population. D) [$^3$H]-thymidine incorporation into effector T cells in the presence or absence of TIL Tregs. Tregs were either in direct contact with the effector cells or were separated by a transwell membrane. The whole tumor leucocytes were isolated and cultured ***ex vivo*** for 72 hours in the presence of AH-1 peptide and PD-1 and CTLA-4 blocking antibodies as indicated. After culture, supernatants were analyzed for secretion of TGF-β (E), IL-10 (F), and IFN-γ (G). Results are from 3 representative experiments. Bar graphs show mean ± SD.

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**Table 1**

Cumulative results of all mice inoculated with either CT26 and ID8-VEGF tumors.

| Treatment Groups | CT26 | ID8-VEGF |
|---|---|---|
| Control IgG | 0 | 0 |
| αPD-1 | 25 | 25 |
| αPD-L1 | 33 | 37.5 |
| αCTLA-4 | 50 | 25 |
| αPD-1+αCTLA-4 | 75* | 50 |
| αPD-L1+αCTLA-4 | 75* | 50 |
| GVAX | 0 | 0 |
| GVAX + αPD-1 | 42 | 37.5 |
| GVAX + αPD-L1 | 50 | 50 |
| GVAX + αCTLA-4 | 75* | 37.5 |
| GVAX + αPD-1+ αCTLA-4 | 100* | 75** |
| GVAX + αPD-L1+ αCTLA-4 | 100* | 75** |

*p* values among different treatments were calculated using the log-rank test

* $P \leq 0.05$;

** $P \leq 0.01$.

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RESEARCH ARTICLE

# Preclinical Development of Ipilimumab and Nivolumab Combination Immunotherapy: Mouse Tumor Models, In Vitro Functional Studies, and Cynomolgus Macaque Toxicology

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## Abstract

The monoclonal antibodies ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) have shown remarkable antitumor activity in an increasing number of cancers. When combined, ipilimumab and nivolumab have demonstrated superior activity in patients with metastatic melanoma (CHECKMATE-067). Here we describe the preclinical development strategy that predicted these clinical results. Synergistic antitumor activity in mouse MC38 and CT26 colorectal tumor models was observed with concurrent, but not sequential CTLA-4 and PD-1 blockade. Significant antitumor activity was maintained using a fixed dose of anti-CTLA-4 antibody with decreasing doses of anti-PD-1 antibody in the MC38 model. Immunohistochemical and flow cytometric analyses confirmed that $CD3^+$ T cells accumulated at the tumor margin and infiltrated the tumor mass in response to the combination therapy, resulting in favorable effector and regulatory T-cell ratios, increased pro-inflammatory cytokine secretion, and activation of tumor-specific T cells. Similarly, *in vitro* studies with combined ipilimumab and nivolumab showed enhanced cytokine secretion in superantigen stimulation of human peripheral blood lymphocytes and in mixed lymphocyte response assays. In a cynomolgus macaque toxicology study, dose-dependent immune-related gastrointestinal inflammation was observed with the combination therapy; this response had not been observed in previous single agent cynomolgus studies. Together, these *in vitro* assays and *in vivo* models comprise a preclinical strategy for the identification and development of highly effective antitumor combination immunotherapies.

support in the form of salaries to individuals listed in the Acknowledgements section (LW MS IC CB).

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## Introduction

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), among other inhibitory T-cell surface molecules, attenuate a variety of activated T-cell functions, including cellular proliferation, cytokine secretion, and cytolysis [1]. Importantly, in the context of oncologic diseases, it has been demonstrated that tumor cells, as well as tumor-infiltrating host cells, express ligands for these inhibitory receptors that permit evasion of immunosurveillance [2]. Antibody blockade of CTLA-4 and PD-1 has resulted in dramatic reductions in tumor burden in many human subjects [3–5].

CTLA-4 has been shown to inhibit T-cell responses by both intrinsic and extrinsic mechanisms [6–10]. With respect to the intrinsic mechanism, engagement of CTLA-4 on T cells by B7 ligands leads to their functional attenuation. There are multiple extrinsic mechanisms that include the ability of CTLA-4-expressing cells to effectively compete with CD28 for B7 ligands or trans-endocytic removal of costimulatory ligands from antigen-presenting cells (APC) [11]. CTLA-4 is critical for the function of regulatory T cells (Tregs), which are essential for suppressing autoimmunity and for maintaining self-tolerance. Blocking antibodies to CTLA-4 have induced antitumor activity in syngeneic mouse tumor models [12]. Treatment of tumor-bearing mice with anti-CTLA-4 antibodies capable of depletion have been shown to substantially reduce Tregs in tumors but not in the periphery, resulting in potentiated antitumor activity as compared to antibodies that lack effector function [13–15].

PD-1 is an additional key inhibitory receptor with immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) intracellular signaling motifs that strongly dampen T cell functions following engagement by its ligands PD-L1 and PD-L2 [16, 17]. PD-1 plays a central role in maintaining T cell tolerance [18]. Persistent high-level PD-1 expression on T cells is a part of a signature for non-responsive exhausted T cells associated with chronic lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV) [19] as well as with tumors [20]. Ligand-blocking anti-PD-1 and anti-PD-L1 antibodies have shown antitumor activity in different models, demonstrating the role this pathway plays in limiting host antitumor responses.

Here we present the rationale and course of preclinical development of anti-CTLA-4 and anti-PD-1 combination immunotherapy. We first assessed whether the combination of mouse surrogate antagonist antibodies to these receptors could promote greater activity in preclinical tumor models both responsive and refractory to the individual therapies. As expression of CTLA-4 and PD-1 appear individually and jointly on multiple T cell subsets with differing levels and kinetics of expression, how these molecules interact to mediate suppression is not completely understood. We investigated different dosing regimens as well as the tumor microenvironment for changes in immune cell subsets and cytokine production as potential indicators of enhanced antitumor response. These data prompted evaluation of human antibodies ipilimumab and nivolumab in *in vitro* assays with human blood cells. Specifically, superantigen staphylococcal enterotoxin B (SEB) and mixed lymphocyte response (MLR) assays were conducted to compare human T cell immune responses in combination therapy and monotherapy. Subsequently, we assessed concurrent dosing of ipilimumab and nivolumab in a non-human primate study for potential immune-related adverse events (AEs). The results and utility of these preclinical experiments were validated by the observed superior clinical efficacy of combined ipilimumab and nivolumab in a phase 3 trial in patients with metastatic melanoma [21].

## Materials and Methods

### Antibodies and Tumor Challenge Experiments

The chimeric anti-mouse PD-1 antibody (4H2) used in this study, engineered as a mouse IgG1 isotype monoclonal antibody (mAb) [22] was shown to bind to CHO transfectants expressing PD-1 and block binding of PD-L1 and PD-L2 to these cells (S1 Fig). The affinity of 4H2 for mouse PD-1, determined by surface plasmon resonance using PD-1-Fc, was $4.68\times10^{-9}$ M. The mIgG2b anti-mouse CTLA-4 mAb (9D9) used in this study was described previously [13]. All mAbs were formulated in sterile PBS and were low in endotoxin (<0.05 EU/mg).

MC38 tumor cells were cultured in DMEM and implanted subcutaneously into female C57/Bl6 from Charles River Laboratories (CRL) or B6.129S7-*Ifng*$^{tm1Ts}$/J C57BL/6 from Jackson Laboratory (JAX) mice. CT26 tumor cells were cultured in DMEM and implanted subcutaneously in female BALB/c mice from CRL or Harlan Laboratories (HAR). Tumor measurements were made 2–3 times weekly using an electronic caliper (reported as either $mm^3$ [L×W×H] or $mm^3$/2). Except where indicated, antibody doses were administered intraperitoneally (IP) in total volumes of 200 μL on days 7, 10, and 13. For T-cell depletion studies, 500 μg of depleting antibodies for CD4 (GK1.5) or CD8 (53.6.72; BioXCell, W. Lebanon, NH) were administered on day 7 following subcutaneous implantation of MC38 tumor cells ($2\times10^6$) in the hind flank. The efficiency of $CD4^+$ or $CD8^+$ T cell depletion (>90%) was confirmed by FACS analysis of blood samples collected four days after administration of the depleting antibodies. Mice were sacrificed with $CO_2$ at either study termination or any of the following clinical endpoints: tumor volume ≥2000 $mm^3$, tumor ulceration, body weight loss ≥20%, or moribund appearance.

### Immune Response Monitoring

Tumors were harvested and processed using GentleMacs cell disruptors (Miltenyi). Resulting cell suspensions were clarified through 70 μM filters, pelleted, resuspended in PBS or DMEM, and counted. Cells were incubated with anti-CD16/32 mAb 24G.2 (BioXCell or BD Biosciences) to reduce background FcγR binding and then stained with antibodies specific for CD8 (BioLegend 53–6.7), CD4 (BioLegend GK1.5), and CD45 (BioLegend 30-F11). Cells were also stained with the LiveDead Aqua fixable viability dye (ThermoFisher L34597). For intracellular staining (ICS), samples were fixed, permeabilized, and stained with antibodies specific for FoxP3 (eBioscience FJK-16s), Ki67 (eBioscience SolA15), CTLA-4 (BD Pharmingen 4F10), IFN-γ (eBioscience XMG1.2), and TNF-α (MP6-XT22). CT26 tumor antigen-specific $CD8^+$ T cells were identified using AH-1 MHC class I tetramers (MBL MuLV gp70 SPSYVYHQF). Tumor and splenic cell suspensions were incubated with tetramer in DMEM and 10% FCS for 30 minutes at 37°C, washed, and stained with surface and intracellular antibodies as above. *Ex vivo* AH-1 peptide stimulation was performed by culturing tumor or splenic cells with 2 μM AH-1 peptide (MBL) in the presence of brefeldin-A for 4 hours at 37°C. *Ex vivo* cytokine staining was performed by fixing and staining cells as described above, directly after tissue harvest. Samples were analyzed on FACS Canto and Fortessa flow cytometers (BD Biosciences).

### Cytokine Assays of Tumor Harvests

Tumors were harvested into 1 mL of complete T-cell media in 24-well microtiter plates and manually dissociated into single-cell suspensions. Tumor homogenates were clarified by centrifugation and resulting supernatants were collected and frozen for later batch sample processing. A volume of 25 μL of neat supernatant from each sample was analyzed in duplicate for concentrations of intratumoral IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-21, IL-22,

IL-27, IP-10, GM-CSF, TNF-α, and IFN-γ using a bead-based cytokine array according to the manufacturer's instructions (FlowCytomix, eBioscience).

## Immunohistochemistry

MC38 tumors were embedded in optimal cutting temperature (OCT) compound, snap-frozen on dry ice, and stored at -80°C until staining. Serial 5 μm tumor sections were placed onto glass slides, dried overnight at room temperature (RT), and then stained or stored at -80°C until use. For immunohistochemical staining, air-dried slides were fixed with acetone at RT, treated with peroxidase block (Abcam) to quench endogenous peroxidase, and then further blocked with a 10% goat serum and 5% BSA solution. Sections were stained with rabbit anti-mouse CD3 antibody (Abcam SP7) at a 1:400 dilution or rat anti-mouse PD-L1 (eBioscience MIH5) at a 1:100 dilution in blocking buffer. $CD3^+$ T cells were detected using the EXPOSE rabbit-specific HRP-DAB detection kit (Abcam ab80437). PD-$L1^+$ cells were detected using goat anti-rat-HRP (1:1000; Jackson ImmunoResearch 112-036-071) and detected with the HRP-DAB detection kit. Slides were co-stained with pre-made hematoxylin (Biocare CATHE-M) to counterstain cell nuclei. A similar protocol was applied for staining of PD-L1.

## *In Vitro* Assays Using Human Lymphocytes

Frozen peripheral blood mononuclear cells (PBMC) from normal healthy leukophoresis donors were seeded at $1\times10^5$ cells/well and stimulated with SEB serially diluted 30-fold from 2.5 μg/mL. Anti-CTLA-4 (ipilimumab), anti-PD-1 (nivolumab), or huIgG4 isotype control (Bristol-Myers Squibb 1D12-g4) was present at a spike concentration of 10 μg/mL. IL-2 secretion was measured by ELISA (BD Biosciences) on day 3.

Mixed lymphocyte response assays were performed by co-culturing $1\times10^5$ cells $CD4^+$ T cells with allogeneic monocyte-derived dendritic cells (DC) at a ratio of 10:1 (T:DC) in flat-bottom 96-well microtiter plates. $CD4^+$ T cells and DC were incubated for 6 days in the presence or absence of nivolumab titrated 1:10 from 50 mg/mL to 5 ng/mL along with ipilimumab at 0, 5, or 50 μg/mL. Culture supernatants were harvested on day 5 for ELISA analysis of IFN-γ secretion.

To assess the potential of nivolumab or ipilimumab, alone or in combination, to induce nonspecific T cell activation, mAbs were mixed with samples of heparinized fresh human whole blood to measure cytokine release. After a 4-hour incubation at 37°C, the cells were pelleted and the plasma fraction collected for measurement of IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 using a cytokine cytometric bead array assay (BD Biosciences). Comparison was made to responses generated by anti-CD3 or isotype control mAb treatment.

## Cynomolgus Monkey Toxicity Study

A 4-week study was conducted in cynomolgus monkeys to evaluate the toxicity of co-administered ipilimumab and nivolumab. The study was conducted at CRL, Sparks, NV in compliance with the Good Laboratory Practice Regulations for nonclinical Laboratory Studies of the US Food and Drug Administration (21 CFR Part 58), the USDA Animal Welfare Act (9 CFR, Parts 1, 2, 3), and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (ILAR publication 1996). The study-specific protocol was approved by the CRL Institutional Animal Care and Use Committee (IACUC). In addition to twice daily cage side observations by CRL staff, weekly physical examinations were performed by a CRL veterinarian.

Thirty purpose-bred cynomolgus monkeys (5/sex/group) were assigned to 3 groups by a stratified randomization scheme designed to achieve similar group mean body weights and the groups were randomly assigned to treatment. The groups were dosed intravenously (IV) with

1) saline control, 2) nivolumab 10 mg/kg plus ipilimumab 3 mg/kg, or 3) nivolumab 50 mg/kg plus ipilimumab 10 mg/kg, once weekly (days 1, 8, 15, and 22), for a total of 4 doses. The animals were housed individually, but were commingled daily to provide for environmental enrichment. Animals were fed Purina Certified Primate Diet No. 5048, and fruits, vegetables, and other treats were provided for environmental enrichment. The animals were evaluated for changes in clinical signs daily and body weights were recorded weekly. Cardiovascular assessments were performed prior to study and on days 1 and 22. Clinical pathology assessments were performed prior to study and on days 7, 28, and 58.

Seventeen monkeys (3/sex/group for Groups 1 and 2, and 2 males/3 females for Group 3) were euthanized 8 days after the last mAb dose, and 12 monkeys (2/sex/group) were euthanized on day 59. Animals were euthanized by exsanguination while under deep anesthesia induced with ketamine and Beuthanasia-D or equivalent. A full necropsy was conducted on all animals, with organs weighed and tissue collected, preserved, and processed for microscopic and histologic evaluations.

### Statistics

Statistical analyses of two groups were conducted using unpaired or paired two-tailed Student's t-tests. Analyses of more than two groups were conducted using Kruskal-Wallis ANOVA with Dunn's method of multiple comparisons against the control condition. Analyses of tumor growth measurements were performed by comparing tumor volumes measured on the last day on which all study animals were alive. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. For each experiment, the number of replicates performed and the number of animals per group are described in the corresponding figure legend(s).

## Results

### Activity of Anti-CTLA-4 and anti-PD-1 and Their Combination in Syngeneic Mouse Tumor Models

We began preclinical assessment of anti-CTLA-4 and anti-PD-1 combination therapy in mouse syngeneic tumor models. While anti-PD-1 functions solely as an antagonist, we and others have shown that anti-CTLA-4 efficacy in preclinical and clinical settings is at least partially dependent on reduction of intratumoral Tregs [13–15]. We treated mice with a non-depleting mIgG1 isotype anti-PD-1 antibody (4H2) and with mIgG2b isotype anti-CTLA-4 (9D9), which most closely resembles the hIgG1 isotype of ipilimumab in its capacity to elicit antibody-dependent cell-mediated cytotoxicity (ADCC) [23].

In a therapeutic treatment model of MC38 colon adenocarcinoma, monotherapy with either anti-CTLA-4 or anti-PD-1 was partially efficacious, with anti-PD-1 eliciting greater rates of tumor rejection (Fig 1A). Consistent with earlier reports [24, 25], combination treatment promoted even greater antitumor activity, with rapid tumor rejection and durable antitumor immunity observed in a majority of mice (Fig 1A).

In the CT26 colon carcinoma model, anti-PD-1 and anti-CTLA-4 combination therapy also elicited tumor rejections that were superior to the individual therapies (Fig 1C and 1D). Notably, antitumor efficacy varied by the source of the mice as tumor-bearing mice from HAR were more responsive to therapy than those from CRL, possibly as a result of intestinal microbiome differences [26, 27] (Fig 1C and 1D). Interrogation of other tumor models such as RENCA renal carcinoma and B16F10 melanoma demonstrated no single agent activity and little or no combinatorial activity.

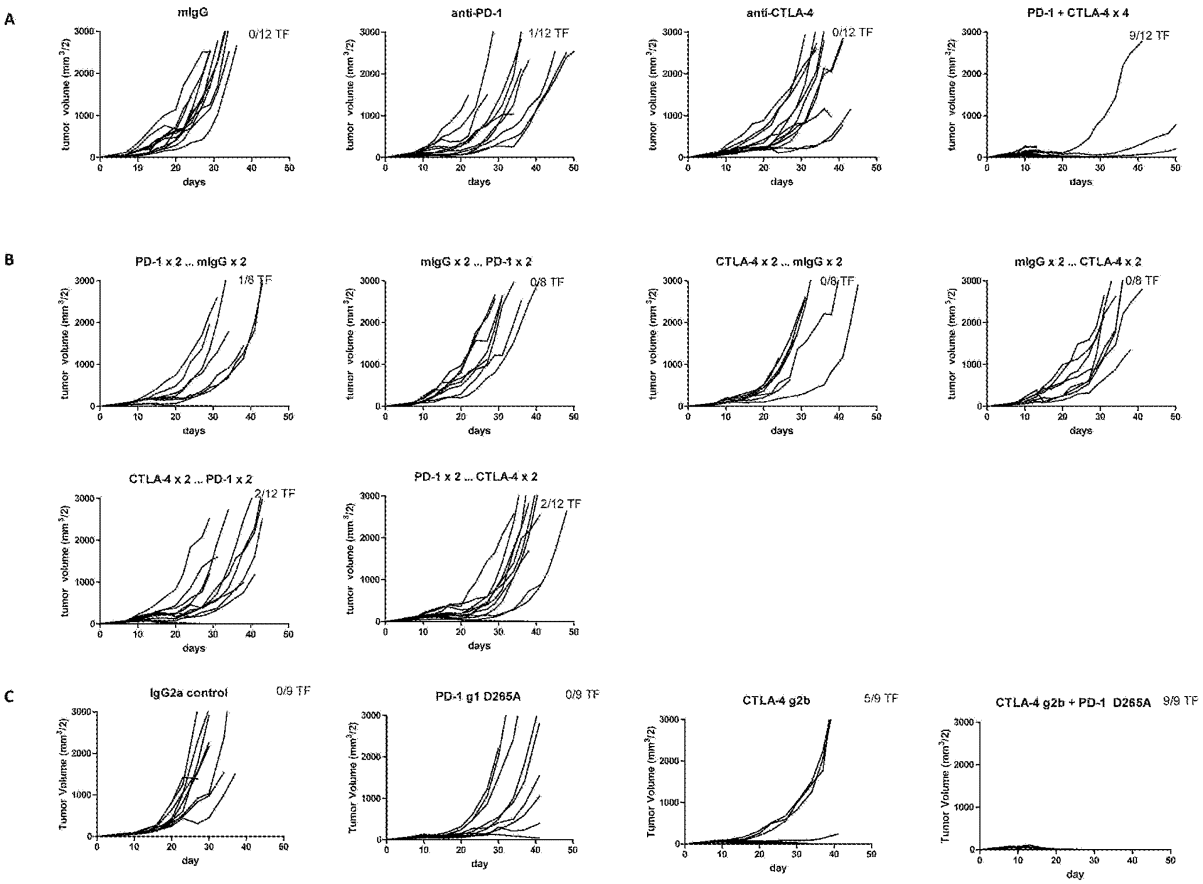



**Fig 1. Antitumor Responses of Anti-CTLA-4 and Anti-PD-1 Antibodies in Staged MC38 and CT26 Tumor Models.** A-B. Groups of 8–12 C57/BL6 mice were sourced from Taconic and subcutaneously injected with $2\times10^6$ MC38 cells. After tumors were measured on day 7, mice were randomized (58 $mm^3$ mean tumor volume per group) and then treated with the designated mAb (200 μg/dose IP) followed by additional doses on days 10, 14, and 17. A. Groups were treated with 4 doses of single or combined agents. Anti-PD-1 vs control p = 0.0176; anti-PD-1 and anti-CTLA-4 vs control p< 0.0001. B. Sequential dosing, where 4 doses were given as 2 doses of one mAb followed by 2 doses of the other mAb and the converse. Anti-CTLA-4 followed by anti-PD-1 vs control p = 0.0250; anti-PD-1 followed by anti-CTLA-4 vs control p = 0.0015. Tumor volumes were measured twice weekly. The number of tumor-free (TF) mice per group is indicated. C-D. Groups of 10 BALB/c mice sourced from CRL (C) or HAR (D) Laboratories were subcutaneously injected with $1\times10^6$ CT26 cells. After tumors were measured on day 7, mice were randomized (C: 56 $mm^3$ and D: 35 $mm^3$ mean tumor volume) and then treated with the designated mAb (200 μg/dose IP) followed by additional doses on days 10, 14 (HAR mice), or 10, 14, 17 (CRL mice). Anti-CTLA-4 vs control p = 0.0035; anti-CTLA-4 and anti-PD-1 vs control p<0.0001. Tumor volumes were measured twice weekly. The number of TF mice per group is indicated.

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In general, the response of different types of tumors to checkpoint blockade is more closely associated with inherent immunogenicity (mutational burden or dominant neoantigens) than with the tissue of tumor origin [28]. For example, CTLA-4 and PD-1 blockade are ineffective in the B16 mouse melanoma tumor model yet have demonstrated activity in human melanoma. Similarly, the antitumor activity observed with anti-PD-1 mAb treatment in mouse colon carcinoma models shown here is not observed in human colorectal cancer patients except for patients with microsatellite unstable tumors [29] or mismatch repair deficient tumors [30].

Alternative dosing schedules in the MC38 model were evaluated to determine their impact on the efficacy of combination immunotherapy. Interestingly, when mAb treatments were

staggered (two doses of anti-PD-1 followed sequentially by two doses of anti-CTLA-4 or *vice versa*), antitumor activity was largely muted (Fig 1B). Pharmacokinetics did not account for the loss of activity since both antibodies have similar half-lives of ~7 days [13]. A "reciprocal" dosing schedule was also evaluated in which the dose of one antibody was fixed while the other was titrated. When anti-PD-1 mAb was dosed at 10 mg/kg Q3D×3, addition of anti-CTLA-4 at the lower doses of 20 or 60 μg (1 or 3 mg/kg, respectively) resulted in reduced antitumor activity (Table 1). In contrast, when CTLA-4 mAb was dosed at 10 mg/kg Q3D×3, antitumor activity was sustained at lower doses (1 or 3 mg/kg) of anti-PD-1 mAb. A requirement for simultaneous co-blockade of both CTLA-4 and PD-1 in effector T cells (Teffs) may be one explanation for the superiority of concurrent over sequential treatment. Indeed, CTLA-4 and PD-1 co-expression was detected in approximately 72% of AH1 antigen-specific $CD8^+$ T cells in CT26 tumors (S2A Fig). In addition, approximately 24% of polyclonal tumor-infiltrating $CD8^+$ T cells in MC38 tumors were double positive (S2B Fig) supporting the notion that these two receptors could directly collaborate in the suppression of Teff responses.

Next we confirmed the role of $CD4^+$ and $CD8^+$ T cells in anti-PD-1 and anti-CTLA-4 treatment of MC38 tumor-bearing mice (S3 Fig). As expected, co-administration of CD8-depleting antibodies with the initial doses of anti-CTLA-4 and anti-PD-1 abrogated antitumor responses in all treatment groups. In contrast, depletion of $CD4^+$ T cells enhanced antitumor responses in tumor-bearing mice treated with control and therapeutic mAbs, likely due to the loss of intratumoral Tregs [31].

## Pharmacodynamic Effects of Combination Therapy on the Mouse Tumor Microenvironment and Infiltrating Lymphocytes

PD-L1 expression and $CD8^+$ T cell tumor infiltration are key markers of tumor sensitivity and responsiveness to immunotherapies [32–34]. Immunohistochemistry (IHC) was employed to examine MC38 tumors for PD-L1 expression and $CD3^+$ T-cell infiltration after treatment. In control mIgG1-treated mice, $CD3^+$ T cells were largely restricted to the periphery of the tumor at the invasive margin (Fig 2) and were coincident with PD-L1 expression. Treatment with

**Table 1. Reciprocal Dosing of Anti-PD-1 and Anti-CTLA-4 Antibodies in a Staged MC38 Tumor Model.**

| Group | Anti-PD-1 (μg) | Anti-CTLA-4 (μg) | % TF Mice | % TGI at Day 22 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 200 | 0 | 0 | 51 |
| 3 | 200 | 20 | 7 | 68 |
| 4 | 200 | 60 | 13 | 82 |
| 5 | 200 | 200 | 47 | 91 |
| 6 | 60 | 200 | 60 | 91 |
| 7 | 20 | 200 | 53 | 94 |
| 8 | 0 | 200 | 0 | 55 |

15 mice/group C57BL/6 mice were subcutaneously injected with $2\times10^6$ MC38 cells. On day 8 tumors were measured and mice randomized to treatment groups (average tumor volume was ~70 $mm^3$). On days 8, 11, and 15, mice in Groups 2–8 were treated with the designated mAbs and Group 1 was injected with 400 μg of isotype control (anti-DT mAb mIgG1; BMS); all groups had a total of 400 μg of total antibody injected, with the balance provided by the isotype control. Tumor volumes were measured twice weekly. The percentage of TF mice per group and the percentage tumor growth inhibition (TGI) are indicated. All combination groups (3–7) exhibited increased TGI relative to the control group (1, $p<0.01$), whereas both single agent groups (2 and 8) did not.

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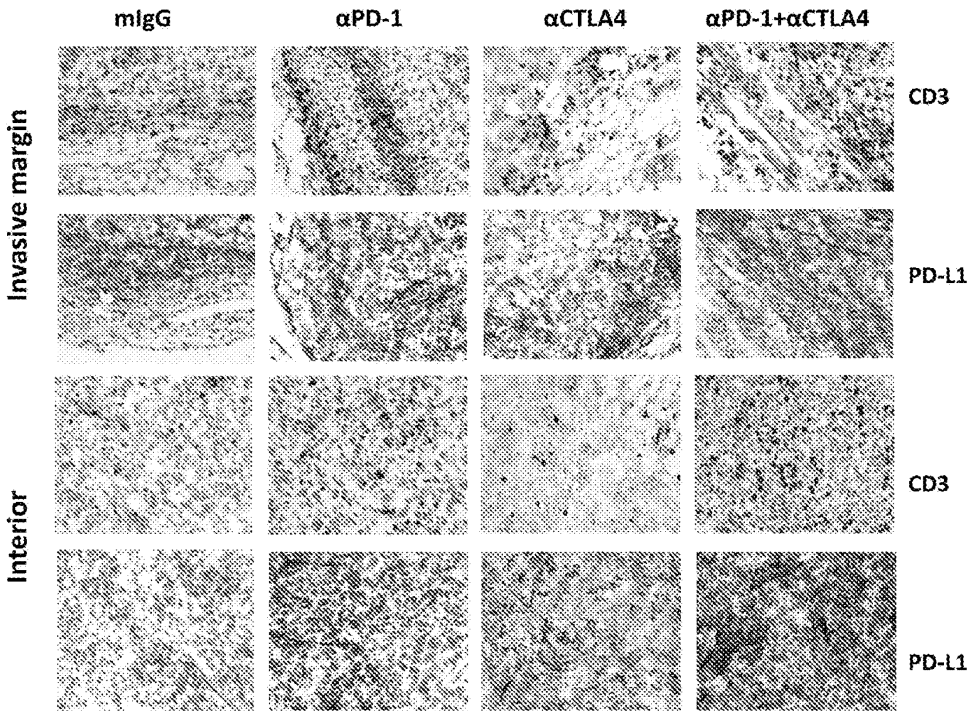



**Fig 2. Immunohistochemistry of MC38 Tumors: Detection of $CD3^+$ T Cells and PD-$L1^+$ Cells on the Tumor Perimeter and Interior.** C57BL/6 mice bearing MC38 tumors (n = 4–5 mice/group) were treated on days 7, 10, and 13 with the indicated antibodies. Tumors were harvested on day 14 and processed for IHC. Sections were stained with anti-CD3 (Abcam SP7 ab16669) or anti-PD-L1 mAbs (14–5982). IHC images of tissue sections derived from the periphery of the tumor (invasive margin) or from within the tumor (interior) are shown.

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anti-PD-1 and anti-CTLA-4 as single agents resulted in T-cell infiltration into tumors; when anti-PD-1 and anti-CTLA-4 were combined, even greater numbers of $CD3^+$ T cells and PD-$L1^+$ cells were found within the tumor mass (Fig 2). In tumors from mice treated with the combined agents, the ratio of intratumoral to peripheral tumor $CD3^+$ T cells increased 475% ($p = 0.03$ as compared to the isotype control) based on mean cell counts from three interior fields (range: 65–569 $CD3^+$ T cells in combined group, 18–99 $CD3^+$ T cells in control group) and three invasive margin fields (range: 26–208 $CD3^+$ T cells in combined group, 24–192 $CD3^+$ T cells in control group) per mouse (n = 5 mice/group). IFN-γ-deficient mice failed to respond similarly (S4 Fig), and we found elevated levels of IFN-γ, IL-10, and IL-13 (among 15 examined) present in tumor lysate supernatants from wild-type mice treated with both antibodies than with either antibody alone (S5 Fig). *In vitro*, IFN-γ stimulation induced upregulation on PD-L1 and MHC-I but not PD-L2 on MC38 tumor cells (S6 Fig). These findings support the notion that treatment with both anti-PD-1 and anti-CTLA-4 antibodies elicits more robust antitumor immune responses, largely driven by IFN-γ and T cells, than can be elicited by either antibody alone.

Flow cytometry was used to directly assess tumor-infiltrating leukocytes (TIL) in these models. In MC38 tumor-bearing mice, treatment with either anti-CTLA-4 or anti-PD-1 antibodies only modestly increased the frequency of effector $CD8^+$ and $CD4^+$ T cells amongst TIL, whereas combination treatment had a more significant effect (550% and 330% increases, respectively, relative to control treatment, $p<0.01$, Fig 3A). There was a concurrent decrease in



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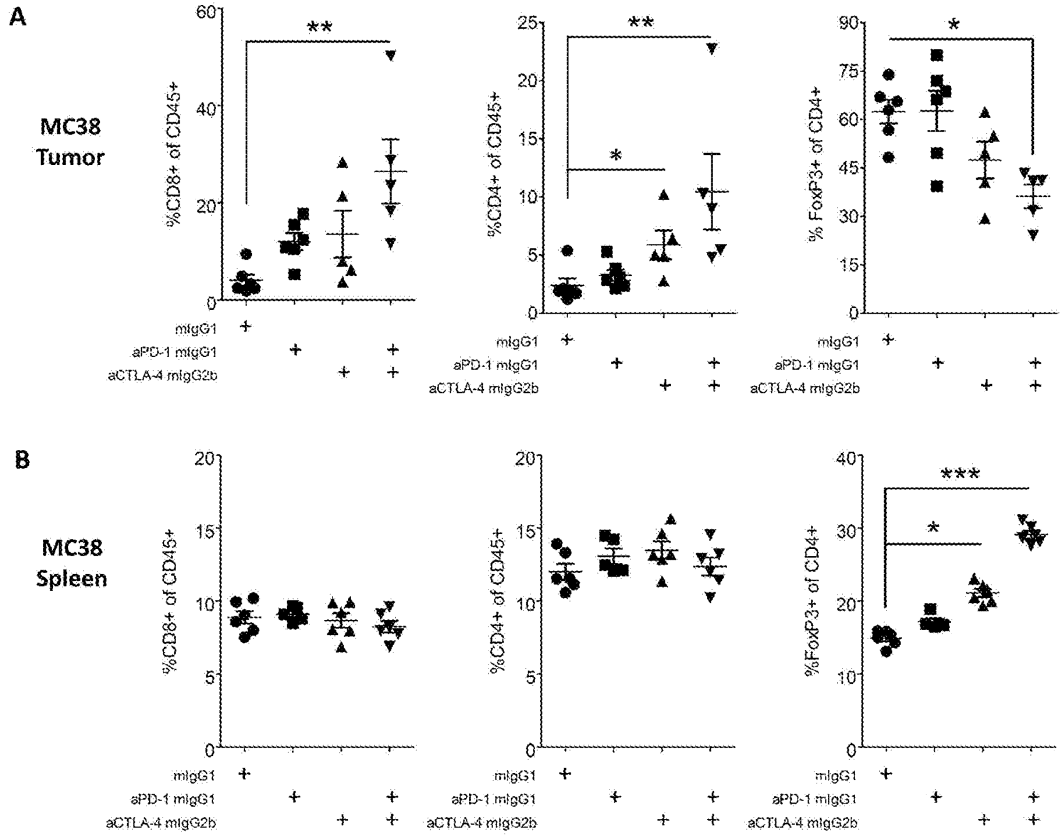



**Fig 3. FACS Analysis of Tumor and Spleen T Cell Populations from MC38 Tumor-Bearing Mice.** MC38 colon tumor cells ($2\times10^6$) were implanted subcutaneously into female C57BL/6 mice. At day 7 post implantation, tumor-bearing mice were randomized and dosed with 10 mg/kg of antibody IP Q3Dx3. On day 15 post implantation, tumors were harvested, dissociated into single-cell suspensions, and stained for flow cytometry. Data are representative of three MC38 independent experiments with ≥5 mice/group/experiment. A-B. Percentages of $CD45^+$ cells that were $CD8^+$ Teffs, $CD4^+$, FoxP3- Teffs, and $CD4^+$ $FoxP3^+$ Tregs found in tumor (A) and matched spleen (B).

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the frequency of Tregs amongst $CD4^+$ TIL in mice receiving combination treatment (45% decrease, $p<0.01$, Fig 3A). In contrast to tumors, analysis of splenic T cells showed no changes in $CD8^+$ or non-regulatory $CD4^+$ T cells, although treatment with either antibody alone or in combination led to an increased frequency of Tregs (Fig 3B), as has previously been reported following anti-CTLA-4 treatment [35].

In the CT26 model, tumor-bearing mice treated with anti-CTLA-4 in combination with anti-PD-1 exhibited an increase in the frequency of $CD8^+$ T cells, but not of $CD4^+$ T cells amongst TIL (72% increase in $CD8^+$ T cell frequency relative to control treatment, $p<0.01$, Fig 4A). Similarly, the frequency of tumor-infiltrating Tregs was decreased in mice receiving combination treatment (50% reduction, $p<0.01$, Fig 4B). Neither pharmacodynamic effect was seen in mice treated with anti-PD-1 alone, consistent with the lack of activity of anti-PD-1 monotherapy in CT26 (Fig 4C), or in mice treated with anti-CTLA-4 alone. In the spleens of CT26 tumor-bearing mice, treatment had no significant effect on total $CD8^+$ or $CD4^+$ T cell frequencies, although anti-CTLA-4 alone or in combination with anti-PD-1 did lead to increased frequencies of Tregs as a percentage of total $CD4^+$ T cells ($p<0.05$, Fig 4D). To track



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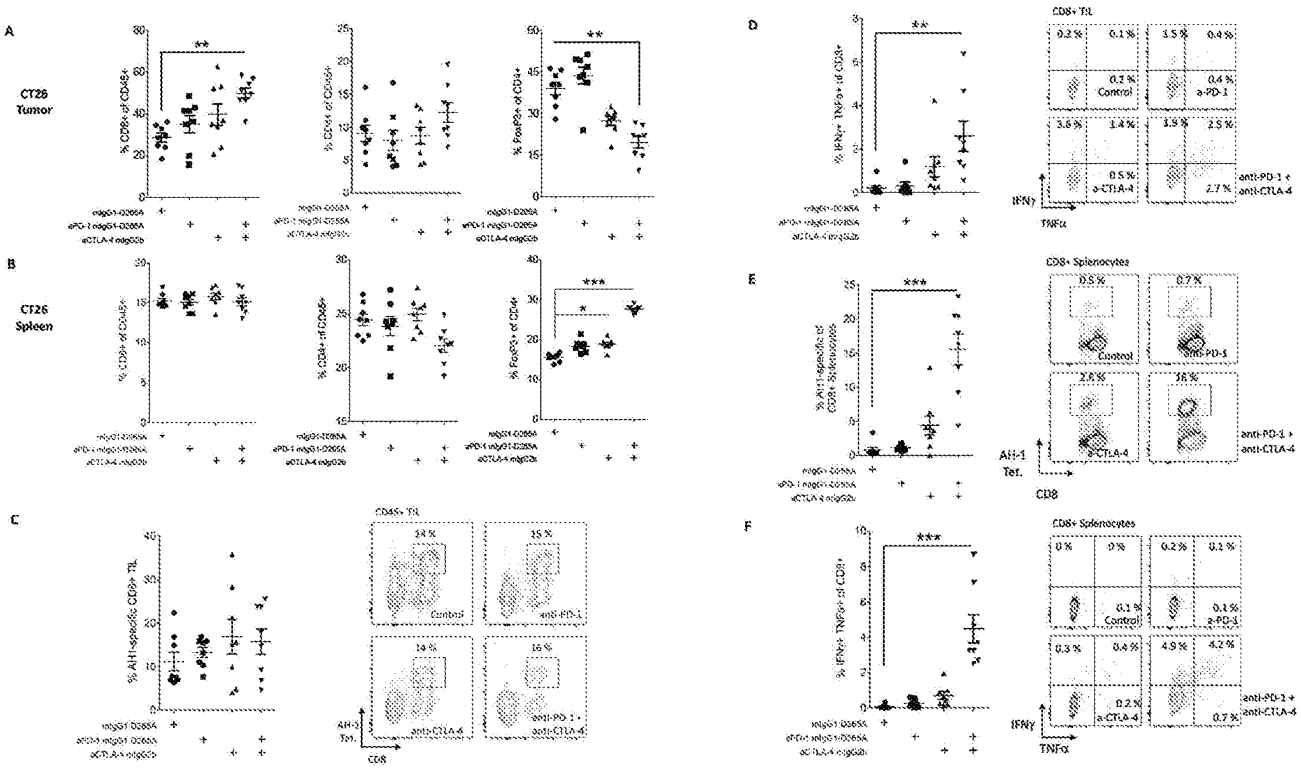


**Fig 4. FACS Analysis of Tumor and Spleen T Cell Populations from CT26 Tumor-Bearing Mice.** CT26 colon tumor cells ($1x10^6$) were implanted subcutaneously into female BALB/c mice. At day 7 post implantation, tumor-bearing mice were randomized and dosed with 10 mg/kg of antibody IP Q3Dx3. On day 15 post implantation, tumors were harvested, dissociated into single-cell suspensions, and stained for flow cytometry (n = 8/group); data are representative of two independent experiments. Cytokine production data are representative of one experiment utilizing *ex vivo* ICS and one experiment utilizing ICS following peptide re-stimulation. A-B. Percentages of polyclonal $CD8^+$ T cells of $CD45^+$ cells (left), $CD4^+$ T cells of $CD45^+$ cells, or $FoxP3^+$ cells of $CD4^+$ T cells (right) in tumors (A) and spleens (B) after the indicated treatments. C. Representative frequencies and plots and of AH1-specific ($tetramer^+$) $CD8^+$ T cells as a percentage of total TIL. D. Representative frequencies and plots of IFN-γ/TNF-$\alpha^+$ cells of total tumor-infiltrating $CD8^+$ T cells following *in vitro* AH1 peptide stimulation. E. Representative frequencies and plots of AH1-specific ($tetramer^+$) $CD8^+$ T cells as a percentage of total splenic $CD8^+$ T cells. F. Representative frequencies and plots of IFN-γ/TNF-$\alpha^+$ cells of total splenic $CD8^+$ T cells following *in vitro* AH1 peptide stimulation. Error bars depict the SEM.

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tumor antigen-specific T cell responses in this model, we utilized AH1 peptide MHC class I tetramers and peptide re-stimulation. In tumors, $tetramer^+$ (AH1-specific) $CD8^+$ T cells constituted approximately 15% of infiltrating leukocytes; this frequency was not consistently altered by treatment with either anti-PD-1 or anti-CTLA-4 (Fig 4E). However, *in vitro* peptide stimulation of TIL did reveal a potent synergistic enhancement of $CD8^+$ TIL effector function by anti-PD-1 and anti-CTLA-4 antibodies (>10-fold increase in the frequency of IFN-$\gamma^+$ TNF-$\alpha^+$ $CD8^+$ T cells relative to control, $p<0.001$, Fig 4F and S7 Fig), consistent with previous reports [25].

AH1-specific $CD8^+$ T cells were also expanded in the spleens of CT26 tumor-bearing mice treated with anti-PD-1 and anti-CTLA-4, increasing from approximately 1% to an average of 15.6% of splenic $CD8^+$ T cells ($p<0.001$, Fig 4E). An increase of similar magnitude was seen in the frequency of splenic $CD8^+$ T cells producing IFN-γ and TNF-α in response to AH1 peptide stimulation ($p<0.001$, Fig 4F and S7 Fig).

The ratio of tumor-infiltrating $CD8^+$ T cells to tumor-infiltrating Tregs was used to assess the state of antitumor T cell responses. In the MC38 model, anti-PD-1 and anti-CTLA-4



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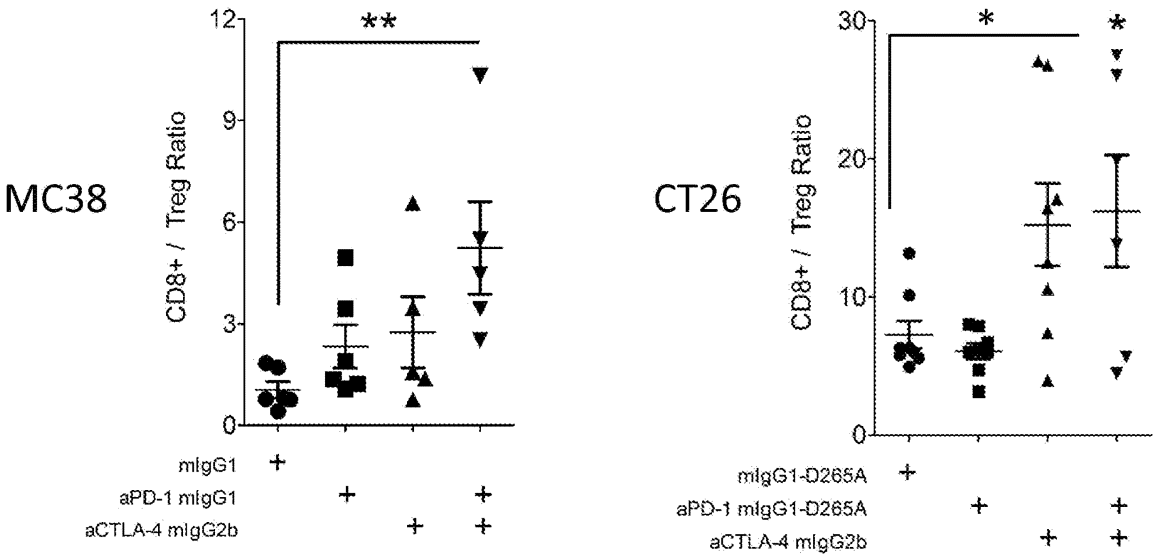



**Fig 5. CD8⁺:Treg TIL Ratios in Treated MC38 and CT26 Tumor-Bearing Mice.** Mice were implanted with MC38/CT26 tumor cells, treated, and analyzed as in Fig 3 and Fig 4. Ratios of $CD8^+$ Teff / $CD4^+$ Treg tumor-infiltrating T cells found in MC38 (left) and CT26 (right) tumors. Error bars depict the SEM.

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combination therapy, but neither monotherapy significantly augmented this ratio ($p<0.01$, Fig 5). In the CT26 model, which is partially responsive to anti-CTLA-4 monotherapy, the $CD8^+$/Treg TIL ratio was significantly increased in mice receiving the combination therapy and trended higher in mice receiving anti-CTLA-4 alone ($p<0.05$, Fig 5). Taken together, these data demonstrated clear enhancement of mouse antitumor T cell responses with anti-PD-1 and anti-CTLA-4 combination treatment.

## *In Vitro* Activity of Ipilimumab and Nivolumab in Human T Cells

The combination activity of the clinical anti-human CTLA-4 and PD-1 antibodies, ipilimumab and nivolumab [36, 37], were assessed in *in vitro* functional assays. In SEB-stimulated PBMC cultures, the addition of either nivolumab or ipilimumab alone enhanced IL-2 production over baseline levels, while the combination of both antibodies stimulated still higher IL-2 release (Fig 6). At a representative SEB concentration of 2.5 μg/mL, ipilimumab and nivolumab each promoted a mean 2-fold increase in IL-2 production, while their combination was 5-fold more potent than baseline (Fig 6B).

In an allogeneic T:DC MLR assay, ipilimumab treatment alone did not augment the T-cell response as measured by IFN-γ production or proliferation (Fig 5). Nivolumab was highly active in this assay, as previously described [37]. While T-cell activity was observed with nivolumab as a single agent, its combination with ipilimumab further enhanced IFN-γ production in two of three donors tested when the concentration of nivolumab was ≥0.5 μg/mL (Fig 7).

Lastly, we performed cytokine release assays to evaluate ipilimumab- and nivolumab-mediated cytokine secretion by naive human peripheral blood cells in the absence of T cell receptor (TCR) stimulation. While an anti-CD3 agonist antibody (UCHT-1) efficiently induced cytokine secretion by these cells, the addition of ipilimumab and/or nivolumab to healthy donor whole blood at concentrations of up to 100 μg/mL did not induce any meaningful cytokine secretion. These data suggested that ipilimumab and nivolumab treatments could



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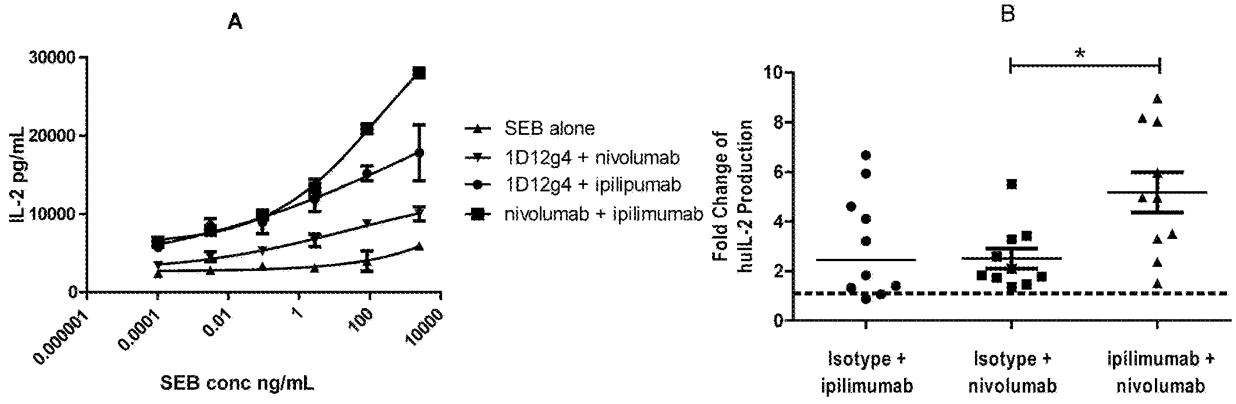



**Fig 6. Ipilimumab (Anti-CTLA-4) and Nivolumab (Anti-PD-1) Antibodies Potentiate IL-2 Secretion in SEB-Stimulated Human PBMC.** Frozen PBMC from 10 normal healthy donors were seeded at $1\times10^5$ cells/well and stimulated with SEB titrated 30-fold from 2.5 μg/mL. Ipilimumab, nivolumab, or isotype control antibody (1D12g4) were present at 10 μg/mL each. IL-2 secretion was measured by ELISA (in triplicate) on day 3. A. Dose titration data from one donor. B. Pooled data from 10 donors are plotted as fold-change over IL-2 values for SEB at 85 ng/mL. Bar = two-tailed T test p = 0.0088.

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collaboratively enhance effector and memory T cell responses without inappropriately activating naive T cells.

## Cynomolgus Macaque Toxicology

Ipilimumab and nivolumab therapy in humans can result in the induction of inflammatory AEs affecting multiple organ systems [21, 38, 39]. To assess the potential safety of combination therapy in the clinic, we conducted a cynomolgus macaque toxicology study. Three groups of animals (5 male and 5 female monkeys per group) consisting of a control group, a low-dose combination group (3 mg/kg ipilimumab and 10 mg/kg nivolumab), and a high-dose combination group (10 mg/kg ipilimumab and 50 mg/kg nivolumab) were treated once weekly for 4 weeks. Six animals per group were necropsied on day 30, while the remainder of the animals (recovery group) was analyzed at day 59.

CD4 and CD8 lymphocyte subsets, B lymphocytes, NK cells, and monocytes in cynomolgus PBMC were analyzed by flow cytometry on days 7, 28, and 58 and no significant alterations of lymphocyte subpopulations were observed. Highly variable increases in T helper lymphocytes

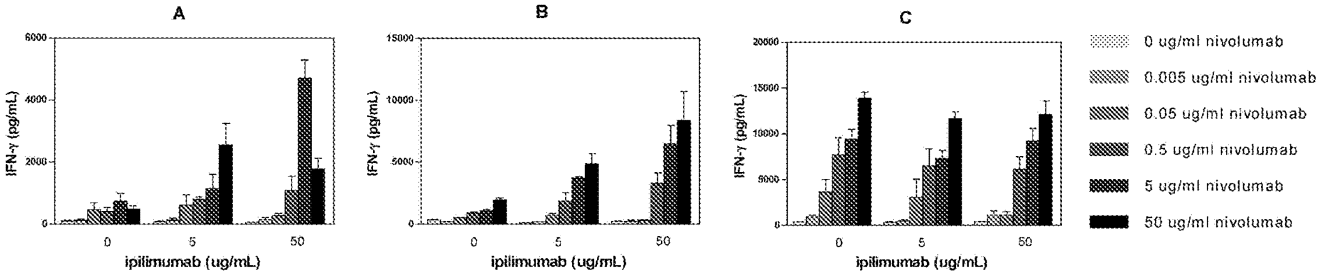



**Fig 7. Ipilimumab (Anti-CTLA-4) and Nivolumab (Anti-PD-1) Antibodies Potentiate IL-2 Release in an Allogeneic MLR Assay.** Allogeneic mixed lymphocyte response (MLR) assays were performed using monocyte-derived DC and $CD4^+$ T cells from 3 donors at a ratio of 1:10 (DC:T cells). $CD4^+$ T cells and DC were incubated for 6 days in the presence of titrated nivolumab with 0, 5, or 50 μg/mL ipilimumab. Culture supernatants were harvested on day 5 for enzyme-linked immunosorbent assay (ELISA) analysis of IFN-γ secretion.

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**Table 2. Cynomolgus Toxicology Signals with Ipilimumab and Nivolumab Combination.**

| Group | M/F | Treatment | Dose | Diarrhea[a] | Mean Spleen Weight[b] (g) | | Spleen Pathology[c] | Gastrointestinal Pathology[d] |
|---|---|---|---|---|---|---|---|---|
| | | | mg/kg | n/N | Day 30 M/F | Day 59 M/F | n/N | n/N |
| 1 | 5/5 | saline control | — | 0/10 | 3.9/2.8 | 3.5/3.7 | 0/6 | 0/6 |
| 2 | 5/5 | nivolumab + ipilimumab | 10 3 | 2/10 | 4.0/3.6 | 4.3/2.4 | 2/6 | 2/6 |
| 3 | 5/5 | nivolumab + ipilimumab | 50 10 | 4/10 | 6.1/4.47 | 7.5/3.2 | 4/5 | 3/5 |

[a] Incidence of repeated diarrhea (number of animals with finding/number of animal examined).

[b] Mean spleen weight on days 30 and 59; at day 30, 3 monkeys per sex per group with the exception of 2 males in Group 3; at day 59, 2 monkeys per sex per group.

[c] Incidence of lymphoid follicle hypertrophy or marginal zone expansion: number of animals with finding (n) / number of animals examined (N).

[d] Minimal, diffuse lymphoplasmacytic inflammation in the lamina propria with concurrent enlargement of the colonic or pelvic lymph nodes: number of animals with finding (n) / number of animal examined (N).

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were detected in the high-dose animals as compared to the untreated control group that reverted to baseline averages at day 58.

In the high-dose combination animals, 40% showed recurrent diarrhea as compared to 20% of animals in the low-dose group. Decreased food consumption was also observed in 20% of the high-dose group. A single male monkey in the high-dose group was found dead on day 23. The animal's early death was attributed to acute gastric dilatation (bloat) based on gross and microscopic findings by the CRL veterinary pathologist. The underlying cause of bloat could not be determined, and therefore a possible direct or indirect relationship to test articles could not be ruled out.

Combination treatment-related effects were observed in the spleen (increased weight and lymphoid follicle hypertrophy and/or marginal zone expansion), and lymph nodes (decreased germinal centers and/or hypocellularity), and were dose dependent. In most high-dose and some low-dose monkeys, gastrointestinal tract inflammation was detected in the lamina propria and was associated with decreased albumin, increased globulin, and/or increased neutrophil and eosinophil counts, and mononuclear inflammation of the large intestine. Acinar cell degranulation was seen in the pancreas of the high-dose group and was attributable to reduced food intake. Toxicology results are summarized in Table 2. Given that similar inflammatory events were not observed in cynomolgus monkeys treated with ipilimumab [36] or nivolumab [37] alone, these findings suggested that combination therapy may further exacerbate self-reactive immune responses in patients.

## Discussion

Checkpoint inhibitor antagonists have transformed the therapeutic landscape for many cancers, and a growing body of evidence indicates that combination therapies have the potential to confer even greater benefits [8]. The two most advanced tumor immunotherapy antagonists target CTLA-4 and PD-1, which promote negative regulation through distinct but complementary signaling pathways in Teffs [40–42]. Co-expression of CTLA-4 and PD-1 has been detected in HCV-specific T cells [43] as well as in melanoma TIL [44]. In the most comprehensive clinical trial to date, Larkin et al [21] explored ipilimumab and nivolumab and their combination in a double-blinded phase 3 study in previously untreated patients with advanced melanoma. The combination showed an objective response rate of 57.6% compared to 43.7% and 19% with nivolumab and ipilimumab monotherapies, respectively, along with a superior progression-free survival of 11.5 months for the combination. These findings in metastatic melanoma have led to the testing of the combination in multiple malignancies in a variety of dosing schedules [39, 45–48].

Here we present the preclinical rationale that led to the combination of ipilimumab and nivolumab immunotherapies. Antibodies to CTLA-4 and anti-PD-1 showed enhanced antitumor activities when used in combination in two mouse syngeneic tumor models. Immunohistochemistry, flow cytometry, and cytokine analysis were used to interrogate the changes in the tumor microenvironment in response to antibody therapy. In the IHC analysis of day 15 MC38 tumors from control IgG-treated mice, $CD3^+$ T cells largely localized around tumors. These IHC images were remarkably similar to those described for human tumors where T cells accumulated at the invasive margins [49–51]. PD-L1 expression was observed to be coincident with T cells surrounding the tumors and likely resulted from local IFN-γ expression after T cell recognition of tumor antigen [50]. Consistent with this suggestion, IHC analysis of MC38 tumors in IFN-γ-deficient mice showed little PD-L1 expression. More T cells were detected within the tumor mass after combination treatment, suggesting that either T cells acquired the ability to migrate into the tumor mass and/or expanded within the tumor.

The potency of combined anti-CTLA-4 and anti-PD-1 is likely due to the effect of the antibodies on different cell populations within the tumor. Treg depletion at the tumor site is a requisite part of the antitumor activity of anti-CTLA-4 whose potency varies by antibody Fc isotype [13]. CTLA-4 blockade in concert with Treg depletion enhances the function and number of $CD4^+$ and $CD8^+$ T cells. Anti-PD-1, which blocks PD-1 interactions with its ligands, functions in the absence of FcαR binding [52]. Anti-PD-1 monotherapy results in modest increases in $CD8^+$ T cells in responsive tumors (herein, [53]). Using anti-CTLA-4 as a mIgG2b isotype, a partial reduction of Tregs at the tumor site was observed in MC38 and CT26 tumors in mice treated with both anti-CTLA-4 and anti-PD-1, and was accompanied by the expansion of tumor-infiltrating $CD8^+$ T cells. An enhanced $CD8^+$ Teff to Treg ratio is considered a favorable correlate of antitumor activity [35] and the combination therapy in these preclinical studies resulted in a higher ratio than the single agents. Interestingly, combination therapy also resulted in a large expansion of cytokine-competent AH1-specific $CD8^+$ T cells in the spleens of CT26 tumor-bearing mice. This novel finding suggests that anti-CTLA-4 and anti-PD-1 act to enhance the priming of new antitumor responses and/or the persistence of existing responses.

Cytokine analysis at the MC38 tumor site revealed that IFN-γ was secreted at higher levels after CTLA-4 or PD-1 treatment compared to isotype control mAb, and IFN-γ secretion was further enhanced when the mAbs were combined. Similarly, in the CT26 tumors, the competency of AH1 tumor antigen-specific $CD8^+$ T cells to produce IFN-γ and TNF-α was increased with combination treatment relative to control or single agent-treated mice. The importance of IFN-γ in antitumor activity was also confirmed by absence of activity in IFN-γ-receptor knockout (KO) mice (herein, [54]). Levels of IL-13 were also increased with PD-1 and combination treatment. In support of a role for IL-13 in promoting antitumor activity, P815 cells expressing IL-13 grew slower than parental tumor cells [55]. IL-10, which is normally associated with reduced antitumor activity [56] was also increased. Of note, Mumm et al [57] reported a positive role for IL-10 in expanding tumor $CD8^+$ cells.

Ipilimumab and nivolumab therapy in humans is characterized by the induction of inflammatory AEs that affect multiple organ systems [21, 38, 39]. Although there are no new types of AEs detected in patients treated with the combination, there is an increased frequency of AEs leading to discontinuation of therapy. These can be controlled by steroids and other agents that do not impact antitumor activity. Notably, similar inflammatory events were not observed in cynomolgus monkeys treated with ipilimumab [36] or nivolumab [37] as monotherapies. However, the combined use of ipilimumab and nivolumab in a cynomolgus macaque toxicity study resulted in a dose-dependent increase in AEs characterized by diarrhea and weight loss. These symptoms were demonstrative of gastrointestinal inflammation, as subsequently

confirmed by pathological findings, and suggest that combination therapy may lead to enhanced inflammation or immune activation as a result of self-reactivity. In mice, we found that antitumor potency could be maintained in mice treated with fixed dose anti-CTLA-4 and titrated doses of anti-PD-1 or, to a lesser extent, with fixed-dose anti-PD-1 and titrated doses of anti-CTLA-4. These observations support further exploration of ipilimumab and nivolumab dosing regimens that will maintain efficacy while minimizing AEs and patient discontinuation in clinical trials.

It is noteworthy that a significant percentage of tumor tetramer$^+$ CD8$^+$ T cells were double positive, suggesting that the simultaneous blockade of the two receptors results in functionality that is superior to singular blockade. Combination of CTLA-4 and PD-1 blockade has only been found effective in a subset of syngeneic tumor models; non-responsive models may require additional therapies such as vaccines or radiation to elicit tumor-reactive T cells. Similarly, human cancers that are not responsive to anti-PD-1 and/or anti-CTLA-4 may require additional immune modulation if patients are to be converted to durable responders [5].

## Supporting Information

**S1 Fig. Binding and Blocking Characteristics of Anti-PD-1 Antibody.** A. Binding of anti-PD-1 mAb to murine PD-1 transfectants. Anti-PD-1 mAb was serially diluted and incubated with CHO-PD-1 cells followed by detection with a FITC-conjugated secondary to mouse IgG Fcγ. B. Inhibition of PD-L1-Fc binding to PD-1 CHO transfectants. CHO-PD-1 cells were pre-incubated with titrated anti-PD-1 mAb followed by addition of PD-L1-Fc at 2 μg/mL. Cell-bound PD-L1-Fc was detected with a FITC-conjugated secondary to human IgG Fcγ. C. Inhibition of PD-L2-Fc binding to PD-1 CHO transfectants CHO-PD-1 cells were preincubated with titrated anti-PD-1 antibody followed by addition of PD-L2-Fc at 15 μg/mL. Cell-bound PD-L-Fc was detected with a FITC-conjugated secondary to human IgG Fcγ.
(TIFF)

**S2 Fig. PD-1/CTLA-4 Co-Expression by Tumor-Infiltrating CD8$^+$ T Cells in the CT26 and MC38 Models.** A. TIL from CT26 tumor-bearing mice were harvested 15 days after tumor implantation. On left, frequencies of PD-1/CTLA-4 co-expression in each population are shown. *** $p<0.001$. On right, representative overlaid plot of PD-1/CTLA-4 (ICS) co-expression in AH-1 tetramer$^+$ CD8$^+$ TIL (red) and AH-1 tetramer- CD8$^+$ TIL (polyclonal, blue). B. PD-1 and CTLA-4 co-expression on polyclonal CD8+ T cells harvested from MC38 tumors 15 days after implantation.
(TIFF)

**S3 Fig. CD4 Depletion Increases and CD8 Depletion Decreases Antitumor Activity of Treatment Antibodies.** A. C57BL/6 mice were with injected $2\times10^6$ MC38 cells and treated on days 7, 10, and 13 with 400 μg of isotype control or single agent, or 200 μg of each agent for the combination therapy. Anti-PD-1 vs control $p = 0.0013$; anti-PD-1 and anti-CTLA-4 vs control $p<0.0001$. B. Treatment of mice as in A with the addition of 500 μg of depleting CD4 mAb (GK1.5 BioXCell) i.p. on day 7. Anti-PD-1 vs control $p = 0.0001$; anti-PD-1 and anti-CTLA-4 vs control $p<0.0001$. C. Treatment of mice as in A with the addition of 500 μg of depleting CD8 mAb (53.6.72 BioXCell) i.p. on day 7. Anti-PD-1 and anti-CTLA-4 vs control $p = 0.0149$. The number of tumor-free (TF) mice per group is shown. FACS analysis of group B and C blood samples confirmed >90% depletion of CD4$^+$ and CD8$^+$ T cells four days after administration of depleting antibodies.
(TIFF)



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**S4 Fig. Antitumor Activities of Therapeutic Antibodies Depend on Interferon-Gamma.** Wild-type (A) or B6.129S7-*Ifng*$^{tm1Ts}$/J (B) C57BL/6 mice were injected with 2×10$^6$ MC38 tumor cells and treated with 400 μg of isotype IgG1 or 200 μg of isotype with 200 μg of anti-PD-1 or anti-CTLA-4, or 200 μg each of anti-PD-1 and anti-CTLA-4 for the combination therapy on days 0, 4, and 7. The number of tumor-free (TF) mice per group is shown. A. anti-PD-1 vs control p = 0.0082; anti-CTLA-4 vs control p = 0.0464; anti-PD-1 and anti-CTLA-4 vs control p<0.0001. B. anti-PD-1 and anti-CTLA-4 vs control p = 0.0264.
(TIFF)

**S5 Fig. Enhancement of Intratumoral Cytokine Secretion by Therapeutic Antibodies.** C57BL/6 mice were injected with 2×10$^6$ MC38 cells and treated on days 7, 10, and 13 with 200 μg of isotype control or single agents, or 200 μg of each agent for the combination therapy. On day 15 post implantation, tumors were harvested, manually dissociated into single-cell suspensions, and levels of intratumoral cytokines were assessed via bead-based cytokine arrays (FlowCytomix, eBioscience). Results are shown for IFN-γ, IL-10, and IL-13.
(TIFF)

**S6 Fig. Interferon Gamma Upregulates PD-L1 and MHC I, But Not PD-L2 or MHC II on MC38 Tumor Cells.** MC38 cells were cultured in the presence of recombinant murine IFN-γ (R&D Systems) at 5 ng/mL for 24 hours and analyzed by FACS for expression of mouse PD-L1 (eBioscience, MIH5), mouse PD-L2 (eBioscience, 122), mouse MHC I (H-2D$^b$; eBioscience 28-14-8), or mouse MHC II (I-A/I-E; eBioscience M5/114.15.2).
(TIFF)

**S7 Fig. Cytokine Production by CT26 TIL and Splenic CD8$^+$ T Cells Following Peptide Stimulation *In Vitro*.** CT26 colon tumor cells (1x10$^6$) were implanted subcutaneously into BALB/c mice, which were then treated and analyzed as described in Fig 3. A. Frequencies of IFN-γ$^+$ TNF-α$^-$ and IFN-γ$^-$ TNF-α$^+$ cells of total tumor-infiltrating CD8$^+$ T cells following *in vitro* AH1 peptide stimulation. B. Frequencies of IFN-γ$^+$ TNF-α$^-$ and IFN-γ$^-$ TNF-α$^+$ cells of total splenic CD8$^+$ T cells following *in vitro* AH1 peptide stimulation. * p<0.05; ** p<0.01; *** p<0.001.
(TIFF)

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# Immunotherapy of Metastatic Colorectal Cancer: Prevailing Challenges and New Perspectives

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## Abstract

Patients with recurring or metastatic colorectal cancer (mCRC) have strikingly low long-term survival, while conventional treatments such as chemotherapeutic intervention and radiation therapy marginally improve longevity. Although, many factors involving immunosurveillance and immunosuppression were recently validated as important for patient prognosis and care, a multitude of experimental immunotherapies designed to combat unresectable mCRC have, in few cases, successfully mobilized antitumor immune cells against malignancies, nor conclusively or consistently granted protection, complete remission, and/or stable disease from immunotherapy – of which benefit less than 10% of those receiving therapy. After decades of progress, however, new insights into the mechanisms of immunosuppression, tolerance, and mutation profiling established novel therapies that circumvent these immunological barriers. This review underlines the most exciting methods to date that manipulate immune cells to curb mCRC, including adoptive cell therapy, dendritic cell vaccines, and checkpoint inhibitor antibodies – of which hint at effective and enduring protection against disease progression and undetected micrometastases.

### Keywords

Metastatic colorectal cancer; T-cell; Cytotoxic T-Lymphocyte; Helper T-Cell; Regulatory T-cell; Immunotherapy; Dendritic cell; Macrophage; Myeloid-derived suppressor cell; Vaccine; Antibody therapy; Adoptive T-cell therapy; Allogeneic stem-cell transplant; Ipilimumab; CTLA-4; PD-1/L1; Immunosuppression; Immunosurveillance; Biomarker; Antitumor immunity; Adoptive Immunotherapy

## INTRODUCTION

Colorectal cancer (CRC) represents 10% of malignancies worldwide, and remains the fourth leading cause of cancer-related mortalities [1]. Although surgical resection of localized tumors greatly improves patient survival past 5 and 10 years [2], liver metastases will develop in over half of all CRC patients further lowering survival [3–5]. A minority of Stage

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III CRC patients benefit from adjuvant chemotherapy (i.e., 5FU/oxaliplatin regimens), yet all endure toxicity [6]. Where chemotherapy and radiation fail, priming the immune system to inhibit metastatic spread may improve patient survival without severe toxicity.

Harnessing the immune system has successfully eradicated malignant cells in animal models, and even though human tumors are more complex, some Phase III trials have been successful [7]. Immunotherapy introduces modulatory agents, such as adjuvants, antibodies, and/or immune cells to initiate or amplify an existing antitumor response. Ipilimumab is an exciting example where immune cell activating antibodies prolong the lives of melanoma patients [8].

The topic of CRC immunotherapy was explored elegantly in previous reviews; this review, however, mainly discusses the inherent immunosuppressive barriers in the local tumor environment that obstruct current immunotherapeutic intervention. In particular, this review will 1) briefly overview the prognostic impact of common tumor-infiltrating immune cells in CRCs, 2) critique the most current and promising therapies that orient the immune system against CRCs, and 3) suggest which immunosuppressive barriers can be targeted to improve immunotherapy efficacy.

## ANTITUMOR IMMUNITY

Before the culmination of seminal studies performed by Freidman, Galon, and Pagès, who demonstrated that effector T-cells infiltrate CRCs and benefit patient survival, Jass and colleagues provided direction for these investigators by connecting the mechanism of protective immunity to infiltrating lymphocytes in advanced rectal cancer [9]. Many studies concur with the supposition that immunosurveillance [10, 11] inhibits CRC tumorigenesis and progression [9, 12–15]. Antitumor activity is highly coordinated, requires specifically primed lymphocytes to eradicate aberrant cells, and is evidenced by high densities of tumor-infiltrating lymphocytes (TILs) in early stage CRCs [16, 17].

### Cytotoxic Immune Response

Rudimentary anti-tumor T-cell responses are driven by anti-proliferative interferon-gamma (IFN-γ) [18]. This cytokine is secreted upon proteasomal degradation and subsequent presentation of antigens to T-cell receptors (TCRs) by major histocompatibility complex (MHC) class I [19]. Cytotoxic $CD8^+$ T-lymphocytes (CTLs) are a major source of IFN-γ [20, 21] as well as apoptosis inducing granzyme B [22] and require cooperative interactions from Type-1 polarized $CD4^+$ T ($T_H1$)-cells [23]. High densities of $CD8^+$ effector memory T-cells in CRCs associate with prolonged survival and lack of early signs of metastasis [13], suggesting $T_H1$ polarization and associated CTLs quell metastasis after curative surgery [15].

**CXCR3 Ligands—**A complex network of chemokines and cognate receptors dictate the type and density of infiltrating T-cells into tumors [24, 25]. The majority of CTLs and active $T_H1$ cells express CXCR3, the receptor for a trio of IFN-γ-induced chemokines, CXCL9, CXCL10, and CXCL11 [26–29]. These chemokines home CXCR3-expressing cells towards inflammation.

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**CCR5 Ligand**—CCR5 is co-expressed with CXCR3 on $T_H1$-cells and CTLs in CRC invasive margins [30], however, is absent from CRCs with lymphatic and liver metastasis and low densities of $CD8^+$ T-cells [31, 32]. Its ligand, CCL5 [33], is released from stored secretory compartments upon $CD8^+$ T-cell stimulation [34], functions to attract T-cells towards inflammation [35], and is strongly secreted from specific CRC subsets [36].

### Natural Killer Cells in CRC

Among the arsenal of cytotoxic lymphocytes, natural killer (NK)-cells and NK T-cells recognize and eliminate MHC negative tumor cells [37, 38]. However, their association with cancer progression is inconsistent across multiple cancer types. Recently, Sconocchia and colleagues demonstrate that certain cancer types harbor low densities of infiltrating NK-cells; breast cancers, melanomas, hepatocellular carcinomas, renal cell carcinomas, and CRCs all stain poorly for NK-cell markers [39, 40]. These cells are not found in CRC-associated liver metastases [41] and have no prognostic relevance for CRC patients [42–44]. These studies suggest that evasion from NK-cell detection and elimination is an obligate prerequisite for tumorigenesis [43]. The prognostic impact of TILs on CRC patient survival is summarized in Table 1.

## MULTIDIMENSIONAL IMMUNOSUPPRESSION

Tumor eradication and host tolerance are dictated by the immune system. The type, polarity, and density of infiltrating immune cells can either reject or accept aberrant cell growth. Each CRC contains a unique composition of immune cells, including those that quench the tumor fighting capacity of effector T-cells described above. Other infiltrating immune cells produce proliferative signals and accelerate metastatic disease.

### Immune-Regulating T-cells

In healthy tissues, regulatory $FoxP3^+CD4^+CD25^+$ T-cells [45, 46] ($T_{regs}$) suppress effector T-cells and protect against autoimmune disease through an array of immune quenching factors that are not limited to, TGF-β, IL-10, and cytotoxic T-lymphocyte antigen 4 (CTLA-4) [47–49]. These factors silence memory $CD8^+$ T-cell activity and IFN-γ production [50]. For example, $T_{regs}$ powerfully suppress T-cells isolated from CRC patients [51] and depletion of $T_{regs}$ via anti-CD25 antibody augments CRC rejection in mice [52].

Contradicting the studies mentioned above, $T_{regs}$ may benefit CRC patients. $FoxP3^+$ cell densities in CRCs positively correlate with patient survival [53–55] and absence of metastases [56]. These data question whether colon and rectum residing $FoxP3^+$ cells truly represent $T_{regs}$. FoxP3 is transiently expressed by intratumoral effector T-cells upon TCR stimulation [57], suggesting that FoxP3 is a poor biomarker for immunosuppressive T-cells. Alternatively, $T_{regs}$ may protect against tumor progression in barrier organs by suppressing tumor promoting inflammation initiated by foreign antigens [58]. Perhaps the true prognostic relevance of $T_{regs}$ lies with their abundance to cytotoxic $CD8^+$ T-cells; tumor progression may be slowed or halted when $CD8^+$ T-cells far outnumber $T_{regs}$.

Considering all the studies mentioned above, it is unlikely that only one factor supplied by $T_{regs}$ solely inhibits of immunosurveillance, nor is it likely that only one cell type promotes



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immunosuppressive environments. Therefore, effective immunotherapy requires blockage of other cell types and their soluble factors. Without clear understanding, we risk over-valuing unreliable immunosuppressive markers and fail personalizing therapy. A better approach is to consider the holistic immune contexture of the tumor environment.

The major hole in immunotherapy research is the lack of mouse models with multidimensional immunosuppression. Mice engineered with a specific immune modulating mechanism do not remotely represent the complexity of the human tumor environment.

## Tumor-promoting Myelogenous Cells

Macrophages residing in human tumors affect disease progression. These myelogenous cells polarize to either promote acute inflammation and adaptive T-cell immunity or immunosuppression and tumor escape [59]. Therefore, the prognostic relevance of tumor-associated macrophages (TAMs) in CRCs is debatable.

**Tumor-Associated Macrophages—**TAMs represent major components of the colorectal intratumoral immune milieu and are classified into heterogeneous subgroups by an array of secreted cytokines [60, 61]. Angiogenesis and metastasis promoting cytokines link these cells to CRC progression and poor prognosis [62]. Immature TAMs, termed myeloid-derived suppressor cells (MDSCs) facilitate immunosuppression, inflammation, and angiogenesis [63–66] by secreting VEGF, and T- and NK-cell inhibiting (and $T_{reg}$ expanding) nitric oxide, reactive oxygen species, arginase, IL-10, and IL-6 [67, 68]. In contrary, the presence of TAMs at CRC invasive margins associate with improved survival [69]. Therefore, fully ascertaining the link between TAMs/MDSCs and patient survival will identify their mitigating effects on therapy.

**Dendritic Cells—**In the periphery, protective immunity requires antigen presentation by dendritic cells (DCs) [70]. These cells are equipped with molecular receptors that take up microbial components and program lymphocytes with the resulting information. Programming requires concurrent ligation of CD28 or OX40 on T-cells to CD80/86 [71]. Like TAMs, DCs form heterogeneous populations that dictate immune responses either away or against tumor antigens [72]. DCs activated without maturation signals will promote tolerance and immunosuppression through the induction of $T_{regs}$ [73].

DC analysis in CRCs is limited to low density immunohistochemistry and tissue microarrays. These methods analyze immune cells within small tissue fragments [74]. Immunohistochemistry is very useful for studying spatial arrangements of immune cells in solid tumors, however only identifies cells by a limited number of markers. Tissue microarrays utilize more markers, but require fragmenting tissues, thereby destroying spatial arrangements of immune cells. Markers such as CD83 [75], S-100 [76], CD208 [61, 77] represent DCs in the stroma and invasive margins. However, no CRC study conclusively characterized the maturation status of DCs via cell surface markers or cytokine secretion using multidimensional approaches. As potential targets for immunotherapeutic intervention, much remains to be learned about the role of DCs in CRC progression. A summary of common immunosuppressive mechanisms is listed in Table 2.

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Novel methods that improve immune infiltrate profiling and prognostic accuracy are encouraging, however counting T-cells, macrophages, and DCs alone does not optimally predict which patients will respond to therapy because the phenotype and activity of infiltrates are equally critical. We are very far from identifying all the immunosuppressive mechanisms, yet our currently knowledge will soon extent and improve the quality of patient lives.

The diversity of CRC environments requires research to develop multiple methods that directly manipulate immune infiltrate phenotype and activity. However, these methods must overcome three major obstacles to eradicate tumors. 1) Interruption of the immunosuppressive environment will strengthen the impact of existing anti-tumor responses. This can be done using simple molecules that target specific immune modulating pathways. 2) Encouraging CTLs to migrate to the invasive margin will augment antitumor immunity. Tumor rejection may simply require overpower the immunosuppressive environment by introducing an overwhelming number of T-cells, cytokines, and cytotoxic mediators. And, 3) Repolarizing myelogneous cells to secrete antitumor cytokines will suppress $T_{regs}$ and encourage tumor antigen specific immunity. Tumor-fighting DCs may vaccinate patients to produce tumor fighting memory T-cells. Immunosuppressive barriers that may inhibit immunotherapy are depicted in Figure 1.

## DIRECTING THE IMMUNE SYSTEM AGAINST METASTATIC CRC

The reminder of this review discusses methods that treat advanced CRC patients through immune manipulation, and includes perspectives of how immunosuppression stymies these methods. In the context of cancer, immunotherapy broadly encompasses any immune manipulation that eliminates tumor cells. Immunotherapy promises to eradicate microscopic metastases more precisely than a surgeon's scalpel and provide lasting protective immunological memory. The current arsenal used to prime the immune system against occult metastases is limited; however major breakthroughs are propelling research to investigate novel avenues.

### Antibody therapy

The monoclonal antibody therapy anti-CTLA-4, or ipilimumab, is prominently used to improve metastatic melanoma patient survival [78]. As a checkpoint blocker, anti-CTLA-4 prevents CTLA-4 from outcompeting the T-cell co-stimulation receptor, CD28. Its effectiveness requires existing immunosuppressed intra-tumoral T-cells, making it the obvious choice for treating highly infiltrated CRCs. However, a pilot study reported no objective responses in 3 colon cancer patients [79], and a larger study using tremelimumab – another anti-CTLA-4 antibody – could not report any conclusive findings other than increased incidence of immune enterocolitis in 45 treatment-refractory CRC patients [80]. Other checkpoint proteins may be more appropriate for CRC therapy.

**Programmed death receptor-1/ligand-1 blockade—**Programmed death receptor-1/ligand-1 (PD-1/PD-1L) are members of the CD28 and B7 families and induce tolerance through regulation of T-cell. Activated $CD8^+$ T-cells express PD-1; however PD-L1/L2 expressing tumor cells, circulating MDSCs [64], and DCs can impart unresponsiveness and

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exhaustion of immune-directed T-cell activities [81]. An initial study reported only marginal responses to anti-PD-1 antibody where one CRC patient demonstrated complete response [82]. This study speculated that anti-PD-1 encourages lymphocyte migration into tumors and tissues [82]. Two larger studies included 19 patients with advanced CRC treated with anti-PD-1 [83] and 18 treated with anti-PD-L1 [84], but did not observe equitable clinical responses when compared to other cancers. Considering other tumor types, the first study witnessed objective responses in nine of the 25 patients with PD-L1-postive tumors and none with PD-L1 negative tumors.

A recent study demonstrated that defective mismatch repair CRCs strongly upregulate PD-1 and CTLA-4, as well as other immune checkpoints: PD-L1, lymphocyte activation gene-3 (LAG-3) and indoleamine 2,3-dioxygenase (IDO) [85••]. While these tumors rarely metastasize, these data provide evidence that multidimensional immunosuppression is important in more aggressive tumors. Two interesting notes: approximately 30% of intratumoral $CD4^+$ $FoxP3^-$ T-cells expressed LAG-3 [86•–88], and IDO expression in CRCs is associated with liver metastasis, tumor progression, and decreased overall survival [89, 90]. These data suggest that strong anti-tumor T-cell responses are concomitantly surmounted by multiple mechanisms of immunosuppression.

Questions still remain as to why patients with metastatic melanoma and other cancers benefit from checkpoint blockade while CRC patients do not. The simple answer is previous CRC studies did not reach statistical significance; fortunately larger clinical trials are underway. The complex answer is checkpoint blockers may not provide durable or prolonged protection. For example, ipilimumab allows $T_{reg}$ numbers to rebound after treatment and fails to increase CRC-specific T-cell abundance in peripheral blood [79], tremelimumab treatment fostered misdirected/nonspecific responses with mostly ineffective or undesirable consequences [80], anti-PD-1/PD-L1 antibodies may only be effective against PD-1/PD-L1 positive tumors [83], and finally, blockading only one axis of immunosuppression may doom almost every CRC clinical trial [83, 84].

## Adoptive T-cell Therapy

Adoptive cell transfer (ACT) introduces overwhelming numbers of tumor-targeting effector T-cells to circumvent the need to reverse tolerance to tumor antigens. Therapy requires the transfer of autologous T-cells – specific to tumor antigens or transfected to encode tumor directing T-cell receptors (TCRs) – directly to the patient. However, recruiting T-cells to tumor beds is challenging because T-cell infiltration is mitigated by immunosuppressive pressures.

Early attempts to elicit protective immunity against CRCs through adoptive transfer have been effective. Six out of 19 patients exhibited complete or partial response after their lymphocytes were isolated, propagated, sensitized to autologous tumor antigen, and then stimulated with a streptococcal immunopotentiator before transferred back [91]. Fourteen CRC patients received reinfusion of IL-2 stimulated TILs, however did not demonstrate meaningful clinical results other than preserved TCR ζ- and ε-chain expression [92]. Three out of 14 gastric and colon cancer patients achieved clinical response with IL-2 stimulated

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T-cells. These patients stably expressed TCR ζ-chain on peripheral blood T-cells, which is normally down-regulated with disease progression and immunosuppression [93].

These data suggest that effective adoptive transfer of IL-2 stimulated T-cells must avoid immunosuppressive preprogramming. To achieve this, sentinel lymph node-derived $CD4^+$ $T_H1$-cells were expanded instead of TILs. Intriguingly, 4 out of 9 stage IV CRC patients achieved complete remission [94]. The study was successful because lymphocytes were acquired from lymph nodes that first received drainage from tumors and therefore were segregated from the immunosuppressive environment [94]. T-cells residing in sentinel lymph nodes have greater proliferative potential upon stimulation [95] and should be the standard mode of therapy. To criticize, however, harvesting sentinel lymph nodes complicates surgery and may not yield enough T-cells.

**Chimeric antigen receptors—**The presumption that TILs are defective through immunosuppressive preprogramming calls for novel approaches that refocus adoptive T-cell therapy. Engineering T-cells to express chimeric antigen receptors (CARs) specific for carcinoembryonic antigen (CEA) – commonly expressed by colorectal tumors – benefits some advanced staged CRC patients. CARs are antigen-specific heavy and light chain antibodies genetically attached to cytoplasmic signaling molecules, such as 4-1BB, OX40, Luk, and TCR ζ-chain [96] and designed to relay activation signals to T-cells without peptide presentation. CAR therapy is advantageous when MHC class I is downregulated [97].

However, an unfortunate case study describes treatment failure for a patient with ERBB2 overexpressing colon cancer. T-cells recognized ERBB2 on normal lung cells and triggered high levels of tumor necrosis factor-alpha (TNF-α) and IFN-γ, pulmonary toxicity, and death [98]. Another study transferred CEA-specific T-cells retrovirally transduced to express murine TCR; although one patient demonstrated regression of lung and liver metastases, all patients demonstrated severe transient inflammatory colitis [99].

These studies highlight how ubiquitously expressed self-antigens in the lungs and throughout the gastrointestinal tract are risky targets for immunotherapy. Future efforts need to consider the perils of targeting self-antigens, or at least weigh the consequences of treatment against clinical benefit.

**Lymphodepletion—**Suppressor cells, as well as endogenous lymphocytes, effectively add another layer of immunosuppression by serving as sinks for T-cell promoting cytokines IL-7 and IL-15 [100]. Therefore, increased access to homeostatic cytokines may be critical for successful ACT. Lymphodepleting chemotherapy prior to immunotherapy reduces the number of circulating $T_{regs}$, promotes successful engraftment of adoptively transferred T-cells, and improves the overall survival of melanoma patients [101]. Cyclophosphamide decreased peripheral $FoxP3^+$ $T_{regs}$ in all six metastatic CRC patients and increased IFN-γ producing T-cells after 22 days [102]. Local ablation of immune cells using fludarabine, cyclophosphamide, and/or temozomide may dramatically increase the durability of ACT by reducing immunosuppressive pressures and widening a niche for tumor-specific T-cells.



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This treatment may best suit tumors devoid of beneficial $CD8^+$ T-cells, and not mismatch repair defective CRCs, which are highly infiltrated by $CD8^+$ T-cells.

### Allogeneic $CD34^+$ stem-cell transplantation

Allogeneic $CD34^+$ stem-cell transplantation (allo-SCT) is common for treating blood-related diseases; however early attempts with CRC have achieved mild success [103, 104]. A preparative regimen of lymphodepleting drugs is administered before matched donor hematopoietic stem-cells (HSCs) are transferred to cancer patients. The ensuing curative graft-versus-tumor (GvT) effect is driven by donor cytotoxic T-cells that proliferate *in vivo* and target malignancies [105]. Injecting 111-Indium-labeled lymphocytes via the hepatic portal artery confirmed that donor $CD3^+$, $CD19^+$, and $CD56^+$ lymphocytes home to liver metastases [106].

Successful allo-SCT requires local cytokine production. Neutralization of TNF-α and IL-1β in target epithelium inhibits acute GvT effect in mice [107], suggesting cytokine-mediated cytotoxicity obviates multiple layers of immunosuppression. One allo-SCT patient with CRC demonstrated increased tumoral expression of HLA-class I-associated β2-microglobulin molecule – an indicator of $CD8^+$ T-cell activity – but, not severe enough for any meaningful clinical benefit. Donor cytotoxic T-cells, however, inadvertently targeted the recipient's lymphoid system [108]. Three out of 15 metastatic colon cancer patients receiving allo-SCT experienced disease stabilization or partial remission. Responders harbored intra-tumoral CEA-specific $CD8^+$ T-cells [109]. Interestingly, patients receiving HSCs from unrelated donors engrafted $CD3^+$ cells faster than those receiving HLA-identical HSCs [110], suggesting non-perfect matching may induce more aggressive GvT effects.

This approach has many obstacles. First, complete T-cell engraftment lags behind myeloid and B-cells and can take over 60 days. Fortunately, patients who fail engraftment can receive infusion of $CD3^+$ T-cells [111]. Second, an overwhelming majority of patients who benefit from engraftment eventually succumb to disease, suggesting allo-SCT fades or becomes immunosuppressed by the tumor environment. Third, a study unrelated to gastrointestinal cancer observed striking up-regulation of IDO activity in colon tissues of patients receiving allo-SCT. This is expected because subsequent tryptophan depletion is a hallmark of intense gut inflammation [112]. Therefore, therapeutic inhibition of IDO activity during allo-SCT must be explored. And forth, the number of T-cells generated after transplant is limited; large and advanced tumors may require an absurd number of generated T-cells.

### Anti-tumor Vaccines

The ideal vaccine is easy to administer, offers prolonged protection, and induces relatively low toxicity; however no vaccine has induced reproducible clinically relevant regression of mCRC. Induction of memory T-cells activates the anti-tumor cascade and provides prolonged protection against existing micrometastases [113]. Cancer vaccines must effectively break immunological tolerance and induce or amplify antigen-directed T-cell assaults.



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Initial efforts to break tolerance to CRC antigens were directed against CEA. When delivered by DNA vaccine, or "naked" plasmid DNA, CEA is presented by MHC class I/CTL pathways [114]. A clinical trial did not detect relevant CEA-specific antibody responses in all 17 metastatic CRC patients, yet 4 patients demonstrated proliferation of peripheral lymphocytes [114]. This proliferation, however, was most likely triggered by CEA expressed by normal tissues. Overcoming tolerance to self-antigens is challenging, especially in profusely immunosuppressive environments, and requires adjuvants to intensify vaccination. Directing an immunological attack against self-antigens while ignoring healthy tissues is fraught with many unidentified obstacles.

**Pathogen Derived Adjuvants—**Inherent tolerance against self-antigens can be broken using bacterial or viral immunopotentiators. Diphtheria toxin (DT) conjugated to beta-human chorionic gonadotropin (β-hCG) peptide – often expressed by CRCs – induced humoral immune responses in 73% of IV CRC patients. Higher antibody responses to β-hCG associated with increased survival; however patients who mounted stronger antibody responses to DT antigen did not benefit from treatment [115]. Another study treated 161 patients with DT conjugated to gastrin-17 (G-17), a growth factor that contributes to gastrointestinal tumor growth. Three percent of patients achieved partial responses while 32% achieved stable disease, and those who generated antibodies to G-17 survive longer [116]. A promising study utilized adenovirus serotype-5 (Ad5) – known to trigger robust T-cell responses – to deliver CEA. This vaccine induced cell-mediated T-cell responses in 61% of advanced CRC patients; however the small study size could not conclude any survival advantage [117].

Alternatively, immune responses can be directed against tumor antigens by combining irradiated autologous tumor cells with pathogen derived adjuvants. Colon cancer patients survived longer when given autologous tumor cell-bacillus Calmette-Guérin (BCG) vaccines. [118]. A trial consisting of 254 colon cancer patients reported that these benefits are limited to stage II disease [119]. Two other studies used Newcastle disease virus (NDV) as an adjuvant because viral challenge induces production of interferons and RANTES (CCL5) and encourages chemotaxis of cytotoxic lymphocytes. Both studies boasted delayed disease reoccurrence and improved survival for colon cancer patients [120, 121]. No explanation was given to why rectal cancer patients did not benefit from either BCG or NDV. These studies strongly suggest that both the route of antigen presentation and the condition of immunostimulation dictate the efficacy of anti-cancer vaccines. These principles were applied to the next generation of vaccines.

**Cytokine Adjuvants—**Specific cytokines activate the adaptive immune response and establish T-cell memory. Among multiple signaling layers of antigen presentation and coreceptor ligation, cytokines mobilize immune cells. Granulocyte macrophage colony-stimulating factor (GM-CSF) – an immune stimulatory cytokine – enhances proliferation of T-cells and IFN-γ and IL-2 secretion in multiple myeloma patients immunized with autologous myeloma immunogen [122]. GM-CSF enhanced antibody response to CEA antigen and protected against metastatic CRC. All nine patients subcutaneously immunized and treated with GM-CSF demonstrated stronger dose-dependent IgG antibody responses,



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T-cell proliferation, and IFN-γ secretion when compared to those not given GM-CSF [123]. Another study inserted epithelial cellular adhesion molecule (Ep-CAM) – commonly expressed on CRC cells – into a replicative-deficient recombinant avipoxvirus to ensure the antigen is presented through MHC class I and CTL pathway. All 6 patients received GM-CSF, and five generated Ep-CAM-specific IFN-$\gamma^+$ T-cells and no IL-4 secreting T-cells [124].

**Dendritic Cell Vaccines—**The previous studies suggest that successful vaccination against tumor antigens may be dependent on GM-CSF-mobilized immune cells. Introduced earlier in this review, DCs reside in dermal tissues and lymph nodes, present antigens, induce protective T-cell responses, and reprogram immunosuppression in the tumor environment and elsewhere in the body. Early attempts to direct a memory response against antigen-presenting tumor cells using DC-based vaccines have been promising [113]. DCs require harvesting, propagation, and specific *ex vivo* manipulation to be coaxed into effective primers of Type-1 T-cells [125].

A series of clinical trials successfully activated CTL responses by vaccinating CRC patients with antigen loaded DCs. Patients with metastatic tumors receiving CEA-pulsed GM-CSF/IL-4-derived DCs (GM/IL4-DCs) demonstrated no signs of autoimmune disease or acute toxicities [126]. Other studies used alternative means to generate DCs with similar results: Flt3-ligand [127] and IL-13 [128]. Either way, the majority of recent DC vaccines are generated using GM-CSF, IL-4, and CEA peptide [129–132].

Theoretically, antigen loading via mRNA introduces a broader range of antigenic epitopes than peptide loading, thereby generating a more potent T-cell repertoire. In contrast however, mRNA transfected GM/IL4-DCs did not produce CEA-reactive T-cells in CRC patients [133]. Regardless of the loading method, GM/IL4-DCs with CEA class I HLA-A2-restricted peptide, CAP-1, or with mRNA encoding CEA, triggered similar antigen-specific T-cell activities *in vitro* [134].

The method of antigen loading may not be most important for effective DC vaccination. Inactivated DCs remain ineffective at promoting protective T-cells and can induce tolerance to target antigens [135]. Immunopotentiators optimize DC-based vaccines and enhance production of the type-1 T-cell mediator IL-12. These include toll-like receptor agonists poly I:C, LPS, Pam3Cys and R848 [136–138]. The final study to be discussed in this review designed a vaccine with hyperstimulatory potential by loading DCs with a poxvector containing genes for CEA and other costimulatory molecules, including CD80. Amazingly, this vaccine induced CEA-specific immune T-cells in 10 out of 12 patients [139]. A summary of all the clinical studies mentioned in this review is listed in Table 3.

Combined, these studies fortify the assumptions that 1) naturally processed peptides via class I MHC to CTLs are sufficient for T-cell priming, 2) DCs can be generated with effective stimulating capacity through multiple means, and 3) properly activated DCs induce protective immunity and avoid inducing tolerance to tumor antigens. DCs vaccines are efficient at generating tumor-specific CD8+ T-cells *in vivo*; however the question still

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remains to whether these are effective at countering the multiple layers of immunosuppression inherent in the tumor environment.

## CONCLUSION

The population of colorectal tumors is heterogeneous in regards to immune cell infiltrates. This explains why directly manipulated only one immune mechanism has had limited success. Most studies described in this review administered experimental treatments to groups of mCRCs only to witness diseases relapse. Confounding treatment, particular mutations within cancer genes distinctly construe different tumor subtypes with unique histology and gene expression. Antibody therapy can only unlock one layer of suppression at a time, CAR T-cell therapy targets only one antigen, and DC-based vaccines activate CTLs that eventually become muted once migrated to the tumor. This Sisyphean cycle of trail and failure mainly proves that tumor environments eventually return to an immunosuppressive state. Improved perception of the multiple immunosuppressive forces interwoven throughout each tumor environment, as seen with mismatch repair defective tumors [85••], will facilitate experimental immunotherapies that progress past the trial stage. Effective immunotherapy needs to counter all layers of immunosuppression, therefore patients may benefit from combinational therapy, barring severe toxicities (Figure 2).

Currently, chemotherapy may provide a better option for altering the tumor environment. Major findings are limited to mouse studies, however MDSCs are inhibited by 5-fluorouracil, which relinquishes its suppressive effects on T-cells [140]. In contrast, irinotecan – a topoisomerase inhibitor [141] used in a cocktail with 5-fluorouracil and leucovorin called FOLFIRI – reverses the inhibitory effects of 5-fluorouracil on MDSCs [142•]. Therefore, the specific effects of combinational chemotherapy on the tumor environment should be fully understood to provide immunotherapies the potency to abrogate all suppressive barriers. Effective immunotherapy must reprogram the tumor environment by either disrupting immune cells that induce tolerance or stromal and tumor cells that anchor immunosuppression.

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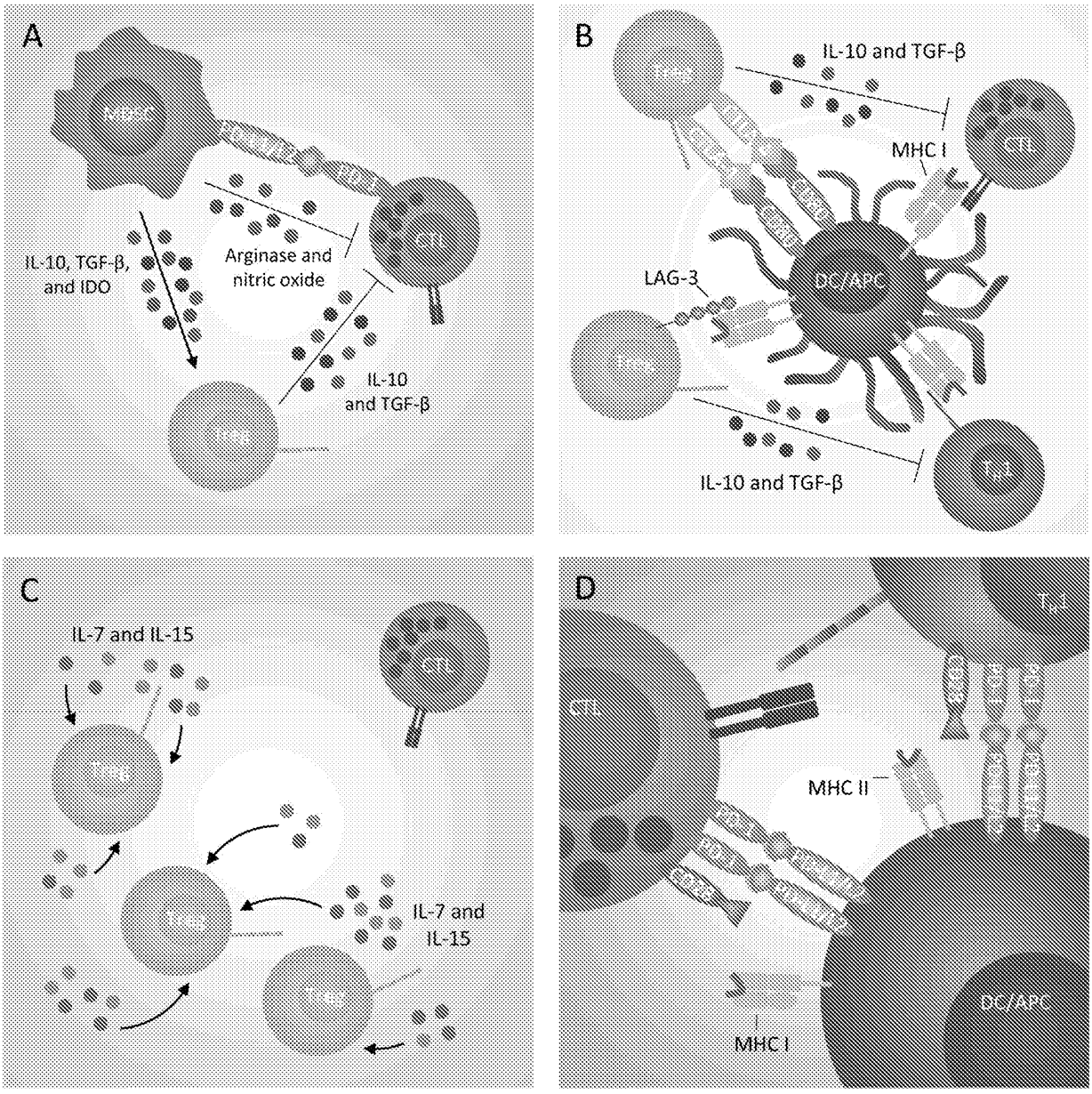



**Figure 1. Common immunosuppressive barriers in CRC that are potential targets for immunotherapy**

Myeloid-derived suppressor cells (MDSCs, brown) release $T_{reg}$ (orange) promoting factors, IL-10, TGF-β, and IDO (grey, purple, and blue), and T-cell suppressing arginase and nitric oxide (olive and brown), A. $T_{regs}$ compete with other T-cells for coreceptor ligation by expressing CTLA-4 and LAG-3. CTLA-4 binds to CD80/CD86 and LAG-3 binds to MHC class II, thereby blocking the transmission of stimulatory signals from DCs (purple) and other antigen-presenting cells (APCs) to effector T-cells, B. $T_{regs}$ and endogenous lymphocytes serve as sinks and limit the amount of homeostatic cytokines, IL-7 and IL-15

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(light blue and red), that are available for effector T-cells, C. Chronic T-cell stimulation through viral challenge and cancer antigens perpetuates PD-1 expression leading to effector T-cell exhaustion, D.

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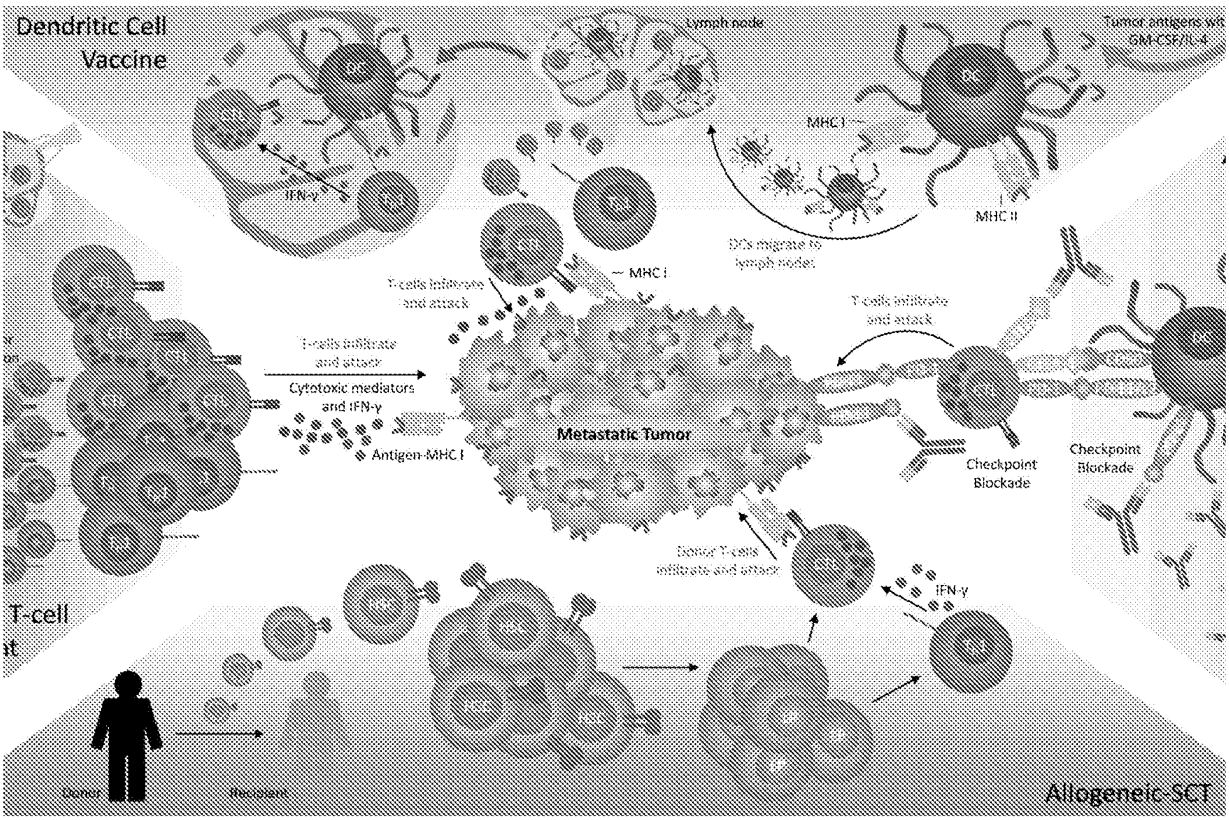



**Figure 2. Immunotherapies used to treat metastatic CRC**

Immunotherapies designed to direct cytotoxic T-cells to eradicate colorectal tumor cells. Antibody therapy against CRC has two popular targets. Anti-CTLA-4 (purple) liberates CD28 to ligate to CD80/CD86 for CTL (red) activation. Anti-PD-1 (purple) prevents tumor cells (gray) and DCs (violet) from inducing tolerance, unresponsiveness, and exhaustion of T-cell activities. Adoptive cell transfer requires autologous peripheral or sentinel lymph node T-cells ($T_H1$-cells, blue) to be isolated and propagated *in vitro* via IL-2 and antigen (red curved bar) stimulation, then delivered back into the patient. Allo-SCT requires donor hematopoietic stem-cells (HSCs, mustard) to be introduced into cancer patients. HSCs mature into lymphoid progenitors (PL, green) and then into functional T-cells (and other lymphocytes) to induce a GvT effect. This effect requires donor CTLs to proliferate *in vivo* and target malignancies. DC vaccines program the immune system to mount a durable memory response against tumor antigens. DCs are derived *ex vivo* from peripheral immune cells precursor, then propagated and challenged with tumor antigens. Mature and activated DCs are delivered back into the patient and migrate to the lymph nodes to prime and educate naive T-cells against tumor antigens. In the lymph node, primed $T_H1$ cells release IFN-γ (dark green) to activate $CD8^+$ T-cells. Once mature, CTLs migrate to tumor cells and release cytotoxic mediators (dark red) upon recognition of antigen-MHC complex (light green) on the tumor cell surface.



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**Table 1**

Ability of common lymphocyte markers to predict CRC patient survival

| Marker | Reference | Survival | Patient Benefit |
|---|---|---|---|
| CD3/Lymphocytes | [143, 144] | CS | Inconclusive |
| | [15, 145–149] | DFS | Positive |
| | [15, 143, 145, 147, 150, 151] | OS | Positive |
| CD8 | [12, 14, 17, 143, 144, 152] | CS | Positive |
| | [14, 17, 145, 153•–156] | DFS | Positive |
| | [14, 16, 143, 145, 153•, 155–160] | OS | Positive |
| FoxP3 | [54, 55, 143] | CS | Inconclusive |
| | [155] | DFS | Inconclusive |
| | [143, 155, 160–162] | OS | Inconclusive |
| CD56/CD57 | [44] | OS | Inconclusive |

CS, Cancer-specific survival; DFS, Disease-free survival; OS, Overall survival

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**Table 2**

Effect of common immune regulating mechanisms on T-cells

| Marker | Effect on effector T-cells |
|---|---|
| $T_{regs}$ | Down-regulate immune responses by secreting modulating cytokines and expressing modulating receptors [46]. |
| MDSCs | Promote angiogenesis and expansion of $T_{regs}$ and suppress effector T-cells [67, 68]. |
| Immature DCs | Promote immunosuppressive secretion from T-cells [73]. |
| PD-1 | Chronic viral challenge and cancer perpetuates PD-1 expression and exhausts T-cell [81]. |
| CTLA-4 | Prevents CD28 binding to co-receptors CD80 and CD86 during T-cell activation [49]. |
| LAG-3 | Expressed by $T_{regs}$ and completes with CD4 binding to MHC class II, thereby inhibiting $T_H1$-cells activation [86•]. |
| IDO | Inhibits T-cell proliferation [89]. |

CTLA-4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cells; IDO, indoleamine 2,3-dioxygenase; LAG-3, lymphocyte activation gene-3; MDSCs, myeloid-derived suppressor cells; PD-1, programmed death receptor-1; $T_{regs}$, regulatory T-cells



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**Table 3**

Summary of important studies that targeted immune cells for treating metastatic CRC

| Immunotherapy | Targeted Antigen | Adjuvants/immune modulator | Study population and size | Remarks | Reference |
|---|---|---|---|---|---|
| Ipilimumab | CTLA-4 | None | 3 colon cancer | $T_{regs}$ were depressed early in treatment, but no increase in $CD8^+$ T-cell responses. | [79] |
| Tremelimumab | CTLA-4 | None | 45 treatment-refractory CRC | One patient demonstrate partial response, while others experienced enterocolitis. | [80] |
| MDX-1106 | PD-1 | None | 14 advanced CRC and 25 other cancers | One patient demonstrated durable complete response. Inflammatory colitis or adverse events occurred in 3 patients. | [82] |
| BMS-936558 | PD-1 | None | 19 advanced CRC | No objective responses in CRC patients. One CRC patient died from treatment. | [83] |
| BMS-936559 | PD-L1 | None | 18 advanced CRC | No objective responses in CRC patients. | [84] |
| AIT (Lymph node and peripheral lymphocytes) | Sonicated tumor extract | T-cell growth factors and OK-432 (Streptococcal preparation) | 19 gastric or colorectal cancers with metastasis to the liver | 2 patients demonstrated complete response, 4 with partial. | [91] |
| AIT (Tumor-associated lymphocytes) | Autologous tumor | IL-2 | 14 stage IV gastric and colorectal cancer | 3 patients were protected from TCR-ζ down-regulation and experienced clinical response. | [93] |
| AIT (TILs) | Untargeted | IL-2 | 14 metastatic CRC | No difference was observed from control group. | [92] |
| AIT ($CD4^+$ T-cells) | Autologous tumor | IL-2 | 16 CRC | 4 of 9 stage IV patients experienced complete remission. | [94] |
| CAR (PBLs) | ERBB2 | Anti-CD3 and IL-2 | 1 colon cancer | Patient died from respiratory distress. | [98] |
| CAR (PBLs) | CEA | Anti-CD3 and IL-2 | 3 metastatic CRC | 1 patient demonstrated regression of lung and liver metastasis. All patients experienced severe inflammatory colitis. | [99] |
| Lymphodepletion | Untargeted | Cyclophosphamide | 27 CRC | Increased IFN-γ-producing T-cells. | [102] |
| Allo-SCT | Untargeted | Busulfan and fludarabine | 4 metastatic CRC | No significant toxicities were detected. | [103] |
| Allo-SCT | Untargeted | Busulfan and fludarabine | 1 chemoradiotherapy resistant CRC | Severe inflammatory cell infiltration into mucosa. Patient did not achieve remission. | [104] |
| Allo-SCT | Untargeted | Fludarabine and total body irradiation | 3 CRC and 4 other cancers | 3 patients demonstrated stable disease for at least 18 months. | [106] |
| Allo-SCT | Untargeted | Fludarabine and total body irradiation | 1 CRC | Patient died of pneumonia 4 months after transplant. Most metastases were necrotic with few remaining tumor cells. | [108] |
| Allo-SCT | Untargeted | Fludarabine and total body | 15 metastatic CRC | 3 patients demonstrated stable disease and 1 | [109] |

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| Immunotherapy | Targeted Antigen | Adjuvants/immune modulator | Study population and size | Remarks | Reference |
|---|---|---|---|---|---|
| | | irradiation | | with partial response. | |
| Allo-SCT | Untargeted | Fludarabine and total body irradiation | 6 colon cancer and 12 other cancers | 2 colon cancer patients demonstrated repression. 4 patients died from transplant. | [110] |
| Allo-SCT | Untargeted | Combinations of cyclophosphamide, busulfan, fludarabine, and total body irradiation | 39 refractory metastatic CRC | 6 stable disease and 2 partial response. | [111] |
| DNA vaccine | CEA | None | 17 metastatic CRC | No clinical response. 4 patients demonstrated increased numbers of circulating CEA-specific lymphocytes. | [114] |
| Peptide vaccine | β-hCG | Diphtheria toxoid | 77 metastatic CRC | The 56 patients who generated antibodies generally lived longer. | [115] |
| Peptide vaccine | G-17 | Diphtheria toxoid | 161 CRC | 3 partial responses and 32 stable disease. Patients who generated antibodies generally lived longer. | [116] |
| Peptide vaccine | CEA | Ad5 | 25 advanced CRC | 12 patients lived past 12 months. Did not report impact on survival. | [117] |
| Autologous tumor cell vaccine | Autologous tumor cells | BCG | 80 CRC | Colon cancer patients demonstrated improved survival. Rectal cancer patients did not improve. | [118] |
| Autologous tumor cell vaccine | Autologous tumor cells | BCG | 128 colon cancer | Vaccinated patients demonstrated fewer reoccurrences. | [119] |
| Autologous tumor cell vaccine | Autologous tumor cells | NDV | 23 metastatic CRC | Vaccinated patients demonstrated fewer reoccurrences. | [120] |
| Autologous tumor cell vaccine | Autologous tumor cells | NDV | 25 metastatic CRC | Vaccinated patients demonstrated improved metastasis-free and overall survival. | [121] |
| Peptide vaccine | CEA | GM-CSF | 9 CRC | All patients receiving GM-CSF generated IFN-γ secreting T-cells. No autoimmunity was reported. | [123] |
| Peptide vaccine | Ep-CAM | GM-CSF and ALVAC | 12 CRC | The GM-CSF patients strongly generated anti-Ep-CAM-specific Type-1 responses. | [124] |
| DC vaccine | CAP-1 | GM-CSF and IL-4 | 21 CRC | 1 patient demonstrated minor response and 1 demonstrated stable disease. No observable autoimmunity. | [126] |
| DC vaccine | CAP-1 | GM-CSF and IL-4 | 17 metastatic CEA-expressing cancer | No difference between peptide and mRNA loading. | [134] |
| DC vaccine | CAP-1 | GM-CSF, IL-4,$PGE_2$, TNF-α, IL-1β, and IL-6 | 16 metastatic CEA-expressing cancer | 8 of 11 receiving peptide demonstrated T-cell responses. None of the mRNA group responded. | [133] |
| DC vaccine | CEA | GM-CSF, IL-4,$PGE_2$, TNF- | 7 CRC and 2 lung | CEA-reactive $CD8^+$ T-cells were expanded. No | [129] |

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| Immunotherapy | Targeted Antigen | Adjuvants/immune modulator | Study population and size | Remarks | Reference |
|---|---|---|---|---|---|
| | | α, IL-1β, and IL-6 | cancer | tumor repression was observed. | |
| DC vaccine | CEA | GM-CSF, IL-4,$PGE_2$, and TNF-α | 10 metastatic CRC | CEA-reactive $CD8^+$ T-cells were expanded in 7 patients. 2 patients demonstrated stable disease for at least 12 months. | [130] |
| DC vaccine | CEA | GM-CSF, IL-4, TNF-α, and IFN-α | 10 metastatic CEA-expressing cancer | 2 patients responded with positive delayed-type hypersensitivity test. | [131] |
| DC vaccine | CAP-1 | GM-CSF, IL-4, TNF-α, and IFN-α | 7 stage III CRC | 4 of 7 patients generated CEA-specific T-cell responses. | [132] |
| DC vaccine | CEA | Flt3L | 9 colon, 1 rectum, and 2 lung | 2 patients demonstrated complete response, and 2 with stable disease. | [127] |
| DC vaccine | CEA and other peptides | GM-CSF, IL-13, Klebsiella-derived cell wall fraction, and IFN-γ | 11 advanced CRC | 3 patients generated CEA-specific T-cells. | [128] |
| DC vaccine | CAP-1 | GM-CSF, IL-4, and poxvector containing B7.1 (CD80), ICAM-1 (CD54), and LFA-3 (CD58) | 11 CRC and 3 non-small lung cancer | 10 patients generated CEA-specific T-cells. No toxicities were reported. | [139] |

AIT, Adoptive Immunotherapy; Allo-SCT, allogeneic stem-cell transplantation; ALVAC, replicative-deficient recombinant avipoxvirus; BCG, bacillus Calmette-Guérin; β-hCG, beta-human chorionic gonadotropin CAP-1, carcinoembryonic antigen peptide-1; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CRC, colorectal cancer; CTLA-4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cells; ERBB2; Ep-CAM, epithelial cellular adhesion molecule; Flt3L, Flt3-ligand G-17, gastrin-17; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; LFA-3, leukocyte function-associated antigen-3; NDV, Newcastle disease virus; PBLs, peripheral blood lymphocytes; PD-1/PD-1L, programmed death receptor-1/ligand-1; $PGE_2$, prostaglandin E2; TCR, T-cell receptor; TILs, Tumor-infiltrating lymphocytes; TNF, tumor necrosis factor; $T_{regs}$, regulatory T-cells

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# Electronic Acknowledgement Receipt

| | |
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| **Confirmation Number:** | 7097 |
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| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Customer Number:** | 115018 |
| **Filer:** | Brian Mason Dudley/Kisha Murray |
| **Filer Authorized By:** | Brian Mason Dudley |
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| 39 | Non Patent Literature | NPL26_POSTOW.pdf | 166678<br>c4f8f8c7782857e790fa07e0dbf63df979e78a86 | no | 8 |
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| **Information:** | | | | | |
| 40 | Non Patent Literature | NPL27_Rosenbaum.pdf | 498132<br>10cd0db9dc267ce983bad6a34391509468 2d156c | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 41 | Non Patent Literature | NPL29_SJOBLOM_SCIENCE_2006_314_268_EFS.PDF | 6558222<br>ce5bedad0b92a4cd91b9426cf1af45e6381cbebb | no | 8 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 42 | Non Patent Literature | NPL30_Overman_2016.pdf | 90716<br>c05a7f4fe65e455981abef4056fd65f1fa1fb401 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 43 | Non Patent Literature | NPL31_TOPALIAN_NENGJMED_2012_366_2443_EFS.PDF | 7439466<br>e1847421ada587475954eaacc3d909abca3fa461 | no | 19 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| 44 | Non Patent Literature | NPL32_TOPALIAN_CURROPINIMMU_2012_24_207_EFS.PDF | 4482081<br>11d6c65585b1949ec69759405c570e67fc5c86b8 | no | 11 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 45 | Non Patent Literature | NPL33_TOPALIAN_JCLIONCOLOGY_2014_32_1020_EFS.PDF | 6275020<br>bc566fb0c8110c439f1e13ccd5396b494d63a303 | no | 12 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 46 | Non Patent Literature | NPL34_WANG_CANCERIMMUNOLOGYRESEARCH_2014_2_846_EFS.PDF | 5793696<br>e15e6c687963119bd6735f8aef002b5fa0ba5d85 | no | 12 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 47 | Non Patent Literature | NPL5_Dudley.pdf | 848860<br>2ee1e0bd71dbfffefa6c876bd5acefa7254e4fcc | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 48 | Non Patent Literature | NPL6_Duraiswamy_2013.pdf | 1480478<br>8ca6460d68d744f323e43bb20f62d6b86d089ff6 | no | 21 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 49 | Non Patent Literature | NPL28_Selby.pdf | 23637126<br>17ac261167cec9feea087ace6ee4e45a7a5928ac | no | 19 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 50 | Non Patent Literature | NPL35_Zumwalt.pdf | 5964037<br>aef921ecedc35f647ac2bd1b023381aed74bbb63 | no | 29 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 232627927 | | |

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**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

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| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | 1644 | 1720 | 3338.0550001/ELE/C-K/BMD | 20 | 2 |

**CONFIRMATION NO. 7097**

**UPDATED FILING RECEIPT**

115018
Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

OC000000107536505

Date Mailed: 04/23/2019

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

**Please verify the accuracy of the data presented on this receipt.** If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

**Inventor(s)**

Michael AXELSON, Princeton, NJ;

**Applicant(s)**

Bristol-Myers Squibb Company, Princeton, NJ;

**Assignment For Published Patent Application**

Bristol-Myers Squibb Company, Princeton, NJ

**Power of Attorney:** The patent practitioners associated with Customer Number 115018

**Domestic Priority data as claimed by applicant**

This application is a 371 of PCT/US2017/035822 06/02/2017
which claims benefit of 62/345,662 06/03/2016

**Foreign Applications** for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
*Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.*

**Permission to Access Application via Priority Document Exchange:** Yes

**Permission to Access Search Results:** Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

**If Required, Foreign Filing License Granted:** 02/10/2019

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/306,290**

**Projected Publication Date:** 05/23/2019

**Non-Publication Request:** No

**Early Publication Request:** No

**Title**

USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER

**Preliminary Class**

424

**Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications:** No

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Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

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## Title 37, Code of Federal Regulations, 5.11 & 5.15

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## PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/306,290

### APPLICATION AS FILED - PART I

| FOR | (Column 1) NUMBER FILED | (Column 2) NUMBER EXTRA | SMALL ENTITY RATE($) | SMALL ENTITY FEE($) | OR | OTHER THAN SMALL ENTITY RATE($) | OTHER THAN SMALL ENTITY FEE($) |
|---|---|---|---|---|---|---|---|
| BASIC FEE (37 CFR 1.16(a), (b), or (c)) | N/A | N/A | N/A | | | N/A | 300 |
| SEARCH FEE (37 CFR 1.16(k), (i), or (m)) | N/A | N/A | N/A | | | N/A | 520 |
| EXAMINATION FEE (37 CFR 1.16(o), (p), or (q)) | N/A | N/A | N/A | | | N/A | 760 |
| TOTAL CLAIMS (37 CFR 1.16(i)) | 20 minus 20 = | * | | | OR | x 100 = | 0.00 |
| INDEPENDENT CLAIMS (37 CFR 1.16(h)) | 2 minus 3 = | * | | | | x 460 = | 0.00 |
| APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | | | 0.00 |
| MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | | | 0.00 |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | TOTAL | | | TOTAL | 1580 |

### APPLICATION AS AMENDED - PART II

| | | (Column 1) CLAIMS REMAINING AFTER AMENDMENT | | (Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3) PRESENT EXTRA | SMALL ENTITY RATE($) | SMALL ENTITY ADDITIONAL FEE($) | OR | OTHER THAN SMALL ENTITY RATE($) | OTHER THAN SMALL ENTITY ADDITIONAL FEE($) |
|---|---|---|---|---|---|---|---|---|---|---|
| AMENDMENT A | Total (37 CFR 1.16(i)) | * | Minus | ** | = | x = | | OR | x = | |
| | Independent (37 CFR 1.16(h)) | * | Minus | *** | = | x = | | OR | x = | |
| | Application Size Fee (37 CFR 1.16(s)) | | | | | | | | | |
| | FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | OR | | |
| | | | | | | TOTAL ADD'L FEE | | OR | TOTAL ADD'L FEE | |

| | | (Column 1) CLAIMS REMAINING AFTER AMENDMENT | | (Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3) PRESENT EXTRA | RATE($) | ADDITIONAL FEE($) | OR | RATE($) | ADDITIONAL FEE($) |
|---|---|---|---|---|---|---|---|---|---|---|
| AMENDMENT B | Total (37 CFR 1.16(i)) | * | Minus | ** | = | x = | | OR | x = | |
| | Independent (37 CFR 1.16(h)) | * | Minus | *** | = | x = | | OR | x = | |
| | Application Size Fee (37 CFR 1.16(s)) | | | | | | | | | |
| | FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | OR | | |
| | | | | | | TOTAL ADD'L FEE | | OR | TOTAL ADD'L FEE | |

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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| U.S. APPLICATION NO. | FIRST NAMED INVENTOR | ATTY. DOCKET NO. |
|---|---|---|
| 16/306,290 | Michael AXELSON | 3338.0550001/ELE/C-K/BMD |

115018
Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| INTERNATIONAL APPLICATION NO. | |
|---|---|
| PCT/US2017/035822 | |
| I.A. FILING DATE | PRIORITY DATE |
| 06/02/2017 | 06/03/2016 |

**CONFIRMATION NO. 7097**
**371 ACCEPTANCE LETTER**

*OC000000107556506*

Date Mailed: 04/23/2019

## NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

11/30/2018
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 11/30/2018
- English Translation of the IA filed on 11/30/2018
- Copy of the International Search Report filed on 11/30/2018
- Preliminary Amendments filed on 04/12/2019
- Information Disclosure Statements filed on 04/12/2019
- Inventor's Oath or Declaration filed on 11/30/2018
- U.S. Basic National Fees filed on 11/30/2018
- Authorize Access to Search Results filed on 11/30/2018
- Priority Documents filed on 11/30/2018
- Power of Attorney filed on 11/30/2018
- Authorization to Permit Access filed on 11/30/2018
- Application Data Sheet (37 CFR 1.76) filed on 11/30/2018



FORM PCT/DO/EO/903 (371 Acceptance Notice)

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

PATRICIA A BOOKER

Telephone: (571) 272-3882



FORM PCT/DO/EO/903 (371 Acceptance Notice)

**To:** ckim@sternekessler.com,e-office@sternekessler.com,pcalvo@sternekessler.com
**From:** PAIR_eOfficeAction@uspto.gov
**Cc:** PAIR_eOfficeAction@uspto.gov
**Subject:** Private PAIR Correspondence Notification for Customer Number 115018

Apr 23, 2019 04:05:29 AM

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1100 New York Avenue NW
Washington, DC 20005
UNITED STATES

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| Application | Document | Mailroom Date | Attorney Docket No. |
|---|---|---|---|
| 16306290 | APP.FILE.REC | 04/23/2019 | 3338.0550001/ELE/C-K/BMD |
| | M903 | 04/23/2019 | 3338.0550001/ELE/C-K/BMD |

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www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ELE/C-K/BMD |

**CONFIRMATION NO. 7097**

115018
Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

**PUBLICATION NOTICE**

*OC000000108415757*

**Title:**USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER

**Publication No.**US-2019-0153100-A1
**Publication Date:**05/23/2019

## NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

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Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

**To:** ckim@sternekessler.com,e-office@sternekessler.com,pcalvo@sternekessler.com
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**Cc:** PAIR_eOfficeAction@uspto.gov
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May 24, 2019 03:50:31 AM

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| Application | Document | Mailroom Date | Attorney Docket No. |
|---|---|---|---|
| 16306290 | NTC.PUB | 05/23/2019 | 3338.0550001/ELE/C-K/BMD |

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Alexandria, Virginia 22313-1450
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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

115018 7590 07/14/2020

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER | |
|---|---|
| LOCKARD, JON MCCLELLAND | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1647 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 07/14/2020 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

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ckim@sternekessler.com
e-office@sternekessler.com
pcalvo@sternekessler.com

| *Office Action Summary* | Application No. 16/306,290 | Applicant(s) AXELSON, Michael | |
|---|---|---|---|
| | Examiner JON M LOCKARD | Art Unit 1647 | AIA (FITF) Status Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

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- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
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- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 12 April 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 1-5,7-8,10-13 and 15-23 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1-5,7-8,10-13 and 15-23 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

***Notice of Pre-AIA or AIA Status***

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

***Election/Restrictions***

REQUIREMENT FOR UNITY OF INVENTION

2. As provided in 37 CFR 1.475(a), a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in a national stage application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim. See 37 CFR 1.475(e).

WHEN CLAIMS ARE DIRECTED TO MULTIPLE CATEGORIES OF INVENTIONS

As provided in 37 CFR 1.475(b), a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

(1) A product and a process specially adapted for the manufacture of said product; or

(2) A product and process of use of said product; or

(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

(4) A process and an apparatus or means specifically designed for carrying out the said process; or

(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

Otherwise, unity of invention might not be present. See 37 CFR 1.475(c).

3. Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claim(s) 1-5, 7-8, 10-13 and 16-23, drawn to a method of treatment, comprising administering a PD-1 antibody and a CTLA-4 antibody.

Group II, claim(s) 15, drawn to a kit comprising a PD-1 antibody and a CTLA-4 antibody.

4. The groups of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Pursuant to 37 C.F.R. § 1.475(B-D), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto, i.e., method of making and a method of

using. However, Groups 1-2 lack unity of invention because even though the inventions of these groups require the technical feature of a PD-1 antibody and a CTLA-4 antibody, this technical feature is not a special technical feature as it does not make a contribution over the prior art in view of Wolchok et al. (N. Engl. J. Med. 369(2):122-133; published 2013) which discloses method of treatment comprising administering a PD-1 antibody and a CTLA-4 antibody.

***Rejoinder:***

5. The examiner has required restriction between product or apparatus claims and process claims. Where applicant elects claims directed to the product/apparatus, and all product/apparatus claims are subsequently found allowable, withdrawn process claims that include all the limitations of the allowable product/apparatus claims should be considered for rejoinder. All claims directed to a nonelected process invention must include all the limitations of an allowable product/apparatus claim for that process invention to be rejoined.

6. In the event of rejoinder, the requirement for restriction between the product/apparatus claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product/apparatus are found allowable, an otherwise proper restriction requirement between product/apparatus claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product/apparatus claim will not be rejoined. See MPEP § 821.04. Additionally, in order for rejoinder to occur, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product/apparatus claims. **Failure to do so**

**may result in no rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

7. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

***Advisory Information***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Jon M. Lockard** whose telephone number is **(571) 272-2717**. The examiner can normally be reached on Monday through Friday, 8:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Joanne Hama**, can be reached on **(571) 272-2911**. The fax number for the organization where this application or proceeding is assigned is **571-273-8300**.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see **http://pair-direct.uspto.gov**. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at **866-217-9197** (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JON M LOCKARD/
Examiner, Art Unit 1647
July 8, 2020

**To:** ckim@sternekessler.com,e-office@sternekessler.com,pcalvo@sternekessler.com
**From:** PAIR_eOfficeAction@uspto.gov
**Cc:** PAIR_eOfficeAction@uspto.gov
**Subject:** Private PAIR Correspondence Notification for Customer Number 115018

Jul 14, 2020 03:43:43 AM

Dear PAIR Customer:

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 115018 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

| Application | Document | Mailroom Date | Attorney Docket No. |
|---|---|---|---|
| 16306290 | CTRS | 07/14/2020 | 3338.0550001/ELE/C-K/BMD |

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| Inventor: Michael AXELSON | Confirmation No.: 7097 |
| Applicant: Bristol-Myers Squibb Company | Art Unit: 1644 |
| Application No.: 16/306,290 | Examiner: LOCKARD, Jon McClelland |
| International Filing Date: June 2, 2017 | Atty. Docket: 3338.0550001/ELE/C-K |

Title: **USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER**

## Reply to Unity of Invention Requirement

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the office action dated July 14, 2020, requesting an election of one group of claims to prosecute in the above-referenced patent application, solely to advance prosecution, and not in acquiescence to the Examiner's arguments, Applicant hereby provisionally elects to prosecute Group I, represented by claims 1-5, 7-8, 10-13, and 16-23. This election is made without prejudice to or disclaimer of the other claims or inventions disclosed.

This election is made **without traverse**. Nonetheless, Applicant submits that the cited reference, Wolchok et al., does not teach or suggest a method of treating a subject afflicted with a tumor derived from a colorectal cancer, comprising administering to the subject: (i) an anti-PD-1 antibody, and (ii) an anti-CTLA-4 antibody; wherein the tumor exhibits a high degree of microsatellite instability ("MSI-H"), as recited by the pending claims.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

/Brian M. Dudley/

Brian M. Dudley
Attorney for Applicant
Registration No. 77,546

Date: September 14, 2020

1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

15478965.1

Atty. Dkt. No. 3338.0550001/ELE/C-K/BMD

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 40551053 |
| **Application Number:** | 16306290 |
| **International Application Number:** | |
| **Confirmation Number:** | 7097 |
| **Title of Invention:** | USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Customer Number:** | 115018 |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Filer Authorized By:** | Brian Mason Dudley |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |
| **Receipt Date:** | 14-SEP-2020 |
| **Filing Date:** | 30-NOV-2018 |
| **Time Stamp:** | 18:18:51 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Response to Election / Restriction Filed | 2020-09-14-Reply-Restriction-Requirement-3338-0550001.pdf | 86947<br>ccaa43a4babfd00fa4dd0aad84cc9a14708fb878 | no | 2 |

**Warnings:**

| Information: | |
|---|---|
| Total Files Size (in bytes): | 86947 |

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**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

115018 7590 12/24/2020

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER | |
|---|---|
| LOCKARD, JON MCCLELLAND | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1647 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 12/24/2020 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ckim@sternekessler.com
e-office@sternekessler.com
pcalvo@sternekessler.com

| *Office Action Summary* | Application No. 16/306,290 | Applicant(s) AXELSON, Michael | |
|---|---|---|---|
| | Examiner JON M LOCKARD | Art Unit 1647 | AIA (FITF) Status Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 14 September 2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 1-5,7-8,10-13 and 15-23 is/are pending in the application.

5a) Of the above claim(s) 15 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1,3-5,7-8,10-13 and 16-23 is/are rejected.

8) ☑ Claim(s) 2 is/are objected to.

9) ☑ Claim(s) 1-5,7-8,10-13 and 15-23 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☑ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 30 November 2018 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

***Notice of Pre-AIA or AIA Status***

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

## DETAILED ACTION

***Election/Restrictions***

2. Applicant's election without traverse of Group I, claims 1-5, 7-8, 10-13 and 16-23, in the reply filed on 14 September 2020 is acknowledged. Claim 15 is withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 14 September 2020.

3. The requirement is still deemed proper and is therefore made FINAL.

***Status of Application, Amendments, and/or Claims***

4. The response filed 14 September 2020 has been entered in full. Claim 15 has been withdrawn as discussed *supra*. Therefore, claims 1-5, 7-8, 10-13 and 15-23 are pending, and claims 1-5, 7-8, 10-13 and 16-23 are the subject of this Office Action.

***Information Disclosure Statement***

5. The information disclosure statement (IDS) submitted on 12 April 2020 has been considered by the examiner.

***Specification***

6. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed.

***Claim Objections***

7. Claims 1 and 3 are objected to because of the following informalities: "MSI-H" should read MSI-H (claim 1), and "MMR genes" should read MMR genes (claim 3). Appropriate correction is required.

***Claim Rejections - 35 USC § 112 (Scope of Enablement)***

8. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

9. Claim 1, 3-5, 7-8, 10-13 and 16-23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for methods of treating a subject with a tumor derived from a colorectal cancer, comprising administering to the subject an anti-PD-1 antibody and an anti-CTLA-4 antibody, wherein the tumor is a colon cancer

or a rectal cancer, and wherein the tumor exhibits a high degree of microsatellite instability (MSI-H), does not reasonably provide enablement for a method of treating any tumor derived from a colorectal cancer. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

10. Factors to be considered in determining whether a disclosure enables one skilled in the art to make and use the claimed invention in its full scope without resorting to undue experimentation include: (1) the quantity of experimentation necessary; (2) the amount of direction or guidance presented; (3) the presence or absence of working examples; (4) the nature or complexity of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims. See In re Wands, 8 USPQ2d. 1400 (Fed. Cir. 1988).

11. In the instant case, the claims are broadly drawn to a method of treating a subject afflicted with a tumor derived from a colorectal cancer, comprising administering to the subject: (i) an anti-PD-1 antibody, and (ii) an anti-CTLA-4 antibody; wherein the tumor exhibits a high degree of microsatellite instability ("MSI-H"). Thus, the claims encompass complex and unpredictable subject matter, involving the effects of a complex biological molecule on a tumor derived from a colorectal cancer. The Specification teaches that a "tumor derived from" a colorectal cancer refers to a tumor that is the result of a metastasized colorectal cancer. Because the distal tumor is derived from the pre- metastasis tumor, the "derived from" tumor can also comprise the pre-metastasis tumor, e.g., a tumor derived from a colorectal cancer can comprise a

colorectal cancer. Therefore, based on the disclosure, a tumor derived from a colorectal does not necessarily comprise a colorectal cancer.

12. As was found in Ex parte Hitzeman, 9 USPQ2d 1821 (BPAI 1987), a single embodiment may provide broad enablement in cases involving predictable factors such as mechanical or electrical elements, but more will be required in cases that involve unpredictable factors such as most chemical reactions and physiological activity. This invention is in a class of invention which the CAFC has characterized as "the unpredictable arts such as chemistry and biology", Mycogen Plant Sci., Inc. v. Monsanto Co., 243 F.3d 1316, 1330 (Fed. Cir. 2001). See also In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 1212, 18 USPQ2d 1016, 1026 (Fed. Cir.), cert, denied, 502 U.S. 856 (1991).

13. The specification provides detailed guidance regarding the effects of the combination therapy of an anti-PD-1 antibody and an anti-CTLA-4 antibody in subjects with metastatic colorectal cancer with high microsatellite instability (See Example 1 pp. 40-50, for example). However, there is no guidance or working examples describing the effects of the combination therapy of an anti-PD-1 antibody and an anti-CTLA-4 antibody on tumors other that colon and rectal cancer. One would not expect to be able to extrapolate inhibition of PD-1 and CTLA-4 across the entire scope of the claims.

14. While the level of skill in the art is high, the specification lacks working examples of, guidance for the treatment of, the scope of tumors encompassed by the claims. To practice the invention in a manner consistent with the breadth of the claims would require a substantial inventive contribution on the part of the skilled practitioner involving the determination of the

tumor types that might be treated with the recited combination therapy. This additional characterization is considered undue.

15. Thus, in view of the breadth of the claims, the unpredictability in the art and the limited teachings and lack of working examples, it would require undue experimentation and making a substantial inventive contribution for the skilled artisan to discover how to use the Applicants' invention in its full scope.

***Summary***

16. Claims 1, 3-5, 7-8, 10-13 and 16-23 stand rejected.

17. Claim 2 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

***Advisory Information***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jon M. Lockard whose telephone number is (571) 272-2717. The examiner can normally be reached on Monday through Friday, 8:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joanne Hama, can be reached on (571) 272-2911. The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

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/JON M LOCKARD/
Examiner, Art Unit 1647
December 19, 2020

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | Examiner | Art Unit |
| | JON M LOCKARD | 1647 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| NONE | | 12/19/2020 | JML |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| EAST (USPAT, US-PGPUB, EPO, DERWENT): See attached search history. | 12/19/2020 | JML |
| STN (MEDLINE, SCISEARCH, EMBASE, BIOSIS): See attached search history. | 12/19/2020 | JML |
| PALM: Inventor search. | 12/19/2020 | JML |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | | | |

**EAST Search History**

**EAST Search History (Prior Art)** /J.L./

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | Time Stamp |
|---|---|---|---|---|---|---|
| L1 | 37,466 | PDCD1 CD279 PD-1 PD1 SLEB2 hPD-1 hPD-l hSLE1 "Programmed cell death 1" | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:30 |
| L2 | 8,347 | l1 near5 (antibody or antibodies) | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:31 |
| L3 | 33,940 | CTLA4 ALPS5 CD152 CELIAC3 CTLA-4 GRD4 IDDM12 "cytotoxic T-lymphocyte associated protein 4" | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:32 |
| L4 | 11,235 | l3 near5 (antibody or antibodies) | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:32 |
| L5 | 37,466 | PDCD1 CD279 PD-1 PD1 SLEB2 hPD-1 hPD-l hSLE1 (Programmed adj cell adj death adj "1") | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:32 |
| L6 | 1,113 | (colorectal adj cancer) same ((microsatellite adj instability) or msi-h) | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:35 |
| L7 | 2,416 | l2 with l4 | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:35 |
| L8 | 37 | l6 and l7 | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:35 |
| L9 | 1 | l6 same l7 | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:36 |
| L10 | 37 | axelson-m$.in. | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:37 |
| L11 | 2 | (l2 or l4) and l10 | US-PGPUB; USPAT; EPO; DERWENT | OR | ON | 2020/12/19 20:37 |

12/19/2020 8:38:07 PM
C:\Users\jlockard\Documents\EAST\Workspaces\16306290.wsp

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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 10

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1644 |
| Examiner Name | *To Be Assigned* |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

## U. S. PATENT DOCUMENTS

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| | US2 | US-6051227-A | 04-18-2000 | ALLISON; James Patrick et al. | |
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| | US10 | US-8008449-B2 | 08-30-2011 | KORMAN; Alan J. et al. | |
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| | US13 | US-8354509-B2 | 01-15-2013 | CARVEN; Gregory John et al. | |
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| | US15 | US-8779108-B2 | 07-15-2014 | QUEVA; Christophe et al. | |
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| | US25 | US-20140294852-A1 | 10-02-2014 | KORMAN; Alan J et al | |
| | US26 | US-20140348743-A1 | 11-27-2014 | KORMAN; Alan J et al | |
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| | US29 | US-20170088615-A1 | 03-30-2017 | KORMAN; Alan J et al | |
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| | US33 | US-9492539-B2 | 11-15-2016 | KORMAN; Alan J et al | |
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| First Named Inventor | AXELSON; Michael |
| Art Unit | 1644 |
| Examiner Name | *To Be Assigned* |
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| First Named Inventor | AXELSON; Michael |
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| | FP5 | WO-2014179664-A2 | 11-06-2014 | ANAPTYSBIO INC [US] | | ☐ |
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| Examiner Signature | | Date Considered | |
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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 7 of 10

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1644 |
| Examiner Name | *To Be Assigned* |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

## NON-PATENT LITERATURE DOCUMENTS

| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
|---|---|---|---|
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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 8 of 10

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1644 |
| Examiner Name | *To Be Assigned* |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

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# INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 9 of 10

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1644 |
| Examiner Name | *To Be Assigned* |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

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| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
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Sheet 10 of 10

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| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1644 |
| Examiner Name | *To Be Assigned* |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

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| Examiner Signature | /JON M LOCKARD/ | Date Considered | 12/19/2020 |
|---|---|---|---|

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L4          7694 S L3 (5A) ANTIBOD?
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# BIB DATA SHEET

**CONFIRMATION NO. 7097**

| SERIAL NUMBER | FILING or 371(c) DATE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018<br>RULE | 424 | 1647 | 3338.0550001/ELE/C-K/BMD |

**APPLICANTS**
Bristol-Myers Squibb Company, Princeton, NJ;

**INVENTORS**
Michael AXELSON, Princeton, NJ;

**** CONTINUING DATA ***************************
This application is a 371 of PCT/US2017/035822 06/02/2017
which claims benefit of 62/345,662 06/03/2016

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
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| Foreign Priority claimed ❑ Yes ☑ No<br>35 USC 119(a-d) conditions met ❑ Yes ❑ No<br>Verified and Acknowledged /JON MCCLELLAND LOCKARD/ Examiner's Signature | ❑ Met after Allowance<br>Initials | STATE OR COUNTRY | SHEETS DRAWINGS | TOTAL CLAIMS | INDEPENDENT CLAIMS |
|---|---|---|---|---|---|
| | | NJ | 12 | 20 | 2 |

**ADDRESS**

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005
UNITED STATES

**TITLE**

USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER

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| | | ❑ Credit |

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/J.L./

# Inventor Information for 16/306290

| Inventor Name | City | State/Country |
|---|---|---|
| AXELSON, MICHAEL | PRINCETON | NEW JERSEY |

Appln Info | Comments | Petition Info | Atty/Agent Info | Continuity Data | Foreign Data | **Inventors** | Applicants | Address | Fees | Post Info | Pre Gr

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|---|---|---|---|
| 16306290 | CTNF | 12/24/2020 | 3338.0550001/ELE/C-K/BMD |
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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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|---|---|
| Inventor: Michael AXELSON | Confirmation No.: 7097 |
| Applicant: BRISTOL-MYERS SQUIBB COMPANY | Art Unit: 1647 |
| Application No.: 16/306,290 | Examiner: LOCKARD, Jon McClelland |
| International Filing Date: June 2, 2017 | Atty. Docket: 3338.0550001/ELE/C-K/BMD |

Title: **USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER**

## Amendment and Reply Under 37 C.F.R. § 1.111

***Mail Stop Amendment***

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

In reply to the Office Action dated December 24, 2020, Applicant submits the following Amendment and Remarks.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.

Remarks and Arguments begin on page 6 of this paper.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any additional fees required to continue prosecution or appeal of this application (including issue fee, fees for net addition of claims or forwarding to appeal) are hereby authorized to be charged to our Deposit Account No. 19-0036.

## *Amendments to the Specification*

Please replace the Title with the following Title:

Methods of Treating Colorectal Cancer

## *Amendments to the Claims*

This listing of claims will replace all prior versions, and listings, of claims in the application.

1. (Currently Amended) A method of treating a subject afflicted with a tumor derived from a colorectal cancer, comprising administering to the subject:

(i) an anti-PD-1 antibody, and

(ii) an anti-CTLA-4 antibody;

wherein the tumor is a colon cancer or a rectal cancer; and

wherein the tumor exhibits a high degree of microsatellite instability ([["]]MSI-H[["]]).

2. (Cancelled)

3. (Currently Amended) The method of claim 1, wherein the tumor exhibits one or more characteristics selected from the group consisting of:

(a) the tumor comprises a germline alteration in at least two DNA mismatch repair genes ([["]]MMR genes[["]]);

(b) the tumor comprises a germline alteration in at least 30% of five or more MMR genes;

(c) at least one protein encoded by DNA MMR genes is not detected in the tumor; and

(d) any combination thereof.

4. (Previously Presented) The method of claim 3, wherein the DNA MMR genes comprise MSH2, MLH1, MSH6, PMS2, PMS1, or any combination thereof.

5. (Previously Presented) The method of claim 1, wherein:

(i) the subject exhibits a progression-free survival of at least about one month after the administration, or

(ii) the subject exhibits an overall survival of at least about one month after the administration.

6. (Cancelled)

7. (Previously Presented) The method of claim 1, wherein the anti-PD-1 antibody is nivolumab or pembrolizumab.

8. (Previously Presented) The method of claim 1, wherein the anti-CTLA-4 antibody is administered at a dose ranging from at least about 0.1 mg/kg to at least about 10.0 mg/kg body weight once about every 1, 2, 3, or 4 weeks.

9. (Cancelled)

10. (Previously Presented) The method of claim 1, wherein the anti-CTLA-4 antibody is ipilimumab or tremelimumab.

11. (Previously Presented) The method of claim 1, wherein the anti-PD-1 antibody is administered at a dose ranging from at least about 0.1 mg/kg to at least about 10.0 mg/kg body weight once about every 1, 2, 3, or 4 weeks.

12. (Previously Presented) The method of claim 1, wherein:
(i) the anti-PD-1 antibody is administered at a dose of about 1 mg/kg body weight and the anti-CTLA-4 antibody is administered at a dose of about 1 mg/kg body weight;
(ii) the anti-PD-1 antibody is administered at a dose of about 1 mg/kg body weight and the anti-CTLA-4 antibody is administered at a dose of about 3 mg/kg body weight;
(iii) the anti-PD-1 antibody is administered at a dose of about 3 mg/kg body weight and the anti-CTLA-4 antibody is administered at a dose of about 1 mg/kg body weight; or
(iv) the anti-PD-1 antibody is administered at a dose of about 3 mg/kg body weight and the anti-CTLA-4 antibody is administered at a dose of about 3 mg/kg body weight.

13. (Previously Presented) The method of claim 12, wherein the administration of the anti-PD-1 antibody and the anti-CTLA-4 antibody is followed by an anti-PD-1 antibody monotherapy.

14. (Cancelled)

15. (Cancelled)

16. (Previously Presented) The method of claim 1, wherein the anti-PD-1 antibody is administered at a dose of about 3 mg/kg body weight and the anti-CTLA-4 antibody is administered at a dose of about 1 mg/kg body weight once about every 3 weeks for a total of 12 weeks.

17. (Previously Presented) The method of claim 16, wherein the administration of the anti-PD-1 antibody and the anti-CTLA-4 antibody is followed by an anti-PD-1 antibody monotherapy.

18. (Previously Presented) The method of claim 17, wherein the anti-PD-1 antibody monotherapy is administered once about every 2 weeks.

19. (Previously Presented) The method of claim 17, wherein the anti-PD-1 antibody monotherapy is administered at a dose of about 3 mg/kg once about every 2 weeks.

20. (Previously Presented) The method of claim 17, wherein the anti-PD-1 antibody monotherapy is administered as a flat dose.

21. (Previously Presented) The method of claim 17, wherein the anti-PD-1 antibody monotherapy is administered at a flat dose of about 240 mg once about every 2 weeks.

22. (Previously Presented) The method of claim 21, wherein the anti-PD-1 antibody is nivolumab.

23. (Previously Presented) The method of claim 22, wherein the anti-CTLA-4 antibody is ipilimumab.

## *Remarks*

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 1, 3-5, 7-8, 10-13, and 16-23 are pending in the application, with claim 1 being the independent claim. Claims 1 and 3 are sought to be amended. Claims 2 and 15 are sought to be canceled without prejudice to or disclaimer of the subject matter therein; and claims 6, 9, 14, and 15 were previously canceled. Applicant reserves the right to pursue the canceled subject matter in one or more continuing applications. Support for the amendments to the claims can be found throughout the specification as filed. In particular, support for amended claim 1 can be found, *inter alia*, at original claim 2 and paragraph [0008]. Accordingly, these changes introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicant respectfully requests that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

***Objections to the Specification***

The Examiner objected to the Specification for allegedly lacking a title that is descriptive of the invention. Office Action, p. 3. We disagree. However, solely to advance prosecution, the title is amended to recite "Methods of Treating Colorectal Cancer." Claim 1 is amended to recite, *inter alia*, a method of treating a subject afflicted with a tumor derived from a colorectal cancer, wherein the tumor is a colon cancer or a rectal cancer. Claims 3-5, 7-8, 10-13, and 16-23 depend directly or indirectly from claim 1 and thus include every element of claim 1. As such, each of the pending claims is directed to a method of treating a subject afflicted with a tumor derived from a colorectal cancer, wherein the tumor is a colon cancer or a rectal cancer. Therefore, the title is descriptive of the invention. Accordingly, Applicant requests the Examiner reconsider and withdraw the objection.

***Objections to the Claims***

The Examiner objected to claims 1 and 3 for reciting "('MSI-H')" and "('MMR genes')," respectively. Office Action, p. 3. We disagree. However, solely to further prosecution, claim 1 is amended to recite "(MSI-H)," and claim 3 is amended to recite "(MMR genes)." Accordingly, Applicant requests the Examiner reconsider and withdraw this objection to claims 1 and 3.

***Rejections under 35 U.S.C. § 112***

The Examiner rejected claims 1, 3-5, 7-8, 10-13, and 16-23 under 35 U.S.C. § 112(a) or 35 U.S.C. § (pre-AIA), first paragraph for allegedly lacking enablement for a method of treating any tumor derived from a colorectal cancer. Office Action, pp. 3-6. Though the Examiner concedes that the specification is "enabling for methods of treating a subject with a tumor derived from a colorectal cancer, comprising administering to the subject an anti-PD-1 antibody and an anti-CTLA-4 antibody, wherein the tumor is a colon cancer or a rectal cancer, and wherein the tumor exhibits a high degree of microsatellite instability (MSI-H)," the Examiner argues that the specification "does not reasonably provide enablement for a method of treating any tumor derived from a colorectal cancer." Applicant respectfully disagrees.

However, solely to advance prosecution, and without acquiescing to the Examiner's arguments, claim 2 has been canceled, rendering the Examiner's rejection of this claim moot. In addition, claim 1 is amended to recite a method of treating a subject afflicted with a tumor derived from a colorectal cancer, comprising administering to the subject: (i) an anti-PD-1 antibody, and (ii) an anti-CTLA-4 antibody; wherein the tumor is a colon cancer or a rectal cancer; and wherein the tumor exhibits a high degree of microsatellite instability (MSI-H). Further, amended claim 1

incorporates the elements of previous claim 2, which the Examiner did not reject for an alleged lack of enablement. As such, the Examiner concedes that amended claim 1 is enabled by the specification as filed. A person of ordinary skill in the art at the time the application was filed would have been able to practice the full scope of amended claim 1 in view of the specification as filed, and therefore amended claim 1 is enabled.

Insofar as the Examiner's rejection applies to amended claims 3-5, 7-8, 10-13, and 16-23 based on their dependency from claim 1, claims 3-5, 7-8, 10-13, and 16-23 depend directly or indirectly from amended claim 1 and thus include every element of amended claim 1. Amended claim 1 is enabled for the reasons provided above, and therefore amended claims 3-5, 7-8, 10-13, and 16-23, are also enabled. Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw this rejection to previous claims 1, 3-5, 7-8, 10-13, and 16-23.

## *Conclusion*

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

/Brian M. Dudley/

Brian M. Dudley
Attorney for Applicant
Registration No. 77,546

Date: March 24, 2021

1100 New York Avenue, N.W.
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(202) 371-2600

16389784.3

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 42273062 |
| **Application Number:** | 16306290 |
| **International Application Number:** | |
| **Confirmation Number:** | 7097 |
| **Title of Invention:** | USE OF ANTI-PD-1 ANTIBODY IN THE TREATMENT OF PATIENTS WITH COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Customer Number:** | 115018 |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Filer Authorized By:** | Brian Mason Dudley |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |
| **Receipt Date:** | 24-MAR-2021 |
| **Filing Date:** | 30-NOV-2018 |
| **Time Stamp:** | 17:50:59 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| Submitted with Payment | no |
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## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | | 2021-03-24-Amendment-Reply-111-3338-0550001.pdf | 142811<br>19c4e069a6f20a4a3d36748b33d80127e55711ca | yes | 9 |

| | Multipart Description/PDF files in .zip description | | |
|---|---|---|---|
| | **Document Description** | **Start** | **End** |
| | Amendment/Req. Reconsideration-After Non-Final Reject | 1 | 1 |
| | Specification | 2 | 2 |
| | Claims | 3 | 5 |
| | Applicant Arguments/Remarks Made in an Amendment | 6 | 9 |
| **Warnings:** | | | |
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PTO/SB/06 (09-11)
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| PATENT APPLICATION FEE DETERMINATION RECORD<br>Substitute for Form PTO-875 | Application or Docket Number<br>16/306,290 | Filing Date<br>11/30/2018 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $100 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $460 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | 03/24/2021 | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * 18 | Minus | ** 20 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 1 | Minus | *** 3 | = 0 | | x $480 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /ANGELA S WHITE/ |
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| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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115018 7590 04/29/2021
Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER | |
|---|---|
| LOCKARD, JON MCCLELLAND | |
| ART UNIT | PAPER NUMBER |
| 1647 | |

DATE MAILED: 04/29/2021

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

TITLE OF INVENTION: METHODS OF TREATING COLORECTAL CANCER

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $0.00 | $1200 | 07/29/2021 |

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

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PTOL-85 (Rev. 02/11)

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| (Typed or printed name) |
|---|
| (Signature) |
| (Date) |

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

TITLE OF INVENTION: METHODS OF TREATING COLORECTAL CANCER

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $0.00 | $1200 | 07/29/2021 |

| EXAMINER | ART UNIT | CLASS-SUBCLASS |
|---|---|---|
| LOCKARD, JON MCCLELLAND | 1647 | 424-139100 |

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

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(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

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Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required) ❑Advance Order - # of Copies _____

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❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

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NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

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Typed or printed name _____ Registration No. _____

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
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115018 7590 04/29/2021

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER | |
|---|---|
| LOCKARD, JON MCCLELLAND | |
| ART UNIT | PAPER NUMBER |
| 1647 | |

DATE MAILED: 04/29/2021

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## OMB Clearance and PRA Burden Statement for PTOL-85 Part B

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8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

| *Notice of Allowability* | **Application No.** 16/306,290 | **Applicant(s)** AXELSON, Michael | |
|---|---|---|---|
| | **Examiner** JON M LOCKARD | **Art Unit** 1647 | **AIA (FITF) Status** Yes |

***-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--***

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the Amendment filed 24 March 2021.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1,3-5,7-8,10-13 and 16-23 (renumbered as claims 1-18, respectively) . As a result of the allowed claim(s ), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   **Certified copies:**

   a) ☐All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   **Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

| /J.L/<br>Examiner, Art Unit 1647 | /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 |
|---|---|

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | Examiner | Art Unit |
| | JON M LOCKARD | 1647 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| NONE | | 12/19/2020 | JML |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| EAST (USPAT, US-PGPUB, EPO, DERWENT): See attached search history. | 12/19/2020 | JML |
| STN (MEDLINE, SCISEARCH, EMBASE, BIOSIS): See attached search history. | 12/19/2020 | JML |
| PALM: Inventor search. | 12/19/2020 | JML |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | EAST (USPAT): See attached search history. | 04/23/2021 | JML |
| | PALM: Inventor search. | 04/23/2021 | JML |

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | **Examiner** | **Art Unit** |
| | JON M LOCKARD | 1647 |

| CPC | | | | | | |
|---|---|---|---|---|---|---|
| **Symbol** | | | | | **Type** | **Version** |
| C07K | / | 16 | / | 2818 | F | 2013-01-01 |
| A61P | / | 35 | / | 00 | I | 2018-01-01 |
| C07K | / | 16 | / | 2827 | I | 2013-01-01 |
| C07K | / | 16 | / | 2878 | I | 2013-01-01 |
| A61K | / | 2039 | / | 507 | A | 2013-01-01 |

| CPC Combination Sets | | | | | | | | |
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| | / | | / | | | | | |

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| /JON M LOCKARD/<br>Examiner, Art Unit 1647 | 23 April 2021 | **Total Claims Allowed:** | |
| (Assistant Examiner) | (Date) | 18 | |
| /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 | 23 April 2021 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20210423

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | Examiner | Art Unit |
| | JON M LOCKARD | 1647 |

| INTERNATIONAL CLASSIFICATION | | | | | |
|---|---|---|---|---|---|
| CLAIMED | | | | | |
| A61K | / | 39 | / | 395 | |
| C07K | / | 16 | / | 28 | |

| NON-CLAIMED | | | | |
|---|---|---|---|---|
| | / | | / | |

| US ORIGINAL CLASSIFICATION | |
|---|---|
| CLASS | SUBCLASS |
| | |

| CROSS REFERENCES(S) | | | | | | |
|---|---|---|---|---|---|---|
| CLASS | SUBCLASS (ONE SUBCLASS PER BLOCK) | | | | | |
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| /JON M LOCKARD/<br>Examiner, Art Unit 1647 | 23 April 2021 | Total Claims Allowed: | |
| (Assistant Examiner) | (Date) | 18 | |
| /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 | 23 April 2021 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20210423

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | Examiner | Art Unit |
| | JON M LOCKARD | 1647 |

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

| Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original |
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|---|---|---|---|
| /JON M LOCKARD/<br>Examiner, Art Unit 1647 | 23 April 2021 | **Total Claims Allowed:** | |
| (Assistant Examiner) | (Date) | 18 | |
| /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 | 23 April 2021 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20210423

# Inventor Information for 16/306290

/J.L./

| Inventor Name | City | State/Country |
|---|---|---|
| AXEL SON, MICHAEL | PRINCETON | NEW JERSEY |

Appln Info | Contents | Petition Info | Atty/Agent Info | Continuity Data | Foreign Data | **Inventors** | Applicants | Address | Fees | Post Info | Pre Gr

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**PCT /** / Search **or PG PUBS #** Search

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**EAST Search History**

**EAST Search History (Interference)** /J.L./

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | Time Stamp |
|---|---|---|---|---|---|---|
| L1 | 12,618 | PDCD1 CD279 PD-1 PD1 SLEB2 hPD-1 hPD-l hSLE1 "Programmed cell death 1" | USPAT | OR | ON | 2021/04/23 14:01 |
| L2 | 1,997 | l1 near5 (antibody or antibodies) | USPAT | OR | ON | 2021/04/23 14:01 |
| L3 | 10,329 | CTLA4 ALPS5 CD152 CELIAC3 CTLA-4 GRD4 IDDM12 "cytotoxic T-lymphocyte associated protein 4" | USPAT | OR | ON | 2021/04/23 14:02 |
| L4 | 3,512 | l3 near5 (antibody or antibodies) | USPAT | OR | ON | 2021/04/23 14:02 |
| L5 | 12,618 | PDCD1 CD279 PD-1 PD1 SLEB2 hPD-1 hPD-l hSLE1 (Programmed adj cell adj death adj "1") | USPAT | OR | ON | 2021/04/23 14:02 |
| L6 | 380 | (colorectal adj cancer) same ((microsatellite adj instability) or msi-h) | USPAT | OR | ON | 2021/04/23 14:03 |
| L7 | 629 | l2 with l4 | USPAT | OR | ON | 2021/04/23 14:03 |
| L8 | 5 | l6 and l7 | USPAT | OR | ON | 2021/04/23 14:04 |
| L9 | 0 | l6 same l7 | USPAT | OR | ON | 2021/04/23 14:05 |
| L10 | 5 | axelson-m$.in. | USPAT | OR | ON | 2021/04/23 14:05 |
| L11 | 0 | (l2 or l4) and l10 | USPAT | OR | ON | 2021/04/23 14:05 |

4/23/2021 2:06:05 PM
C:\Users\jlockard\Documents\EAST\Workspaces\16306290.wsp

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# BIB DATA SHEET

**CONFIRMATION NO. 7097**

| SERIAL NUMBER | FILING or 371(c) DATE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018<br>RULE | 424 | 1647 | 3338.0550001/ELE/C-K/BMD |

**APPLICANTS**
Bristol-Myers Squibb Company, Princeton, NJ;

**INVENTORS**
Michael AXELSON, Princeton, NJ;

**** CONTINUING DATA ***************************
This application is a 371 of PCT/US2017/035822 06/02/2017
which claims benefit of 62/345,662 06/03/2016

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
02/10/2019

| Foreign Priority claimed ❑ Yes ☑ No<br>35 USC 119(a-d) conditions met ❑ Yes ❑ No<br>Verified and Acknowledged /JON MCCLELLAND LOCKARD/ Examiner's Signature | ❑ Met after Allowance<br>Initials | STATE OR COUNTRY | SHEETS DRAWINGS | TOTAL CLAIMS | INDEPENDENT CLAIMS |
|---|---|---|---|---|---|
| | | NJ | 12 | 20 | 2 |

**ADDRESS**

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005
UNITED STATES

**TITLE**

METHODS OF TREATING COLORECTAL CANCER

| FILING FEE RECEIVED | FEES: Authority has been given in Paper | ❑ All Fees |
|---|---|---|
| 1720 | No._____ to charge/credit DEPOSIT ACCOUNT | ❑ 1.16 Fees (Filing) |
| | No._____ for following: | ❑ 1.17 Fees (Processing Ext. of time) |
| | | ❑ 1.18 Fees (Issue) |
| | | ❑ Other _____ |
| | | ❑ Credit |

BIB (Rev. 05/07).

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# FIRST SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD; JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

## FOREIGN PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Foreign Patent Document<br>Country Code[3]-Number[4]-Kind Code[5] (if known) | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear | T[6] |
|---|---|---|---|---|---|---|
| | FP16 | WO-2003072822-A2 | 09-04-2003 | INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM) | | ☐ |
| | FP17 | WO-2005021743-A1 | 03-10-2005 | TANAKA; NORIAKI | | ☒ |
| | FP18 | WO-2013004618-A1 | 01-10-2013 | HELSINGIN YLIOPISTO | | ☐ |

| Examiner Signature | | Date Considered | |
|---|---|---|---|

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# FIRST SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 2 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD; JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

| NON-PATENT LITERATURE DOCUMENTS | | | |
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| | NPL36 | Anonymous: "An Investigational Immuno-therapy Study of Nivolumab. and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread - Full Text View- Clinical Trials.gov". Retrieved from the Internet: URL: ttps://clinicaltrials.gov/ct2/history/NCT02060188?V_50=View#StudyPageTop [retrieved on June 23, 2021], 9 pages. | ☐ |

17117986.1

| Examiner Signature | | Date Considered | |
|---|---|---|---|

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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(22) International Filing Date: 26 February 2003 (26.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02290483.3 28 February 2002 (28.02.2002) EP

(71) Applicant *(for all designated States except US)*: **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)** [FR/FR]; 101, rue de Tolbiac, F-75013 PARIS (FR).

(72) Inventors; and
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(54) Title: MONONUCLEOTIDE REPEATS MICROSATELLITE MARKERS FOR DETECTING MICROSATELLITE INSTABILITY.

(57) Abstract: The invention relates to new microsatellite markers, and their use for the evaluation of microsatellite instability in the diagnosis of tumours.

**MONONUCLEOTIDE REPEATS MICROSATELLITE MARKERS FOR DETECTING MICROSATELLITE INSTABILITY**

The invention concerns new microsatellite markers and their use for the detection of the microsatellite instability (MSI) associated with some tumours.

Microsatellites are short DNA motifs (1-10 base pairs), which occur as tandem repeats at numerous loci throughout the genome.

The microsatellite instability (MSI) phenotype is defined as the presence in tumour DNA of alternative sized microsatellites that are not seen in the corresponding germline DNA (AALTONEN *et al.*, Science, 260(5109), 812-816, 1993; IONOV *et al.*, Nature, 363(6429), 558-561, 1993; THIBODEAU *et al.*, Science, 260(5109), 816-819, 1993; IACOPETTA *et al.*, Hum. Mutat., 12(5), 355-360, 1998).

The MSI phenotype is a characteristic of the hereditary non-polyposis colorectal cancer (HNPCC) syndrome, wherein it can be detected in more than 90% of all HNPCC tumours (LIU *et al.*, Nature Med., 2, 169-174, 1996); it also occurs in approximately 15% of sporadic colon and gastric tumours. It has also been detected in other tumours, such as pancreatic carcinomas (HAN *et al.*, Cancer Res., 53, 5087-5089, 1993), prostate carcinomas (GAO *et al.*, Oncogene, 9, 2999-3003, 1994), carcinomas of the endometrium (RISINGER *et al.*, Cancer Res., 53, 5100-5103, 1993; PELTOMAKI *et al.*, Cancer Res., 53, 5853-5855, 1993).

MSI reflects an underlying mismatch repair (MMR) defect that fails to recognize errors introduced during the replication of microsatellite sequences. In the familial cancer syndrome HNPCC, the MSI phenotype is caused by germline mutations in the mismatch repair (MMR) genes *hMSH2*, *hMLH1* and less frequently in *hPMS1*, *hPMS2* and *hMSH6* (KINZLER *et al.*, Cell, 87, 159-170, 1996). In sporadic cancers it is often caused by methylation of the *hMLH1* promoter leading to the transcriptional silencing of this gene (HERMAN *et al.*, Proc. Natl. Acad. Sci. USA, 95(12), 6870-6875, 1998).

MSI colonic and gastric tumours have distinctive molecular and clinicopathological profiles and are often

associated with favourable prognosis (LOTHE et al., Cancer Res., 53, 5849-5852, 1993; KIM et al., Am. J. Pathol., 145, 148-156, 1994; OLIVEIRA et al., Am. J. Pathol., 153, 1211-1219, 1998). There is also evidence to suggest that colorectal cancer patients with MSI tumours show good survival benefit from 5FU-based chemotherapy (ELSALEH et al., The Lancet, 355, 1745-1750, 2000; LIANG et al., Int. J. Cancer, 101, 519-525, 2002) and therefore MSI might be a useful molecular predictive marker for response to this type of adjuvant therapy. Routine analysis of MSI status also has clinical application for assisting in the diagnosis of suspected HNPCC cases (AALTONEN et al., N. Eng. J. Med., 338, 1481-1487, 1998). Indeed, tumours from HNPCC patients lack phenotypic features that readily distinguish them from sporadic tumours and hence the diagnosis of this disease was historically based on family history of cancer using for example the Amsterdam criteria (VASEN et al., Dis. Colon Rectum, 34, 424-425, 1991; VASEN et al., Gastroenterology, 115, 1453-1456, 1999). Such criteria are too restrictive however and identify only a fraction of HNPCC families so that the true incidence of this disease is not known and estimates vary from 0.5 to 13%. Given that familial carriers of MMR defects have a greater than 80% risk of developing cancer, it is important to devise efficient and cost-effective ways to detect this condition. For this purpose, molecular-based laboratory approaches are now being developed that may help in establishing HNPCC diagnosis. Two methods are generally proposed: microsatellite genotyping and immunohistochemistry of the main mismatch repair proteins. The use of one or the other, or both of these methods is still a matter of debate, based on their relative efficiency, specificity and cost (LINDOR et al., J. Clin. Oncol., 20, 1043-1048, 2002; WAHLBERG et al., Cancer Res., 62, 3485-3492, 2002; TERDIMAN et al., Gastroenterology, 120, 21-30, 2001; LOUKOLA et al., Cancer Res., 61, 4545-4549, 2001). It appears so far that microsatellite genotyping has a higher sensitivity than IHC, but is more expensive and more difficult to set up in routine laboratories. It is thus

important to develop simple and accurate methods to determine MSI tumours for predisposition and prognostic diagnosis informations.

Numerous different microsatellites have been studied by investigators with the aim of identifying MSI tumours.

Depending on the type and number of microsatellites analysed, widely variable results for the frequency of MSI in different tumour types have been published (PERUCHO, Cancer Res., 59(1), 249-256, 1999).

The use of a BAT-25 and BAT-26 marker combination has been proposed for the detection of MSI (ZHOU *et al.*, Genes, Chromosomes & Cancer, 21(2), 101-107, 1998; HOANG *et al.*, Cancer Res., 57(2), 300-303, 1997).

The BAT-25 and BAT-26 are mononucleotide repeats respectively located in intron 16 of *c-kit* and intron 5 of *hMSH2*. These two repeats are quasimonomorphic in Caucasian populations (HOANG *et al.*, Cancer Res., 57(2), 300-303, 1997; ZHOU *et al.*, Oncogene, 15(14), 1713-1718, 1997). This property allows ready classification of the large allelic size variations seen in MSI tumour DNA as being due to somatic alteration. In the large majority of tumours, analysis of BAT-25 and BAT-26 is sufficient to establish their MSI status without reference to the germline DNA (ZHOU *et al.*, Genes, Chromosomes & Cancer, 21(2), 101-107, 1998).

However, alternative sized BAT-25 and BAT-26 alleles have been identified in 18.4 and 12.6%, respectively, of African Americans (PYATT *et al.*, Am. J. Pathol., 155(2), 349-353, 1999; SAMOWITZ *et al.*, Am. J. Pathol., 154(6), 1637-1641, 1999). Thus, analysis of additional repeats may be needed in order to avoid the occasional false positive result arising from these germline polymorphisms.

Accordingly it has been proposed to complete the analysis of these mononucleotide repeats by an additional analysis of dinucleotide repeats in both the tumour and germline DNA.

For instance, US Patent 6,150,100 proposes the use of 2 mononucleotide repeats selected from BAT25, BAT26

and BAT40, associated with 2 or 3 dinucleotide repeats selected from APC, Mfd15, D2S123, and D18S69, and optionally with the pentanucleotide repeat TP53Alu. Preferred combinations of microsatellite markers disclosed in US Patent 6,150,100 are BAT25, BAT26, APC, Mfd15 and D2S123 or BAT26, BAT40, APC, Mfd15 and D2S123.

In 1997 an international consensus meeting on the detection of MSI recommended a panel of five markers for the uniform analysis of MSI status (BOLAND *et al.*, Cancer Res., 58(22), 5248-5257, 1998). This included two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats. Tumours with instability at two or more of these markers were defined as being MSI-H. Tumours with instability at one marker, and without instability were defined as MSI-L and MSS respectively. MSI-H cancers have distinct clinicopathological features from MSI-L and MSS tumours.

Some of the characteristics of dinucleotide repeats make their use as markers of the MSI status somewhat problematical. The dinucleotide repeats in the above panels generally show instability in only 60-80% of MSI-H tumours (SUTTER *et al.*, Mol. Cell Probes, 13(2), 157-165, 1999). There is some evidence to suggest that loss of MMR and subsequent alteration of mononucleotide repeats occurs earlier in the MSI-H tumour progression pathway than the mutation of dinucleotide repeats (PERUCHO *et al.*, Cold Spring Harb. Symp. Quant. Biol., 59, 339-348, 1994). Furthermore, some MSI cell lines with MMR deficiency caused by *hMSH6* mutation do not show alteration in dinucleotide repeats (AKIYAMA et al., Cancer Res., 57(18), 3920-3923, 1997). Therefore the underlying MMR deficiency affecting mononucleotide and dinucleotide repeats may be different and the analysis of both may lead to misinterpretation of the MSI status of some tumours. In many instances the analysis of dinucleotide repeats adds no further information to the results obtained by analysis of mononucleotide repeats (DIETMAIER *et al.*, Cancer Res., 57(21), 4749-4756, 1997; LOUKOLA *et al.*, Cancer Res., 61(11), 4545-4549, 2001).

In addition, unlike mononucleotide repeats such as BAT-25 and BAT-26, dinucleotide repeats are highly polymorphic. Therefore, their use for the identification of MSI in tumour DNA always requires the analysis of corresponding germline DNA.
This makes the MSI screening process considerably more time-consuming and expensive, as well as introducing potential errors due to mixing of germline and tumour DNA samples. Moreover, the interpretation of size alterations in these dinucleotide repeats is difficult and can lead to misclassification (PERUCHO, Cancer Res., 59(1), 249-256, 1999). Finally, in many situations germline DNA from cancer patients is not readily available.

For all these reasons, the methods using BAT-26 and BAT-25 either alone, or in combination with dinucleotide repeats are not completely satisfactory for the accurate determination of MSI status in human tumours and there is an urgent need for improvement.

Thus, multiple, quasimonomorphic mononucleotide repeats are needed for the accurate diagnosis of MSI tumours.

The inventors have now identified new mononucleotide repeats that are conserved in germline DNA from Caucasian and African subjects and that, similar to BAT-25 and BAT-26, are highly sensitive to somatic deletion in MSI-H tumours.

Three of these new microsatellite markers are poly(T) repeats hereinafter referred as NR21, NR22, and NR24.

The NR21 marker is a 21T repeat identified in the 5′ untranslated region of the SLC7A8 gene (cDNA sequence GenBank XM_033393).

The NR22 marker is a 22T repeat identified in the 3′ untranslated region of the putative trans-membrane precursor protein B5 gene (cDNA sequence GenBank L38961).

The NR24 marker is a 24T repeat identified in the 3′ untranslated region of the zinc finger-2 gene (cDNA sequence GenBank X60152).

A fourth microsatellite marker, hereinafter referred as NR27, is a 27A repeat identified in the 5′

untranslated region of the inhibitor of apoptosis protein-1 gene (cDNA sequence GenBank AF070674).

The NR21, NR22, NR24 and NR27 markers are useful for the evaluation of microsatellite instability in the diagnosis of tumours.

The invention thus provides a method for evaluating the microsatellite instability associated with a tumour, by amplification of microsatellite loci in a biological sample comprising genomic DNA from said tumour and determination of the sizes of the DNA amplification products, characterized in that said method comprises the amplification of at least one microsatellite locus selected among NR21, NR22, NR24 and NR27.

According to a preferred embodiment of the invention, said method comprises the amplification of the two microsatellite loci NR21 and NR24, and the amplification of a third microsatellite locus selected among NR22 and NR27.

Advantageously, said method further comprises the amplification of at least one microsatellite locus different from NR21, NR22, NR24 and NR27. Preferably, said microsatellite locus is a mononucleotide repeat locus. More preferably this mononucleotide repeat locus is selected among BAT-25 and BAT-26.

According to a particular embodiment, the method of the invention comprises the amplification of the five microsatellite loci BAT-25, BAT-26, NR21, NR22, and NR24.

According to another particular embodiment, the method of the invention comprises the amplification of the five microsatellite loci BAT-25, BAT-26, NR21, NR27 and NR24.

Microsatellite instability at each of these loci is evaluated by comparison of the size of the amplification product obtained from tumoral DNA with the size of the amplification product obtained from normal (i.e. non-tumoral) DNA with the same set of primers.

This comparison can be performed in the conventional way, by obtaining an amplification product from normal DNA from the same subject with the same set of primers, and using it as a reference.

However, the present invention makes it possible, in most of cases, to avoid the need to amplify normal DNA from the same subject. Instead, the comparison can be made by reference to the average size of amplification products obtained from normal DNAs of a pool of subjects with the same set of primers. In these cases, microsatellite instability is assumed in the case of locus BAT-26 if the size of the amplification product obtained from tumoral DNA is shorter of more than 3 bp than the average size of the amplification product obtained from normal DNA using the same set of primers, and in the case of loci BAT-25, NR21, NR22, NR24 and NR27, if the size of the amplification product obtained from tumoral DNA is shorter of more than 2 bp than the average size of the amplification product obtained from normal DNA using the same set of primers.

Tumoral genomic DNA can be obtained from different sources including principally biopsies or tumoral tissues, or body fluids or secretions containing disseminated tumour cells, paraffin embedded tissue.

The invention also provides reagents for carrying out the method of the invention.

In particular, the invention provides pairs of primers suitable for the amplification of a microsatellite locus selected among NR21, NR22, NR24 and NR27.

Suitable primers can be derived from the genomic sequences surrounding said microsatellite loci.

For instance:

- primers allowing the amplification of NR21 can be derived from the genomic sequence GenBank AL117258, and preferably from the portion thereof represented by SEQ ID NO: 1;

- primers allowing the amplification of NR22 can be derived from the genomic sequence GenBank AP001132, and preferably from the portion thereof represented by SEQ ID NO: 2;

- primers allowing the amplification of NR24 can be derived from the genomic sequence GenBank AC092835, and

preferably from the portion thereof represented by SEQ ID NO: 3;

- primers allowing the amplification of NR27 can be derived from the genomic sequence GenBank AP001167, and preferably from the portion thereof represented by SEQ ID NO: 16.

By way of example:

- a pair of primers suitable for the amplification or NR21 consists of the following oligonucleotides:

TAAATGTATGTCTCCCCTGG (SEQ ID NO: 4)

ATTCCTACTCCGCATTCACA (SEQ ID NO: 5)

- a pair of primers suitable for the amplification or NR22 consists of the following oligonucleotides:

GAGGCTTGTCAAGGACATAA (SEQ ID NO: 6)

AATTCGGATGCCATCCAGTT (SEQ ID NO: 7)

- a pair of primers suitable for the amplification or NR24 consists of the following oligonucleotides:

CCATTGCTGAATTTTACCTC (SEQ ID NO: 8)

ATTGTGCCATTGCATTCCAA (SEQ ID NO: 9).

The above primers give when used on normal DNA, amplification products of 104, 143, and 134 bp for NR21, NR22 and NR24 respectively.

They can advantageously be labelled with fluorescent dyes and used in multiplex PCR assays. Preferably, different fluorescent dyes will be used for primers that give amplification products of similar size (i.e having sizes differing of less than 15-20 pb). This allows to avoid uncertainties that might result from overlapping of PCR products due to the average deletion of 5-12 bp for these markers in MSI tumors.

If one prefers not to use different fluorescent dyes, primers can be designed in order to give amplification products of clearly distinct size (i.e having sizes differing of at least 15 pb and preferably of at least 20 pb between different markers). This allows a clear separation between

markers on a size basis by standard electrophoresis techniques, even when deleted due to microsatellite instability in tumor DNA.

Primers giving amplification products of clearly distinct size for NR21, NR24, and NR27 are by way of example:

- a pair of primers suitable for the amplification or NR21 consists of the following oligonucleotides:

GAGTCGCTGGCACAGTTCTA (SEQ ID NO: 17);

CTGGTCACTCGCGTTTACAA (SEQ ID NO: 18);

- a pair of primers suitable for the amplification or NR24 consists of the following oligonucleotides:

GCTGAATTTTACCTCCTGAC (SEQ ID NO: 19);

ATTGTGCCATTGCATTCCAA (SEQ ID NO: 9);

- a pair of primers suitable for the amplification or NR27 consists of the following oligonucleotides:

AACCATGCTTGCAAACCACT (SEQ ID NO: 20);

CGATAATACTAGCAATGACC (SEQ ID NO: 21).

When used on normal DNA, the above primers give amplification products of 131, 109 and 87 bp for NR24, NR21 and NR27 respectively.

The invention also provides a kit for the analysis of microsatellite instability, characterized in that it comprises at least two pairs of primers suitable for the amplification of at least two microsatellite loci selected among NR21, NR22, NR24, and NR27.

Advantageously said kit comprises at least:

- one pair of primers suitable for the amplification of NR21;
- one pair of primers suitable for the amplification of NR24;
- one pair of primers selected among a pair of primers suitable for the amplification of NR22 and a pair of primers suitable for the amplification of NR27.

According to a preferred embodiment said kit further comprises at least one pair of primers suitable for

the amplification of at least one microsatellite locus different from NR21, NR22, NR24 and NR27. Preferably, said microsatellite locus is a mononucleotide repeat locus. More preferably this mononucleotide repeat locus is selected among BAT-25 and BAT-26.

Primers allowing the amplification of BAT-26 can be derived from the genomic sequence GenBank AC0799775, and preferably from the portion thereof represented by SEQ ID NO: 10

Primers allowing the amplification of BAT-25 can be derived from the genomic sequence GenBank AC092545, and preferably from the portion thereof represented by SEQ ID NO: 11

By way of example, a kit of the invention can comprise:

- a pair of primers suitable for the amplification of BAT-25, consisting of the following oligonucleotides:

TCGCCTCCAAGAATGTAAGT (SEQ ID NO: 12)

TCTGCATTTTAACTATGGCTC (SEQ ID NO: 13);

- a pair of primers suitable for the amplification of BAT-26, consisting of the following oligonucleotides:

TGACTACTTTTGACTTCAGCC (SEQ ID NO: 14)

AACCATTCAACATTTTTAACCC (SEQ ID NO: 15);

When used on normal DNA, the above primers amplify a fragment of 121 bp for BAT-26, and 124 bp for BAT-25.

They can be used in particular in a multiplex PCR assay using different fluorescent dyes, for instance in combination with the NR21 primers SEQ ID NO: 4 and 5, the NR22 primers SEQ ID NO: 6 and 7, and the NR24 primers SEQ ID NO: 8 and 9.

If one prefers to obtain amplification products of clearly distinct size for BAT-25 and BAT-26, one can use for instance:

- a pair of primers suitable for the amplification or BAT-25 consisting of the following oligonucleotides:

TACCAGGTGGCAAAGGGCA (SEQ ID NO: 22);

TCTGCATTTTAACTATGGCTC (SEQ ID NO: 13);

- a pair of primers suitable for the amplification or BAT-26, consisting of the following oligonucleotides:

CTGCGGTAATCAAGTTTTTAG (SEQ ID NO: 23);

AACCATTCAACATTTTTAACCC (SEQ ID NO: 15).

When used on normal DNA, the above primers give respectively amplification products of 153 and 183 bp for BAT-25 and BAT-26.

They can advantageously be used in combination with NR21 primers (SEQ ID NO: 17 and 18) and/or NR24 primers (SEQ ID NO: 19 and 9) and/or NR27 primers (SEQ ID NO: 20 and 21), allowing a clear separation of the five markers on a size basis.

Optionally, the kits of the invention can further comprise appropriate reagents and materials useful to carry out DNA amplification.

The method, reagents and kits of the invention can be used in the same applications as the prior art methods of evaluation of microsatellite instability. This includes mainly the diagnosis of the MSI phenotype of tumours, in particular tumours of the gastrointestinal tract, and more specifically colorectal or gastric tumours, or tumours of the endometrium. Tumours with instability at three or more of the BAT-25, BAT-26, NR21, NR22 (or NR27), or NR24 loci are defined as being MSI-H.

The method of the invention has the advantage over the prior art methods of allowing to establish the MSI status without ambiguity in particular in the case of tumours of the gastrointestinal tract, without needing a simultaneous analysis of corresponding germline DNA from each patient.

We propose that concurrent use of these mononucleotide markers in a single pentaplex PCR system allows accurate evaluation of tumour MSI status with 100%

sensitivity, 100% specificity. This assay is simpler to use than those involving dinucleotide markers, and is more specific than using BAT-25 and BAT-26 alone. This test could be routinely used in the hospital to provide information on prognosis, as a possible predictor of response to adjuvant therapies, and for the detection of new HNPCC family members.

The invention will be further illustrated by the additional description which follows, which refers to an example of use of the mononucleotide markers of the invention in multiplex PCR analysis. It should be understood however that this example is given only by way of illustration of the invention and does not constitute in any way a limitation thereof.

**EXAMPLE:**

**Material and Methods**

Mononucleotide Repeats and Multiplex Polymerase Chain Reaction (PCR)

Three new poly(T) repeats and one new poly(A) repeat were identified respectively in the 3' or 5' untranslated regions of the SLC7A8 (NR21, 21T), trans-membrane precursor protein B5 (NR22, 22T), zinc finger-2 (NR24, 24T), and inhibitor of apoptosis protein-1 (NR27, 27A) genes. Details including primer sequences for these repeats and BAT 25 and BAT 26 are shown in Table I below.

TABLE I

| Name | Gene | GenBank accession (cDNA) | Location of the repeat | Fluorescent marker | colour | Primers | SEQ ID NO | PCR product (bp)* |
|---|---|---|---|---|---|---|---|---|
| BAT 26 | hMSH2 | U04045 | 26(A)intron 5 | FAM[a] | blue | TGACTACTTTTGACTTCAGCC<br>AACCATTCAACATTTTTAACCC | 14<br>15 | 121 |
| | | | | | | CTGCGGTAATCAAGTTTTTAG<br>AACCATTCAACATTTTTAACCC | 23<br>15 | 183 |
| BAT 25 | c-kit | X06182 | 25(T)intron 16 | NED[s] | yellow | TCGCCTCCAAGAATGTAAGT<br>TCTGCATTTTAACTATGGCTC | 12<br>13 | 124 |
| | | | | | | TACCAGGTGGCAAAGGGCA<br>TCTGCATTTTAACTATGGCTC | 22<br>13 | 153 |
| NR21 | SLC7A8 | XM_033393 | 21(T)5'UTR | HEX[a] | green | TAAATGTATGTCTCCCCTGG<br>ATTCCTACTCCGCATTCACA | 4<br>5 | 104 |
| | | | | | | GAGTCGCTGGCACAGTTCTA<br>CTGGTCACTCGCGTTTACAA | 17<br>18 | 109 |
| NR22 | Putative transmembrane precursor protein B5 | L38961 | 22(T)3'UTR | FAM[a] | blue | GAGGCTTGTCAAGGACATAA<br>AATTCGGATGCCATCCAGTT | 6<br>7 | 143 |
| NR24 | ZINC FINGER 2 (ZNF-2) | X60152 | 24(T)3'UTR | HEX[a] | green | CCATTGCTGAATTTTACCTC<br>ATTGTGCCATTGCATTCCAA | 8<br>9 | 134 |
| | | | | | | GCTGAATTTTACCTCCTGAC<br>ATTGTGCCATTGCATTCCAA | 19<br>9 | 131 |
| NR27 | Inhibitor of apoptosis protein-1 | AF070674 | 27 (A) 5'UTR | | | AACCATGCTTGCAAACCACT<br>CGATAATACTAGCAATGACC | 20<br>21 | 87 |

[s]: sense primer

[a]: anti-sense primer

*: theorical size deduced from GenBank sequence

Primers were designed to allow different PCR product sizes to be resolved on 5% denaturing gels run in an ABI PRISM 377 automated DNA sequencer. GENESCAN software (GENOTYPER 2.1) was used to calculate the size, height and area of each fluorescent PCR product.

The following primers were used in the experimentations described below:

- BAT 26 primers : SEQ ID NO: 14 and SEQ ID NO: 15;
- BAT 25 primers: SEQ ID NO: 12 and SEQ ID NO: 13;
- NR21 primers: SEQ ID NO: 4 and SEQ ID NO: 5;
- NR22 primers: SEQ ID NO: 6 and SEQ ID NO: 7;
- NR24 primers: SEQ ID NO: 8 and SEQ ID NO: 9.

One primer in each pair was labelled with one of the fluorescent markers FAM, HEX or NED (PE APPLIED BIOSYSTEMS). The five mononucleotide repeats were amplified in one multiplex PCR containing 20 µM of each primer, 200 µM dNTP, 1.5 mM $MgCl_2$ and 0.75 units of *Taq* DNA polymerase. The PCR was performed using the following conditions: denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, followed by an extension step for 72°C for 7 min.

DNA samples

Germline DNA was obtained from 128 Caucasian individuals at the Centre d'Etudes du Polymorphisme Humain (CEPH) in Paris and from 56 individuals of African descent.

A total of 124 colon, 50 gastric tumours, 20 endometrial tumours and 16 colon cell lines that had previously been tested for MSI using several dinucleotide markers and BAT-25 and BAT-26 mononucleotide markers (147 cases) or BAT26 and BAT25 alone (63 cases) (HOANG *et al.* Cancer Res., 57(2), 300-303, 1997 ; SERUCA *et al.*, Int. J. Cancer, 64, 32-36, 1995; TIBELETTI *et al.*, Gynecol. Oncol., 73(2), 247-252, 1999). Of these a total of 81 primary colon tumours, 42 primary gastric cancers, 20 primary endometrial

tumours and 5 colon tumour cell lines were considered to be MSI-H based on deletions in the above repeats.

**RESULTS**

Fluorescent Multiplex PCR

The five mononucleotide markers BAT-25, BAT-26, NR21, NR22 and NR24 were co-amplified in a single multiplex PCR mix using the PCR conditions described above, and analysed for size in an automated DNA sequencer. In these conditions, no non-specific bands within the 100-142 bp size range were observed, thus allowing accurate identification of the five markers.

Figure 1 shows typical allelic profiles of (a), BAT25, BAT26, NR21, NR22 and NR24 in DNA from the germline or from MSS tumours, (b) MSI-H primary tumour showing both deleted and normal sized alleles, and (c) MSI-H cell line showing homozygous deletions.

Figure 1a shows an example of the fluorescent peaks observed for each marker, in this case representing the most common allele size found in germline DNA.

The size of PCR products and the corresponding fluorescent labels were chosen so as to allow simultaneous analysis of normal sized alleles with the smaller sized alleles containing deletions that are typically seen in MSI-H tumours (Fig. 1b). In addition to the smaller alleles most MSI-H primary tumours also showed normal sized alleles that presumably originate from contaminating non-cancer cells. These were absent in the homozygous mutant MSI-H cell line shown in Figure 1c.

The most common allelic sizes for BAT-25, BAT-26, NR21, NR22 and NR24 were 124, 120, 103, 142 and 132 bp respectively, although for each repeat, a slight variation in the position of the peaks representing the size of the PCR product was observed (Figure 2).

Legend of Figure 2:

▨ = BAT-25

■ = BAT-26

▩ = NR21

▩ = NR22

▩ = NR24

In order to account for these variations, for BAT-25, NR21, NR22 and NR24 alleles of ≥ 3 pb and for BAT-26 allelic sizes of ≥ 4 pb were considered to be polymorphisms or somatic alterations.

Polymorphisms in Germline DNA

As shown in Table IIa below, each marker was at least 95% monomorphic in 128 germline DNA samples from unrelated Caucasians (CEPH samples).

Table IIa

| | | BAT-26 | BAT-25 | NR21 | NR22 | NR24 |
|---|---|---|---|---|---|---|
| germline Caucasian | germline DNA | 128/128 (100%) | 126/128 (98.4%) | 122/128 (95.3%) | 128/128 (100%) | 128/128 (100%) |
| germline African | germline DNA | 51/56 (91.1%) | 44/56 (78.6%) | 54/56 (96.1%) | 56/56 (100%) | 52/56 (92.9%) |

Furthermore, 121 (94.5%) of this population was monomorphic in all five repeats and the remaining 7 individuals (5.5%) were monomorphic in 4/5 markers. No CEPH DNA sample contained a polymorphism in more than 1 of the 5 repeats. Polymorphisms were more common in African germline DNA, with BAT 25 having the highest level of polymorphism at 21.4%. Although interestingly, of 56 African germline DNA samples tested, 37 (66.1%) were monomorphic in all five repeats, 15 (26.8%) showed a polymorphism in 1/5 markers and 4 (7.1%) in 2/5 markers. None of the germline DNA samples were polymorphic in > 2/5 markers. These data are also shown in Figure 3.

Identification of MSI-H tumours

Using the above criteria the average deletion observed for each mononucleotide repeat was calculated in MSI-H gastric and colon tumours. The sensitivity, specificity and the average deletions of the five markers in different types of DNA are shown in Table IIb below.

Table II

| | | BAT-26 | BAT-25 | NR21 | NR22 | NR24 |
|---|---|---|---|---|---|---|
| Colon | average deletion | 11.9 | 7.3 | 6.8 | 5.1 | 5.6 |
| | MSI-H (sensitivity) | 77/77 (100%) | 72/72 (100%) | 78/78 (100%) | 77/79 (97.5%) | 72/75 (96%) |
| | MSS (specificity) | 42/43 (97.7%) | 44/44 (100%) | 43/44 (97.7%) | 45/45 (100%) | 43/44 (97.7%) |
| Colon Cell Line | average deletion | 11 | 7.8 | 7.4 | 4.4 | 7.6 |
| | MSI-H (sensitivity) | 4/4 (100%) | 5/5 (100%) | 5/5 (100%) | 5/5 (100%) | 5/5 (100%) |
| | MSS (specificity) | 11/11 (100%) | 11/11 (100%) | 11/11 (100%) | 11/11 (100%) | 10/10 (100%) |
| Gastric | average deletion | 12 | 7.2 | 6.8 | 4.9 | 5 |
| | MSI-H (sensitivity) | 39/39 (100%) | 39/39 (100%) | 37/39 (94.9%) | 38/39 (97.4%) | 26/30 (86.7%) |
| | MSS (specificity) | 8/11 (72.7%) | 10/11 (90.9%) | 10/10 (100%) | 10/11 (90.9%) | 11/11 (100%) |
| Endometrial | average deletion | 5.9 | 4.6 | 3.9 | 2.9 | 2.4 |
| | MSI-H (sensitivity) | 15/17 (88.2%) | 18/19 (94.7%) | 17/19 (89.5%) | 14/19 (73.7%) | 10/16 (62.5%) |

For BAT-26, the average deletion was almost 12 bp, or approximately twice the average length of deletion seen with the other markers. Each mononucleotide repeat was deleted in MSI-H tumours with a sensitivity >86%. Allelic shifts due to polymorphisms or somatic mutation were infrequent in non-MSI tumours, resulting in a high degree of specificity for the detection of MSI by each of these markers, with the exception of BAT-26 whose specificity was lowered due to the previous misclassification of 6 tumours as discussed below.

A total of 104 colon and gastric tumours and cell lines which were previously identified as MSI-H showed amplification data for all five markers. Tumours showed deletions in either all (88 tumours) or 4/5 (9 tumours) mononucleotide repeats (Figure 3). Only one sample showed deletions in 3/5 markers. In 5 cases, previously defined as MSI-H, size alterations were found in only Bat-26 or Bat-25; these samples were considered as misclassified due to ethnic polymorphisms (4 cases) or borderline shortening (1 case). Finally, an additional tumour sample was previously classified as MSI-H using dinucleotide repeats but not BAT-26 (HOANG *et al.*, Cancer Res., 59(1), 300-303, 1997). This sample was monomorphic for all five mononucleotide repeats used in this study, suggesting that it was misclassified with

dinucleotide repeats, possibly due to the fact that germline DNA did not match with tumour DNA. In Table II and Figure 3, these 6 samples were considered as MSS.

Of 55 colon and gastric tumours and cell lines previously classified as MSS and containing data for all 5 repeats, 53 (96.4%) were monomorphic at all 5 markers. One tumour was monomorphic at 4 markers (2%) and 1 tumour was monomorphic at 3/5 (2%) repeats. None of the 55 MSS tumours showed allelic shifts in 3 or more repeats (Figure 3), still complying with the MSI identification criteria of 2/5 repeat polymorphisms described earlier.

These results are illustrated by Figure 3, which shows the percentage of samples with allelic size shifts in the five mononucleotide repeats in Caucasian germline DNA (□), African germline DNA (■), MSS tumours (▩), MSI-H colorectal tumours (▨) and MSI-H gastric tumours (▧).

The three mononucleotide repeats NR21, NR22, and NR24 are quasimonomorphic in germline DNA and, similar to BAT-25 and BAT-26, are highly sensitive to somatic deletion in MSI-H tumours. Distinction of MSI-H from MSS tumours is unambiguous when these three new markers are used in conjunction with BAT-25 and BAT-26. Multiplex PCR of these 5 markers has the additional advantage of avoiding the need for simultaneous analysis of corresponding germline DNA from each patient.

Although the quasimonomorphic nature of the three new mononucleotide repeats remains to be fully established in different populations, none of 128 Caucasian and 56 African germline DNA cases had polymorphisms in more than 2 of the repeats. Since all 98 true MSI-H tumours examined here, with successful amplification of the 5 markers, showed deletions in at least 3 markers, the probability of misinterpretation of an MSI result because of polymorphisms in 3 or more of the 5 markers is statistically insignificant. Therefore when the results from all five repeats were analysed together, the MSI status of this entire 159 tumour series was determined unambiguously with 100% sensitivity and specificity.

Moreover, this was achieved without the need to analyse corresponding germline DNA.

Table 3 below indicates tumours where polymorphism or borderline deletion on BAT-26 or BAT-25 would have misclassified the MSI status of the corresponding tumour. In all cases, the use of the multiplex panel allowed to unambiguously classify the tumour.

Table III: MSI-H tumours reclassified as MSS with the pentaplex PCR

| MSS | BAT-26 | BAT-25 | NR21 | NR22 | NR24 |
|---|---|---|---|---|---|
| Polymorphisms | 11 | 2 | 0 | 0 | 0 |
| | 11 | 0 | 0 | 0 | 0 |
| | 11 | 0 | 0 | 0 | 0 |
| | 12 | 0 | 0 | 0 | 0 |
| Borderline deletions | 0 | 4 | 0 | 0 | 0 |
| Unstable only in dinucleotide repeats | 0 | 0 | 0 | 0 | 0 |

In a further 31 DNA samples from colon and gastric tumours, only four out of the five markers were correctly amplified. This was probably due to the quality of DNA extracted from formalin-fixed and paraffin-embedded tissues. Twenty four of these cases showed 4/4 or 3/4 unstable loci and were correctly identified as MSI-H, the remaining 7 cases showing 0/4 or 1/4 unstable loci were correctly identified as MSS (results not shown, but included in Table II). Therefore, incomplete amplification of the five repeats could still be used effectively to identify the MSI status of difficult DNA.

We have previously observed that endometrial MSI-H tumours show significant quantitative and qualitative differences in instability compared to gastro-intestinal MSI-H tumours (DUVAL *et al.*, Cancer Res., 62, 1609-1612, 2002). In the present study, 20 endometrial tumours known to be MSI-H were also tested with the same fluorescent pentaplex assay. These tumours showed significantly shorter average lengths of deletions in all five mononucleotide repeats markers compared to MSI-H gastro-intestinal tumours (Table II). Using the MSI detection criteria established with this microsatellite panel, 17/20 endometrial tumours were identified as MSI-H and 1 tumour was identified as MSS (data not shown). It was not possible to conclusively identify the

MSI status of the remaining 2 tumours due to the very small allelic shifts observed in all five markers.

Thus, it was possible to effectively identify the MSI status of all 190 colon and gastric tumours and cell lines tested in this experiment, including six samples which were previously misclassified. Because of the smaller size of allelic shifts found in MSI-H endometrial tumours, we recommend the continued analysis of matching germline DNA for routine MSI screening of this cancer type.

Accumulating evidence suggests that MSI status defines a subset of colorectal cancers with distinctive biological and clinical properties, emphasizing the importance of simple and accurate markers for detection. This set of five mononucleotide markers, determines the MSI status of tumours with higher sensitivity and specificity than BAT-25 and BAT-26 alone, and is technically simpler to use than the panel recommended at the Bethesda consensus meeting.

CLAIMS

1) A method for evaluating the microsatellite instability associated with a tumour, by amplification of microsatellite loci in a biological sample comprising genomic DNA from said tumour and determination of the sizes of the DNA amplification products, characterized in that said method comprises the amplification of at least one microsatellite locus selected among NR21, NR22, NR24 and NR27.

2) The method of claim 1, characterized in that it comprises the amplification of the two microsatellite loci NR21 and NR24, and the amplification of a third microsatellite locus selected among NR22 and NR27.

3) The method of any of claims 1 or 2, characterized in that it further comprises the amplification of at least one mononucleotide repeat locus selected among BAT-25 and BAT-26.

4) The method of claim 3, characterized in that it comprises the amplification of the four microsatellite loci BAT-25, BAT-26, NR21, NR24, and the amplification of a fifth microsatellite locus selected among NR22 and NR27.

5) A pair of primers suitable for the amplification of a microsatellite locus selected among NR21, NR22, NR24 and NR27.

6) A pair of primers of claim 5 selected among:

- a pair of primers suitable for the amplification of NR21, consisting of the oligonucleotides SEQ ID NO: 4 and SEQ ID NO: 5;
- a pair of primers suitable for the amplification of NR21, consisting of the oligonucleotides SEQ ID NO: 17 and SEQ ID NO: 18;
- a pair of primers suitable for the amplification of NR22, consisting of the oligonucleotides SEQ ID NO: 6 and SEQ ID NO: 7;
- a pair of primers suitable for the amplification of NR24, consisting of the oligonucleotides SEQ ID NO: 8 and SEQ ID NO: 9;

- a pair of primers suitable for the amplification of NR24, consisting of the oligonucleotides SEQ ID NO: 19 and SEQ ID NO: 9;

- a pair of primers suitable for the amplification of NR27, consisting of the oligonucleotides SEQ ID NO: 20 and SEQ ID NO: 21.

7) A kit for the analysis of microsatellite instability, characterized in that it comprises at least a pair of primers of any of claims 5 or 6 suitable for the amplification of NR21, and a pair of primers of any of claims 5 or 6 suitable for the amplification of NR24.

8) A kit of Claim 7, characterized in that it further comprises a pair of primers of any of claims 5 or 6 selected among:

- a pair of primers suitable for the amplification of NR22 ;

- a pair of primers suitable for the amplification of NR27.

9) A kit of any of claims claim 7 or 8, characterized in that it further comprises at least one pair of primers selected among:

- a pair of primers suitable for the amplification of the microsatellite locus BAT-25;

- a pair of primers suitable for the amplification of the microsatellite locus BAT-26.

10) A kit of claim 9, characterized in that:

- the pair of primers suitable for the amplification of BAT-25, is selected among a pair of primers consisting of the oligonucleotides SEQ ID NO: 12 and SEQ ID NO: 13, and a pair of primers consisting of the oligonucleotides SEQ ID NO: 22 and SEQ ID NO: 13;

- the pair of primers suitable for the amplification of BAT-26, is selected among a pair of primers consisting of the oligonucleotides SEQ ID NO: 14 and SEQ ID NO: 15, and a pair of primers consisting of the oligonucleotides SEQ ID NO: 23 and SEQ ID NO: 15.

11) A method for diagnosis of the MSI-H phenotype of a tumour, wherein said method comprises evaluating the microsatellite instability according to any of claims 1 to 4.

12) The method of claim 11 wherein said tumour is a tumour of the gastrointestinal tract.

13) The method of claim 12, wherein said tumour is a colorectal or gastric tumour.

14) The method of claim 11, wherein said tumour is a tumour of the endometrium.

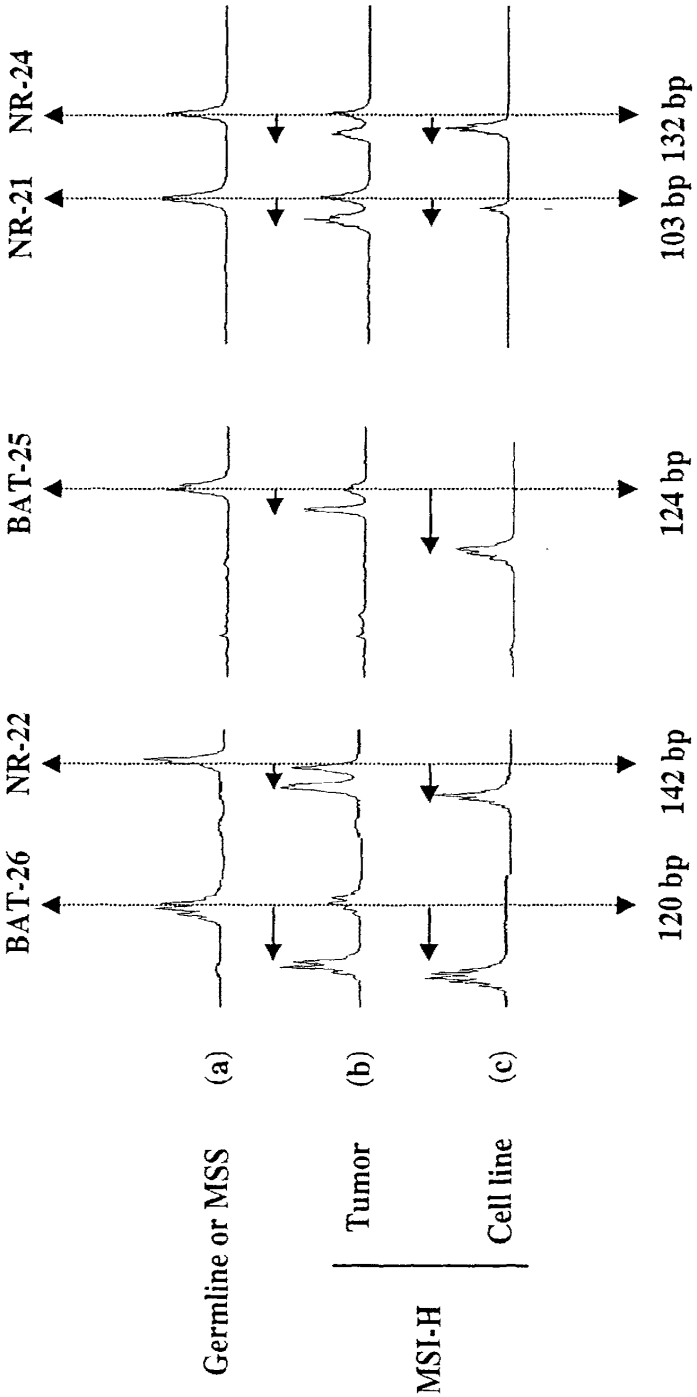

BAT-26
NR-22
BAT-25
NR-21
NR-24
120 bp
142 bp
124 bp
103 bp
132 bp
(a)
(b)
(c)
Germline or MSS
MSI-H
Tumor
Cell line

Figure 1

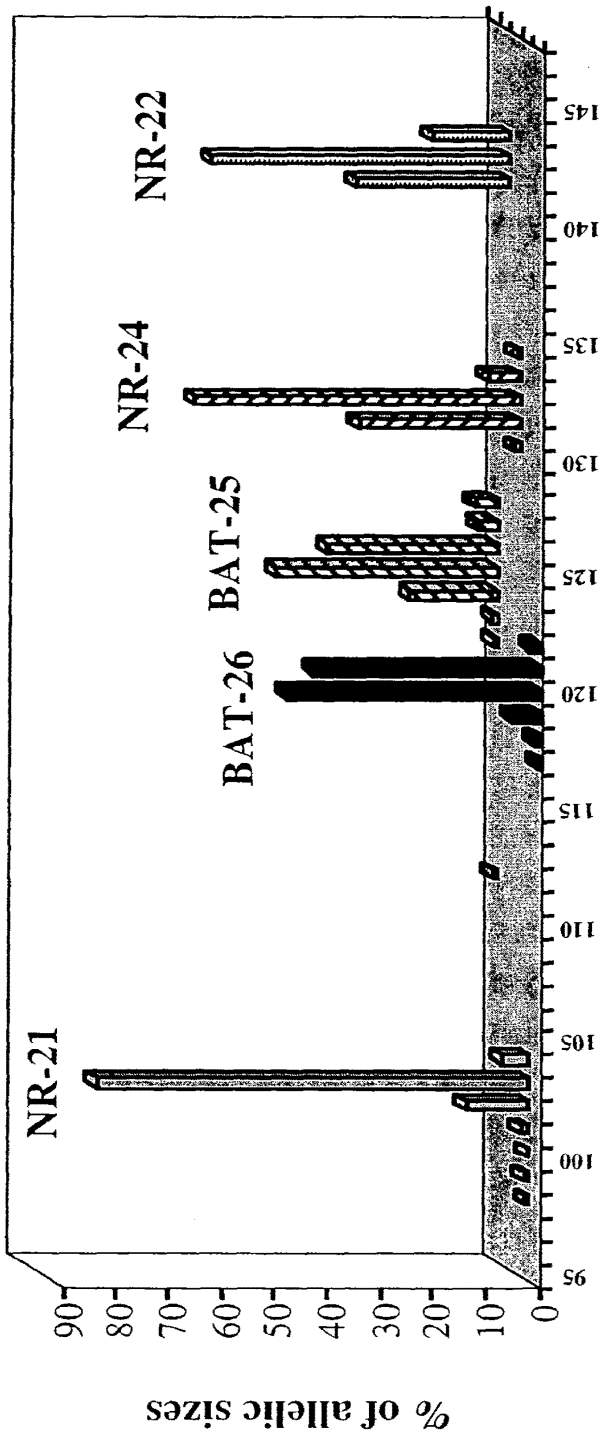

NR-21
BAT-26
BAT-25
NR-24
NR-22
% of allelic sizes
0
10
20
30
40
50
60
70
80
90
95
100
105
110
115
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125
130
135
140
145
Size in bp

Figure 2

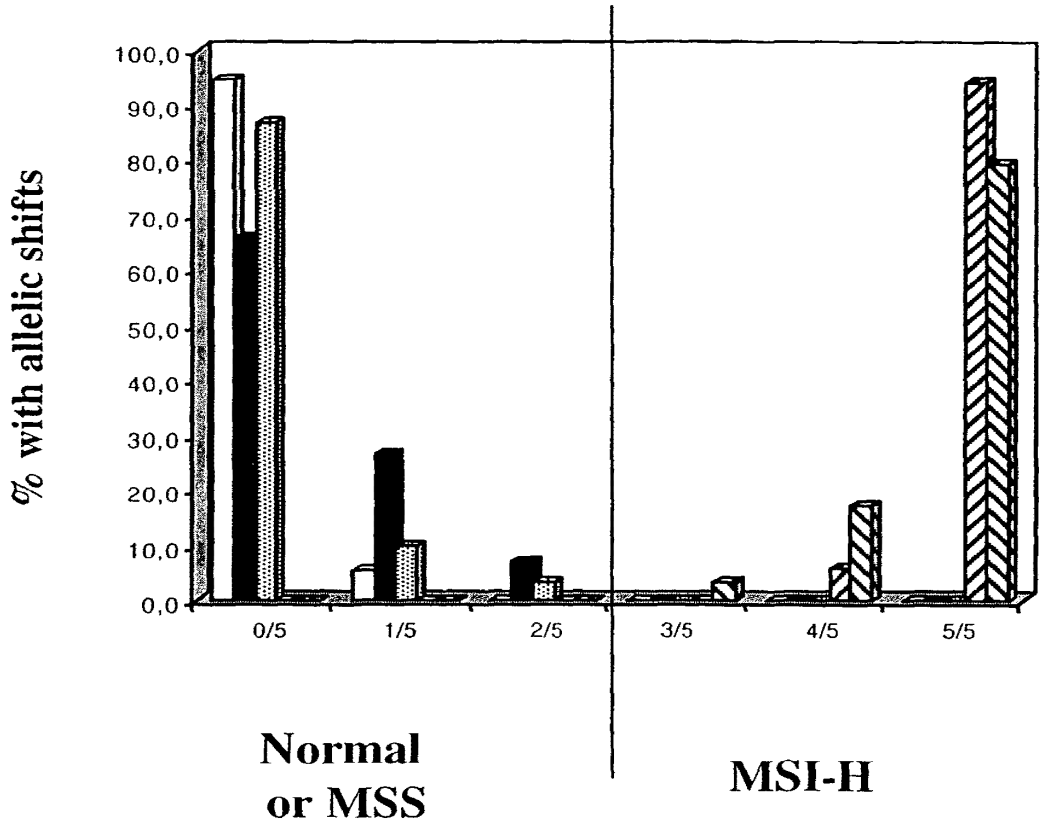

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50,0
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20,0
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1/5
2/5
3/5
4/5
5/5
Normal or MSS
MSI-H

Figure 3

SEQUENCE LISTING

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HAMELIN, Richard
SURAWEERA, Nirosha

<120> MONONUCLEOTIDE REPEATS MICROSATELLITE MARKERS FOR DETECTING MICROSATELLITE INSTABILITY

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<400> 23
ctgcggtaat caagttttta g 21

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(54) Title: PRIMERS FOR NUCLEIC ACID AMPLIFICATION AND METHOD OF EXAMINING COLON CANCER USING THE SAME

(54) 発明の名称: 核酸増幅用プライマー及びこれを用いた大腸癌の検査方法

(57) Abstract: It is intended to provide a method whereby the presence or absence of methylation in a CpG region of a gene, in particular, a CpG region in a promoter region of a gene encoding hMLH1 can be conveniently discriminated; primers to be used in this method; a primer set; an examination reagent; and an examination reagent kit. A method comprising using at least two types of primers including a primer specifically recognizing methylated cytosine in a CpG region in a gene (DNA) to be analyzed such as hMLH1 gene and a primer binding to a region in the target gene regardless of the presence or absence of the methylation and forming an amplification product through the nucleic acid amplification in the region including the CpG region as described above, with another primer non-specific to methylation whereby the region including the CpG region as described above can be amplified together with these two types of primers.

(57) 要約: 本発明は、遺伝子中のＣｐＧ領域、特にｈＭＬＨ１をコードする遺伝子のプロモーター領域中のＣｐＧ領域のメチル化の有無を簡便に識別できる方法、この方法に用いるためのプライマー、プライマーセット、検査用試薬及び検査用試薬キットを提供する。 本発明の方法は、ｈＭＬＨ１遺伝子のような解析対象遺伝子（ＤＮＡ）中のいずれかのＣｐＧ領域のメチル化されたシトシンを特異的に認識するプライマーと、前記メチル化の有無によらず目的遺伝子中のいずれかの領域に結合し、前記いずれかのＣｐＧ領域の存在する領域で核酸増幅により増幅産物を形成するプライマーの少なくとも２種のプライマーを、これらの２種のプライマーと一緒になって前記ＣｐＧ領域を含む領域を増幅することができる別のメチル化非特異的プライマーとともに用いる。

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# 明 細 書

## 核酸増幅用プライマー及びこれを用いた大腸癌の検査方法

## 技術分野

[0001] 本発明は、遺伝子のCpG領域のメチル化の検出方法及びそれを利用した方法、これらの方法に使用するためのプライマー、プライマーセット、検査用試薬及び検査用試薬キットに関する。特に、本発明は、大腸癌の臨床検査の分野において、散発性大腸癌と遺伝性非腺腫性大腸癌とを識別(スクリーニング)する方法、これに用いるためのプライマー、プライマーセット、検査用試薬及び検査用試薬キットに関する。

## 背景技術

[0002] ヒトの大腸癌は、散発性(非遺伝性)大腸癌と遺伝性大腸癌の2種類に大別される。そして、遺伝性大腸癌には、家族性腺腫性大腸癌(以下適宜「FAP」という。)と遺伝性非腺腫性大腸癌(以下「HNPCC」という。)の2種類が存在する。

[0003] FAPはAPC遺伝子の異常によるものであることが分かっており、多量のポリープを産生するという形質的特徴により診断は比較的容易である。

[0004] 一方、HNPCCはFAPのような形質的特徴を示さないため、その診断は困難であった。これまでは、HNPCCの診断は、専ら家族歴を追跡することにより行われていたが、依然として信頼できる診断基準(diagnostic criteria)が確立されていない。

[0005] 1993年にHNPCCの原因が、DNAミスマッチ修復タンパク質(mismatch repair protein; MMR proteins)をコードする遺伝子群(MMR genes; 以下適宜「MMR遺伝子」という。)に変異が起こって、その遺伝子修復タンパク質が機能しなくなり、その結果、ヒトの遺伝子修復機構が機能しなくなることであることが分かった。具体的には、HNPCCの病理検体においては、hMLH1、hMSH2、hMSH6、hPMS1、hPMS2の5種のMMRタンパク質をコードするMMR遺伝子のいずれか1つ以上に生殖細胞変異が認められ、通常は、前3種のタンパク質をコードする遺伝子のいずれか1つ以上に変異が認められる。このうち、hMSH2をコードする遺伝子(以下適宜「hMSH2遺伝子」という。)とhMLH1をコードする遺伝子(以下適宜「hMLH1遺伝子」という。)の2つの遺伝子の変異が主因であることが明らかになった。

[0006] また、免疫染色によるタンパク発現の検査において、この3種のうちhMSH2とhMSH6のタンパク質発現が陰性である場合は、まず、間違いなくHNPCCであることが分かっている。

[0007] 一方、hMLH1タンパク質の異常は、HNPCCのみでなく、散発性(非遺伝性)大腸癌の場合にも認められ、この両者をhMLH1タンパク質の免疫染色によって識別することは困難である。もし識別するとするならば、hMLH1タンパク質の免疫染色のみでなく、hMLH1タンパク質をコードする遺伝子領域すべてをシーケンスするといった、hMLH1遺伝子の突然変異の確認を行わなければならない。しかし、現在のところ、HNPCCと臨床診断された患者のうち、遺伝子突然変異解析で遺伝子突然変異を検出できるのは約50%程度であり、残りについては原因の変異が検出できない。また、この検出法には高い検出費用もかかる。HNPCCは遺伝性疾患であり、その診断には社会的・倫理的・法的に注意を必要とする。そのため、効率良く、低コストで、かつ、精度の高い検出方法が求められている。

[0008] 近年、hMLH1遺伝子等のMMR遺伝子の変異は、DNA中のマイクロサテライト(microsatellite)反復配列の異常と関連していることが分かってきた。即ち、ある種の癌細胞においてはCAの繰り返し配列等に代表されるマイクロサテライトの反復回数が、正常細胞のそれに比べて多くなったり、少なくなったりしている。こうしたDNA内の塩基配列の異常をマイクロサテライト不安定性(microsatellite instability, MSI+, MSI−H)と呼ぶ。一方、マイクロサテライト反復配列の異常を示さないことをマイクロサテライト安定性(microsatellite stability, MSS, MSI−, MSI−L)という。

[0009] このMSI+はIINPCCに認められる特徴である。一方、IINPCCとは関係のない散発性の大腸癌、胃癌などの多くの腫瘍でMSI+の性質を示す例が12ー30%の頻度で存在することが明らかになった(以下においてMSI+を示す散発性大腸癌を適宜「散発性MSI+大腸癌」という)。これら非遺伝性のMSI+腫瘍においては、hMLH1やhMSH2遺伝子の突然変異の頻度が非常に低く、別の機序が考えられたが、近年、これらの腫瘍では75ー90%以上の頻度でhMLH1遺伝子のプロモーター領域にメチル化が起こり、hMLH1タンパク質の発現が消失していることが明らか

になった。

[0010] また、散発性MSI＋大腸癌細胞株においては、hMLH1をコードする遺伝子のプロモーター領域内にある、hMLH1を発現させるタンパク質であるCBP結合因子（CBP binding factor）であるCCAAT boxの上流のCpG領域のメチル化が多く見られることが分かった。一方、HNPCCの細胞株においては、この領域のメチル化は、低い割合で起こっていることも分かってきた。

[0011] さらに、大腸癌の約10ー20％を占めるCIMP（CpG Island Methylator Phenotype）癌においては、hMLH1をコードする遺伝子を含む特定の遺伝子又は遺伝子座（例えば、MINT2、MINT31、及びp16）において高頻度のメチル化が観察されることもわかっている。

[0012] また、hMLH1をコードする遺伝子のプロモーター領域内にあるCpG領域の部位によっては正常細胞にもメチル化が存在することも分かってきた。

[0013] 一般に、遺伝子のメチル化を検出する方法としては、MSP法（WO97／46705、特表2000−511776）、MCA法（WO00／26401、特表2002−533061）、COBRA法などが存在する。しかし、MCA法は、メチル化感受性制限酵素で切断し、末端にリンカーを付加した遺伝子断片をランダムに増幅するものであり、「未知の」メチル化されている遺伝子を探索する目的に適しているが、特定の解析対象遺伝子について行うには不向きである。また、COBRA法は、制限酵素による切断の工程を必要とし、手間及びコストがかかり、使用する制限酵素の認識部位のみしか調べることができず、そのため、メチル化の有無を検討したいCpG領域を必ずしも解析できないという欠点を持つ。MSP法は、制限酵素を用いないが、メチル化の解析を行う遺伝子領域ひとつにつき、メチル化特異的プライマーのみを用いたPCRと非メチル化特異的プライマーのみを用いたPCRという2回のPCRを必要とする。また、そのメチル化の有無の判定は、そのPCR産物の有無によって行うため、PCR自体のエラーを確認できないという欠点を持つ。

## 発明の開示

[0014] 本発明者らは、そこで、hMLH1をコードする遺伝子のプロモーター領域のCpG領域のメチル化の有無、特にCCAAT box上流のCpG領域のメチル化の有無を簡便

に検出できる方法があれば、そのメチル化を検出することにより、散発性MSI＋大腸癌を識別することが可能と考え、また、散発性MSI＋大腸癌を除外することによりHNPCCを効率よく識別することが可能と考えた。

[0015] そこで、本発明の目的は、制限酵素による切断の工程を必要とせず、遺伝子のメチル化状態、特にhMLH1をコードする遺伝子のプロモーター領域中のCpG領域のメチル化の有無を簡便に識別できる方法を提供することである。さらに、この検査方法に用いるためのプライマー、プライマーセット、検査用試薬及び検査用試薬キットを提供することである。

[0016] かかる実情において、本発明者らは、hMLH1をコードする遺伝子（DNA）のプロモーター領域中のいずれかのCpG領域のメチル化されたシトシンを特異的に認識するプライマーと、前記メチル化の有無によらずhMLH1をコードする遺伝子のプロモーター領域中のいずれかの領域（前記いずれかのCpG領域以外の領域が好ましい。）に結合し、前記いずれかのCpG領域の存在する領域で核酸増幅によりオリゴヌクレオチドを形成するプライマーの少なくとも2種のプライマーとを用いれば、前記CpG領域のメチル化の有無を識別でき、これにより、MSI＋大腸癌であるか、MSI−大腸癌であるかを精度よく識別することができることを見出した。そして、この検出方法は、他のHNPCCの遺伝子診断よりも簡便で、かつ、低コストである。

[0017] また、検出原理は基本的にあらゆる遺伝子に適応可能であって、メチル化状態を調べたい解析対象遺伝子は、何であってもよい。さらに、本発明の方法は、他の一般的な遺伝子のメチル化状態検出方法と異なり、制限酵素での切断の工程を含まないので、それらの方法と比較しても迅速・簡便であり、臨床検査において特に有利である。即ち、本発明の方法によれば、従来の検出技術の欠点をすべて補うことが可能であり、核酸増幅は1回のみでよく、メチル化の有無を検討したい任意のCpG領域の検討が可能であり、増幅過程自体のエラーはメチル化非特異的プライマーによるPCR産物によって確認することが可能である。COBRA法のように制限酵素を用いないため、手間及びコストもかからない。また、MSP法よりも核酸増幅の回数が少なく、増幅過程自体のエラーの確認も可能なため、簡便かつ確実である。

[0018] また、この方法に、マイクロサテライト反復配列分析（以下適宜「MSI分析」という。）

を組み合わせることにより、散発性MSI＋大腸癌であるか、HNPCCであるかをより精度よく識別することができることを見出した。

[0019] その上にまた、DNAミスマッチ修復タンパク質の免疫染色分析（immunohistochemical analysis；以下適宜「IHC」という。）を組み合わせることにより、散発性MSI＋大腸癌であるか、HNPCCであるかを精度よく識別することができることを見出した。

[0020] また、前記プライマーを用いた検査用試薬及び検査用試薬キットと合わせて本発明を完成した。

[0021] 即ち、（1）本発明の第1の態様は、以下の1）ー10）のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択する核酸増幅用プライマーである：

1）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

2）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

3）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

4）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

5）配列番号2ー7に表される塩基配列からなるオリゴヌクレオチド；

6）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

7）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

8）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

9）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；及び

10）前記1）から9）のいずれか1に記載のオリゴヌクレオチドの塩基配列中の1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列からなり、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[0022] （2）本発明の第2の態様は、以下の1）ー10）のいずれか1に記載のオリゴヌクレオチドのうち2つ以上を選択する核酸増幅用プライマーセットである：

1）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

2）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

3）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

4）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

5）配列番号2ー7に表される塩基配列からなるオリゴヌクレオチド；

6）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

7）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

8）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

9）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

10）前記1）から9）のいずれか1に記載のオリゴヌクレオチドのうち1又は複数のオリゴヌクレオチドについて、1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列を含み、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[0023] （3）本発明の第3の態様は、組織中のhMLH1をコードする遺伝子のプロモーター領域の中にある5'領域又はCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を識別するために用いられるプライマーセットであって、少なくとも1つのメチル化シトシン非特異的プライマーの融解温度が、メチル化シトシン特異的プライ

マーの融解温度より1ー5℃高いことを特徴とする前記(2)記載の核酸増幅用プライマーセットである。

[0024] (4)本発明の第4の態様は、組織中のhMLH1をコードする遺伝子のプロモーター領域の中にあるCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を識別するために用いられるプライマーセットであって、プライマー同士のGC含有率の差が20%以内であることを特徴とする前記の(2)又は(3)記載の核酸増幅用プライマーセットである。

[0025] (5)本発明の第5の態様は、配列番号3に記載のオリゴヌクレオチド及び配列番号4に記載のオリゴヌクレオチドを選択することを特徴とする前記(2)又は(3)記載の核酸増幅用プライマーセットである。

(6)本発明の第6の態様は、配列番号2に記載のオリゴヌクレオチド及び配列番号4に記載のオリゴヌクレオチドを選択することを特徴とする前記(2)又は(3)記載の核酸増幅用プライマーセットである。

[0026] (7)本発明の第7の態様は、配列番号6に記載のオリゴヌクレオチド及び配列番号7に記載のオリゴヌクレオチドを選択することを特徴とする前記(2)から(4)のいずれか1項記載の核酸増幅用プライマーセットである。

(8)本発明の第8の態様は、配列番号5に記載のオリゴヌクレオチド及び配列番号7に記載のオリゴヌクレオチドを選択することを特徴とする前記(2)から(4)のいずれか1項記載の核酸増幅用プライマーセットである。

[0027] (9)本発明の第9の態様は、前記(1)記載の核酸増幅用プライマーを使用し、hMLH1をコードする遺伝子のプロモーター領域におけるCpG領域のシトシンのメチル化の有無を検査することを特徴とする散発性大腸癌と遺伝性非腺腫大腸癌の識別方法である。

(10)本発明の第10の態様は、組織中のhMLH1をコードする遺伝子のプロモーター領域の中にある5'領域又はCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を指標とすることを特徴とする散発性大腸癌と遺伝性非腺腫大腸癌の識別方法である。

[0028] (11)本発明の第11の態様は、前記シトシンのメチル化の有無の検査を核酸増幅

方法により行う前記(9)又は(10)に記載の識別方法である。

(12)本発明の第12の態様は、マイクロサテライト反復配列分析を組み合わせたことを特徴とする前記(9)から(11)のいずれか1項に記載の識別方法である。

(13)本発明の第13の態様は、DNAミスマッチ修復タンパク質の免疫染色分析を組み合わせたことを特徴とする前記(9)から(12)のいずれか1項に記載の識別方法である。

[0029] (14)本発明の第14の態様は、前記(1)記載のプライマーを含む検査用試薬である。

(15)本発明の第15の態様は、前記(1)記載のプライマーを含む検査用試薬キットである。

[0030] (16)本発明の第16の態様は、以下の1)ー9)のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択する核酸増幅用プライマーであって、解析対象遺伝子のCpG領域中のシトシンのメチル化の有無を検出するためのプライマーである:

1)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

2)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

3)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

4)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

5)前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

6）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

7）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

8）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；及び

9）前記1）から8）のいずれか1に記載のオリゴヌクレオチドの塩基配列中の1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列からなり、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[0031] （17）本発明の第17の態様は、前記（16）記載のプライマーを少なくとも3つ含む解析対象遺伝子中のCpG領域のメチル化の有無を検出するための核酸増幅用プライマーセットであって、この少なくとも3つのプライマーが、

a）前記解析対象遺伝子中の少なくとも1つの検出対象CpG領域を含む部分が増幅されるように選択され、かつCpG領域中のシトシンが前記修飾剤による修飾の後にシトシンのままであっても、ウラシル又はチミンに転換されても、前記解析対象遺伝子に結合する、1対のメチル化非特異的プライマー、及び

b）前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にシトシンである場合にのみ前記解析対象遺伝子に結合し、前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にウラシル又はチミンに変換されれば前記解析対象遺伝子に結合しない、少なくとも1つのメチル化シトシン特異的プライマー

である、プライマーセットである。

[0032] （18）本発明の第18の態様は、解析対象遺伝子のCpG領域中のシトシンのメチル化を検出する方法であって、

（1）試料由来の核酸を修飾剤によって修飾し、

(2)前記核酸を鋳型として、以下のプライマー:

a)前記解析対象遺伝子中の少なくとも1つの検出対象CpG領域を含む部分が増幅されるように選択され、かつCpG領域中のシトシンが前記修飾剤による修飾の後にシトシンのままであっても、ウラシル又はチミンに転換されても、前記解析対象遺伝子に結合する、1対のメチル化非特異的プライマー、及び

b)前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にシトシンである場合にのみ前記解析対象遺伝子に結合し、前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にウラシル又はチミンに変換されれば前記解析対象遺伝子に結合しない、少なくとも1つのメチル化シトシン特異的プライマー

を用いて前記解析対象遺伝子核酸を増幅し、

(3)前記増幅産物を検出する

工程を含むことを特徴とする方法である。

[0033] (19)本発明の第19の態様は、前記メチル化シトシン特異的プライマー及び前記メチル化シトシン非特異的プライマーが、それぞれ以下の1)ー9)のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択するプライマーである、前記(18)記載の方法である:

1)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

2)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

3)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

4)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

5）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

6）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

7）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

8）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；及び

9）前記1）から8）のいずれか1に記載のオリゴヌクレオチドの塩基配列中の1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列からなり、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[0034] （20）本発明の第20の態様は、前記解析対象遺伝子が、hMLH1、hMSH2、hMSH6、hPMS1、hPMS2を含むDNAミスマッチ修復タンパク質をコードする遺伝子群及びその他の大腸癌関連遺伝子群から選択される、前記（18）記載の方法である。

[0035] （21）本発明の第21の態様は、前記解析対象遺伝子における検出対象CpG領域におけるシトシンのメチルの有無を、試料の由来する個体における大腸癌の存在又は分類と関連づけるための、前記（18）記載の方法である。

[0036] （22）本発明の第22の態様は、前記検出対象CpG領域が2ヵ所以上であり、前記b）のメチル化シトシン特異的プライマーを2種類以上用いる、前記（18）記載の方法である。

## 図面の簡単な説明

[0037] [図1]は、hMLH1をコードする遺伝子のプロモーター領域においてメチル化された

シトシンの有無を示す塩基配列の例である。

[図2]は、本発明の検査方法の概要を示す模式図である。

[図3]は、hMLH1の免疫染色の実施例である。

[図4]は、本発明の実施に係る核酸増幅の産物の例を示す写真図である。

## 発明を実施するための最良の形態

[0038] 以下、hMLH1遺伝子を解析対象遺伝子とした場合について詳細に説明するが、本発明は任意の遺伝子に対して適用可能であり、本発明における解析対象遺伝子はhMLH1遺伝子に限定されない。

(検出原理)

核酸を修飾剤によって修飾すると、メチル化されている塩基は修飾されない。

[0039] 例えば、hMLH1をコードする遺伝子(一本鎖DNA)のプロモーター領域中のCpG領域においては、メチル化されたシトシンは重亜硫酸修飾によって脱アミノ化されにくく、シトシンのままで存在するとされる。一方、メチル化されていないシトシンは、そのすべて又はその殆んどが重亜硫酸修飾によって一旦ウラシルに転換され、この転換されたウラシルは、これを含む一本鎖DNAを増幅すると、チミンとして複製される。

[0040] そこで、基本的に、いずれかのCpG領域中のシトシンが重亜硫酸修飾の後にシトシンである場合にのみ前記hMLH1をコードする遺伝子に結合し、前記いずれかのCpG領域CpG領域中のシトシンが重亜硫酸修飾の後にウラシルに変換されれば前記hMLH1をコードする遺伝子に結合しなくなるプライマー(メチル化シトシン特異的プライマー、以下適宜「メチル化特異的プライマー」という。)と、前記いずれかのCpG領域CpG領域中のシトシンが重亜硫酸修飾の後にシトシンのままであっても、ウラシル(遺伝子増幅により増幅されたオリゴヌクレオチドにおいては、このウラシルの部位(位置)にはチミンが置かれる。)に転換されても、前記hMLH1をコードする遺伝子に結合し、前記シトシンの存在するCpG領域を含む領域で核酸増幅によりオリゴヌクレオチドを形成するプライマー(メチル化シトシン非特異的プライマー、以下適宜「メチル化非特異的プライマー」という。)との少なくとも2種を、これらの両方の各々と対になって前記CpG領域を含む領域を増幅し得る別のメチル化非特異的プライマーとともに用いて、核酸増幅方法にて増幅した産物を検出すると、前記メチル化されたシト

シンを含む遺伝子に対しては、メチル化特異的プライマーとメチル化非特異的プライマー由来の2種の増幅産物が検出されるが、メチル化されていないシトシンのみを含む遺伝子に対しては、メチル化非特異的プライマー由来の1種の増幅産物のみが検出される。よって、検出される増幅産物の違いに基づいて、前記CpG領域のメチル化の有無を識別することができる。

[0041] 本発明においては、メチル化特異的プライマーであれ、メチル化非特異的プライマーであれ、プライマーが少なくとも1つのCpG領域(そのCpG領域中にメチル化シトシンが存在する)を含むオリゴヌクレオチドを選択する。ここで、「プライマーが少なくとも1つのCpG領域を含むオリゴヌクレオチドを選択する」とは、そのプライマーが核酸中の前記少なくとも1つのCpG領域を含むオリゴヌクレオチドの領域に結合し、前記少なくとも1つのCpG領域を含むオリゴヌクレオチドを含む核酸を鋳型として、核酸増幅により前記CpG領域に対応する領域(一般には前記CpG領域に対して相補的である。)を含む核酸を形成することをいう。したがって、プライマーと、そのプライマーが選択する(即ち結合する)オリゴヌクレオチドとは、基本的に互いに相補的な配列を有する。

[0042] 本発明においては、メチル化特異的プライマーは、検出しようとするメチル化シトシンの存在するCpG領域に結合するように設計される。一方、メチル化非特異的プライマーは、前記メチル化シトシンの存在するCpG領域に結合しないように設計されるが、前記メチル化シトシンの存在するCpG領域に結合するものとなってもよい。

[0043] 本発明においては、配列番号1で表されるhMLH1をコードする遺伝子(一本鎖DNA)のプロモーター領域中で、塩基番号1031から1216の間の領域(以下、この領域を適宜「5'領域」若しくは「5'region」という。)のCpG領域のシトシンのメチル化の有無を識別できるプライマーセットと、塩基番号1369から1600の間の領域(以下この領域を適宜「CCAAT領域」若しくは「CCAAT region」という。)のCpG領域のシトシンのメチル化の有無を識別できるプライマーセットが見出された。特に、CCAAT領域のシトシンのメチル化はhMLH1の発現に深く関与している可能性があるので、後者のプライマーセットは、HNPCCの診断において重要であると考えられる。

[0044] 図1に、散発性大腸癌と診断された試料とHNPCCと診断された試料についてCC

AAT領域において重亜硫酸処理をして核酸増幅した部分の塩基配列を示す。図中、CCAAT boxの上流の矢印で示される位置の塩基は、散発性MSI＋大腸癌の試料では、いずれもシトシンとして検出されているのに対して、HNPCCの試料では、いずれもチミンとして検出されている。即ち、散発性大腸癌の試料では、いずれもこの部位のシトシンがメチル化されているため、重亜硫酸処理の後もシトシンのまま存在し、シトシンとして検出されるのに対して、HNPCCの試料では、いずれもこの部位のシトシンがメチル化されていないため、重亜硫酸処理の後に一旦ウラシルに転換され、核酸増幅の後にチミンとして検出されている。

(プライマーとプライマー用のオリゴヌクレオチド)

本発明においては、メチル化特異的プライマーは、メチル化解析の対象となるhMLH1をコードする遺伝子のプロモーター領域のCpG領域中のシトシンに対応する部位のみがシトシンに対応した塩基であり、前記プロモーター領域中のそれ以外のシトシンに対応する部位は、ウラシル若しくはチミンに対応するように設計される。メチル化特異的プライマーは、擬陽性を低減させるためには、好ましくは2ヵ所以上のCpG領域を含むように設計する。一方、メチル化非特異的プライマーは、hMLH1をコードする遺伝子のプロモーター領域のCpG領域のシトシンがウラシル若しくはチミンに転換された配列、シトシンのままの配列の双方に対応するように設計される。

[0045] 一般に、メチル化特異的PCRでは、プライマー配列と重亜硫酸修飾後のDNA配列とが完全に相補的にアニーリングしたときにのみDNAの増幅が起こり、プライマー配列と重亜硫酸修飾後のDNA配列との間に1塩基以上のミスマッチが存在するときにはDNAの増幅がおこらないようにプライマー及びPCRの反応系を設計する。このことによって、メチル化特異的PCRでは、1塩基のメチル化の有無も識別することができる。

[0046] 本発明においてプライマーとして使用されるオリゴヌクレオチドは、各種の核酸合成反応において必要な特異性を維持しながら相補鎖との塩基対結合を行うことができる。

[0047] 具体的には、プライマーは、以下の1)ー10)のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択する核酸増幅用プライマーである。

1）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、例えば、生体試料から採取した遺伝子の2本鎖DNAのうちの重亜硫酸修飾後による1本鎖DNA由来のものである。メチル化特異的プライマーはこのオリゴヌクレオチドを選択し得るが、このオリゴヌクレオチドを選択するプライマーは、メチル化特異的プライマーに限られず、メチル化非特異的プライマーであってもよい。

2）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、1）で表されるオリゴヌクレオチドを含む核酸を鋳型として、核酸増幅したときに形成される増幅物の有する塩基配列の一部と同じ塩基配列を有する。前記増幅物は、核酸増幅の次の段階で鋳型として用いられる。メチル化特異的プライマーはこのオリゴヌクレオチドを選択し得るが、このオリゴヌクレオチドを選択するプライマーは、メチル化特異的プライマーに限られず、メチル化非特異的プライマーであってもよい。

3）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、生体試料から採取した遺伝子の2本鎖DNAのうちの重亜硫酸修飾後による1本鎖DNA由来のものである。このオリゴヌクレオチドを選択するプライマーはメチル化非特異的プライマーとされる。

4）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、3）で表されるオリゴヌクレオチドを含む核酸を鋳型として、核酸増幅したときに形成される増幅物の有する塩基配列の一部と同じ塩基配列を有する。前記増幅物は、核酸増幅の次の段階で鋳型として用いられる

。このオリゴヌクレオチドを選択するプライマーはメチル化非特異的プライマーとされる。

5）配列番号2ー7に表される塩基配列からなるオリゴヌクレオチド。プライマーが別のプライマーを認識して結合すれば、核酸増幅に好適である。

6）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、例えば、生体試料から採取した遺伝子の2本鎖DNAのうちの1）において示した1本鎖DNAに相補的に結合していた1本鎖（反対鎖）DNA由来のものである。メチル化特異的プライマーはこのオリゴヌクレオチドを選択し得るが、このオリゴヌクレオチドを選択するプライマーは、メチル化特異的プライマーに限られず、メチル化非特異的プライマーであってもよい。

7）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、6）で表されるオリゴヌクレオチドを鋳型として、核酸増幅したときに形成される増幅物の有する塩基配列と同じ塩基配列を有する。前記増幅物は、核酸増幅の次の段階で鋳型として用いられる。メチル化特異的プライマーはこのオリゴヌクレオチドを選択し得るが、このオリゴヌクレオチドを選択するプライマーは、メチル化特異的プライマーに限られず、メチル化非特異的プライマーであってもよい。

8）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、例えば、生体試料から採取した遺伝子の2本鎖DNAのうちの3）において示した1本鎖DNAに相補的に結合していた1本鎖（反対鎖）DNA由来のものである。このオリゴヌクレオチドを選択するプライマーはメチル化非特異的プライマーとされる。

9）配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド。このオリゴヌクレオチドは、8）で表されるオリゴヌクレオチドを鋳型として、核酸増幅したときに形成される増幅物の有する塩基配列と同じ塩基配列を有する。前記増幅物は、核酸増幅の次の段階で鋳型として用いられる。このオリゴヌクレオチドを選択するプライマーはメチル化非特異的プライマーとされる。

10）前記1）から9）のいずれか1に記載のオリゴヌクレオチドのうち1又は複数のオリゴヌクレオチドについて、1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列を含み、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[0048] より具体的には、ある実施態様においては、5'領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化特異的プライマーは、5'領域中の少なくとも1つのCpG領域中のシトシン以外のシトシンをウラシル又はチミンに変換した配列を持ったオリゴヌクレオチドを選択するものである。

[0049] 詳しくは、このプライマーは、5'領域中の前記少なくとも1つのCpG領域中のシトシンは重亜硫酸修飾後もシトシンのままで存在して、5'領域中の前記少なくとも1つのCpG領域中のシトシン以外のシトシンは、重亜硫酸修飾後にウラシルに変換されても（遺伝子増幅により増幅されたオリゴヌクレオチドにおいては、このウラシルの部位にはチミンが置かれる）、変換されずにシトシンのままで存在しても、その配列をもったオリゴヌクレオチドを選択するものである。

[0050] 本実施形態においては、5'領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化特異的プライマーとしては、配列番号3で表されるオリゴヌクレオチドがある。このプライマーは、hMLH1タンパク質をコードする遺伝子の塩基配列1097から1118の部位を認識して、その遺伝子に結合する。

[0051] 5'領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化非特異的プライマーは、5'領域中におけるCpG領域以外の領域に結合する

ように設計されることが好ましい。もし、CpG領域を含む領域に結合するようにせざるを得ない場合には、前記CpG領域中のシトシンのメチル化の有無によらず、前記CpG領域を含む領域に結合するように設計されるものである。

[0052] 詳しくは、このメチル化非特異的プライマーは、5'領域中におけるCpG領域以外の領域に結合するように設計されることが好ましい。もし、CpG領域を含む領域に結合するようにせざるを得ない場合には、前記CpG領域中のシトシンのメチル化の有無に影響を受けないように、すなわち、CpG領域中のシトシンがウラシルに変換された場合、また、シトシンのまま変換されない場合の双方の配列を増幅するように設計されることが好ましい。具体的には、前記CpG領域中のシトシンの部位に対応するメチル化非特異的プライマーの部位に混合塩基、又はイノシン3リン酸(ITP)由来のイノシン(ヒポキサンチン)を配置した塩基配列からなるオリゴヌクレオチドがこの種のメチル化非特異的プライマーである。

[0053] 本実施形態においては、5'領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化非特異的プライマーとしては、配列番号2又は4で表されるオリゴヌクレオチドがある。配列番号2で表されるプライマーは、hMLH1タンパク質をコードする遺伝子の塩基配列1031から1055の部位を認識して、その遺伝子に結合する。また、配列番号4で表されるプライマーは、hMLH1タンパク質をコードする遺伝子の塩基配列1191から1216の部位を認識して、その遺伝子に結合する。

[0054] 上記配列番号3で表されるメチル化特異的プライマーと、配列番号2で表されるメチル化非特異的プライマーと、配列番号4で表されるメチル化非特異的プライマーとの合計3種のプライマーセットを用いれば、5'領域中のCpG領域のシトシンのメチル化の有無を識別できる。

[0055] 即ち、図2の模式図に示されるように、5'領域において、メチル化特異的プライマーである配列番号3で表される5'−MSプライマー(メチル化特異的上流プライマー)と、メチル化非特異的プライマーである配列番号2で表される5'−Sプライマー(メチル化非特異的上流プライマー)と、メチル化非特異的プライマーである配列番号4で表される5'−ASプライマー(メチル化非特異的下流プライマー)の3種のプライマーを用いて、核酸増幅法にて核酸を増幅する。そのとき、このCpG領域のシトシンがメチ

ル化された試料においては、5'－MSプライマーも含めた3種のプライマーがすべて遺伝子に結合する。そして、5'－MSプライマーと5'－ASプライマーから核酸増幅して形成される118塩基のメチル化断片と、5'－Sプライマーと5'－ASプライマーから核酸増幅して形成される184塩基の非メチル化断片の2つのDNA断片が形成される。一方、このCpG領域のシトシンがメチル化されていない試料においては、5'－MSプライマーを除いた2種のプライマーのみが遺伝子に結合するため、結果的に、5'－Sプライマーと5'－ASプライマーから核酸増幅して形成される184塩基の非メチル化断片のみが形成される。よって、検出される増幅産物の違いに基づいて、前記CpG領域のメチル化の有無を識別することができる。

[0056] また、別の実施態様によれば、CCAAT領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化特異的プライマーは、CCAAT領域中の少なくとも1つのCpG領域中のシトシン以外のシトシンをウラシル又はチミンに変換した配列を持ったオリゴヌクレオチドを選択する(即ち、前記オリゴヌクレオチドに結合する)ものである。

[0057] 詳しくは、このプライマーは、CCAAT領域中の前記少なくとも1つのCpG領域中のシトシンは重亜硫酸修飾後もシトシンのままで存在して、CCAAT領域中の前記少なくとも1つのCpG領域中のシトシン以外のシトシンは、重亜硫酸修飾後にウラシルに変換されても(遺伝子増幅により増幅されたオリゴヌクレオチドにおいては、このウラシルの部位にはチミンが置かれる)、変換されずにシトシンのままで存在しても、その配列をもったオリゴヌクレオチドを選択するものである。

[0058] 本実施形態においては、CCAAT領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化特異的プライマーとしては、配列番号6で表されるオリゴヌクレオチドがある。このプライマーは、hMLH1タンパク質をコードする遺伝子の塩基配列1479から1502の部位を認識して、その遺伝子に結合する。

[0059] CCAAT領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化非特異的プライマーは、CCAAT領域中におけるCpG領域以外の領域に結合するように設計されることが好ましい。もし、CpG領域を含む領域を認識してその領域に結合するようにせざるを得ない場合には、前記CpG領域中のシトシン

のメチル化の有無によらず、前記CpG領域を含む領域に結合するように設計されるものである。

[0060] 詳しくは、このメチル化非特異的プライマーは、CCAAT領域中におけるCpG領域以外の領域に結合するように設計されることが好ましい。もし、CpG領域を含む領域に結合するようにせざるを得ない場合には、前記CpG領域中のシトシンのメチル化の有無に影響を受けないように、すなわち、CpG領域中のシトシンがウラシルに変換された場合、また、シトシンのまま変換されない場合の双方の配列を増幅するように設計されることが好ましい。具体的には、前記CpG領域中のシトシンの部位に対応するメチル化非特異的プライマーの部位に混合塩基、又はイノシン3リン酸(ITP)由来のイノシン(ヒポキサンチン)を配置した塩基配列からなるオリゴヌクレオチドがこの種のメチル化非特異的プライマーである。

[0061] 本実施形態においては、CCAAT領域中のCpG領域のシトシンのメチル化の有無を識別するために用いられるメチル化非特異的プライマーとしては、配列番号5又は7で表されるオリゴヌクレオチドがある。配列番号5で表されるプライマーは、hMLH1タンパク質をコードする遺伝子の塩基配列1368から1394の部位を認識して、その遺伝子に結合する。また、配列番号7で表されるプライマーは、hMLH1タンパク質をコードする遺伝子の塩基配列1575から1600の部位を認識して、その遺伝子に結合する。

[0062] 上記配列番号6で表されるメチル化特異的プライマーと、配列番号5で表されるメチル化非特異的プライマーと、配列番号7で表されるメチル化非特異的プライマーとの合計3種のプライマーセットを用いれば、CCAAT領域中のCpG領域のシトシンのメチル化の有無を識別できる。

[0063] 即ち、図2の模式図に示されるように、CCAAT領域において、メチル化特異的プライマーである配列番号6で表されるCCAAT−MSプライマー(メチル化特異的上流プライマー)と、メチル化非特異的プライマーである配列番号5で表されるCCAAT−Sプライマー(メチル化非特異的上流プライマー)と、メチル化非特異的プライマーである配列番号7で表されるCCAAT−ASプライマー(メチル化非特異的下流プライマー)の3種のプライマーを用いて、核酸増幅法にて核酸を増幅する。そのとき、このC

pG領域のシトシンがメチル化された試料においては、CCAAT−MSプライマーも含めた3種のプライマーがすべて遺伝子に結合する。そして、CCAAT−MSプライマーとCCAAT−ASプライマーから核酸増幅して形成される122塩基のメチル化断片と、CCAAT−SプライマーとCCAAT−ASプライマーから核酸増幅して形成される232塩基の非メチル化断片の2つのDNA断片が形成される。一方、このCpG領域のシトシンがメチル化されていない試料においては、CCAAT−MSプライマーを除いた2種のプライマーのみが遺伝子に結合するため、結果的に、CCAAT−SプライマーとCCAAT−ASプライマーから核酸増幅して形成される232塩基の非メチル化断片のみが形成される。よって、検出される増幅産物の違いに基づいて、前記CpG領域のメチル化の有無を識別することができる。

[0064] プライマーオリゴヌクレオチドは、公知の方法により製造することができ、例えば、リン酸トリエステル法や、リン酸アミダイト法、リン酸基部位無保護法といった固相化学的合成法を用いることができる。具体的には、オリゴヌクレオチド合成装置(Applied Biosystem社製、Expedite Model 8909)等を用いて合成することができる。また、1ないし複数個の塩基を置換、欠失、挿入若しくは付加といった変異させたオリゴヌクレオチドも公知の固相化学的合成法で合成することができる。

(プライマーセット)

本発明のプライマーセットは、上述の本発明のプライマーを2つ以上、好ましくは少なくとも3つ含む。この少なくとも3つのプライマーは、

a)解析対象遺伝子中の少なくとも1つの検出対象CpG領域を含む部分が増幅されるように選択され、かつCpG領域中のシトシンが前記修飾剤による修飾の後にシトシンのままであっても、ウラシル又はチミンに転換されても、前記解析対象遺伝子に結合する、1対のメチル化非特異的プライマー、及び

b)前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にシトシンである場合にのみ前記解析対象遺伝子に結合し、前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にウラシル又はチミンに変換されれば前記解析対象遺伝子に結合しない、少なくとも1つのメチル化シトシン特異的プライマー

であると好都合である。

[0065] 解析対象遺伝子がhMLH1である実施態様について説明すれば、本発明で組織中のhMLH1をコードする遺伝子のプロモーター領域の中にある5'領域又はCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を識別するために用いられる核酸増幅用プライマーセットにおいては、少なくとも1つのメチル化シトシン非特異的プライマーの融解温度を、メチル化シトシン特異的プライマーの融解温度より1ー5℃高くなるようにプライマーを設計することが好ましい。また、いずれのメチル化シトシン非特異的プライマーの融解温度も、いずれのメチル化シトシン特異的プライマーの融解温度より1ー5℃高くなるようにプライマーを設計するとより好ましい。ここに、プライマーの融解温度とは、プライマーがオリゴヌクレオチドに結合する至適温度のことをいう。このように、プライマーを設計した後、核酸増幅のアニーリング時の温度を、いずれかのメチル化シトシン非特異的プライマーの融解温度よりも低く、またメチル化シトシン特異的プライマーの融解温度より高くすれば、あるCpG領域中のシトシンが重亜硫酸修飾の後にシトシンでないにもかかわらず、メチル化特異的プライマーが鋳型のオリゴヌクレオチドと結合して、擬陽性の結果を生ずることを防止することができるからである。また、このようにプライマーを設計すればメチル化の有無を考慮しない、PCRにおけるコントロール産物(すなわち、2種類のメチル化非特異的プライマーにより合成される産物)を検出しやすくなる。

[0066] 本発明で組織中のhMLH1をコードする遺伝子のプロモーター領域の中にあるCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を識別するために用いられる核酸増幅用プライマーセットにおいては、プライマー同士のGC含有率の差を20%以内にすることが好ましい。ここに、プライマーのGC含有率とは、プライマーの全塩基配列中グアニンとシトシンを合わせた塩基数の割合のことをいう。例えば、それぞれ100塩基からなる2つのプライマーがあり、1つ目のプライマーのGCの数が45で、もう1つのプライマーのGCの数が50であればGC含有率の差は5%であるとされる。このとき、プライマーのGC含有率は、メチル化シトシン特異的プライマーを低くするとよい。このように、プライマーを設計すれば、擬陽性の結果を生ずることを防止することができ、かつ再現性のよい結果を得やすくなるので好ましい。

(検査方法)

本発明における検査方法は、具体的には、試料の準備、DNAの抽出、DNAの修飾、プライマーを用いた遺伝子増幅、DNAの検出の過程を含む。

[0067] （検査用試料・病理検体の準備）

本発明の検査方法に供される、ヒトの試料はhMLH1タンパク質をコードする遺伝子を含むものであればよく、特に限定されない。具体的には、生体から採取した組織の他、血液、血清、糞便、精液、唾液、脳脊髄液等が挙げられる。生体から採取した組織としては、例えば、手術により切除した大腸癌の組織や、手術前の内視鏡検査等に用いる生体検査材料等が、試料の有効利用の点で好適に用いられる。

[0068] （DNAの抽出）

本発明の検査方法に供される、ヒトの試料は、ブレンダーを用いて組織を破砕し、次いで、フェノール・クロロフォルム法等の公知の遺伝子抽出法により、DNAを抽出し、検査用試料として用いる。

[0069] （DNAの修飾）

前記DNAは重亜硫酸塩によって修飾される。これにより、メチル化されたシトシンは、シトシンのまま存在するが、メチル化されていないシトシンは、一旦ウラシルに転換された後、チミンとして発現することになる。本発明に使用される重亜硫酸塩は特に限定されないが、重亜硫酸ナトリウムが好適に用いられる。また、上記のような重亜硫酸塩と同等の機能を有するものを修飾剤として用いることができる。

[0070] さらに具体的には、例えば、まず、200pgから50μgのDNAをアルカリ条件下にて変性させる。変性の条件は、PCRの測定系に適した条件であればよいが、例えば、0．2Mー0．3MのNaOH溶液中にて、37℃で5ー30分インキュベートする。次いで、ハイドロキノン溶液及びpH5．0の重亜硫酸ナトリウム溶液を終濃度がそれぞれ0．5mM及び3．1mMになるように添加し、50ー55℃にて16ー40時間インキュベートすることで、非メチル化シトシンが修飾される。さらに、段階的な透析を行うことで余剰の重亜硫酸を除去する。透析された試料は、真空オーブンによる濃縮又はエタノール沈殿の後、適当な緩衝液又は純水に溶解し、通常のPCR又はメチル化特異的PCRに用いることができる。

[0071] （遺伝子・核酸増幅）

本発明において、遺伝子増幅方法としては、公知の方法を使用することができ、例えば、PCR法、NASBA法、LAMP法等が挙げられる。好ましくは、PCR法が用いられる。

[0072] PCR法により増幅された産物の塩基配列を決定する方法としては、Frommer et. al. (Proc. Natl. Acad. Sci. USA 89, 1827-1831(1992))及びClark et. al. (Nucleic Acids Research 22, 2990-2997 (1994))等の方法がある。

[0073] (DNAの検出)

メチル化特異的PCRによって増幅されたDNAの検出は、例えば、一般的なアガロースゲル又はポリアクリルアミドゲル電気泳動の後、DNAの染色によって行われる。染色方法としては、銀染色による方法、エチジウムブロマイド、SYBRR Green等の蛍光性染色剤等による方法がある。また、電気泳動以外の方法として、5'エキソヌクレアーゼアッセイや蛍光共鳴エネルギー転移を利用したホモジニアス検出も可能である。

[0074] (MSI分析)

本発明の検査方法は、まず、マイクロサテライト反復配列分析(MSI分析)を行って、マイクロサテライト不安定性(MSI+)を示す散発性MSI+大腸癌とHNPCCの検体を識別(スクリーニング)した後に用いれば、hMLH1をコードする遺伝子の変異に基づく前記MSI+が、散発性MSI+大腸癌であるか、HNPCCであるかを、より精度よく識別することできる。MSI分析は、例えば、癌細胞の遺伝子と正常細胞の遺伝子とでマイクロサテライト領域を含む領域をPCR増幅し、電気泳動によって増幅産物の鎖長を調べることにより解析できる。即ち、癌細胞の遺伝子と正常細胞の遺伝子とでは、増幅産物の鎖長が異なるので、これを基に塩基配列を決定し、マイクロサテライトが不安定である(MSI+)か、安定である(MSI-又はMSS)かを判定できる。

[0075] (hMLH1の免疫染色)

本発明の検査方法は、前記MSI分析に加えて、hMLH1の免疫染色分析(IHC)を組み合わせることにより、前記CpG領域のメチル化の有無を識別できて、hMLH1をコードする遺伝子の変異が、散発性大腸癌であるか、HNPCCであるかをさらに精度よく識別することできる。図3に示すように、hMLH1の免疫染色分析においては、

CpG領域がメチル化されていれば、hMLH1が発現されにくくなり、免疫染色において陰性となる率が高くなる。一方、CpG領域がメチル化されていなければ、hMLH1は発現されやすくなるので、免疫染色において陽性となる率が高くなる。hMLH1の免疫染色分析においては、抗体としてhMLH1のモノクローナル抗体を用い、色素としてダイアミノベンチジン(diaminobenzidine)を用いるのが好適である。

[0076] (生体試料の判定)

本発明において、メチル化特異的PCR検査を実施すると、MSI+を示す散発性大腸癌の検体においては、5'領域とCCAAT領域の2箇所において、シトシンのメチル化が80%以上の高い割合で検出されるのに対して、MSI+を示すHNPCCの検体においては、5'領域とCCAAT領域の2箇所において、シトシンのメチル化が20%以下の低い割合で検出される。よって、hMLH1をコードする遺伝子のプロモーター領域におけるCpG領域のシトシンのメチル化の有無を識別することで、散発性MSI+大腸癌とHNPCCを識別することができる。

[0077] (検査用試薬及び検査用試薬キット)

本発明はまた、組織中のhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域におけるCpGのシトシンのメチル化の有無を散発性大腸癌と遺伝性非腺腫大腸癌の判定のための指標とする検査方法に用いられる検査試薬及び検査試薬キットを含む。検査試薬としては、メチル化特異的核酸増幅用のプライマー、エキソヌクレアーゼ、核酸検出用の標識等、本発明の方法に使用されるあらゆる試薬のいずれであってもよい。

[0078] また、検査用試薬キットは、本発明の検査方法に使用されるあらゆる試薬のうち少なくとも2以上をキットとして使用するものであればよい。例えば、hMLH1をコードする遺伝子(DNA)のプロモーター領域中のCpG領域のメチル化特異的プライマーとメチル化非特異的プライマーセット等が例示される。その他、蛍光標識をプローブしたDNAも本キットに含めてもよい。

実施例

[0079] 以下、実施例に基づいて本発明をより詳細に説明するが、本発明はこれら実施例に限定されるものではない。

（実施例1）

採取された大腸癌234症例の組織試料について、MSI分析を行い、MSI＋とMSI－の2種類に大腸癌を分類した。MSI分析は、公知の手法にしたがって行った。

[0080] MSI＋、MSI－と診断された癌患者について、hMLH1をコードする遺伝子のプロモーター領域の5'領域中及びCCAAT領域中の2箇所のCpG領域のシトシンのメチル化の有無を調べた。具体的には以下のようにして行った。この実施例の方法の概要、即ちこの実施例において使用したプライマー及び増幅産物の関係、及び結果の判定の概略を図2に示す。

[0081] 1．試料の用意

生体試料からDNAを通常のフェノール・クロロホルム法で抽出した。生体試料より抽出したDNA 100ngに、DNA修飾キット「CpGenome（Intergen社）」を用いて重亜硫酸ナトリウムによる非メチル化シトシンの修飾を行った。

[0082] 2－A 5'領域のCpG領域のシトシンのメチル化の検出

上記1．のように用意した重亜硫酸修飾済みのDNAの一部に対してメチル化特異的PCR及びメチル化非特異的PCRを実施した。重亜硫酸修飾済みのDNA（鋳型）を、1×PCR緩衝液（15mM $MgCl_2$を含む）、1．25ユニットのDNAポリメラーゼ「HotStarTaq DNA Polymerase（Qiagen社）」、各々200μMのdATP、dGTP、dTTP及びdCTP、及び2μMプライマー（後述）を含む50μLのPCR反応液に加え、5'領域のメチル化の検出のために、95℃／0．5分、54℃／0．5分、72℃／0．5分、35サイクルのPCR反応を実施した。

[0083] 本実施例においては、hMLH1をコードする遺伝子のプロモーター領域の5'領域のCpG領域のシトシンのメチル化を検出するためのメチル化特異的なプライマーとしては、配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の5'領域のCpG領域以外にあるシトシン以外のすべてのシトシンをチミン又はウラシルに変換した配列及びその相補鎖から、下記のオリゴヌクレオチドを選択した（以下、塩基番号は配列番号1中の対応する塩基の番号を表す）。

[0084] メチル化特異的上流プライマー（5'－MSプライマー）

（塩基番号1097－1118）（配列番号3）

5'−CGTTCGTCGTTCGTTCGTTATATATC−3'

また、hMLH1をコードする遺伝子のプロモーター領域の5'領域のCpG領域のシトシンのメチル化を検出するためのメチル化非特異的なプライマーとしては、配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の5'領域のCpG領域にあるすべてのシトシンをチミン又はウラシルに変換した配列及びその相補鎖から、下記のオリゴヌクレオチドを選択した。

[0085] メチル化非特異的上流プライマー(5'−Sプライマー)

(塩基番号1031−1055)(配列番号2)

5'−TTTTTTTTAGGAGTGAAGGAGGTTA−3'

メチル化非特異的下流プライマー(5'−ASプライマー)

(塩基番号1191−1216)(配列番号4)

5'−CRATAAAACCCTATACCTAATCTATC−3'

なお、配列番号4中の2番目のRは、アデニンA又はグアニンG(つまりプリン)を表す。

[0086] 例えば、5'−Sプライマー及び5'−ASプライマーのペアによりhMLH1をコードする遺伝子の5'領域のDNAが増幅され、図2に示される184塩基のDNA断片(「No-specific fragment」)が検出されるが、5'−MSプライマー及び5'−ASプライマーのペアによりhMLH1をコードする遺伝子の5'領域のDNAが増幅されず、図2に示される118塩基のDNA断片(「Methylated fragment」)が検出されない場合には、前記プライマーで増幅される領域中にメチル化されたシトシンが存在しないか、存在しても検出限界を下回るほど微量であり、メチル化は陰性と判断した。

[0087] 一方、5'−Sプライマー及び5'−ASプライマーのペアによりhMLH1をコードする遺伝子の5'領域のDNAが増幅され、図2に示される184塩基のDNA断片が検出され、5'−MSプライマー及び5'−ASプライマーのペアによりhMLH1をコードする遺伝子の5'領域のDNAも増幅され、図2に示される118塩基のDNA断片も検出される場合には、前記プライマーで増幅される領域中にメチル化されたシトシンとメチル化されていないシトシンが共存していると考えられ、この場合、メチル化は陽性と判断した。

[0088] 2-B CCAAT領域のCpG領域のシトシンのメチル化の検出

上記1．のように用意した重亜硫酸修飾済みのDNAの一部に対してメチル化特異的PCR及びメチル化非特異的PCRを実施した。重亜硫酸修飾済みのDNA（鋳型）を、1×PCR緩衝液（15mM $MgCl_2$を含む）、1．25ユニットのDNAポリメラーゼ「HotStarTaq DNA Polymerase（Qiagen社）」、各々200μMのdATP、dGTP、dTTP及びdCTP、及び2μMプライマー（後述）を含む50μLのPCR反応液に加え、CCAAT領域のメチル化の検出のために、95℃／0．5分、58℃／0．5分、72℃／0．5分、35サイクルのPCR反応を実施した。

[0089] 本実施例においては、hMLH1をコードする遺伝子のプロモーター領域のCCAAT領域のCpG領域のシトシンのメチル化を検出するためのメチル化特異的なプライマーとしては、配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域のCCAAT領域のCpG領域以外にあるシトシン以外のすべてのシトシンをチミン又はウラシルに変換した配列及びその相補鎖から、下記のオリゴヌクレオチドをプライマーとして選択した。

[0090] メチル化特異的上流プライマー（CCAAT-MSプライマー）

（塩基番号1479-1502）（配列番号6）

5'-YGTATTTTTCGAGTTTTTAAAAAC-3'

なお、配列番号6中の1番目のYはシトシンC又はチミンT（つまりピリミジン）を表す。

[0091] また、hMLH1をコードする遺伝子のプロモーター領域のCCAAT領域のCpG領域のシトシンのメチル化を検出するためのメチル化非特異的なプライマーとしては、配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域のCCAAT領域のCpG領域にあるすべてのシトシンをチミン又はウラシルに変換した配列及びその相補鎖から、下記のオリゴヌクレオチドを選択した。

[0092] メチル化非特異的上流プライマー（CCAAT-Sプライマー）

（塩基番号1369-1394）（配列番号5）

5'-GAGGAGGAGTTTGAGAAGIGTTAAGT-3'

メチル化非特異的下流プライマー（CCAAT-ASプライマー）

（塩基番号1575−1600）（配列番号7）

5’−TAAATCTCTTCITCCCTCCCTAAAAC−3’

なお、配列番号7中の12番目のIはイノシン3リン酸（ITP）由来のイノシン（ヒポキサンチン）である。

[0093] 例えば、CCAAT−Sプライマー及びCCAAT−ASプライマーのペアによりhMLH1をコードする遺伝子のCCAAT領域のDNAが増幅され、図2に示される232塩基のDNA断片（「No-specific fragment」）が検出されるが、CCAAT−MSプライマー及びCCAAT−ASプライマーのペアによりhMLH1をコードする遺伝子のCCAAT領域のDNAが増幅されず、図2に示される122塩基のDNA断片（「Methylated fragment」）が検出されない場合には、前記プライマーで増幅される領域中にメチル化されたシトシンが存在しないか、存在しても検出限界を下回るほど微量であり、メチル化は陰性と判断した。

[0094] 一方、CCAAT−Sプライマー及びCCAAT−ASプライマーのペアによりhMLH1をコードする遺伝子のCCAAT領域のDNAが増幅され、図2に示される232塩基のDNA断片が検出され、CCAAT−MSプライマー及びCCAAT−ASプライマーのペアによりhMLH1をコードする遺伝子のCCAAT領域のDNAも増幅され、図2に示される122塩基のDNA断片も検出される場合には、前記プライマーで増幅される領域中にメチル化されたシトシンとメチル化されていないシトシンが共存していると考えられ、この場合、メチル化は陽性と判断した。

[0095] 3. hMLH1タンパクの免疫染色法

免疫染色は、合計75例の試料について行った。

[0096] 定法にしたがってホルマリン固定し、パラフィンに包埋した組織試料に、Tyramide Signal Amplification Biotin System （Perkin Elmer, Boston, MA）を使用した。

[0097] 4. 遺伝子修復タンパク質遺伝子の変異分析（mutation analysis）

hMLH1、hMSH2、hPMS1、hPMS2、及びhMSH6の5個の遺伝子変異分析は、MSI＋と診断された35例の試料についてダイレクトシーケンス法及び免疫染色

法によって行った。

[0098] 5. 結果

上記の手法により、234症例の大腸癌全体に対して検討を行った。MSI分析の結果、234症例の大腸癌は、35例のMSI＋癌（HNPCCを含む）及び199例のMSI－癌に分類された。すなわち、35例のMSI＋癌（HNPCCを含む）及び199例のMSI－癌の生体試料についてhMLH1をコードする遺伝子のプロモーター領域の5'領域とCCAAT領域のCpG領域のシトシンのメチル化及び非メチル化を調べた。

[0099] その結果を表1に示す。

[0100] [表1]

| | MSI＋（HNPCC を含む） | MSI- |
|---|---|---|
| CCAAT メチル化 (+) | 18 (51.4%) | 0 (0%) |
| CCAAT メチル化 (-) | 17 (48.6%) | 199 (100%) |

$p$ <0.0001

| | MSI＋（HNPCC を含む） | MSI- |
|---|---|---|
| 5' メチル化 (+) | 21 (60.0%) | 28 (14.1%) |
| 5' メチル化 (-) | 14 (40.0%) | 171 (85.9%) |

$p$ <0.0001

5'領域及びCCAAT領域のメチル化は有為に（フィッシャーの正確検定p＜0.0001）MSI＋癌に認められた。一方、5'領域のメチル化がMSI－癌においても認められたのに対し、CCAAT領域のメチル化はMSI－癌においては一切認められなかった。

[0101] また、この5'領域及びCCAAT領域のメチル化検出検査におけるMSI＋癌のMSI－癌に対する感度及び特異度、陽性尤度比及び陰性尤度比も併せて表1に示す。5'領域及びCCAAT領域のメチル化陽性の場合は共に感度は50%から60%である。これは後述するようにMSI＋癌にHNPCC及び散発性MSI＋癌が含まれるためである。一方、特異度は、5'領域のメチル化検出では85.9%であるのに対し、CCAAT領域のメチル化検出では100%であった。

[0102] このことよりCCAAT領域のメチル化検出検査を行うことは、MSI＋癌を見逃す(false-negative)可能性が5'領域のメチル化検出よりもやや高いが、特異度が極めて高いため、誤診(false-positive)を避けることが可能であると考えられる。逆に、5'領域のメチル化検出検査を行うことは、false-negativeの可能性がCCAAT領域のメチル化検出よりもやや低いが、特異度がやや劣るため、わずかながらfalse-positiveが生じる可能性を有すると考えられる。いずれの検査も、MSI＋癌の発見に有用であることが明らかになった。

[0103] また、hMLH1タンパクの免疫染色(IHC)との関係を表2に示す。図3に、hMLH1タンパクの免疫染色(IHC)の陽性例及び陰性例を示す。

[0104] [表2]

| | hMLH1 IHC | |
|---|---|---|
| | 陽性 | 陰性 |
| CCAAT メチル化 (+) | 0 (0%) | 18 (72.0%) |
| CCAAT メチル化 (-) | 50 (100%) | 7 (28.0%) |

$p$ <0.0001

| | hMLH1 IHC | |
|---|---|---|
| | 陽性 | 陰性 |
| 5' メチル化 (+) | 6 (12.0%) | 19 (76.0%) |
| 5' メチル化 (-) | 44 (88.0%) | 6 (24.0%) |

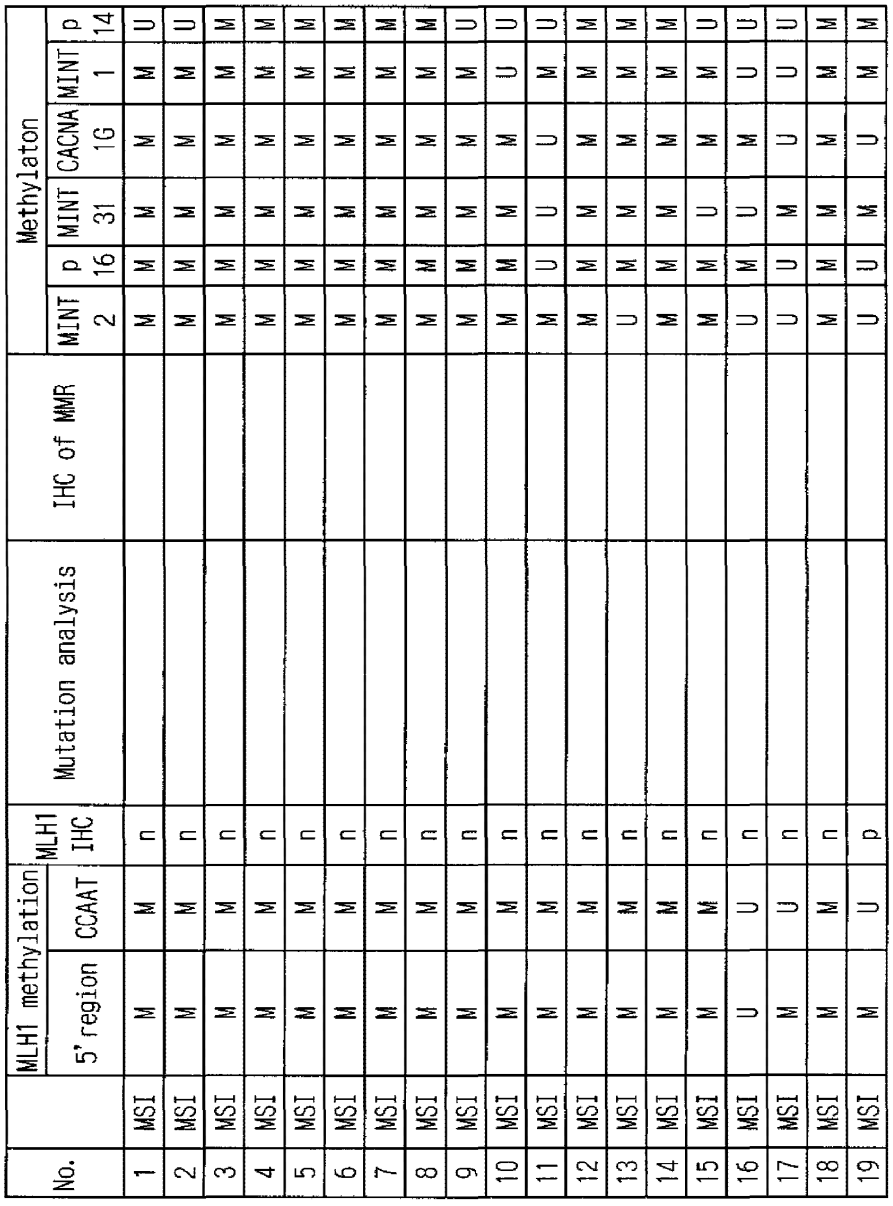
$p$ <0.0001

免疫染色は合計75症例に対して行われた。5'領域及びCCAAT領域のメチル化はhMLH1タンパク発現との間に強い相関関係を示した(フィッシャーの正確検定p＜0.0001)。しかし、5'領域にメチル化を認めた症例25例のうち6症例にhMLH1免疫染色陽性が認められたのに対し、CCAAT領域にメチル化を認めた症例18症例はすべて陰性であった。なお、この免疫染色陰性症例の中にはhMLH1遺伝子に突然変異を持つHNPCC症例も含まれている。このため、5'領域にメチル化を認めない、かつ、hMLH1免疫染色陰性例6症例のうち2症例にhMLH1遺伝子に突

然変異を認めた。また、同様にCCAAT領域にメチル化を認めない、かつ、hMLH1免疫染色陰性例7症例のうち、2症例にhMLH1遺伝子に突然変異を認めた（後出の表3、表4参照）。

[0105] 次に、MSI＋と診断された35症例に対して検討を行った。MSI＋と診断された35症例のうち、散発性大腸癌と診断された19症例と、家族歴の追跡等によりHNPCCと診断された16症例に対して、上記と同様の解析を行った。結果を表3及び表4の左側の欄にそれぞれ示す。

[0106] [表3]



| No. | | MLH1 methylation | | MLH1 IHC | Mutation analysis | IHC of MMR | Methylaton | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5' region | CCAAT | | | | MINT 2 | p 16 | MINT 31 | CACNA 1G | MINT 1 | p 14 |
| 1 | MSI | M | M | n | | | M | M | M | M | M | U |
| 2 | MSI | M | M | n | | | M | M | M | M | M | U |
| 3 | MSI | M | M | n | | | M | M | M | M | M | M |
| 4 | MSI | M | M | n | | | M | M | M | M | M | M |
| 5 | MSI | M | M | n | | | M | M | M | M | M | M |
| 6 | MSI | M | M | n | | | M | M | M | M | M | M |
| 7 | MSI | M | M | n | | | M | M | M | M | M | M |
| 8 | MSI | M | M | n | | | M | M | M | M | M | M |
| 9 | MSI | M | M | n | | | M | M | M | M | M | U |
| 10 | MSI | M | M | n | | | M | M | M | M | U | U |
| 11 | MSI | M | M | n | | | M | U | U | U | M | U |
| 12 | MSI | M | M | n | | | M | M | M | M | M | M |
| 13 | MSI | M | M | n | | | U | M | M | M | M | M |
| 14 | MSI | M | M | n | | | M | M | M | M | M | M |
| 15 | MSI | M | M | n | | | M | M | U | M | M | U |
| 16 | MSI | U | U | n | | | U | M | U | M | U | U |
| 17 | MSI | M | U | n | | | U | U | M | U | U | U |
| 18 | MSI | M | M | n | | | M | M | M | M | M | M |
| 19 | MSI | M | U | p | | | U | U | M | U | M | M |

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[0107] [表4]

| No. | | MLH1 methylation | | MLH1 IHC | Mutation analysis | IHC of MMR | Methylaton | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5' region | CCAAT | | | | MINT 2 | p 16 | MINT 31 | CACNA 1G | MINT 1 | p 14 |
| 1 | HNPCC | U | U | n | no mutation | Absent of PMS2 | U | M | U | M | U | M |
| 2 | HNPCC | U | U | p | no mutation | Absent of MSH2 | U | U | U | U | U | U |
| 3 | HNPCC | U | U | p | exon5 of MSH6 | Absent of MSH6 | U | U | M | U | U | U |
| 4 | HNPCC | M | M | n | no mutation | | U | U | U | U | U | U |
| 5 | HNPCC | M | U | p | exon1 of MSH2 | Absent of MSH2 | U | U | M | U | M | M |
| 6 | HNPCC | U | U | p | no mutation | Absent of MSH2 | U | U | U | U | M | U |
| 7 | HNPCC | U | U | p | no mutation | Absent of MSH2 | U | U | U | U | M | U |
| 8 | HNPCC | U | U | p | whole gene deletion of MSH2 | Absent of MSH2 | U | U | U | U | M | U |
| 9 | HNPCC | U | U | p | no mutation | Absent of PMS2 | U | U | U | U | U | U |
| 10 | HNPCC | U | U | n | no mutation | | U | U | U | U | M | U |
| 11 | HNPCC | U | U | n | exon16 of MLH1 | | U | U | U | U | U | M |
| 12 | HNPCC | U | U | n | no mutation | | U | U | U | U | M | U |
| 13 | HNPCC | U | U | n | exon17 of MLH1 | | U | U | U | U | U | U |
| 14 | HNPCC | M | M | n | | | U | U | U | M | U | U |
| 15 | HNPCC | U | U | p | | Absent of MSH6 | U | U | U | U | U | U |
| 16 | HNPCC | U | U | p | | | U | U | M | U | U | U |

HNPCC16症例の解析

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表3及び表4において、「M」＝メチル化検出、「U」＝メチル化検出せず、「n」＝陰性、「p」＝陽性を表す。

[0108] 表3に示す散発性MSI＋大腸癌の19症例においては、5'領域のシトシンのメチル化(「M」)が18症例で見られ、CCAAT領域のシトシンのメチル化(「M」)が16症例で見られた。一方、表4に示すHNPCCの16症例においては、5'領域のシトシンのメチル化は3症例でしか見られず、CCAAT領域のシトシンのメチル化(「M」)も2症例でしか見られなかった。このことから、散発性MSI＋大腸癌と5'領域及びCCAAT領域のシトシンのメチル化(「M」)との間には有為な相関関係が認められた(フィッシャーの正確検定p＜0．0001)。一方、HNPCCと5'領域及びCCAAT領域のシトシンの非メチル化(「U」)との間にも有為な相関関係が認められることが分かった(フィッシャーの正確検定p ＜0．0001)。

[0109] また、表3における散発性MSI＋大腸癌の症例No．1からNo．7までの7例の5'領域及びCCAAT領域の核酸増幅の産物を図4(a)に示す。これは、散発性MSI＋大腸癌の症例No．1からNo．7までの7例がすべて、5'領域及びCCAAT領域においてメチル化されたシトシンを有したことを示すものである。なお、図4(a)において、「5' region」及び「CCAAT box」の各々について、最上段の「1」ー「7」はぞれぞれ表3における症例No．を示し、その下の段の「T」は腫瘍部分から抽出されたDNAを、「N」はその正常粘膜部分から抽出されたDNAより得られた解析結果を示す。右端の「M」はメチル化DNAのコントロールを、「U」は非メチル化DNAのコントロールを示す。写真の下の「M」は、メチル化シトシンが検出されたことを示す。

[0110] また、表4におけるHNPCC大腸癌の症例No．3からNo．9までの7例の5'領域及びCCAAT領域の核酸増幅の産物を図4(b)に示す。これは、IINPCC大腸癌の症例No．4と5の2例の5'領域、及びNo．4の1例のCCAAT領域においてメチル化されたシトシンを有したことを示すものである。図4(b)中の記号については上記と同様である。

[0111] また、上記35症例について、遺伝子修復(MMR)タンパク質であるhMLH1、hMSH2、hPMS1、hPMS2、及びhMSH6の5個について、遺伝子の変異分析(mutation analysis)及びタンパク質の免疫染色分析(IHC)を行った。表3の中央(「

Mutation Analysis」の欄及び「IHC
of MMR」の欄）に示すように、散発性MSI＋大腸癌の19症例においては、遺伝子の変異及びhMLH1以外の遺伝子修復タンパク質の消失を示すデータは現れなかった（空欄は変異又はタンパク質の消失が検出されなかったことを示す）。一方、表4に示すように、HNPCCの16症例においては、比較的多くの症例において、遺伝子の変異とそれによる遺伝子修復タンパク質の消失が見られた。

[0112] さらに、上記35症例について、大腸癌と関連する他の6種の遺伝子又は遺伝子座、即ち、MINT2、p16、MINT31、CACNA1G、MINT1、p14をコードする遺伝子又は遺伝子座のメチル化の状態を調べた。表3の右側（「Methylation」）の欄に示すように、散発性MSI＋大腸癌の19症例においては、どの遺伝子又は遺伝子座においても高い割合でメチル化（「M」）が検出された（表中、「U」は非メチル化を示す）。一方、表4に示すように、HNPCCの16症例においては、どの遺伝子又は遺伝子座においてもメチル化の検出された割合は低かった。

[0113] この35症例の検査結果のもとに、MSI＋大腸癌症例に対してhMLH1をコードする遺伝子の5'領域及びCCAAT領域のメチル化の検出検査におけるMSI＋癌のHNPCCに対する感度及び特異度、陽性尤度比及び陰性尤度比を求めた。結果を表5の上2段に示す。

[0114] [表5]

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/CCAAT メチル化 (+) | 16 | 2 | 84.21052632 | 87.5 | 6.736842105 | 0.180451128 |
| MSI+/CCAAT メチル化 (-) | 3 | 14 | | | | |

$p$ <0.0001

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/5' メチル化 (+) | 18 | 3 | 94.73684211 | 81.25 | 5.052631579 | 0.064777328 |
| MSI+/5' メチル化 (-) | 1 | 13 | | | | |

$p$ <0.0001

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MMR IHC(-)/CCAAT メチル化 (+) | 16 | 2 | 88.88888889 | 86.66666667 | 6.666666667 | 0.128205128 |
| MMR IHC(-)/CCAAT メチル化 (-) | 2 | 13 | | | | |

$p$ <0.0001

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MMR IHC(-)/5' メチル化 (+) | 17 | 3 | 94.44444444 | 80 | 4.722222222 | 0.069444444 |
| MMR IHC(-)/5' メチル化 (-) | 1 | 12 | | | | |

$p$ <0.0001

表5に示すように、MSI＋大腸癌において、散発性MSI＋大腸癌を見逃さずに検出する場合は、5'領域のメチル化検出検査の方がCCAAT領域のメチル化の検出

検査よりも優れていることが感度より示された。一方、散発性MSI＋大腸癌を誤診せずに検出する場合は、CCAAT領域のメチル化の検出検査の方が5'領域のメチル化検出検査よりも優れていることが特異度より示された。

[0115] 以上から、目的に応じていずれか一方の検査でも充分有用であるが、双方の検査を行うことにより、MSI＋大腸癌患者から散発性MSI＋大腸癌患者を精度よく検出することが可能であり、そしてこのMSI＋大腸癌患者から散発性MSI＋大腸癌患者を識別することによりHNPCC患者を精度よく識別できることが確認された。

[0116] また、遺伝子修復タンパク質であるhMLH1、hMSH2、hPMS1、hPMS2、及びhMSH6の5個についての免疫染色分析（MMR IHC）において、どれかに陰性（－）を示す症例において、hMLH1をコードする遺伝子の5'領域及びCCAAT領域のメチル化の検出検査におけるMSI＋癌のHNPCCに対する感度及び特異度、陽性尤度比及び陰性尤度比を求めた。結果を表5の下2段に示す。

[0117] 表5に示すように、MMR IHC（－）大腸癌において、散発性MSI＋大腸癌を見逃さずに検出する場合は、5'領域のメチル化検出検査の方がCCAAT領域のメチル化の検出検査よりも優れていることが感度より示された。一方、散発性MSI＋大腸癌を誤診せずに検出する場合は、CCAAT領域のメチル化の検出検査の方が5'領域のメチル化検出検査よりも優れていることが特異度より示された。

[0118] 以上から、遺伝子修復タンパク質であるhMLH1、hMSH2、hPMS1、hPMS2、及びhMSH6の5個の免疫染色分析（MMR IHC）を行った際においても、5'領域及びCCAAT領域のメチル化検出検査を行うことによって、MSI＋大腸癌患者から散発性MSI＋大腸癌患者を精度よく検出することが可能であり、そしてこのMSI＋大腸癌患者から散発性MSI＋大腸癌患者を識別することによりIINPCC患者を精度よく識別できることが確認された。

2－C 他の遺伝子のメチル化状態の検出

さらに、MSI＋大腸癌症例に対してMINT2、p16、MINT31、CACNA1G、MINT1、p14をコードする遺伝子座及び遺伝子のメチル化の検出感度及び特異度、陽性尤度比及び陰性尤度比を求めた。結果を表6に示す。

[0119] この例においても散発性MSI＋大腸癌患者のHNPCC患者に対するMINT2、p1

6、MINT31、CACNA1G、MINT1、p14をコードする遺伝子座及び遺伝子のメチル化の検出感度及び特異度は高いことが分かった。

[0120] [表6]

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/MINT2 メチル化 (+) | 15 | 0 | 78.94736842 | 100 | ∞ | 0.210526316 |
| MSI+/MINT2 メチル化 (-) | 4 | 16 | | | | |

$p$ =0.0002

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/p16 メチル化 (+) | 16 | 1 | 84.21052632 | 93.75 | 13.47368421 | 0.168421053 |
| MSI+/p16 メチル化 (-) | 3 | 15 | | | | |

$p$ <0.0001

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/MINT1 メチル化 (+) | 16 | 6 | 84.21052632 | 62.5 | 2.245614035 | 0.252631579 |
| MSI+/MINT1 メチル化 (-) | 3 | 10 | | | | |

$p$ <0.0001

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/MINT31 メチル化 (+) | 16 | 3 | 84.21052632 | 81.25 | 4.49122807 | 0.194331984 |
| MSI+/MINT31 メチル化 (-) | 3 | 13 | | | | |

$p$ <0.0001

| | MSI＋(散発性) | HNPCC | 感度 (%) | 特異度 (%) | 陽性尤度比 | 陰性尤度比 |
|---|---|---|---|---|---|---|
| MSI+/p14 メチル化 (+) | 11 | 3 | 57.89473684 | 81.25 | 3.087719298 | 0.518218623 |
| MSI+/p14 メチル化 (-) | 8 | 13 | | | | |

$p$ <0.0001

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上記の各実施例に於ける陽性尤度比及び陰性尤度比であるが、陽性尤度比は、感度／（100%－特異度）で算出され、陰性尤度比は（100%－感度）／特異度で算出される。例えばある疾患を検出する検査方法を行うとした場合、その検査前におけるその疾患である可能性（検査前確率）があるとすると、尤度比により、その検査を行った後のその疾患である可能性（検査後確率）を求めることが可能である。よって、陽性尤度比の数値は大きければ大きい程、陰性尤度比の数値は小さければ小さい程、その検査を行った後の診断に誤りがなくなる。上記の各実施例における陽性尤度比はすべて比較的高い値を示し、陰性尤度比はすべて比較的低い値を示した。

[0121] このように、本実施例によれば、MSI＋という表現型を持つ、又はMMR IHC（－）が認められる大腸癌の検体に対し、hMLH1をコードする遺伝子のプロモーター領域の5'領域とCCAAT領域のCpG領域のシトシンのメチル化の検出を行うことにより、散発性MSI＋大腸癌を精度よく識別することによって、MSI＋という表現型を持つ、又はMMR IHC （－）が認められる大腸癌症例から散発性MSI＋大腸癌を除外することにより、HNPCC症例を精度よく識別することができることが確認された。

[0122] 以上説明したように、本発明に係る核酸増幅用プライマー及びこれを用いた遺伝子のメチル化検出方法によれば、解析対象遺伝子中のCpG領域のメチル化の有無を簡便・迅速に検出することができる。特に、大腸癌の検査方法としての態様においては、hMLH1をコードする遺伝子のプロモーター領域中のCpG領域のメチル化の有無を簡便に識別でき、それに基づいて、hMLH1タンパク質の失活がメチル化によるものなのかどうかを判定することが可能である。この判定を行うことにより、散発性MSI＋大腸癌であるか、HNPCCであるかを精度よく識別することができる。

[0123] この出願は、平成15年8月29日出願の日本特許出願、特願2003－209838に基づくものであり、特願2003－209838の明細書及び特許請求の範囲に記載された内容は、すべてこの出願明細書に包含される。

# 請求の範囲

[1] 以下の1)ー10)のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択する核酸増幅用プライマー:

1)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

2)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

3)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

4)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

5)配列番号2ー7に表される塩基配列からなるオリゴヌクレオチド;

6)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

7)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

8)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌ

クレオチド;

9)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;及び

10)前記1)から9)のいずれか1に記載のオリゴヌクレオチドの塩基配列中の1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列からなり、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[2] 以下の1)ー10)のいずれか1に記載のオリゴヌクレオチドのうち2つ以上を選択する核酸増幅用プライマーセット:

1)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

2)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

3)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

4)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

5)配列番号2ー7に表される塩基配列からなるオリゴヌクレオチド;

6)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外

のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

7)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

8)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

9)配列番号1で表されるhMLH1をコードする遺伝子のプロモーター領域の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

10)前記1)から9)のいずれか1に記載のオリゴヌクレオチドのうち1又は複数のオリゴヌクレオチドについて、1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列を含み、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[3] 組織中のhMLH1をコードする遺伝子のプロモーター領域の中にある5'領域又はCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を識別するために用いられるプライマーセットであって、少なくとも1つのメチル化シトシン非特異的プライマーの融解温度が、メチル化シトシン特異的プライマーの融解温度より1ー5℃高いことを特徴とする請求の範囲2記載の核酸増幅用プライマーセット。

[4] 組織中のhMLH1をコードする遺伝子のプロモーター領域の中にあるCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を識別するために用いられるプライマーセットであって、プライマー同士のGC含有率の差が20%以内であることを特徴とする請求の範囲2又は3記載の核酸増幅用プライマーセット。

[5] 配列番号3に記載のオリゴヌクレオチド及び配列番号4に記載のオリゴヌクレオチドを選択することを特徴とする請求の範囲2又は3記載の核酸増幅用プライマーセット。

[6] 配列番号2に記載のオリゴヌクレオチド及び配列番号4に記載のオリゴヌクレオチドを選択することを特徴とする請求の範囲2又は3記載の核酸増幅用プライマーセット。

[7] 配列番号6に記載のオリゴヌクレオチド及び配列番号7に記載のオリゴヌクレオチドを選択することを特徴とする請求の範囲2から4のいずれか1項記載の核酸増幅用プライマーセット。

[8] 配列番号5に記載のオリゴヌクレオチド及び配列番号7に記載のオリゴヌクレオチドを選択することを特徴とする請求の範囲2から4のいずれか1項記載の核酸増幅用プライマーセット。

[9] 請求の範囲1記載の核酸増幅用プライマーを使用し、hMLH1をコードする遺伝子のプロモーター領域におけるCpG領域のシトシンのメチル化の有無を検査することを特徴とする散発性大腸癌と遺伝性非腺腫大腸癌の識別方法。

[10] 組織中のhMLH1をコードする遺伝子のプロモーター領域の中にある5'領域又はCCAAT領域におけるCpG領域におけるシトシンのメチル化の有無を指標とすることを特徴とする散発性大腸癌と遺伝性非腺腫大腸癌の識別方法。

[11] 前記シトシンのメチル化の有無の検査を核酸増幅方法により行う請求の範囲9又は10に記載の識別方法。

[12] マイクロサテライト反復配列分析を組み合わせたことを特徴とする請求の範囲9から11のいずれか1項に記載の識別方法。

[13] DNAミスマッチ修復タンパク質の免疫染色分析を組み合わせたことを特徴とする請求の範囲9から12のいずれか1項に記載の識別方法。

[14] 請求の範囲1記載のプライマーを含む検査用試薬。

[15] 請求の範囲1記載のプライマーを含む検査用試薬キット。

[16] 以下の1)ー9)のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択する核酸増幅用プライマーであって、解析対象遺伝子のCpG領域中のシトシンのメチル化の有無を検出するためのプライマー:

1)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

2）前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

3）前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

4）前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

5）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

6）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；

7）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド；

8）前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド；及び

9）前記1）から8）のいずれか1に記載のオリゴヌクレオチドの塩基配列中の1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列からなり、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

[17] 請求の範囲16記載のプライマーを少なくとも3つ含む解析対象遺伝子中のCpG領域のメチル化の有無を検出するための核酸増幅用プライマーセットであって、この少なくとも3つのプライマーが、

a）前記解析対象遺伝子中の少なくとも1つの検出対象CpG領域を含む部分が増幅されるように選択され、かつCpG領域中のシトシンが前記修飾剤による修飾の後にシトシンのままであっても、ウラシル又はチミンに転換されても、前記解析対象遺伝子に結合する、1対のメチル化非特異的プライマー、及び

b）前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にシトシンである場合にのみ前記解析対象遺伝子に結合し、前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にウラシル又はチミンに変換されれば前記解析対象遺伝子に結合しない、少なくとも1つのメチル化シトシン特異的プライマー

である、プライマーセット。

[18] 解析対象遺伝子のCpG領域中のシトシンのメチル化を検出する方法であって、

（1）試料由来の核酸を修飾剤によって修飾し、

（2）前記核酸を鋳型として、以下のプライマー：

a）前記解析対象遺伝子中の少なくとも1つの検出対象CpG領域を含む部分が増幅されるように選択され、かつCpG領域中のシトシンが前記修飾剤による修飾の後にシトシンのままであっても、ウラシル又はチミンに転換されても、前記解析対象遺伝子に結合する、1対のメチル化非特異的プライマー、及び

b）前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にシトシンである場合にのみ前記解析対象遺伝子に結合し、前記検出対象CpG領域中のシトシンが前記修飾剤による修飾の後にウラシル又はチミンに変換されれば前記解析対象遺伝子に結合しない、少なくとも1つのメチル化シトシン特異的プライマー

を用いて前記解析対象遺伝子核酸を増幅し、

（3）前記増幅産物を検出する

工程を含むことを特徴とする方法。

[19] 前記メチル化シトシン特異的プライマー及び前記メチル化シトシン非特異的プライマーが、それぞれ以下の1）ー9）のいずれか1に記載のオリゴヌクレオチドのうち少なくとも1つを選択するプライマーである、請求の範囲18記載の方法：

1）前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリ

ゴヌクレオチド;

2)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

3)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

4)前記解析対象遺伝子におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

5)前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

6)前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;

7)前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列からなるオリゴヌクレオチド;

8)前記解析対象遺伝子の配列の相補鎖におけるCpG領域を1以上含み、前記CpG領域中に存在するシトシン及びCpG領域以外のシトシンがチミン又はウラシルに変換された塩基配列に相補的な塩基配列からなるオリゴヌクレオチド;及び

9)前記1)から8)のいずれか1に記載のオリゴヌクレオチドの塩基配列中の1ないし複数個の塩基が置換、欠失、挿入若しくは付加といった変異された塩基配列からなり、CpG領域のシトシンのメチル化を検出可能なプライマー機能を有するオリゴヌクレオチド。

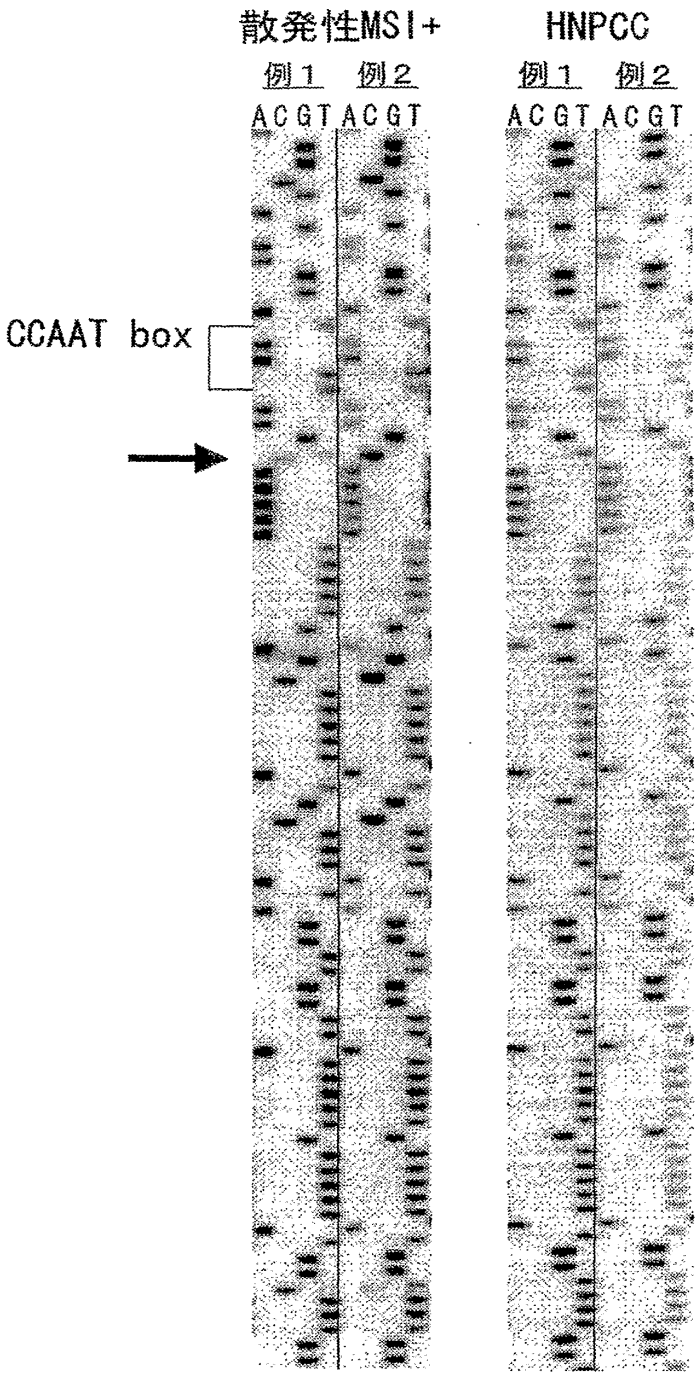
[20] 前記解析対象遺伝子が、hMLH1、hMSH2、hMSH6、hPMS1、hPMS2を含むDNAミスマッチ修復タンパク質をコードする遺伝子群及びその他の大腸癌関連遺

伝子群から選択される、請求の範囲18記載の方法。

[21] 前記解析対象遺伝子における検出対象CpG領域におけるシトシンのメチルの有無を、試料の由来する個体における大腸癌の存在又は分類と関連づけるための、請求の範囲18記載の方法。

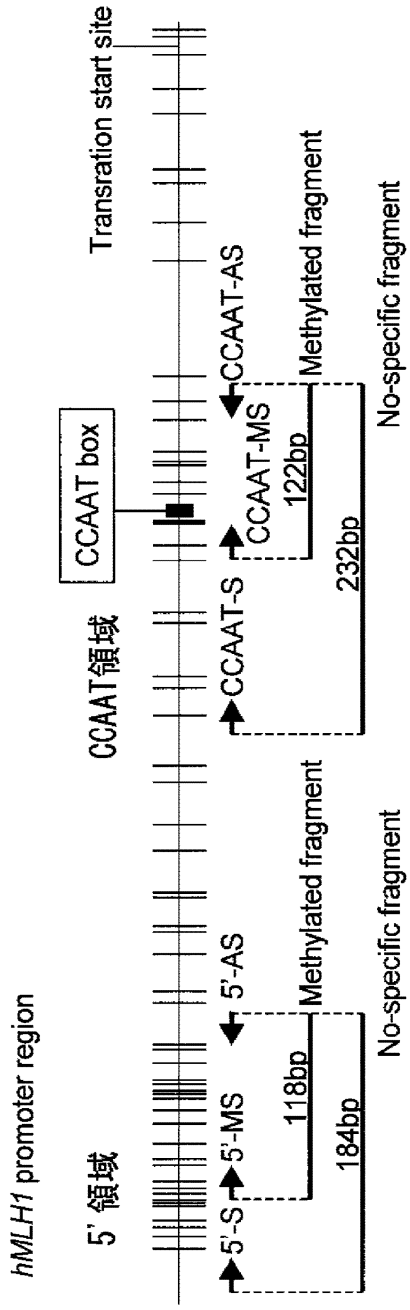
[22] 前記検出対象CpG領域が2ヵ所以上であり、前記b)のメチル化シトシン特異的プライマーを2種類以上用いる、請求の範囲18記載の方法。

[図１]



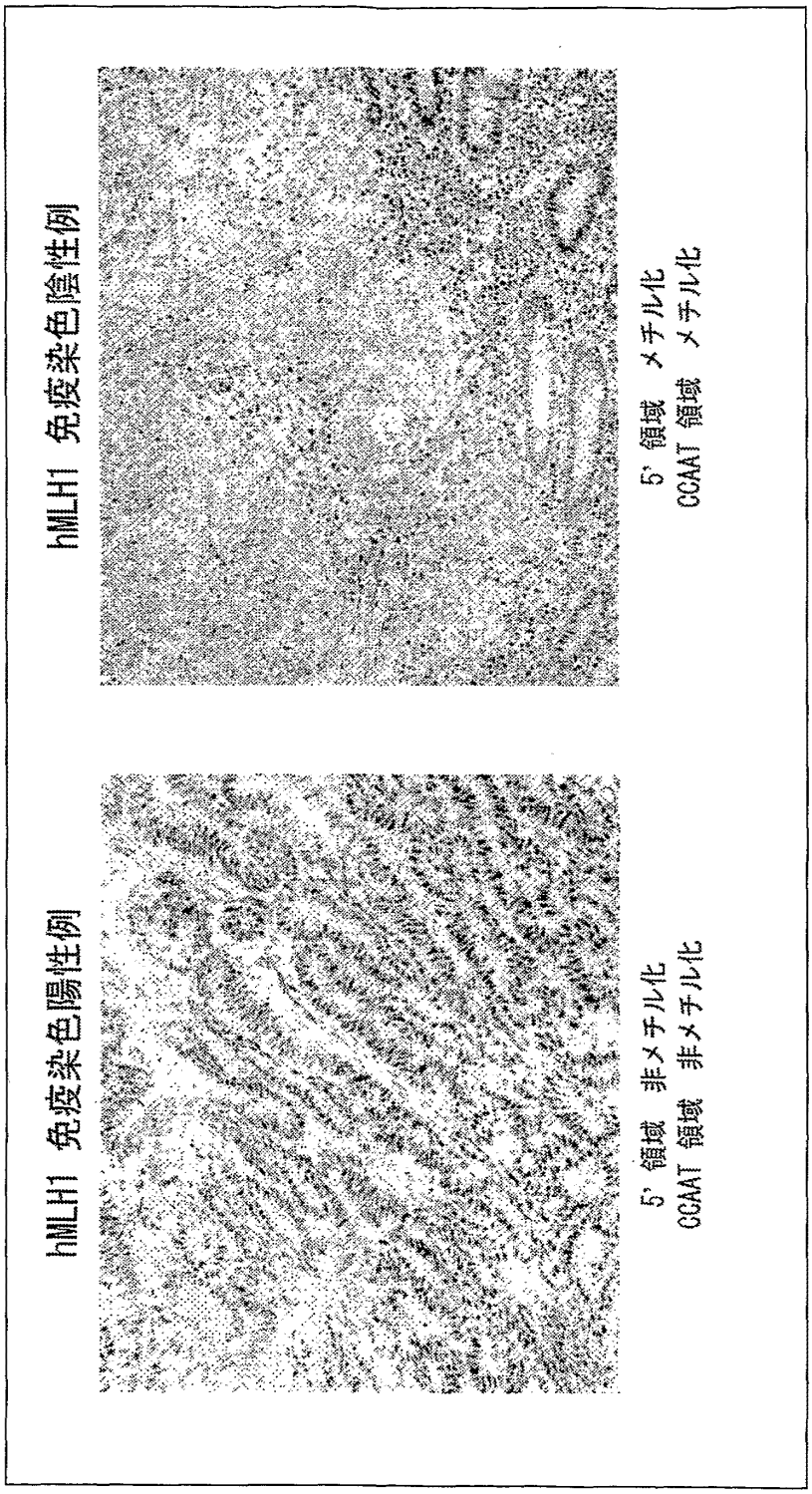

差替え用紙（規則26）

[図2]



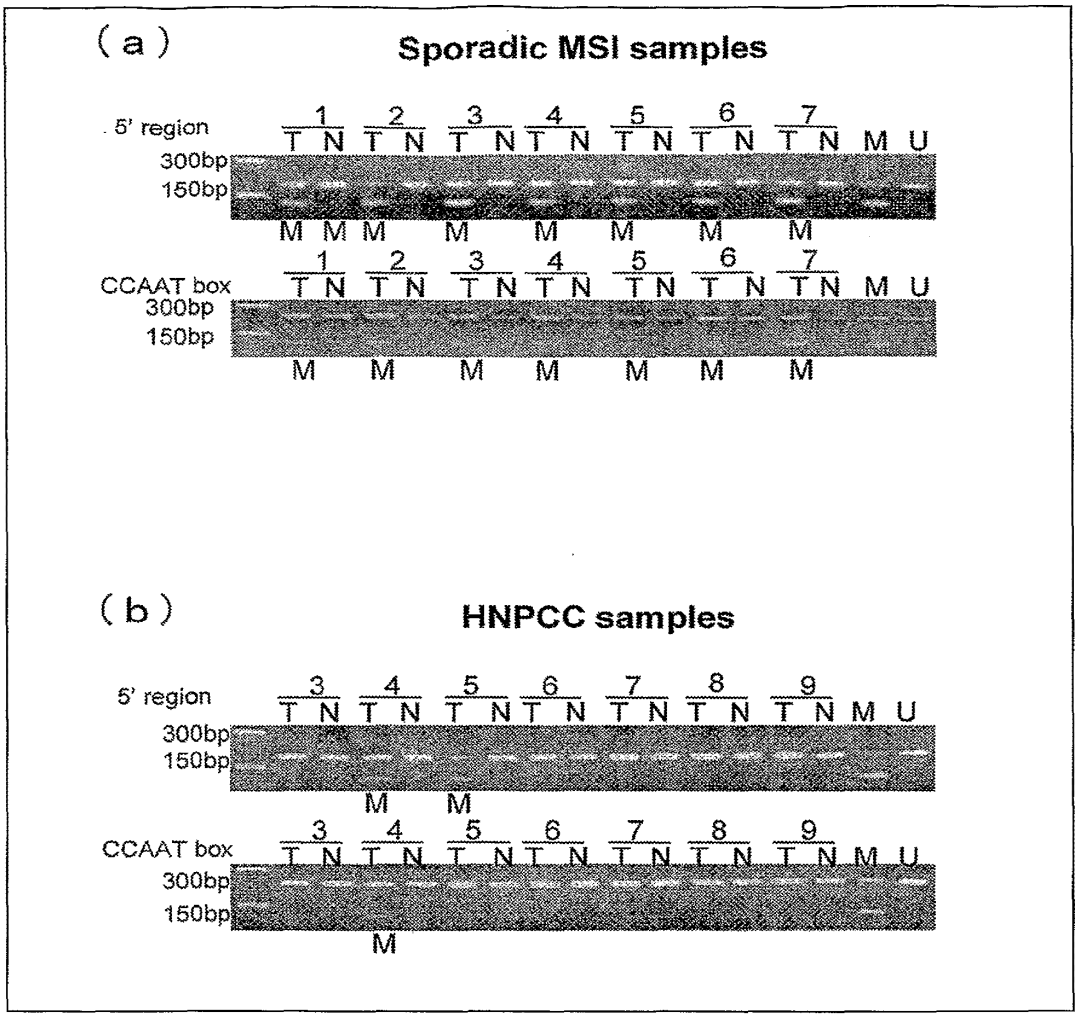

EX1002 - Page 2687

［図３］




差替え用紙（規則26）

[図４]




差替え用紙(規則26)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2004/012256

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N15/00, C12Q1/68, G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12N15/00, C12Q1/68, G01N33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA/MEDLINE/BIOSIS/EMBASE/BIOTECHABS/WPIDS(STN),
GenBank/EMBL/DDBJ/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | JP 2000-511776 A (THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE),<br>12 September, 2000 (12.09.00),<br>Full text<br>& WO 97/46705 A | 16-22<br>1-9,14,15 |
| Y | ITO, E., YANAGISAWA, Y., IWAHASHI, Y., SUZUKI, Y., NAGASAKI, H., AKIYAMA, Y., SUGANO, S., YUASA, Y. and MARUYAMA, K., A core promoter and a frequent single-nucleotide polymorphism of the mismatch repair gene hMLH1, Biochem. Biophys.Res.Commun. (1999), 256, 488 to 494 | 1-9,14,15 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 04 November, 2004 (04.11.04) | 22 November, 2004 (22.11.04) |
| Name and mailing address of the ISA/<br>Japanese Patent Office<br><br>Facsimile No. | Authorized officer<br><br>Telephone No. |

Form PCT/ISA/210 (second sheet) (January 2004)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2004/012256

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Potocnik, U.; Glavac, D.; Golouh, R.; Ravnik-Glavac, M., Causes of microsatellite instability in colorectal tumors. Implications for hereditary non-polyposis colorectal cancer screening, Cancer Genetics and Cytogenetics (2001), 126(2), 85 to 96 | 10-13 |
| X | Wheeler, J.M.D.; Loukola, A.; Aaltonen, L.A.; McC Mortensen, N.J.; Bodmer, W.F., The role of hypermethylation of the hMLH1 promoter region in HNPCC versus MSI+ sporadic colorectal cancers, Journal of Medical Genetics (2000), 37(8), 588 to 592 | 10-13 |
| P,X | JP 2003-259874 A (Nagahide MATSUBARA), 16 September, 2003 (16.09.03), Full text (Family: none) | 16-19,21,22 |

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2004/012256

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:
(See extra sheet.)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Continuation of Box No.III of continuation of first sheet(2)

The inventions as claimed in independent claim 1 and independent claim 2 (invention group A) relate to primers for nucleic acid amplification for selecting the oligonucleotides as described in 1) to 10).

The invention as claimed in independent claim 10 (invention group B) relates to a method of discriminating sporadic colon cancer from hereditary nonlinear colon cancer with the use of the presence or absence of methylation of cytosine in a definite CpG region in a promoter region of a gene encoding hMLH1 in a tissue as an indication.

The invention as claimed in independent claim 16 (invention group C) relates to primers for nucleic acid amplification for selecting the oligonucleotides as described in 1) to 9) and these primers are to be used for detecting the presence or absence of methylation in a CpG region of a gene to be analyzed.

The invention as claimed in independent claim 18 (invention group D) relates to a method of detecting methylation of cytosine in a CpG region of a gene to be analyzed with the use of a pair of a methylation-nonspecific primer and a metylated cytosine-specific primer in a definite step.

Invention group A and invention group C (invention groups AC)

The primers according to the invention group A and the invention group C are each to be employed for selecting an oligonucleotide in which cytosine except cytosine existing in a CpG region of a gene to be analyzed is converted into thymine or uracil.

Invention groups AC, invention group B and invention group D

It appears that the technical matter common to the invention groups AC, the invention group B and the invention group D resides in detecting the presence or absence of methylation of cytosine in a definite CpG region in a promoter region of a gene. As the results of search, however, it is found out that detection of the presence or absence of methylation of cytosine in a definite CpG region in a promoter region of the gene is not novel because of having been disclosed in WO 97/46705 A1 (Johns Hopkins University School of Medicine), 11 December, 1997 (11.12.97).

Accordingly, detection of the presence or absence of methylation of cytosine in a definite CpG region in a promoter region of the gene falls within the category of prior art and, therefore, this common matter cannot be regarded as a special technical feature in the meaning within the second sentence of PCT Rule 13.2.

Since there is no other matter seemingly being a special technical feature in the meaning within the second sentence of PCT Rule 13.2 common to the invention groups AC, the invention group B and the invention group D, no technical relevancy can be found out among these inventions differing from each other in the meaning within PCT Rule 13.

Such being the case, there is no technical feature common to the invention groups AC (claims 1 to 9 and 11 to 17), the invention group B (claim 10) and the invention group D (claims 18 to 22).

| 国際調査報告 | 国際出願番号　PCT／JP2004／012256 |
|---|---|

A.　発明の属する分野の分類（国際特許分類（IPC））

Int cl7 C12N 15/00 C12Q 1/68 G01N 33/50

B.　調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int cl7 C12N 15/00 C12Q 1/68 G01N 33/50

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）
CA／MEDLINE／BIOSIS／EMBASE／BIOTECHABS／WPIDS（STN），
GenBank／EMBL／DDBJ／GeneSeq

C.　関連すると認められる文献

| 引用文献のカテゴリー＊ | 引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示 | 関連する請求の範囲の番号 |
|---|---|---|
| X<br>Y | JP　2000－511776　A（ザ　ジョーンズ　ホプキンス　ユニバーシティー　スクール　オブ　メディシン）2000．09．12，文献全体　＆　WO　97／46705　A | 16-22<br>1-9、14, 15 |
| Y | Ito, E., Yanagisawa, Y., Iwahashi, Y., Suzuki, Y., Nagasaki, H., Akiyama, Y., Sugano, S., Yuasa, Y. and Maruyama, K., A core promoter and a frequent single-nucleotide polymorphism of the mismatch repair gene hMLH1, Biochem. Biophys. Res. Commun. (1999), 256, 488-494 | 1-9、14, 15 |

☒ C欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「T」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリー文献

| 国際調査を完了した日 | 国際調査報告の発送日 |
|---|---|
| 04．11．2004 | 22.11.2004 |
| 国際調査機関の名称及びあて先<br>日本国特許庁（ISA／JP）<br>郵便番号100－8915<br>東京都千代田区霞が関三丁目4番3号 | 特許庁審査官（権限のある職員）　4B　8615<br>内藤　伸一<br>電話番号　03－3581－1101　内線　3448 |

様式PCT／ISA／210（第2ページ）（200-4年1月）

Ｃ（続き）．　関連すると認められる文献

| 引用文献のカテゴリー＊ | 引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示 | 関連する請求の範囲の番号 |
|---|---|---|
| X | Potocnik, U.; Glavac, D.; Golouh, R.; Ravnik-Glavac, M., Causes of microsatellite instability in colorectal tumors. Implications for hereditary non-polyposis colorectal cancer screening, Cancer Genetics and Cytogenetics (2001), 126 (2), 85-96 | １０－１３ |
| X | Wheeler, J. M. D.; Loukola, A.; Aaltonen, L. A.; McC Mortensen, N. J.;Bodmer, W. F., The role of hypermethylation of the hMLH1 promoter region in HNPCC versus MSI+ sporadic colorectal cancers, Journal of Medical Genetics (2000), 37(8), 588-592 | １０－１３ |
| ＰＸ | ＪＰ　２００３－２５９８７４　Ａ（松原長秀）２００３．０９．１６，文献全体（ファミリーなし） | 16-19, 21, 22 |

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２００４／０１２２５６

第Ⅱ欄　請求の範囲の一部の調査ができないときの意見（第１ページの２の続き）

法第８条第３項（ＰＣＴ１７条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

1．☐ 請求の範囲 _____ は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、

2．☐ 請求の範囲 _____ は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、

3．☐ 請求の範囲 _____ は、従属請求の範囲であってＰＣＴ規則6.4(a)の第２文及び第３文の規定に従って記載されていない。

第Ⅲ欄　発明の単一性が欠如しているときの意見（第１ページの３の続き）

次に述べるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。

別紙参照

1．☒ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求の範囲について作成した。

2．☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求の範囲について調査することができたので、追加調査手数料の納付を求めなかった。

3．☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求の範囲のみについて作成した。

4．☐ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求の範囲について作成した。

追加調査手数料の異議の申立てに関する注意

☐ 追加調査手数料の納付と共に出願人から異議申立てがあった。

☒ 追加調査手数料の納付と共に出願人から異議申立てがなかった。

第III欄の続き
　独立した請求の範囲１及び独立した請求の範囲２に記載された発明（発明群Ａ）は、1)－10)に記載されたオリゴヌクレオチドを選択する核酸増幅用プライマーに関するものである。
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　独立した請求の範囲１８に記載された発明（発明群Ｄ）は、所定の１対のメチル化非特異的プライマーとメチル化シトシン特異的プライマーを、所定の工程で用いる、解析対象遺伝子のＣｐＧ領域中のシトシンのメチル化を検出する方法に関するものである。

・発明群Ａと発明群Ｃ（発明群ＡＣ）について
　発明群Ａと発明群Ｃに係るプライマーは、いずれも解析対象遺伝子のＣｐＧ領域中に存在するシトシン以外のシトシンがチミン又はウラシルに変換されたオリゴヌクレオチドを選択するものである。

・発明群ＡＣ、発明群Ｂ、発明群Ｄについて
　発明群ＡＣ、発明群Ｂ、発明群Ｄに共通する技術的特徴は、遺伝子のプロモーター領域の中にある所定のＣｐＧ領域におけるシトシンのメチル化の有無を検出することと認められるが、調査の結果、当該遺伝子のプロモーター領域の中にある所定のＣｐＧ領域におけるシトシンのメチル化の有無を検出することは、ＷＯ　９７／４６７０５　Ａ１（Johns Hopkins University School of Medicine），１９９７．１２．１１に開示されているから、新規でないことが明らかになった。
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　また、発明群ＡＣ、発明群Ｂ及び発明群Ｄにおいて、ＰＣＴ規則１３．２の第２文の意味において特別な技術的特徴と考えられる他の共通の事項は存在しないので、それらの相違する発明の間にＰＣＴ規則１３の意味における技術的な関連を見いだすことができない。
　したがって、発明群ＡＣ（請求の範囲１－９、１１－１７）、発明群Ｂ（請求の範囲１０）及び発明群Ｄ（請求の範囲１８－２２）において、共通の技術的特徴はない。

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(54) Title: METHOD TO DETERMINE DNA MISMATCH REPAIR FUNCTION

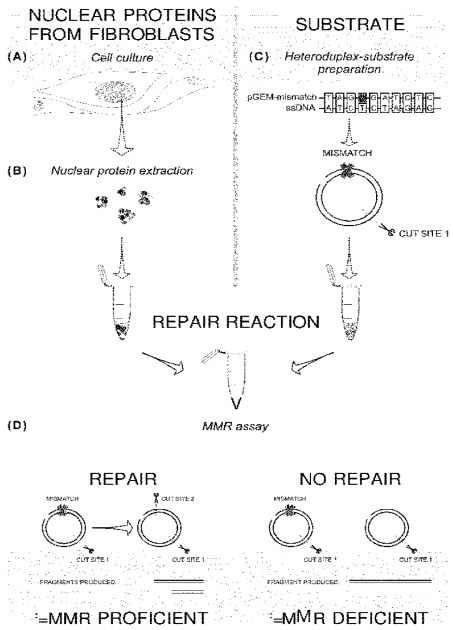



*Fig. 1*

(57) Abstract: This invention relates to a quantitative method for determining whether a human subject has an impaired DNA mismatch repair function;providing a diagnostic sample taken from said human and producing a nuclear extract from said sample;providing MMR proficient and MMR deficient nuclear extracts as positive and negative controls, respectively;combining each nuclear extract with at least one mismatch bearing substrate DNA molecule; performing a mismatch repair assay;and determining whether said sample nuclear extract is capable of repairing said substrate DNA molecule; wherein said sample comprises normal, non-malignant constitutive cells, such as fibroblasts. The invention further relates to a kit providing necessary reagents for use in said method.

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# METHOD TO DETERMINE DNA MISMATCH REPAIR FUNCTION

## FIELD OF THE INVENTION

This invention relates to cancer diagnostics and provides methods based on a DNA mismatch repair assay for determining whether a human subject has an impaired DNA mismatch repair function; and for identifying a mismatch repair gene having a defective function and thus an increased risk of developing hereditary cancers, especially colorectal cancer. The method also provides a novel method of diagnosing Lynch syndrome. The method is performed on a sample derived from normal tissue, such as fibroblasts.

## BACKGROUND OF THE INVENTION

Colorectal cancer (CRC) is the second most common cause of cancer-related death in the USA and Europe. According to a recent review article by Pino, M.S. and Chung, D.D. in Expert Rev Mol Diagn, 10(5):651 -665,201 0, CRC is responsible for an estimated 52,000 deaths per year in the USA and 146,000 per year in the EU. Approximately 2-5% of newly diagnosed cases of CRC can be attributed to Lynch syndrome.

Lynch syndrome (LS), often also referred to as hereditary nonpolyposis colorectal cancer syndrome (HNPCC), is manifested by early-onset colorectal and endometrial cancer, and an increased risk of certain extracolonic cancers, including tumors elsewhere in the gastrointestinal tract (e.g., stomach, small bowel, biliary tract), urinary collecting system (renal pelvis, ureter) and the female reproductive system (ovaries).

Studies of selected LS families have estimated a 70-80% lifetime risk of colon cancer with a mean age at diagnosis in the mid-40s. The second most common cancer in LS is endometrial cancer, and women with LS have a cumulative lifetime risk of developing endometrial and ovarian cancer, with a mean age at diagnosis approximately 10 years earlier than sporadic cases. The lifetime risk for gastric cancer in LS patients varies between populations, with a particularly high incidence in areas such as China and Korea, where there is a high endemic risk of gastric cancer, with a lifetime risk of approximately 30%. In these areas, the risk for gastric cancer exceeds that for endometrial cancer.

Lynch syndrome is highly associated with autosomal dominant inheritance of mutations in genes fundamental to the DNA mismatch repair (MMR) mechanism, and it is caused by germline mutations in one of four DNA mismatch repair (MMR) genes, *MLH1, MSH2, MSH6* and *PMS2.* Moreover, 25% of sporadic colon tumours, as well as a number of tumours of endometrium, ovary and some other organs and tissues, are deficient in MMR. MMR proteins normally recognize and repair mismatched nucleotides and insertion/deletion loops caused by the slippage of DNA polymerase during replication of short repeat sequences known as microsatellites.

The five proteins involved in the human mismatch repair (MMR) mechanism to maintain genomic integrity function as heterodimers are MutLa (MLH1 +PMS2), MutSa (MSH2+MSH6) and MutSβ (MSH2+MSH3). MMR proteins correct base/base mismatches and small insertion/deletion loops (IDLs) that arise on the newly synthesised strand during DNA replication and recombination.

The most frequently affected genes include MLH1 , MSH2, MSH6 and PMS2, whose germline variations are reported in the LOVD database (http://www.insight-group.org/; http://www.lovd.nl/). Although, the majority of mutations affecting MMR genes are truncating, a significant proportion of mutations result in a single amino acid substitution or an in-frame deletion and are difficult to distinguish from harmless polymorphisms. Such alterations are often referred to as variants of uncertain significance (VUS) due to the uncharacterized effect of the variation on the function of the polypeptide.

MMR-deficient tumours are strongly associated with microsatellite instability (MSI). However, the degree and type of MSI differ depending on the MMR gene affected (Kantelinen et al., British J Cancer, 1-6, 201 0).

The mean age of cancer onset in LS is significantly lower than that of sporadic colorectal cancer based on the fact that in LS, an individual has already inherited susceptibility through a mutated allele and only needs a second hit in a somatic cell to lose MMR activity and start tumorigenesis. Hence, LS tumors are characterized by the lack or lowered level of a causative MMR protein as well as impaired DNA repair causing MSI.

The timely recognition of LS is essential to identify patients at high-risk who will require intensive cancer surveillance. Colonoscopy screening and removal of precursor lesions, adenomas, significantly reduces cancer morbidity and mortality in MMR gene mutation carriers (Jarvinen et al., Gastroenterology

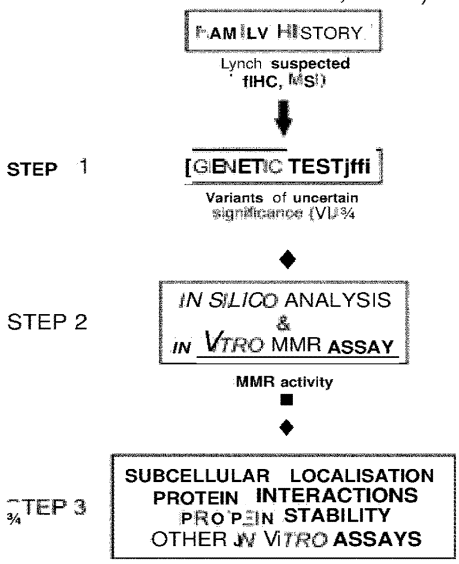
118: 829-834, 2000). Analyses of cost-effectiveness indicate that colorectal cancer surveillance in MMR gene mutation carriers is effective and considerably less costly than the consequences of no surveillance. The Centers for Disease Control and Prevention (CDC) in the United States recommends that all individuals with a new diagnosis of colorectal cancer, regardless of age or family history, should be offered genetic testing for Lynch syndrome. The CDC report further states that there is currently not enough evidence to recommend any specific screening strategy vs. alternative strategies.

However, the wide variety of clinical phenotypes complicates LS diagnostics and several clinical guidelines have been established to distinguish LS families from the general CRC burden. Currently, the clinical diagnosis of LS greatly relies on the Amsterdam criteria or the revised Bethesda guidelines, which take into account the age of cancer onset, the number and segregation of affected individuals in a family, and the level of MSI (Pino & Chung, *Supra).* However, many putative LS families do not fit these criteria and could be recognised as LS families only by characterizing a pathogenic germline MMR gene mutation in them.

The traditional diagnostic workflow to identify MMR gene mutation carriers, i.e., subjects afflicted by Lynch syndrome, involves several phases, such as tumor studies, DNA analyses, in vitro tests for mutation pathogenicity and proceeds from detecting a genetic defect to evaluating if it is associated with reduced MMR capacity. The first clinical step in diagnosing LS associated tumors includes immunohistochemistry (IHC) and MSI analysis followed by mutation screening dictated by the IHC and MSI results. One strategy for screening mutations is analyzing all four MMR genes (MLH1 , MSH2, MSH6 and PMS2). When a pathogenic mutation is found, LS can be confirmed or in the absence of a MMR gene variation, considered unlikely but not ruled out (since no method, whether used alone or in combination, is 100% sensitive and specific).

A recent publication by the present inventors (Kansikas et al., Hum Mutat 32:1 07-1 15, 201 1) discloses that testing *in vitro* synthesized mutant proteins for MMR capacity forms the cornerstone for pathogenicity testing. The published *in vitro* MMR assay studies the phenotypic consequences of LS mutations in a homologous human MMR system. Construction of the LS mutant protein requires that the genetic defect in question is found and fully character-

ized on DNA sequence level. A three-step decision tree was thus proposed (Couch et al., Hum Mutat 29:1 314-1 326, 2008):




However, there are several disadvantages associated with the present tests used for the establishment of a clinical diagnosis:

MSI testing is labour-intensive and requires expert molecular pathologic services, and while it is a hallmark for LS, it is not specific for it. Approximately 10-25% of sporadic CRCs and many extracolonic cancers also exhibit MSI. Furthermore LS-associated and sporadic MSI-positive CRCs have many histopathologic features in common, but differ in that sporadic MSI CRCs are not associated with a positive family history.

An inherent potential shortcoming of the IHC test is that the technique is somewhat subjective and depends upon the quality of tissue preparation, staining and interpretation of the results. Interestingly, abnormal staining patterns may be due to tissue preservation and the tumor microenvironment. For example, tissue hypoxia or oxidative stress may diminish the function of MMR proteins, even in genetically MMR-proficient tissues, leading to a focal loss or weak staining. Secondary abnormalities in MMR genes may also lead to rare staining patterns.

However, the most obvious limitation related to present methods of diagnosing LS, is that the tests are based on genetic testing and mutation analysis on subjects from a family with a known, or suspected, LS history, or on subjects already affected by cancer. This fact does not make present diagnostic methods suitable for routine screening of healthy subjects suspected of

being carriers. Furthermore, the present testing schemes are very laborious requiring well-equipped laboratories as well as highly skilled laboratory personnel. It has been estimated that the steps including IHC, MSI and mutation analysis amount to about 3500 € per test, which is expensive for a routine test.

There is thus a well-established and recognized need for accurate, simpler, but cheaper and clinically suitable tests for identifying, whether a person is a carrier of MMR gene mutations related to Lynch syndrome and thus subjected to a high risk of colorectal cancer, as well as other cancers.

## BRIEF DESCRIPTION OF THE INVENTION

The object of the present invention is to provide novel methods and means for achieving accurate, simple, cheap and clinically suitable tests for detecting decrease in MMR efficiency without preliminary knowledge of LS history or cancers in subjects, and thus solving the problems of current methods of diagnostics. The present invention relates to a quantitative method for determining whether a human subject has an impaired DNA mismatch repair function, wherein said method comprises a) producing a nuclear extract from a sample obtained from said human subject, b) providing MMR proficient and MMR deficient nuclear protein extracts as positive and negative controls, respectively, c) combining in separate vials each nuclear extract with at least one mismatch-bearing heteroduplex DNA substrate, d) performing a mismatch repair assay on said sample and control nuclear protein extracts and said substrate, and e) determining whether said sample nuclear extract is capable of repairing said substrate DNA molecule. Preferably said sample comprises normal cells obtained from a constitutive tissue, more preferably fibroblast cells or blood-derived cells.

In one embodiment of the invention, said substrate is derived from a plasmid having SEQ ID NO:1 , and preferably said substrate further comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

In another embodiment the MMR deficient nuclear extract is derived from a cell line selected from the group consisting of HCT1 16 and LoVo cells. In a specific embodiment the total amount of nuclear extract per vial is in the range of 50-500 µg, preferably 50-200 µg, more preferably 75-100 µg.

The present invention further relates to a quantitative method for identifying a mismatch repair gene having a defective function, wherein said method comprises a) producing a nuclear protein extract from a sample ob-

tained from a human subject, b) providing MMR proficient and MMR deficient nuclear protein extracts as positive and negative controls, respectively, c) mixing at least one MMR deficient nuclear protein extract with said sample extract, d) combining in separate vials each nuclear extract provided in steps a-c with at least one mismatch-bearing heteroduplex DNA substrate, e) performing a mismatch repair assay on said nuclear extracts and said substrate, and f) identifying said defective gene by determining whether said sample nuclear extract is capable of repairing said substrate and complementing said MMR deficient nuclear extract. Preferably said sample comprises normal cells obtained from a constitutive tissue, more preferably fibroblast cells or blood-derived cells.

In one embodiment of the invention, said substrate is derived from a plasmid having SEQ ID NO:1 , and preferably said substrate further comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

In another embodiment the MMR deficient nuclear extract is derived from a cell line selected from the group consisting of HCT1 16 and LoVo cells. In a specific embodiment the total amount of nuclear extract per vial is in the range of 50-200 µg, preferably 75-100 µg.

Furthermore, the present invention relates to a novel method for diagnosing Lynch syndrome in a human, based on the identification of a defective DNA mismatch repair gene using an MMR assay according to the present invention, from a clinical sample obtained from a constitutive tissue of a human subject.

The present invention also relates to a kit for use the methods according to the present invention, comprising at least one mismatch-bearing heteroduplex DNA substrate, at least one MMR deficient nuclear extracts, and an MMR proficient nuclear extract as a positive control. Preferably said MMR deficient nuclear extracts are derived from a cell line having an MMR deficiency in respect of either MLH1 , MSH2 or MSH6, preferably from HCT1 16 or LoVo cells.

In a specific embodiment of the present invention, the kit comprises at least two MMR deficient nuclear extracts.

In a further embodiment the kit may also include additional reagents necessary for performing the MMR assay, such as 10X MMR buffer comprising 200 mM Tris-HCL pH 7.6, 400 mM KCl, 50 mM MgCl2,1 0 mM Glutathione, BSA 500 Mg/ml, 1 mM each dNTP and 15 mM ATP; and a STOP solution for

MMR assay comprising 42 mM EDTA, 1.2% SDS, 50.4 µg/ml Proteinase K. Optionally, the kit may comprise reagents for clarifying the sample, such as TE buffer, phenol/chloroform/isoamyl alcohol and chloroform; for precipitating said sample, such as NaCl and ethanol; and for detecting the repair, such as RNase A and restriction enzymes *BglW* and *Eco31*\ with buffer and loading dye.

## BRIEF DESCRIPTION OF THE FIGURES

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings, in which

Figure 1 is an overview of the method of the present invention;

Figure 2 is an MMR analysis of healthy fibroblast nuclear extract (FB-WT, wild type) compared to that obtained from an MMR proficient cell line (HeLa) and an MMR deficient assay control (MOCK) without nuclear proteins;

Figure 3 is a Western blot analysis showing a) the MMR protein content (MLH1 and its counterpart PMS2) of a healthy fibroblast nuclear extract (FB-WT) compared to the fibroblast derivative cell line FB-M1 where MLH1 has been partially silenced and b) the MMR protein content (MSH2 and its counterpart MSH6) of FB-WT compared to the fibroblast derivative cell line FB-SH1 3 where MSH2 has been partially silenced.

Figure 4 is a quantitative MMR analysis showing that the deficiency of MLH1 in FB-M1 and MSH2 in FB-SH1 3 is detected as a decrease in repair efficiency with varying intensity. Relative repair percentages are shown in respect to the repair efficiency of FB-WT; and

Figure 5 is a quantitative MMR analysis showing that different MMR capability of FB-WT and FB-M1 can be detected when used in combination with an MLH1 deficient nuclear extract HCT1 16); and

Figure 6 is a schematic presentation of the substrate preparation, including the plasmid presentations of pGEM-IDL40, pGEM-IDLGT and pGEM-IDL39, and their derivative constructs 5'IDLGT and 5'IDL1 .

## DETAILED DESCRIPTION OF THE INVENTION

It has now surprisingly been found, that a functional assessment of DNA mismatch repair (MMR) efficiency can be used to recognize individuals with an inherited MMR deficiency, and thus at notable risk to develop cancer. The present invention provides a novel MMR assay, which is aimed at identifying deficient ability for MMR (Figure 1). The assay according to the present

invention is applicable without any prior knowledge of the underlying MMR gene sequence or regulatory change, family history, cancer history or IHC results and therefore constitutes a novel approach for diagnostic purposes. The assay is ideal for screening purposes of MMR mutation carriers in large populations, and thus for finding persons with high risk for cancer development.

The present invention relates to a simple test protocol consisting of only one assay, whereas the traditional diagnostic workflow to identify MMR gene (Lynch syndrome) mutation carriers involves several tests, and proceeds from detecting a genetic defect to evaluating if it is associated with reduced MMR capacity. The present invention provides a one-step procedure that evaluates whether or not an individual carries a MMR defect in the germline, determined by a quantitative MMR assay. The method according to the present invention is based on detection of decreased MMR capacity in a normal tissue sample, such as blood, mucosa or fibroblasts, preferably fibroblasts or lymphoblasts obtained from the subject to be tested.

As used herein, the term "normal" tissue is intended to include any healthy, non-malignant tissue, preferably a constitutive tissue.

As used herein, the term "constitutive tissue" refers to tissues of non-malignant cells which substantially represent the genetic composition inherited at birth and which may be derived from any part of the human body accessible to sampling, e.g. mucosa or fibroblasts.

The test protocol of the present invention relates to a quantitative method. As used herein, the term "quantitative method" refers to a method, which gives results of MMR efficiency with varying intensity, i.e. it is not an on/off test. The present MMR assay detects deficiencies of MMR proteins as a decrease in repair efficiency (see Figure 4 for MLH1 and MSH2). As used herein, MMR genes or proteins refer to any genes or proteins participating in the MMR process, for example selected from the group consisting of MLH1, MSH2, MSH6, MLH3, MSH3, PMS1, PMS2 and EXO1. In a specific embodiment, deficiencies of MMR proteins MLH1, MSH2 and/or MSH6 are detected by the method or means of the present invention. Indeed, there is still some ability to correct mismatches, if for example only one normal MMR allele is present, as is the case in normal cells of all MMR gene mutation carriers in LS families, or if a MMR gene is partly inactivated. In comparison, tests of prior art used for cancer cells reveal whether MMR occurs (both wild type alleles pre-

sent) or is absent (both wild type alleles absent) representing an on/off test for MMR.

MMR of the DNA takes place in the nucleus of the cell, and only the MMR proteins of the nucleus take part in the repair process. By the method of the present invention it is possible to assay the functionality of those MMR proteins which have headed to the nucleus for repair activity *in vivo,* because a starting material of the method is a nuclear extract. On the contrary, in the traditional workflow, the MMR assay is used for studying MMR efficiency of the mutated MMR protein products constructed *in vitro.* In these *in vitro* tests, the proteins to be tested are artificially added to the nuclear extract without the possibility to study only the proteins naturally present in nuclei. Thus, the pathogenicity test of the present invention differs from functional tests of the prior art by revealing the actual MMR ability of the nucleus. And therefore, by the method of the present invention, it is also possible to find out problems of nuclear localization of the MMR proteins.

The methods of the prior art utilize cell extracts i.e. cytoplasmic extracts (CE) instead of nuclear extracts. Tests with cytoplasmic extracts give wrong information of amounts and proportions of MMR proteins, which are capable for repair processes in reality. Therefore, tests with cytoplasmic extracts are suitable only for on/off test and not for quantitative testing of MMR.

In the methods of the present invention, MMR ability is detected directly from the agarose gel without using any indirect detection method of the prior art such as complementation assays in *E.coli,* wherein detection is arranged by expression of lacZa.

The one-step assay according to the present invention may, optionally, be followed by or include steps for identifying an underlying genetically or epigenetically defected gene if one so wishes, but this is not necessary for a clinical diagnosis. Contrary to traditional tests, the method according to the present invention allows for the recognition of individuals with increased cancer susceptibility due to deficient MMR even in cases where no family member has, yet, developed cancer, where mutation tests result in no detectable change, and/or where the underlying change is not genetic but epigenetic (regulatory).

The basic principle, as well as different embodiments of the proposed assay is shown in an array format in Table 1, wherein Y denotes an

MMR deficient nuclear protein extract, X a test sample nuclear protein extract and Z an MMR proficient positive control nuclear protein extract.

**Table 1**

| MMR +/- | MMR gene 1 +/- | MMR gene 2 +/- | MMR gene 3 +/- |
|---|---|---|---|
| Y | $Y_1$ | $Y_2$ | $Y_3$ |
| X | $X + Y_1$ | $X + Y_2$ | $X + Y_3$ |
| Z | Z | Z | Z |

In one embodiment of the present invention a test sample nuclear protein extract (X) is prepared from cultured normal cells, such as fibroblasts, obtained from the subject to be tested, and an MMR assay according to the present invention is performed in vials comprising Y (MMR deficient nuclear extract), X and Z (MMR proficient positive control nuclear extract), respectively. The repair reaction converts heteroduplexes not susceptible to cleavage by the restriction endonuclease to homoduplexes, which can be cleaved. If sample X is MMR proficient, the substrate heteroduplex will thus be cleaved and linearized into three fragments, whereas only one fragment will result because of linearization, if X is MMR deficient. Correspondingly, Y will produce only one fragment, whereas Z will produce three. This embodiment of the present invention, the MMR +/- assay (+/- indicating that one allele of the MMR gene is functioning and the other one is deficient), will allow determination of whether the human subject tested has an impaired/deficient MMR function or a normal MMR function.

In another embodiment of the present invention, the sample nuclear extract (X) is used to complement an MMR deficient nuclear extract (Y) to form X + Y. The complemented test samples (X + Y), as well as the positive (Z) and negative (Y) controls, are then used in an MMR assay according to the present invention to study the ability to repair mismatches. The repair efficiency is quantified by measuring the cleavage efficiency using the repair efficiency of the proficient fibroblast nuclear extract (Z) as a reference level, as exemplified in example 8 and figure 4.

The number of MMR deficient nuclear extracts ($Y_n$) included in the assay may be varied depending on if a defective (s) are considered important to identify. In one preferred embodiment of the method according to the present invention $Y_1$ is MLH1 deficient and $Y_2$ is MSH2/MSH6 deficient. In a fur-

ther embodiment of the present invention $Y_3$ could be PMS2 deficient. However, any one of said MMR deficient nuclear extracts ($Y_n$) could be omitted or added and used, in any combination. A person skilled in the art would be able to construct a test panel suitable for the clinical situation in question.

A typical diagnostic assay according to the present invention is performed as described in more detail below.

### A. Tissue samples

The tissue sample to be tested may be any normal tissue, which is easily obtained and processed. In one specific embodiment of the present invention, the tissue sample of choice is a human skin punch or excision biopsy, which may have been obtained by any method well known to a person skilled in medical care.

Other tissue samples, which may be used in a method according to the present invention, include blood samples, and mucosal swabs.

### B. Expansion of the sample cells

In order to examine the mismatch repair (MMR) ability of the cells, a sufficient amount of cells have to be grown prior to extracting nuclear proteins from them. Cells are grown in standard cell culture conditions, suitable for the type of cells used and well known in the art, and thereafter collected by gently spinning them down.

Any cell type derived from a clinical sample taken from a human subject, including malignant cells derived from a tumor, may be used in a method according to the present invention. Preferred cell types for use in a test according to the present invention are human fibroblasts. Another preferred cell type is blood-derived human cells, such as lymphoid cells.

Most preferably, cells for the test of the present invention are normal primary cells or transformed normal cells.

### C. Extraction of nuclear proteins from human fibroblast cells

MMR proteins are extracted from the nuclei of the cells. Cells are spun down, counted and washed with saline buffer in order to remove any remaining trypsin. The cells are further washed with isotonic buffer before they are spun down and swollen with the osmotic effect of a subsequent hypotonic wash. A narrow needle is used to disrupt the cell membranes until the majority of the cells are confirmed to have lysed under the microscope.

After spinning the cells down, the supernatant containing the cytoplasmic fraction is removed. The nuclear proteins are extracted from the remaining nuclear fraction by incubation in cold extraction buffer with salt after which any remaining nuclear debris is spun down and removed as the remaining pellet. The extracted nuclear protein sample is thereafter dialysed in the presence of proteinase inhibitors, and further cleared by centrifugation prior to storing in -80°C. The protein content of the samples is quantified by a fluorometer.

### D. Substrate preparation

A circular heteroduplex plasmid substrate for use in the MMR assay according to the present invention is made by reannealing a single stranded DNA (ssDNA) and a denatured matching plasmid DNA with one non-homologue site.

The ssDNA is produced from a plasmid with the help of bacterial cells and a commercially available helper phage under heavy antibiotic selection. Cell debris is spun down and phage particles are collected by precipitation and further centrifugation. Bacterial cells are also removed by centrifugation and any remaining chromosomal DNA originating from the bacteria is removed with enzymatic treatments. Upon clearing of the sample, the ssDNA is released from the phage capsid proteins with the help of a proteinase-enzyme.

A double stranded (ds) plasmid template containing the mismatch error is amplified in and purified from bacterial cells. In order to have a 5' nick upstream of the site of the error, a specific restriction enzyme is used to linearize the construct after which the sample is precipitated and dissolved in an appropriate volume of buffer.

In order to make the mismatch-bearing heteroduplex substrate for the MMR assay according to the present invention, the ssDNA and the matching plasmid DNA described above are denatured and reannealed together. Subsequent washing steps, including the removal of salts and unhybridized linear dsDNA as well as unhybridized ssDNA followed by ethanol precipitation ensure the purity of the final product. The concentration is measured with a spectrophotometer as well as by gel electrophoresis which also serves to verify the purity of the sample.

## E. The MMR assay according to the present invention

An MMR assay according to the present invention may be used only for determining whether the MMR function of the test sample is deficient or not, but can also distinguish between MMR capability of nuclear proteins extracted from cells with deficient MMR and of those extracted from MMR proficient cells, as described above. To identify a MMR deficient gene, the sample nuclear extracts (X) are used to complement separately MLH1 **(Yi)** and MSH2/MSH6 **($Y_2$)** deficient nuclear extracts.

An MMR assay according to the present invention is carried out by combining about 50-500 µg, preferably about 50-200 µg, more preferably about 75-100 µg of nuclear protein extract with an excessive amount of circular plasmid substrate (heteroduplex), whereas in the case of complementing a well characterised MMR deficient cell line to determine the MMR gene causing the deficiency, the total amount of the two extracts used in the assay should not exceed that of 200 µg. The repair reaction is induced in the presence of an MMR buffer which ensures optimal salt concentration whilst also providing ATP and deoxyribonucleotides. The repair reaction is terminated enzymatically and the proteins are removed from the sample by a phenol/chloroform/isoamyl alcohol extraction.

After precipitation, the sample is subjected to a double digestion, which cleaves the substrate heteroduplex DNA into two smaller fragments if the repair process was successful. These two smaller fragments are visualized by agarose gel electrophoresis. As the substrate DNA is added in excess, a linearized full length product will also always be visible. Where no repair has occurred, only the full length linearized plasmid DNA will be apparent.

It is a further object of the present invention to provide a kit comprising the necessary reagents for performing a method according to the present invention. A kit according to the present invention includes standard reagents, such as a substrate comprising necessary mismatch bearing heteroduplex DNA substrate(s); at least one, but preferably two, MMR deficient nuclear extracts); and an MMR proficient nuclear extract as a positive control. In a specific embodiment of the present invention, said MMR deficient nuclear extracts are derived from cell lines deficient for MLH1 and MSH2/MSH6, respectively. More specifically, said MMR deficient nuclear extracts are derived from HCT1 16 and LoVo cells.

The reagents included in a kit according to the present invention should be chosen according to the basic principle disclosed in Table 1. In one embodiment, a first nuclear extract (Y1) is deficient of MLH1, whereas a second nuclear extract (Y2) is deficient of MSH2/MSH6. If a tested sample does not complement said first nuclear extract, but does complement said second nuclear extract, said test sample reveals a MLH1 deficiency. However, if said tested sample does complement said first nuclear extract, but not said second nuclear extract, said test sample reveals that the tested subject has either an MSH2 or an MSH6 deficiency.

Optionally, a kit according to the present invention may include further MMR deficient nuclear extracts, which specifically distinguish between deficiencies in the *MSH2* and *MSH6* genes. Such nuclear extracts may be derived from cell lines, such as MSH6 deficient HCT-15/DLD-1 (ATCC CCL-225/221).

Optionally, a kit according to the present invention may also comprise additional reagents necessary for performing the MMR assay, such as necessary buffers and nucleotides.

One example of a kit according to the present invention is given in the examples below.

EXAMPLES

The following examples are given to further illustrate embodiments of the present invention, but are not intended to limit the scope of the invention. It will be obvious to a person skilled in the art, as technology advances, that the inventive concept can be implemented in various ways. The invention and its embodiments are thus not limited to the examples described herein, but may vary within the scope of the claims.

**Example 1. Culturing fibroblasts taken from the subject to be tested**

Fibroblast cells are grown in Dulbecco's Modified Eagle Medium (Invitrogen, CAT# 31966-021) in a 37°C 5% $CO_2$ incubator. The growth medium is supplemented with 10% fetal bovine serum (Invitrogen, CAT# 10106-169), 2 X Non Essential Amino Acids (Invitrogen, CAT# 10370-070) and 2% Penicillin-Streptomycin (Invitrogen, CAT# 15070-063). The cells are subcultured 1:2-1:6 when approximately 80-90% confluent with the help of Trypsin 0.25% EDTA (Invitrogen, CAT# 25200-072). To maintain the silencing in the MLH1-depleted derivative cell line FB-M1 or MSH2-depleted derivative cell line

FB-SH1 3, 150 µg/µl hygromycin B (Invitrogen, CAT# 10687-01 0) is added to the growth medium. Cells are grown in cell culture treated filter-capped 175 bottles (NUNC, CAT# 145-1 78883) until approximately 5 x $10^8$-$10^9$ cells are gathered for the extraction of nuclear proteins.

**Example 2. Cell lines and nuclear extracts**

Adherent human cancer cell lines HeLa, LoVo and HCT1 16 (American Type Culture Collection, Manassas, VA, USA) are cultured according to instructions of manufacturers. HeLa cells (ATCC CCL-2) are MMR proficient epithelial cells derived from the cervix of a female individual, whereas HCT1 16 (ATCC CCL-247) and LoVo cells (ATCC CCL-229) are MMR deficient epithelial cells derived from the colon of a male individual. HCT1 16 cells lack MLH1 whereas in LoVo cells, the *MSH2* gene is inactivated causing a deficiency of MSH2, MSH3 and MSH6 proteins. The lack of MSH2 has been associated with the proteolytic degradation of its counterparts MSH3 and MSH6.

HeLa cells are grown in DMEM medium complemented with 2% L-glutamine, 10% FBS and 5% PS. LoVo cells are grown in F12 (or F12:DMEM) medium (Invitrogen, CAT# 31765-027/31331-028) complemented with 2% L-glutamine, 10-20% FBS and 5% PS whereas HCT1 16 cells are grown in McCoys 5A medium (Invitrogen, CAT# 22330-021 ) complemented with 2% L-glutamine, 10% FBS and 5% PS.

The FB-M1 cell line was produced as described in McDaid et al, BJC, 101:441-451 , 2009. In brief, overlapping oligonucleotides producing siRNA targeting MLH1 at AACTGTTCTACCAGATACTCA were designed and ligated into a commercially available shRNA vector pSilencer™ (Ambion CAT# AM7209). The construct was verified by sequencing before linearising and electroporating 1 x $10^7$ cells with 1 µg DNA. Serum enriched medium was immediately added and the cells were plated a 5 x $10^5$ before adding hygromycin selection (150 µg/µl) for 10-14 days. Similarly, the FB-SH1 3 cell line was produced using overlapping oligonucleotides producing siRNA targeting MSH2 at TCTGCAGAGTGTTGTGCTT into the pSilencer™ shRNA vector.

Correspondingly, fibroblast cell lines comprising other MMR gene-mutations, such as silenced MSH6 may be prepared, using shRNA vector pSilencer™ 4.1 - CMV Hygro (Cat# AM5777). The fibroblast nuclear extracts were prepared according to the following protocol (Lahue et al, Science, 245:1 60-1 64,1 989):

1. Harvest 5 x $10^8$-$10^9$ log phase cells with trypsin.

2. Count the cells.

3. Centrifuge at 500xg for 10 min +4°C.

4. Resuspend the cell pellet in 20-30 ml of cold 1 x isotonic buffer (20 mM Hepes pH 7.5, 5 mM KCl, 1.5 mM MgCl, 250 mM sucrose, 0.2 mM PMSF, 1 × complete EDTA-free protease inhibitor mixture (Roche Diagnostics GmbH, Mannheim, Germany), 0.25 µg ml-1 aprotinin, 0.7 µg ml-1 pepstatin, 0.5 µg ml-1 leupeptin, 1 mM DTT) and centrifuge as before.

5. Estimate the packed cell volume and resuspend it in 20-30 ml cold 1 x hypotonic buffer (isotonic buffer without sucrose) and centrifuge immediately as before.

6. Remove the supernatant and resuspend the cell pellet in 1 x cold hypotonic buffer to obtain a cell density of 1-2 x $10^8$ cells/ml. The packed cell volume is substracted from the resuspension volume.

7. Disrupt cells by a syringe with a narrow gauge needle by drawing cells slowly into the syringe and then ejecting them with a single rapid stroke. Cell disruption is to be carried out until 80-90% of the cells have lysed, as verified with under the microscope (50 µl of 0.4% Trypan blue in PBS with 45 µl PBS and 5 µl cells).

8. Centrifuge the disrupted cells at 3000xg for 10 min +4°C and remove the supernatant.

9. Estimate the nucleic pellet volume and add 1/3 to 1/5 of cold extraction buffer (25 mM Hepes pH 7.5, 10% sucrose, 1 mM PMSF, 0.5 mM DTT, 1 µg ml-1 leupeptin) to the volume of the pellet. Resuspend the pellet well by pipetting up and down.

10. Measure the new volume and add NaCl to a final concentration of 0,1 55 M whilst mixing.

11. Rotate the mixture for 60 min at 4°C.

12. Centrifuge at 14500xg for 20 min at 2°C.

13. Dialyse the sample for 2 x 50 min in a 3.500 MWCO cassette in 1L of cold dialysis buffer (25 mM Hepes pH 7.5, 50 mM KCl, 0.1 mM EDTA pH 8, 10% sucrose, 1 mM PMSF, 2 mM DTT, 1 µg ml-1 leupeptine). Change the buffer after the first 50 minutes.

14. Clarify the extract by centrifugation at 20000xg for 15 min at 2°C.

15. Snap freeze nuclear proteins in liquid nitrogen and store at -80°C.

**Example 3. Western blot analysis**

Protein expression levels in the nuclear extracts (NEs) were studied by western blot analysis using 50 mg of NE and 0.1-5 µl of wild type total protein extract by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis. The proteins were blotted into nitrocellulose membranes (Amersham Hypond™, PVDF, Amersham Pharmacia Biotech, Uppsala, Sweden), which were subsequently incubated with monoclonal antibodies anti-MSH2 (Calbiochem, San Diego, CA, USA, MSH2-Ab1 , NA-26, 0.2 mg/ml), anti-MSH3 (BD Transduction Laboratories, Lexington, KY, USA, M941 20, 250 mg/ml), anti-MSH6 (BD Transduction Laboratories, clone 44, 0.02 mg/ml), anti-PMS2 (Calbiochem/Oncogene Research, San Diego, CA, USA, Ab-1 , 0.2 mg/ml) and anti-MLH1 (BD Biosciences/Pharmingen, San Diego, CA, USA, clone 168-1 5, 0.5 mg/ml). Ubiquitously expressed a-tubulin was used as a loading control to estimate the MMR protein levels in the extracts (anti-a-tubulin; Sigma, Louis, MO, USA, DM1A, 0.2 mg/ml).

**Example 4. Production of wild-type heterodimer protein complexes.**

*Spodoptera frugiperda (Sf9)* (Invitrogen, Carlsbad, CA, USA) insect cells were transfected with bacmid DNA carrying wild-type (WT) MSH2, MSH3, MSH6, PMS2 or MLH1 cDNA fragments afterwhich the cells were re-infected to obtain a higher yield of recombinant baculoviruses (Nystrom-Lahti et al, 2002). These WT-recombinant baculoviruses were used to co-infect *Sf9* cells for protein production forming the heterodimer complexes assayed: MutLa (MLH1 + PMS2), MutSa (MSH2 + MSH6) and MutSβ (MSH2 + MSH3). The heterodimeric complexes were extracted as total protein extracts (TE) at 50 h (MutLa) or 72 h (MutSa and MutSβ) by lysing and centrifuging the cells down.

**Example 5. Substrate preparation**

**Preparation of heteroduplex molecules.** The heteroduplex DNA molecule is a circular 3192 bp long molecule with a single-strand nick 445 bp upstream from the site of the mismatch. This contains a complete *BglW* restriction site. The molecule is made by annealing single stranded DNA from a pGEM-IDL40 plasmid (Figure 6) together with an error introducing plasmid based on the same construct (pGEM-IDL GT, for 5'GT substrate or pGEM-IDL-39 for 5'IDL1 substrate).

Single-stranded DNA (ssDNA) was prepared by infecting pGEM IDL40 trans-formed XL1-blue bacteria cells with the M13K07 bacteriophage (Amersham Biosciences, Piscataway, NJ, USA), which replicates the anti-sense strand that can thereafter be extracted from the cells. The lower (-) strand amplified by the M13 phage has the sequence depicted in SEQ ID NO: 1, where the *BglW* restriction site is annotated.

Two different heteroduplex constructs were prepared; a G-T mis-match (5'GT) and a single nucleotide 5'IDL1. Site-directed mutagenesis was carried out to a pGEM-IDL40 according to manufacturer's instructions (Quik-Change® Site-directed mutagenesis, Stratagene, La Jolla, CA, USA) to pro-duce the error containing pGEM IDLGT and pGEM IDL39 plasmids.

The 5'GT substrate was made by annealing the ssDNA with the mismatch containing pGEM-IDLGT. This 3192bp long base plasmid (pGEM-IDLGT) for linear dsDNA contains an incomplete *BglW* restriction site (GGATCT). The upper (+) strand is complementary to ssDNA produced from IDL40, except for the G which is replacing the A that would be complementary in the BglII site. The sequence of the pGEM-IDLGT is depicted in SEQ ID NO: 2 and the incomplete *BglW* restriction site annotated.

The 5'IDL1 substrate was made by annealing the ssDNA with the error containing pGEM-IDL1. This 3191bp long base plasmid (pGEM-IDL39) for linear dsDNA contains an incomplete *BglW* restriction site (-GATCT). Upper (+) strand is complementary to ssDNA produced from IDL40, except for delA in the *BglW* site. To create the 5' nick the circular substrate was linearized with *BamW* or DraIII before reannealing. The sequence of pGEM-IDL39 is depicted in SEQ ID NO: 3 and the incomplete *BglW* restriction site is annotated.

**Example 6. MMR assay according to the present invention**

The present invention is a modification of the previously described *in vitro* MMR assay (Nystrom-Lahti et al, 2002), allowing to assay the MMR capability directly of a nuclear extract. Furthermore, complementation can be used to characterise the defected MMR gene in the sample by complementing a characterised MMR deficient extract as described in Table 1. Repair reac-tions are standardised to include a total of 75-100 µg of NE. The excess amount of the heteroduplex DNA substrate (5'GT or 5'IDL1) is set to 100 ng. The repair reaction is performed in 20 µl volume with the following reagents:

- 100 ng heteroduplex substrate
- 75-100 µg nuclear extract

- 2 µl of 10X MMR buffer (200 mM Tris-HCL pH 7.6, 400 mM KCl, 50 mM MgCl2 ,10 mM Glutathione, BSA 500 pg/ml, 1 mM each dNTP, 15 mM ATP)
- KCl to get 110mM final concentration
- add $H_2O$ up to 20 µl.

As the reaction is to be done in the total volume of 20 µl and a final KCl concentration of 110mM, the KCl content of the nuclear extract needs to be taken into consideration (2 µl of 10x MMR buffer brings KCl content of the reaction mix to 40 mM). The KCl content of nuclear extract is assumed to be 75 mM/µl.

The typical protocol of the MMR assay according to the present invention is:

1. Pipette the components of the repair reaction together on ice and incubate 30 min at 37°C.
2. Stop the reaction by adding 30 µl of fresh stop solution (42 mM EDTA, 1.2% SDS, 50.4 µg/ml Proteinase K). Incubate at 37°C for 20 min.
3. Add 1 volume (50 µl) of TE buffer pH 8.0.
4. Add 1 volume of phenol-chlorophorm-isoamylic alcohol (25:24:1 ) and vortex for 10s. Centrifuge at maximum speed for 10 min at room temperature.
5. Transfer the upper phase (approximately 90 µl) to a new tube and add an equal volume of chloroform. Vortex for 10 s and centrifuge at maximum speed for 10 min at room temperature.
6. Transfer 85 µl of the upper phase to a fresh tube for ethanol precipitation.
7. Add NaCl to obtain a final concentration of 150mM followed by 2.5 volumes of cold absolute ethanol. Incubate at -20°C for 30 minutes.
8. Pellet the DNA by centrifuging the sample at full speed for 30 minutes at +4°C.
9. Wash the pellet with 150 µl of 70% ethanol, centrifuge at full speed for 10 minutes dry and resuspend the pellet in 6 µl of ddH2O.
10. Add 4 µl of freshly prepared 10 x digestion mix (2.5 µl FastDigest® *BglW* (Fermentas, CAT# FD0083), 2.5 µl FastDigest® *Eco31*\ (Fermentas, CAT# FD0293), 10 µl FastDigest® Buffer and 25 µl ddH2O) and incubate at 37°C for 10 minutes.
11. Add RNAse A up to 40 µg/ml and incubate at 37°C for 10 minutes.
12. Load samples onto a 1.0% agarose gel made with IxTAE to see if repair has occurred.

Repair percentages are analysed using Image-Pro® 4.0 (Media Cybernetics, Silver Spring, MD, USA).

MMR-proficient HeLa NE is used as a positive control, whereas uncomplemented MMR deficient NEs are used as negative controls. The substrates are linearised with *Eco31*\ restriction enzyme. As the repair reaction converts a GT heteroduplex to an AT homoduplex or fills the 1 or 2 nt loop structures recreating the *BglW* restriction site, the repair efficiency can be measured by the efficiency of the double digestion.

**Example 7. Fibroblast cells used in the MMR assay according to the present invention**

This example was performed to show that the MMR assay can be performed on normal tissue, such as fibroblast cells. Wild-type fibroblast nuclear extract (FB-WT) prepared as described in example 2, nuclear extract from MMR proficient HeLa cells and a negative control (MOCK) containing no proteins were subjected to an MMR assay according to the present invention as described in example 3.

After allowing the repair reaction to take place, a double digestion of the substrate DNA will yield a single larger band of approximately 3000 bp where no repair has occurred, and further two smaller fragments (of approximately 1800 and 1300 bp) where repair has occurred. As the substrate DNA is always added in excess, even MMR proficient extracts will have the linearized substrate present in addition to the double cleavage fragments.

The result of this assay is presented in Figure 2, which shows that lane 1 containing MOCK cells has only one large band (= no repair), whereas lanes 2 and 3, containing FB-WT and HeLa nuclear extracts, respectively, show two additional, smaller bands, indicative of a repair reaction.

Thus, this example unambiguously shows that nuclear extracts from normal fibroblast cells have a MMR capability comparable to that of a MMR proficient control cell line (HeLa), which can be confirmed by an assay according to the present invention.

**Example 8. Human fibroblast cells with a partly silenced MMR gene show decreased MMR capability in the MMR assay according to the present invention**

This example shows that human fibroblast cell lines FB-M1 and FB-SH1 3 (described in example 2), where the *MLH1* gene or *MSH2* gene, respec-

tively, is partly silenced by (sh)RNA technology, display a clear decrease in MMR capability.

Firstly, a Western blot was performed on equal amounts of nuclear extracts from wild-type fibroblast (FB-WT) cells and FB-M1 cells or FB-WT and FB-SH1 3 cells. As a result (Figure 3) it can be seen, that the amount of MLH1 protein is clearly decreased in FB-M1 cells as compared to FB-WT, as is the level of MSH2 in FB-SH1 3 cells. a-tubulin is included as a loading control.

Secondly, the MMR assay was performed as described in example 6, using the same nuclear extracts in equal amounts:

MOCK - Nuclear extract free negative assay control;

FB-WT - Wild type human fibroblast cell nuclear extract (positive control); and

FB-M1 - Nuclear extract from human fibroblast cells with partly silenced MLH1 ; or

FB-SH1 3 - Nuclear extract from human fibroblast cells with partially silenced MSH2.

The result of this assay shows that a lowered concentration of one of the key MMR proteins is associated with a defected MMR mechanism. The result is shown in Figure 4 and it clearly demonstrates that compared to the wild type human fibroblast nuclear extract, the MMR assay according to the present invention detects the deficiency of MLH1 and MSH2 as a decrease in repair efficiency. Furthermore, the MMR capability of an MMR deficient nuclear extract can be differentially restored when assayed together with wild-type or partially MMR deficient nuclear extract as presented in Figure 5 (HCT1 16 with FB-WT or FB-M1 in this case) and described by the test presented in Table 1 and described on pages 10-1 1.

The repair efficiency in the MMR assay according to the present invention is regarded as the percentage of repaired DNA of all substrate DNA in the reaction. The shortage of a key MMR protein reduces the repair efficiency with varying intensity whereas the complete absence of key MMR proteins reduce the repair efficiency to a non-detectable level.

**Example 9. A kit for use in an assay for diagnosing defects in MMR**

A kit according to the present invention is preferably presented as vials preloaded with all reagents for performing the MMR assay. Only test sample nuclear extract is added. In one embodiment of the present invention separate vials for the different MMR deficiencies to be tested are presented.

This example describes a kit for determining whether a subject has Lynch syndrome or not, by determining whether a said subject has a defect in the MLH1 gene or in either of the MSH2 or MSH6 genes, without distinguishing between the latter. Such a kit has at least five separate vials.

**Table 2**

| Vial | Reagent | |
|---|---|---|
| 1 | HCT116 | = MLH1 deficient control NE |
| 2 | LoVo | = MSH2/6 deficient control NE |
| 3 | HCT116 | = to be complemented by sample NE |
| 4 | LoVo | = to be complemented by sample NE |
| 5 | FB-WT | = MMR proficient NE, positive control |

The reagents included in said vials are:

- Substrate Heteroduplex
- Nuclear extract (HCT1 16, LoVo or FB-WT)
- 10X MMR buffer (200 mM Tris-HCL pH 7.6, 400 mM KCl, 50 mM MgCl2 ,10 mM Glutathione, BSA 500 pg/ml, 1 mM each dNTP, 15 mM ATP).

In addition to this, a STOP solution for MMR assay (42 mM EDTA, 1.2% SDS, 50.4 µg/ml Proteinase K) is required, and could thus be provided with the kit.

Furthermore, said kit may, optionally, contain reagents required for clarifying the sample (TE buffer, phenol/chloroform/isoamyl alcohol and chloroform), for precipitating it (NaCl and ethanol) and for detecting the repair (RNase A and restriction enzymes *BglW* and *Eco31*\ with buffer and loading dye).

Further optional components of the kit are reagents required for the culturing of the cells the sample nuclear proteins are to be extracted from as well as the reagents required for the extraction of the nuclear proteins from it (see example 2).

A kit according to the present invention is not restricted to any specific form or type of vials. Any type of vials suitable for handling and incubations as described in example 6, such as eppendorf® tubes are suitable.

CLAIMS

1. A quantitative method for determining whether a human subject has an impaired DNA mismatch repair function, wherein said method comprises a) producing a nuclear protein extract from a sample obtained from said human subject, b) providing MMR proficient and MMR deficient nuclear protein extracts as positive and negative controls, respectively, c) combining in separate vials each nuclear extract with at least one mismatch-bearing heteroduplex DNA substrate, d) performing a mismatch repair assay on said sample and control nuclear protein extracts and said substrate, and e) determining whether said sample nuclear extract is capable of repairing said substrate DNA molecule.

2. The method according to claim 1, wherein said sample comprises normal cells obtained from a constitutive tissue.

3. The method according to claim 2, wherein said normal cells are fibroblast cells.

4. The method according to claims 2, wherein said normal cells are blood-derived cells.

5. The method according to any one of claims 1-4, wherein said substrate is derived from a plasmid having SEQ ID NO:1 .

6. The method according to claim 5, wherein said substrate further comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

7. The method according to any one of claims 1-6, wherein said MMR deficient nuclear extract is derived from a cell line selected from the group consisting of HCT1 16 and LoVo cells.

8. The method according to claim 7, wherein the total amount of nuclear extract per vial is in the range of 50-500 µg, preferably 50-200 µg, more preferably 75-1 00 µg.

9. A quantitative method for identifying a mismatch repair gene having a defective function, wherein said method comprises a) producing a nuclear protein extract from a sample obtained from a human subject, b) providing MMR proficient and MMR deficient nuclear protein extracts as positive and negative controls, respectively, c) mixing at least one MMR deficient nuclear protein extract with said sample extract, d) combining in separate vials each nuclear extract provided in steps a-c with at least one mismatch-bearing heteroduplex DNA substrate, e) performing a mismatch repair assay on said

nuclear extracts and said substrate, and f) identifying said defective gene by determining whether said sample nuclear extract is capable of repairing said substrate and complementing said MMR deficient nuclear extract.

10. The method according to claim 9, wherein said sample comprises normal cells obtained from a constitutive tissue.

11. The method according to claim 10, wherein said normal cells are fibroblast cells.

12. The method according to claims 10, wherein said normal cells are blood-derived cells.

13. The method according to any one of claims 9-1 2, wherein said substrate is derived from a plasmid having SEQ ID NO:1 .

14. The method according to claim 13, wherein said substrate further comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

15. The method according to any one of claims 9-14, wherein said MMR deficient nuclear extract is derived from a cell line selected from the group consisting of HCT1 16 and LoVo cells.

16. The method according to claim 15, wherein the total amount of nuclear extract per vial is in the range of 50-500 µg, preferably 50-200 µg, more preferably 75-1 00 µg.

17. A method for diagnosing Lynch syndrome in a human by identifying a defective DNA mismatch repair gene using a method according to any one of claims 9-1 6, wherein said sample is a clinical sample obtained from a constitutive tissue of a human subject.

18. A kit for use in a method according to any one of claims 1-1 7, comprising at least one mismatch-bearing heteroduplex DNA substrate, at least one MMR deficient nuclear extracts, and an MMR proficient nuclear extract as a positive control.

19. The kit according to claim 18, wherein said MMR deficient nuclear extracts derived from a cell line having an MMR deficiency in respect of either MLH1 , MSH2 or MSH6.

20. The kit according to claim 19, comprising at least two MMR deficient nuclear extracts.

21. The kit according to claim 20, wherein said MMR deficient nuclear extracts are derived from cell lines having an MMR deficiency in respect of MLH1 and MSH2/MSH6, respectively.

22. The kit according to any one of claims 18-21 , wherein said MMR deficient nuclear extracts are derived from HCT1 16 and LoVo cells, respectively.

23. The kit according to any one of claims 18-22 further including additional reagents necessary for performing the MMR assay.

24. The kit according to claim 23, further comprising 10X MMR buffer comprising 200 mM Tris-HCL pH 7.6, 400 mM KCl, 50 mM MgCl2 ,10 mM Glutathione, BSA 500 Mg/ml, 1 mM each dNTP and 15 mM ATP; and a STOP solution for MMR assay comprising 42 mM EDTA, 1.2% SDS, 50.4 µg/ml Proteinase K.

25. The kit according to any one of claims 23-24 comprising reagents for clarifying the sample, such as TE buffer, phenol/chloroform/isoamyl alcohol and chloroform; for precipitating said sample, such as NaCl and ethanol; and for detecting the repair, such as RNase A and restriction enzymes *BglW* and *Eco31*\ with buffer and loading dye.

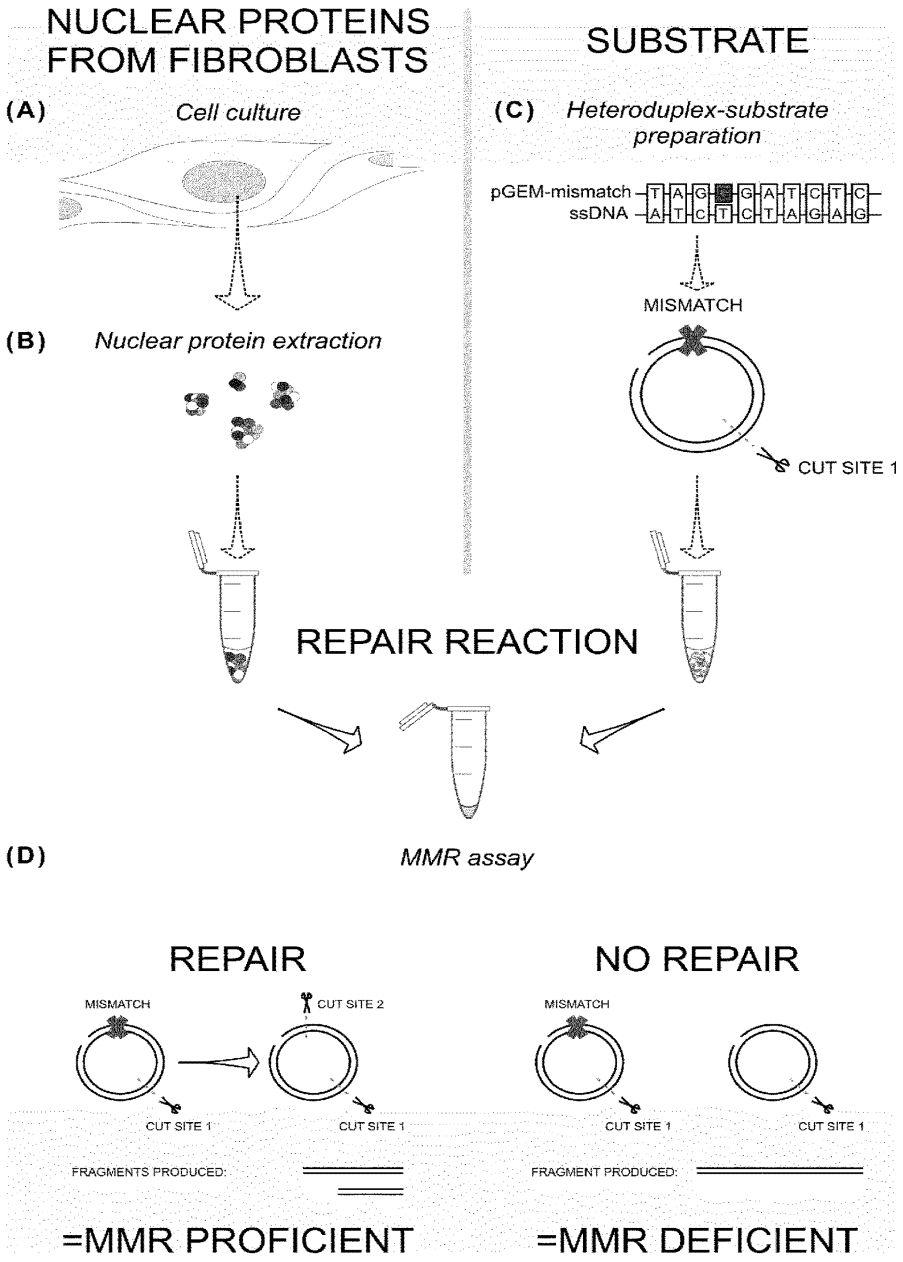

NUCLEAR PROTEINS FROM FIBROBLASTS
SUBSTRATE
(A) Cell culture
(C) Heteroduplex-substrate preparation
pGEM-mismatch
ssDNA
MISMATCH
(B) Nuclear protein extraction
CUT SITE 1
REPAIR REACTION
(D) MMR assay
REPAIR
NO REPAIR
MISMATCH
CUT SITE 2
CUT SITE 1
CUT SITE 1
FRAGMENTS PRODUCED:
MISMATCH
CUT SITE 1
CUT SITE 1
FRAGMENT PRODUCED:
=MMR PROFICIENT
=MMR DEFICIENT

***Fig. 1***

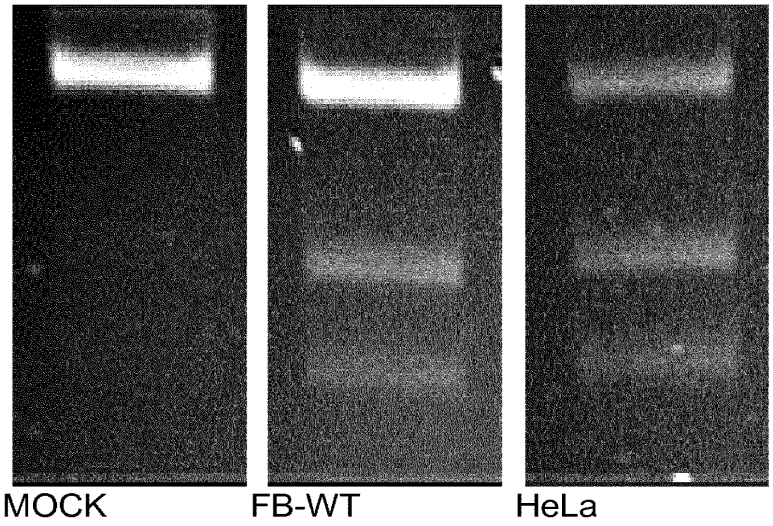



***Fig. 2***

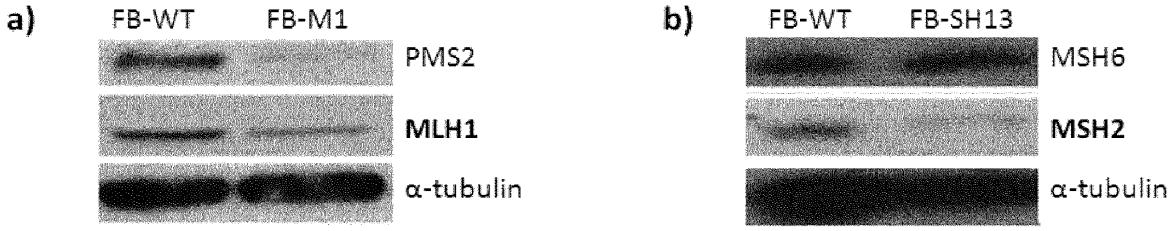



***Fig 3.***

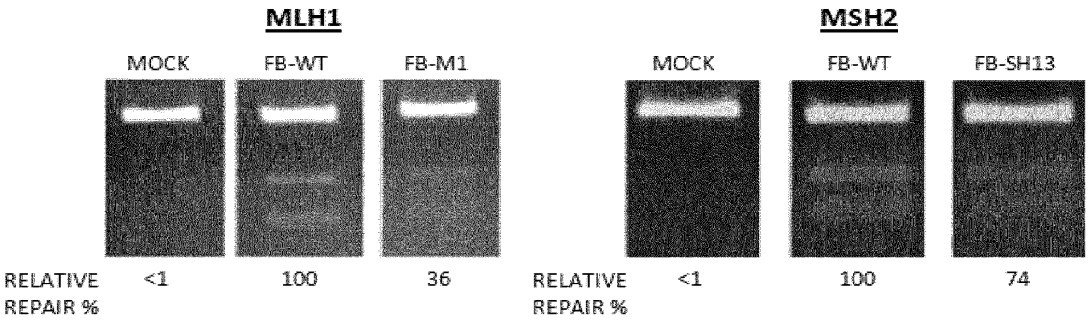



***Fig 4.***

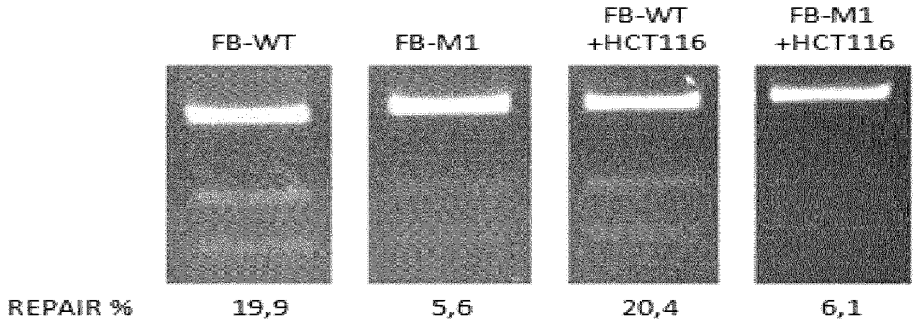
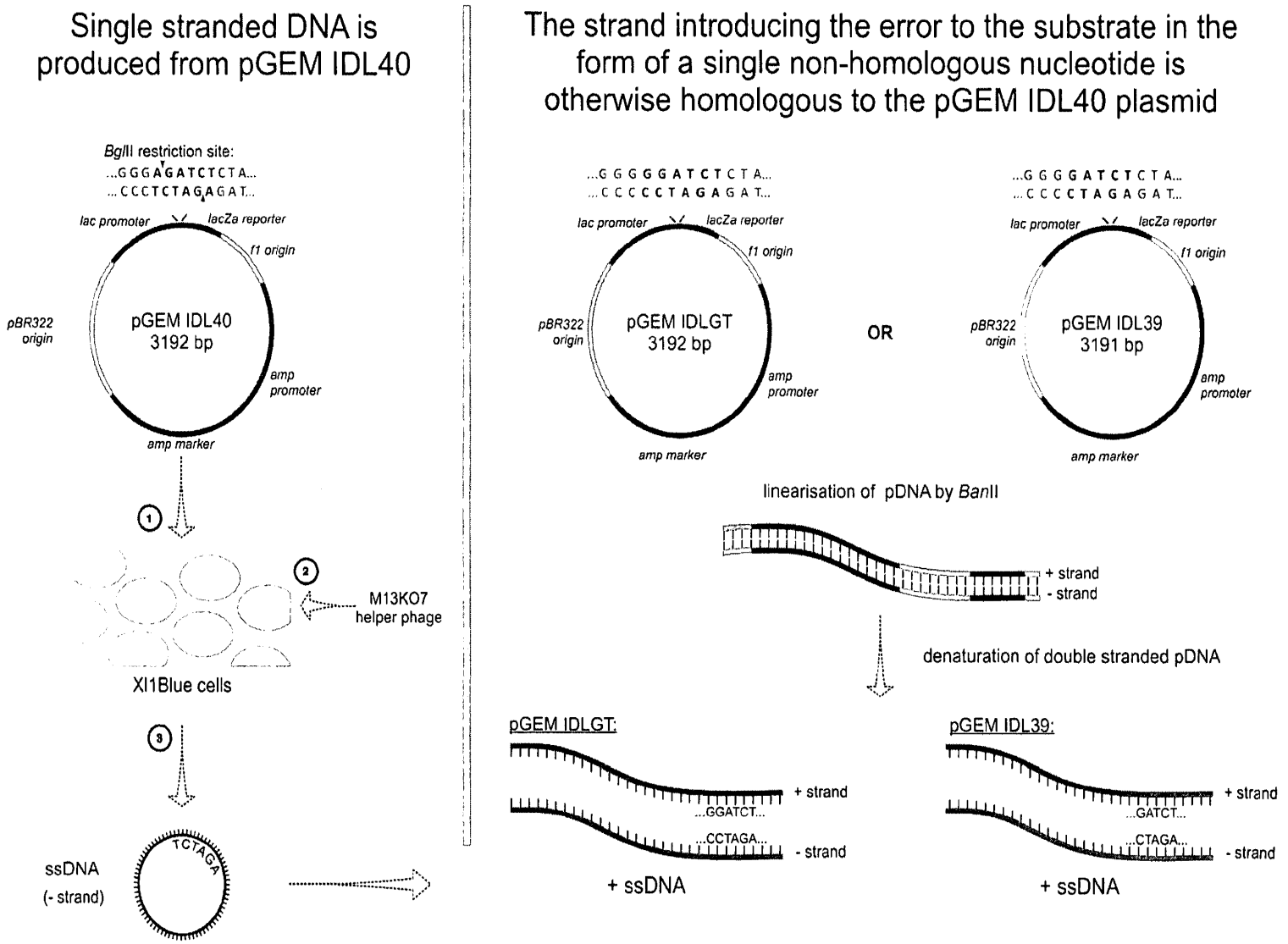



***Fig 5.***

SUBSTITUTE SHEET (RULE 26)

***Fig. 6***



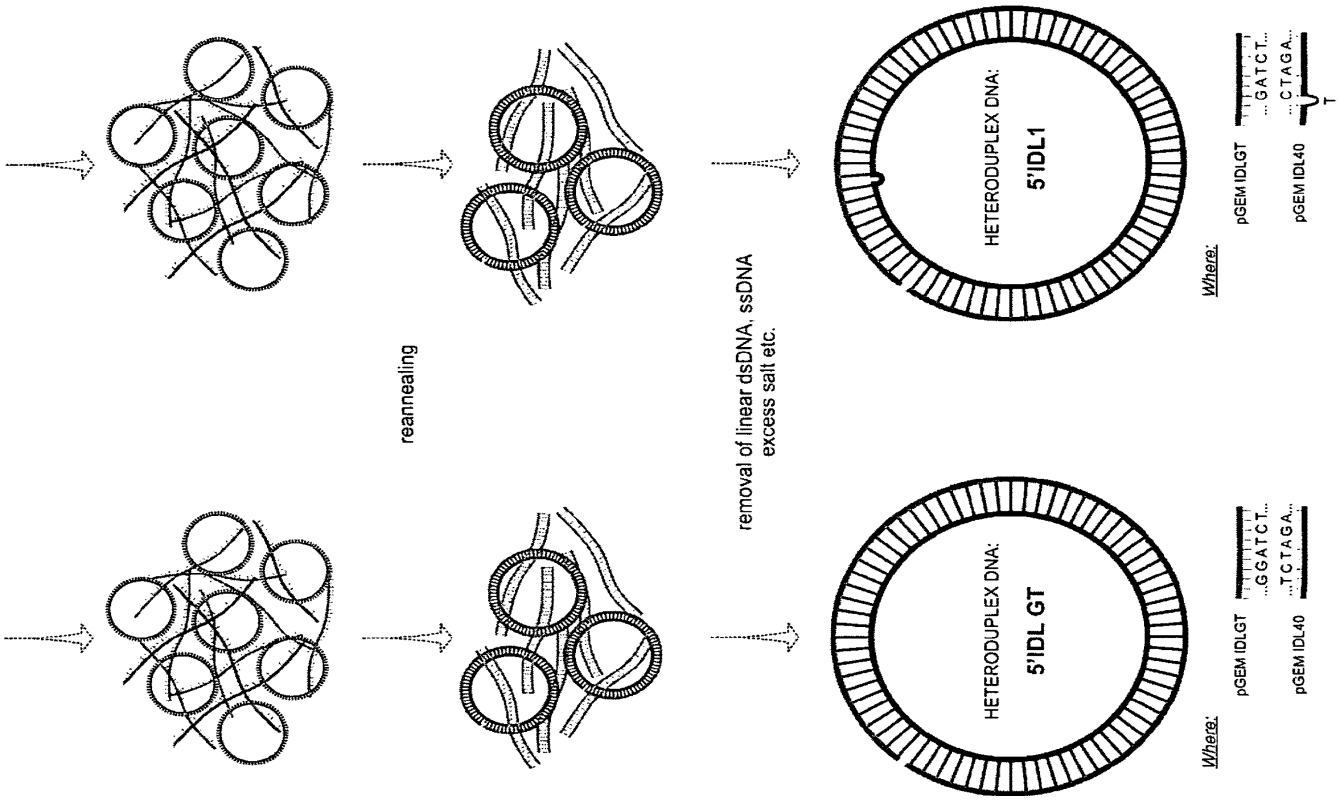

reannealing
removal of linear dsDNA, ssDNA
excess salt etc.
HETERODUPLEX DNA:
5'IDL GT
Where:
pGEM IDLGT
...GGATCT...
...TCTAGA...
pGEM IDL40
HETERODUPLEX DNA:
5'IDL1
Where:
pGEM IDLGT
...GATCT...
...CTAGA...
pGEM IDL40
T

*Fig. 6 cont.*

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/062708

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, Sequence Search , EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KARIOLA R ET AL: "Functional analysis of MSH6 mutations linked to kindreds with putative heredi tary non-polyposis colorectal cancer syndrome", HUMAN MOLECULAR GENETICS, OXFORD UNIVERSITY PRESS, SURREY, vol. 11, no. 11, 15 May 2002 (2002-05-15), pages 1303-1310, XP002507088, ISSN: 0964-6906, DOI: 10.1093/HMG/11.11.1303 Materials and Methods; the whole document<br>-----<br>-/- · | 1-25 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 September 2012 | 05/10/2012 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Dolce, Luca |

1

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2 012/062708

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/062708

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JUKKA KANTELINEN ET AL: "A putati ve Lynch syndrome fami l y carryi ngandvari ants of uncertai n si gni f i cances functi onal analysi s reveal s the pathogeni c one", FAMI LIAL CANCER, KLUWER ACADEMIC PUBLISHERS, DO, vol . 10, no. 3, 24 March 2011 (2011-03-24) , pages 515-520, XP019954550, ISSN: 1573-7292 , D0I : 10. 1007/S10689-011-9436-Z Materi al s and Methods | 1-25 |
| X | NYSTR0M-LAHTI M ET AL: "Functi onal analysi s of MLHI mutati ons l inked t o heredi tary nonpolyposi s col on cancer", GENES CHROMOSOMES & CANCER, JOHN WI LEY & SONS, INC, US, vol . 33, no. 2, 1 January 2002 (2002-01-01) , pages 160-167 , XP002507087 , ISSN: 1045-2257 , D0I : 10. 1002/GCC. 1225 Materi al s and Methods | 1-25 |
| X | W0 2008/142521 A2 (ACADEMISCH ZI EKENHUIS LEIDEN L [NL] ; DE WIND NI ELS [NL] ; DR0ST MARK [N) 27 November 2008 (2008-11-27) Materi al s and Methods | 1-25 |
| X | WANG H ET AL: "Mi smatch repai r i n human nucl ear extracts . Quanti tati ve analyses of exci si on of ni cked ci rcul ar mi smatched DNA substrates , constructed by a new techni que empl oyi ng syntheti c ol i gonucl eoti des", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCI ETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol . 277, no. 29, 19 July 2002 (2002-07-19) , pages 26136-26142 , XP002507089 , ISSN: 0021-9258, D0I : 10. 1074/JBC.M200357200 [retri eved on 2002-05-10] Materi al s and Methods | 1-25 |

1

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/062708

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2008142521 A2 | 27-11-2008 | NON E | |

Form PCT/ISA/210 (patent family annex) (April 2005)

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U.S. National Library of Medicine

*ClinicalTrials.gov archive*

## History of Changes for Study: NCT02060188

### A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)

Latest version (submitted November 4, 2019) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare. One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of the study.
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in red.
- Study additions are displayed in green.

### Study Record Versions

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 1 | ○ | ○ | February 10, 2014 | None (earliest version on record) |
| 2 | ○ | ○ | March 25, 2014 | Recruitment Status, Study Status, Contacts/Locations and Study Description |
| 3 | ○ | ○ | March 28, 2014 | Contacts/Locations and Study Status |
| 4 | ○ | ○ | April 23, 2014 | Contacts/Locations and Study Status |
| 5 | ○ | ○ | May 2, 2014 | Study Status |
| 6 | ○ | ○ | May 7, 2014 | Contacts/Locations, References and Study Status |
| 7 | ○ | ○ | May 27, 2014 | Contacts/Locations and Study Status |
| 8 | ○ | ○ | May 30, 2014 | Study Status |
| 9 | ○ | ○ | June 12, 2014 | Contacts/Locations and Study Status |
| 10 | ○ | ○ | June 25, 2014 | Contacts/Locations and Study Status |
| 11 | ○ | ○ | July 9, 2014 | Contacts/Locations and Study Status |
| 12 | ○ | ○ | July 23, 2014 | Contacts/Locations and Study Status |
| 13 | ○ | ○ | August 11, 2014 | Contacts/Locations and Study Status |
| 14 | ○ | ○ | August 28, 2014 | Contacts/Locations and Study Status |
| 15 | ○ | ○ | September 15, 2014 | Contacts/Locations and Study Status |
| 16 | ○ | ○ | October 13, 2014 | Contacts/Locations and Study Status |
| 17 | ○ | ○ | October 30, 2014 | Contacts/Locations and Study Status |
| 18 | ○ | ○ | November 13, 2014 | Contacts/Locations and Study Status |
| 19 | ○ | ○ | November 28, 2014 | Contacts/Locations and Study Status |

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| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 20 | ○ | ○ | December 5, 2014 | Contacts/Locations and Study Status |
| 21 | ○ | ○ | December 31, 2014 | Study Status |
| 22 | ○ | ○ | January 27, 2015 | Contacts/Locations and Study Status |
| 23 | ○ | ○ | February 6, 2015 | Contacts/Locations and Study Status |
| 24 | ○ | ○ | February 19, 2015 | Study Status |
| 25 | ○ | ○ | March 5, 2015 | Study Status |
| 26 | ○ | ○ | March 23, 2015 | Study Status |
| 27 | ○ | ○ | April 13, 2015 | Study Status and Contacts/Locations |
| 28 | ○ | ○ | April 20, 2015 | Contacts/Locations and Study Status |
| 29 | ○ | ○ | June 3, 2015 | Study Status |
| 30 | ○ | ○ | June 4, 2015 | Study Status and Contacts/Locations |
| 31 | ○ | ○ | June 22, 2015 | Study Status |
| 32 | ○ | ○ | July 3, 2015 | Study Status |
| 33 | ○ | ○ | July 17, 2015 | Contacts/Locations and Study Status |
| 34 | ○ | ○ | August 6, 2015 | Contacts/Locations and Study Status |
| 35 | ○ | ○ | September 3, 2015 | Contacts/Locations and Study Status |
| 36 | ○ | ○ | September 16, 2015 | Study Status |
| 37 | ○ | ○ | October 15, 2015 | Study Status |
| 38 | ○ | ○ | November 4, 2015 | Study Status |
| 39 | ○ | ○ | December 8, 2015 | Study Status |
| 40 | ○ | ○ | December 21, 2015 | Study Status |
| 41 | ○ | ○ | January 5, 2016 | Study Status |
| 42 | ○ | ○ | January 21, 2016 | Contacts/Locations and Study Status |
| 43 | ○ | ○ | January 28, 2016 | Contacts/Locations and Study Status |
| 44 | ○ | ○ | February 10, 2016 | Study Status |
| 45 | ○ | ○ | February 25, 2016 | Study Status |
| 46 | ○ | ○ | March 11, 2016 | Study Status |
| 47 | ○ | ○ | March 28, 2016 | Study Status |
| 48 | ○ | ○ | April 12, 2016 | Study Status |
| 49 | ○ | ○ | May 10, 2016 | Study Status |
| 50 | ◉ | ◉ | May 25, 2016 | Contacts/Locations and Study Status |
| 51 | ○ | ○ | June 24, 2016 | Study Status |
| 52 | ○ | ○ | June 28, 2016 | Study Status |
| 53 | ○ | ○ | July 11, 2016 | Study Status |
| 54 | ○ | ○ | July 26, 2016 | Study Status |

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| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 55 | ○ | ○ | August 8, 2016 | Study Status and Study Design |
| 56 | ○ | ○ | August 23, 2016 | Study Status |
| 57 | ○ | ○ | September 7, 2016 | Study Status |
| 58 | ○ | ○ | September 20, 2016 | Study Status |
| 59 | ○ | ○ | October 14, 2016 | Arms and Interventions, Study Description, Study Status, Contacts/Locations and Eligibility |
| 60 | ○ | ○ | October 25, 2016 | Contacts/Locations, Study Design and Study Status |
| 61 | ○ | ○ | December 2, 2016 | Contacts/Locations and Study Status |
| 62 | ○ | ○ | December 13, 2016 | Study Status and Contacts/Locations |
| 63 | ○ | ○ | December 23, 2016 | Contacts/Locations and Study Status |
| 64 | ○ | ○ | January 6, 2017 | Arms and Interventions, Outcome Measures, Study Status, Study Identification, Eligibility, Study Design and Study Description |
| 65 | ○ | ○ | January 27, 2017 | Study Status |
| 66 | ○ | ○ | February 16, 2017 | Contacts/Locations and Study Status |
| 67 | ○ | ○ | February 28, 2017 | Contacts/Locations, Study Status and References |
| 68 | ○ | ○ | March 3, 2017 | Study Status, References and Contacts/Locations |
| 69 | ○ | ○ | March 23, 2017 | Study Status, Oversight, Study Identification, References, Contacts/Locations and Study Description |
| 70 | ○ | ○ | March 28, 2017 | Study Status, References and Contacts/Locations |
| 71 | ○ | ○ | April 17, 2017 | Study Status, Contacts/Locations and Study Design |
| 72 | ○ | ○ | May 18, 2017 | Study Status, References, Contacts/Locations, Eligibility, Conditions and Study Description |
| 73 | ○ | ○ | June 1, 2017 | Conditions and Study Status |
| 74 | ○ | ○ | June 8, 2017 | References, Contacts/Locations and Study Status |
| 75 | ○ | ○ | June 26, 2017 | Contacts/Locations, References and Study Status |
| 76 | ○ | ○ | July 24, 2017 | Study Status |
| 77 | ○ | ○ | August 2, 2017 | Study Status |
| 78 | ○ | ○ | September 1, 2017 | Study Status and Contacts/Locations |
| 79 | ○ | ○ | September 14, 2017 | Contacts/Locations and Study Status |
| 80 | ○ | ○ | October 3, 2017 | Study Status |
| 81 | ○ | ○ | November 3, 2017 | Study Status |
| 82 | ○ | ○ | December 11, 2017 | Contacts/Locations and Study Status |
| 83 | ○ | ○ | January 9, 2018 | Study Status and Contacts/Locations |
| 84 | ○ | ○ | January 18, 2018 | Contacts/Locations and Study Status |
| 85 | ○ | ○ | January 29, 2018 | Contacts/Locations and Study Status |
| 86 | ○ | ○ | February 22, 2018 | Study Status and Contacts/Locations |
| 87 | ○ | ○ | March 26, 2018 | Study Status and Contacts/Locations |
| 88 | ○ | ○ | April 27, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 89 | ○ | ○ | November 6, 2018 | Study Status and References |

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| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 90 | ○ | ○ | November 19, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 91 | ○ | ○ | December 3, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 92 | ○ | ○ | June 6, 2019 | Study Status |
| 93 | ○ | ○ | November 4, 2019 | Study Status |

Compare　Comparison Format: ◉ Merged ○ Side-by-Side

Scroll up to access the controls

**Study NCT02060188**
**Submitted Date: May 25, 2016 (v50)**

**Study Identification**

Unique Protocol ID: CA209-142
Brief Title: A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)
Official Title: A Phase 2 Clinical Trial of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Microsatellite High (MSI-H) Colon Cancer
Secondary IDs: 2013-003939-30 [EudraCT Number]

**Study Status**

Record Verification: December 2015
Overall Status: Recruiting
Study Start: March 2014
Primary Completion: September 2016 [Anticipated]
Study Completion: August 2017 [Anticipated]
First Submitted: December 18, 2013
First Submitted that Met QC Criteria: February 10, 2014
First Posted: February 11, 2014 [Estimate]
Last Update Submitted that Met QC Criteria: May 25, 2016
Last Update Posted: May 27, 2016 [Estimate]

**Sponsor/Collaborators**

Sponsor: Bristol-Myers Squibb
Responsible Party: Sponsor
Collaborators:

**Oversight**

U.S. FDA-regulated Drug:
U.S. FDA-regulated Device:
Data Monitoring: No

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## Study Description

**Brief Summary:** The purpose of this study is to examine if Nivolumab alone or in combination with Ipilimumab will demonstrate a meaningful objective response rate in patients with recurrent and metastatic colon cancer who also have a specific biomarker in their tumors.

**Detailed Description:** Allocation: The Microsatellite Instability High (MSI-High) Part of the trial is Non-randomized, The Non-MSI high part of the trial contains a randomized portion

## Conditions

**Conditions:** MSI Positive Colorectal Cancer
MSI Negative Colorectal Cancer

**Keywords:**

## Study Design

**Study Type:** Interventional
**Primary Purpose:** Treatment
**Study Phase:** Phase 1/Phase 2
**Interventional Study Model:** Parallel Assignment
**Number of Arms:** 2
**Masking:** None (Open Label)
**Allocation:** Non-Randomized
**Enrollment:** 96 [Anticipated]

## Arms and Interventions

| Arms | Assigned Interventions |
|---|---|
| Experimental: Nivolumab Monotherapy<br>Nivolumab administered as IV infusion at a dose of 3mg/kg every 2 weeks until disease progression | Drug: Nivolumab<br>Other Names:<br>• BMS-936558 |
| Experimental: Nivolumab + Ipilimumab<br>Dose Escalation Phase:<br>Dose Level -1: Nivolumab 0.3mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 1: Nivolumab 1mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 2a: Nivolumab 1mg/Kg IV combined with Ipilimumab 3 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 2b: Nivolumab 3mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression | Drug: IpilimumabDrug: Nivolumab<br>Other Names:<br>• BMS-936558 |

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## Outcome Measures

Primary Outcome Measures:

1. Objective response rate (ORR) in all MSI-High subjects as determined by Investigators
   (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later)) (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later))

   [Time Frame: The final analysis of the primary endpoint will occur at least 6 months after the last enrolled subject's first dose of study therapy (Approximately up to 34 months)]

Secondary Outcome Measures:

2. ORR in all MSI-H subjects based on IRRC determination
   Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/- wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued(whichever occurs later)

   [Time Frame: The final analysis of the secondary endpoint will occur the time of the primary endpoint analysis (Approximately up to 34 months)]

## Eligibility

Minimum Age: 18 Years

Maximum Age:

Sex: All

Gender Based:

Accepts Healthy Volunteers: No

Criteria: For more information regarding BMS clinical trial participation, please visit www.BMSStudyConnect.com

Inclusion Criteria:

- Men and women ≥ 18 years of age
- Eastern Cooperative Oncology Group (ECOG) performance status 0 to 1
- Histologically confirmed colorectal cancer
- Measurable disease by CT or MRI
- Testing for MSI Status
- Adequate organ function as defined by study-specific laboratory tests
- Must use acceptable form of birth control throughout the study. After the final dose of study drug, an acceptable form of birth control must be used for 23 weeks for women of childbearing potential (WOCBP) and 31 weeks for men who are sexually active with WOCBP
- Signed informed consent
- Willing and able to comply with study procedures

Exclusion Criteria:

- Active brain metastases or leptomeningeal metastases are not allowed
- Prior treatment with an anti-Programmed Death Receptor (PD)-1, anti-PD-L1, anti-PD-L2, anti-Cytotoxic T-Cell Lymphoma-4 Antigen (CTLA-4) antibody, or any other antibody or drug specifically targeting T-cell co-stimulation or immune checkpoint pathways
- Prior malignancy active within the previous 3 years except for locally curable cancers
- Subjects with active, known or suspected autoimmune disease
- Subjects with a condition requiring systemic treatment with either corticosteroids or other immunosuppressive medications within 14 days of study drug administration

## Contacts/Locations

Central Contact Person: Recruiting sites have contact information. Please contact the sites directly. If there is no contact information, please email:
Email: Clinical.Trials@bms.com

Central Contact Backup: First line of the email MUST contain NCT# and Site #.

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Study Officials: Bristol-Myers Squibb
Study Director
Bristol-Myers Squibb

Locations: **United States, Arizona**

Banner MD Anderson Cancer Center
[Recruiting]
Gilbert, Arizona, United States, 85234
Contact: Contact: Tomislav Dragovich Site 0028 480-256-3429

**United States, California**

USC Norris Comprehensive Cancer Center
[Recruiting]
Los Angeles, California, United States, 90033
Contact: Contact: Heinz-Josef Lenz, Site 0004 323-865-0620

Pacific Hematology Oncology Associates
[Recruiting]
San Francisco, California, United States, 94115
Contact: Contact: Ari Baron, Site 0001 415-600-1775

**United States, Georgia**

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Atlanta, Georgia, United States, 30322
Contact: Contact: Sassel El-Rayes, Site 0008 404-778-1900

**United States, Massachusetts**

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Contact: Contact: Franklin Chen, Site 0029 336-718-8461

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Contact: Contact: Todd Crocenzi, Site 0005 503-215-2492

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**United States, Tennessee**

Vanderbilt-Ingram Cancer Ctr
[Recruiting]
Nashville, Tennessee, United States, 37232
Contact:　Contact: Emily Chan, Site 0006 615-936-5647

**United States, Texas**

The University Of Texas Md Anderson Cancer Center
[Recruiting]
Houston, Texas, United States, 77030
Contact:　Contact: Michael Overman, Site 0003 713-563-7757

**Australia, New South Wales**

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[Recruiting]
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Toronto, Ontario, Canada, M5G 1X5
Contact:　Contact: Albruni Razak, Site 0016 416-946-4501

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Contact: Contact: Site 0022

Local Institution
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Dublin 9, Ireland
Contact: Contact: Site 0023

Local Institution
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Galway, Ireland
Contact: Contact: Site 0033

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代理人:弁護士 鈴木 信
整合官:渡邉 光
出願番号:特願2018-565110
アドレス:各下参照

Doc Code: TRAN.LET

Document Description: Transmittal Letter

PTO/SB/21 (07-09)
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# TRANSMITTAL FORM

*(to be used for all correspondence after initial filing)*

Total Number of Pages in This Submission

| | |
|---|---|
| Application Number | 16/306,290 |
| Filing Date | November 30, 2018 |
| First Named Inventor | Michael AXELSON |
| Art Unit | 1647 |
| Examiner Name | LOCKARD, JOHN MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

## ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
- ☑ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☑ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☑ Other Enclosure(s) (please Identify below):

E-Payments: $1200 Issue Fee; Issue Fee Transmittal; $260 IDS Fee; IDS Refs: FP16-FP18; and NPL36.

**Remarks**

The Office may charge any fee deficiency for any submission made with this transmittal to Deposit Account 19-0036.

## SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

| | | | |
|---|---|---|---|
| Firm Name | Sterne, Kessler, Goldstein & Fox P.L.L.C. | | |
| Signature | /Brian M. Dudley/ | | |
| Printed name | Brian Dudley | | |
| Date | July 29, 2021 | Reg. No. | 77,546 |

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| Inventor: Michael AXELSON | Confirmation No.: 7097 |
| Applicant: Bristol-Myers Squibb Company | Art Unit: 1647 |
| Application No.: 16/306,290 | Examiner: LOCKARD; Jon McClelland |
| International Filing Date: June 2, 2017 | Atty. Docket: 3338.0550001/ELE/C-K/BMD |

Title: **METHODS OF TREATING COLORECTAL CANCER**

## First Supplemental Information Disclosure Statement

***Mail Stop Issue Fee***

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Listed on accompanying IDS Forms PTO/SB/08a; and PTO/SB/08b equivalent are documents for consideration in view of 37 C.F.R. §§ 1.56, 1.97, and 1.98. The numbering on this First Supplemental Information Disclosure Statement is a continuation of the numbering in Applicant's Information Disclosure Statement filed on **April 12, 2019** in connection with the above-captioned application.

Where the publication date of a listed document does not provide a month of publication, the year of publication of the listed document is sufficiently earlier than the effective U.S. filing date and any foreign priority date so that the month of publication is not in issue. Applicant has listed publication dates on the attached IDS Form based on information presently available to the undersigned. However, the listed publication dates should not be construed as an admission that the information was actually published on the date indicated.

Applicant reserves the right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered.

This statement should not be construed as a representation that a search has been made, or that information more material to the examination of the present patent application does not exist. The Examiner is specifically requested not to rely solely on the material submitted herewith.

This is a filing under 37 C.F.R. § 1.97(d). This Information Disclosure Statement is being filed more than three months after the U.S. filing date and after the mailing date of a Final Rejection or Notice of Allowance, but on or before payment of the Issue Fee. The required fee is provided through online credit card payment authorization in the amount of $260.00 in payment of the fee under 37 C.F.R. § 1.17(p).

I hereby state that each item of information contained in this Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure (37 C.F.R. § 1.97(e)(1)) and not more than thirty days prior to the filing of this Information Disclosure Statement (37 C.F.R. § 1.704(d)(1)(i)).

A concise explanation of the relevance of the non-English language document appears below in accordance with 37 C.F.R. § 1.98(a)(3).

Document **FP17**, WO-2005021743-A1, is in a foreign language. An English language abstract is appended to the face page of document **FP17**.

Copies of documents **FP16-FP18** and **NPL36** are submitted.

Atty. Dkt. No. 3338.0550001/ELE/C-K/BMD

It is respectfully requested that the Examiner initial and return a copy of the enclosed IDS Forms, and indicate in the official file wrapper of this patent application that the documents have been considered.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

/Brian M. Dudley/

Brian M. Dudley
Attorney for Applicant
Registration No. 77,546

Date: July 29, 2021

1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

17118134.1

Atty. Dkt. No. 3338.0550001/ELE/C-K/BMD

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 16306290 |
| **Filing Date:** | 30-Nov-2018 |
| **Title of Invention:** | METHODS OF TREATING COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |

Filed as Large Entity

**Filing Fees for U.S. National Stage under 35 USC 371**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |
| UTILITY APPL ISSUE FEE | 1501 | 1 | 1200 | 1200 |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| SUBMISSION- INFORMATION DISCLOSURE STMT | 1806 | 1 | 260 | 260 |
| | | **Total in USD ($)** | | **1460** |

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 43381030 |
| **Application Number:** | 16306290 |
| **International Application Number:** | |
| **Confirmation Number:** | 7097 |
| **Title of Invention:** | METHODS OF TREATING COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Customer Number:** | 115018 |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Filer Authorized By:** | Brian Mason Dudley |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |
| **Receipt Date:** | 29-JUL-2021 |
| **Filing Date:** | 30-NOV-2018 |
| **Time Stamp:** | 18:13:11 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

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| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Issue Fee Payment (PTO-85B) | Issue-Fee-3338-0550001.PDF | 1619147<br>b0baafdc88adce5be7217851be8e6c8c7be3c99c | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Information Disclosure Statement (IDS) Form (SB08) | 2021-07-29-1st-Supp-IDS-SB08A-B-3338-0550001.pdf | 125438<br>a571fd3a459f57b11ce1220b6caa6ecc6ccf1209 | no | 2 |
| **Warnings:** | | | | | |
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| This is not an USPTO supplied IDS fillable form | | | | | |
| 3 | Foreign Reference | FP16_WO2003072822A2.pdf | 1344545<br>2389b9a4ca7e9bd288cd775e238334b8d72ed2ef | no | 35 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Foreign Reference | FP17_WO2005021743A1.pdf | 3510400<br>bc4da1b92778dfa9364f862c8efbb0119adb4737 | no | 62 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | Foreign Reference | FP18_WO2013004618A1.pdf | 1136762<br>fe8dbe8227e10f29eee8af67d7e4fc606cc9b79e | no | 36 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Non Patent Literature | NPL36_NCT02060188.pdf | 1119439<br>9ce3738d669cf291011a55666a6a731939750f5a | no | 9 |
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| **Information:** | | | | | |
| 7 | Transmittal Letter | 2021-07-29-Transmittal-3338-0550001.PDF | 188701<br>27be71857292a306baa664b19b0982b25df a6612 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 8 | Transmittal Letter | 2021-07-29-1st-Supp-IDS-Pleading-3338-0550001.PDF | 110033<br>1cc22ea5d85e71c07c3ed254c1216fa631f2 c378 | no | 3 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 9 | Fee Worksheet (SB06) | fee-info.pdf | 40435<br>14d465cd68738e2b3fe2eaf627c1460b648 b6530 | no | 2 |
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**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

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| (Signature) |
| (Date) |

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

TITLE OF INVENTION: METHODS OF TREATING COLORECTAL CANCER

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $0.00 | $1200 | 07/29/2021 |

| EXAMINER | ART UNIT | CLASS-SUBCLASS |
|---|---|---|
| LOCKARD, JON MCCLELLAND | 1647 | 424-139100 |

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

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(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Sterne, Kessler, Goldstein & Fox P.L.L.C.
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Bristol-Myers Squibb Company

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Princeton, NJ

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

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5. **Change in Entity Status** (from status indicated above)

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☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

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NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
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Authorized Signature /Brian M. Dudley/ Date July 29, 2021

Typed or printed name Brian M. Dudley Registration No. 77,546

# PRINTER RUSH
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| | | |
|---|---|---|
| Application: 16306290 | Examiner: Lockard | GAU: 1647 |
| From: Chuck Bowen | Location: RTFM | Creation Date: 08/02/2021 |

Week Date: 4/26/2021

| DOC CODE | DOC DATE | MISCELLANEOUS |
|---|---|---|
| [ ] 1449 | | [ ] Continuing Data |
| [X] IDS | 07/29/2021 | [ ] Foreign Priority |
| [ ] CLM | | [ ] Document Legibility |
| [ ] IIFW/FWCLM | | [ ] Fees |
| [ ] SRFW | | [ ] Petition (TC) |
| [ ] DRW | | [ ] Other |
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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

115018 7590 08/17/2021

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER | |
|---|---|
| LOCKARD, JON MCCLELLAND | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1647 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 08/17/2021 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ckim@sternekessler.com
e-office@sternekessler.com
pcalvo@sternekessler.com

| ***CORRECTED*** ***Notice of Allowability*** | **Application No.** 16/306,290 | **Applicant(s)** AXELSON, Michael | |
|---|---|---|---|
| | **Examiner** JON M LOCKARD | **Art Unit** 1647 | **AIA (FITF) Status** Yes |

***-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--***

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the Information Disclosure Statement filed 29 July 2021.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1,3-5,7-8,10-13 and 16-23 (renumbered as claims 1-18, respectively) . As a result of the allowed claim(s ), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   **Certified copies:**

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   **Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

| /J.L/<br>Examiner, Art Unit 1647 | /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 |
|---|---|

***Notice of Pre-AIA or AIA Status***

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

***Information Disclosure Statement***

2. The information disclosure statement (IDS) submitted on 25 June 2021 was filed after the mailing date of the Notice of Allowability on 26 March 2021. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

**REASONS FOR ALLOWANCE**

3. The following is an examiner's statement of reasons for allowance: The information disclosure statement (IDS) filed 29 July 2021 has been considered by the Examiner. After careful consideration, the Examiner has determined that none of the information contained therein raises new issues of patentability.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

***Advisory Information***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Jon M. Lockard** whose telephone number is **(571) 272-2717**. The examiner can normally be reached on Monday through Friday, 8:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Joanne Hama**, can be reached on **(571) 272-2911**. The fax number for the organization where this application or proceeding is assigned is **571-273-8300**.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see **http://pair-direct.uspto.gov**. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at **866-217-9197** (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/J.L/
Examiner, Art Unit 1647
August 12, 2021

/Christine J Saoud/
Primary Examiner, Art Unit 1647

# PRINTER RUSH
(PTO ASSISTANCE)

| | | |
|---|---|---|
| Application: 16306290 | Examiner: Lockard | GAU: 1647 |
| From: Chuck Bowen | Location: RTFM | Creation Date: 08/02/2021 |

Week Date: 4/26/2021

| DOC CODE | DOC DATE | MISCELLANEOUS |
|---|---|---|
| [ ] 1449 | | [ ] Continuing Data |
| [X] IDS | 07/29/2021 | [ ] Foreign Priority |
| [ ] CLM | | [ ] Document Legibility |
| [ ] IIFW/FWCLM | | [ ] Fees |
| [ ] SRFW | | [ ] Petition (TC) |
| [ ] DRW | | [ ] Other |
| [ ] OATH | | |
| [ ] 312 | | |
| [ ] SPEC | | |

**[RUSH] Message:**

Please respond to the 07/29/21 IDS.

Thank you

**[XRUSH] Response:**

The IDS filed 07/29/21 has been considered, and a Corrected Notice of Allowability has been posted for review.

Initials: /J.L/

Examiner: PUBS contact - James Leija, (703) 756-1197
NOTE: This form will be included as part of the official USPTO record with the response document coded as XRUSH.
REV: AUG 16

Receipt date: 07/29/2021

Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO

# FIRST SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD; JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

## FOREIGN PATENT DOCUMENTS

| Examiner Initials* | Cite No.[1] | Foreign Patent Document<br>Country Code[3]-Number[4]-Kind Code[5] (if known) | Publication Date MM-DD-YYYY | Name of Patentee or Applicant of Cited Document | Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear | T[6] |
|---|---|---|---|---|---|---|
| /J.L/ | FP16 | WO-2003072822-A2 | 09-04-2003 | INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM) | | ☐ |
| /J.L/ | FP17 | WO-2005021743-A1 | 03-10-2005 | TANAKA; NORIAKI | | ☒ |
| /J.L/ | FP18 | WO-2013004618-A1 | 01-10-2013 | HELSINGIN YLIOPISTO | | ☐ |

| Examiner Signature | | Date Considered | |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.*

Receipt date: 07/29/2021

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

# FIRST SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD; JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

| NON-PATENT LITERATURE DOCUMENTS | | | |
|---|---|---|---|
| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
| /J.L/ | NPL36 | Anonymous: "An Investigational Immuno-therapy Study of Nivolumab. and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread - Full Text View- Clinical Trials.gov". Retrieved from the Internet: URL: ttps://clinicaltrials.gov/ct2/history/NCT02060188?V_50=View#StudyPageTop [retrieved on June 23, 2021], 9 pages. | ☐ |

17117986.1

| Examiner Signature | /JON M LOCKARD/ | Date Considered | 08/12/2021 |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.*

**To:** ckim@sternekessler.com,e-office@sternekessler.com,pcalvo@sternekessler.com
**From:** PAIR_eOfficeAction@uspto.gov
**Cc:** PAIR_eOfficeAction@uspto.gov
**Subject:** Private PAIR Correspondence Notification for Customer Number 115018

Aug 17, 2021 04:41:20 AM

Dear PAIR Customer:

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005
UNITED STATES

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The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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| Application | Document | Mailroom Date | Attorney Docket No. |
|---|---|---|---|
| 16306290 | NOA | 08/17/2021 | 3338.0550001/ELE/C-K/BMD |
| | 1449 | 08/17/2021 | 3338.0550001/ELE/C-K/BMD |

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

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Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | ISSUE DATE | PATENT NO. | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 09/14/2021 | 11117966 | 3338.0550001/ELE/C-K/BMD | 7097 |

115018 7590 08/25/2021

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

# ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 142 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Bristol-Myers Squibb Company, Princeton, NJ;
Michael AXELSON, Princeton, NJ;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

IR103 (Rev. 10/09)

**To:** ckim@sternekessler.com,e-office@sternekessler.com,pcalvo@sternekessler.com
**From:** PAIR_eOfficeAction@uspto.gov
**Cc:** PAIR_eOfficeAction@uspto.gov
**Subject:** Private PAIR Correspondence Notification for Customer Number 115018

Aug 26, 2021 03:58:52 AM

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1100 New York Avenue NW
Washington, DC 20005
UNITED STATES

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The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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| Application | Document | Mailroom Date | Attorney Docket No. |
|---|---|---|---|
| 16306290 | ISSUE.NTF | 08/25/2021 | 3338.0550001/ELE/C-K/BMD |

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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# REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
## (Submitted Only via EFS-Web)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Application Number | 16/306,290 | Filing Date | 2018-11-30 | Docket Number (if applicable) | 3338.0550001/ELE/C-K/BMD | Art Unit | 1647 |
| First Named Inventor | Michael AXELSON | | | Examiner Name | LOCKARD; Jon McClelland | | |

**This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.**
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

### SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☐ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☒ Other E-Petition to Withdraw an Application from Issue After Payment of the Issue Fee Under 37 CFR 1.313(c)

### MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

### FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 190036

### SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

EFS - Web 2.1.16

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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| Signature of Registered U.S. Patent Practitioner | | | |
|---|---|---|---|
| Signature | /Brian M. Dudley/ | Date (YYYY-MM-DD) | 2021-09-07 |
| Name | Brian M. Dudley | Registration Number | 77546 |

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

# Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| Inventor: Michael AXELSON | Confirmation No.: 7097 |
| Applicant: Bristol-Myers Squibb Company | Art Unit: 1647 |
| Application No.: 16/306,290 | Examiner: LOCKARD; Jon McClelland |
| International Filing Date: June 2, 2017 | Atty. Docket: 3338.0550001/ELE/C-K/BMD |

Title: **METHODS OF TREATING COLORECTAL CANCER**

## Second Supplemental Information Disclosure Statement

***Mail Stop RCE***

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Listed on accompanying IDS Form PTO/SB/08b equivalent are documents for consideration in view of 37 C.F.R. §§ 1.56, 1.97, and 1.98. The numbering on this Second Supplemental Information Disclosure Statement is a continuation of the numbering in Applicant's First Supplemental Information Disclosure Statement filed on **July 29, 2021** in connection with the above-captioned application.

Where the publication date of a listed document does not provide a month of publication, the year of publication of the listed document is sufficiently earlier than the effective U.S. filing date and any foreign priority date so that the month of publication is not in issue. Applicant has listed publication dates on the attached IDS Form based on information presently available to the undersigned. However, the listed publication dates should not be construed as an admission that the information was actually published on the date indicated.

Applicant reserves the right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered.

This statement should not be construed as a representation that a search has been made, or that information more material to the examination of the present patent application does not exist. The Examiner is specifically requested not to rely solely on the material submitted herewith.

This is a filing under 37 C.F.R. § 1.97(b). This Information Disclosure Statement is being filed before the mailing of a first Office Action after the filing of a request for continued examination under 37 C.F.R. § 1.114. No statement or fee is required.

Copies of documents **NPL37-NPL46** are submitted.

It is respectfully requested that the Examiner initial and return a copy of the enclosed IDS Forms, and indicate in the official file wrapper of this patent application that the documents have been considered.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

/Brian M. Dudley/

Brian M. Dudley
Attorney for Applicant
Registration No. 77,546

Date: September 7, 2021

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Substitute for form 1449/PTO

# SECOND SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD, JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

**NON-PATENT LITERATURE DOCUMENTS**

| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
|---|---|---|---|
| | NPL37 | Anonymous: "A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)". Retrieved from the Internet: URL: https://clinicaltrials.gov/ct2/history/NCT02060188?V_17=View#StudyPageTop [retrieved on August 30, 2021], 9 pages. | ☐ |
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| | NPL41 | EROGLU, Z., et al., "Checkpoint Inhibition of PD-1: The Promise of Pembrolizumab (MK-3475) and Beyond," Perzonalied Medicine in Oncology: 13 pages, The Lynx Group, United States (2014). | ☐ |
| | NPL42 | GATALICA, Z., et al., "Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type," Cancer Epidemiol Biomarkers Prev 23(12):2965-2970, American Association of Cancer Research, United States (2014). | ☐ |
| | NPL43 | LE, D., et al., "Phase 2 study of programmed death-1 antibody (anti-PD-1, MK-3475) in patients with microsatellite unstable (MSI) tumors," Journal of Clinical Oncology, 32(15): 4 pages, American Society of Clinical Oncology Journal, United States (2014). | ☐ |

| Examiner Signature | | Date Considered | |
|---|---|---|---|

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# SECOND SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 2 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD, JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

| NON-PATENT LITERATURE DOCUMENTS | | | |
|---|---|---|---|
| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
| | NPL44 | LIPSON, E.J., et al., "Durable Cancer Regression off-treatment and effective Reintroduction Therapy with an Anti-PD-1 Antibody," Clinical Cancer Research 19(2):462-468, American Association for Cancer Research, United States (2013). | ☐ |
| | NPL45 | LLOSA, N.J., et al., "Immune checkpoints expression in MSI versus MSS colorectal cancers and their potential therapeutic implications," Journal of Clinical Oncology 32(15): 3620-3620, American Society of Clinical Oncology Journal, United States (2014). | ☐ |
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17255030_1

| Examiner Signature | | Date Considered | |
|---|---|---|---|

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Doc Code: TRAN.LET

Document Description: Transmittal Letter

PTO/SB/21 (07-09)
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# TRANSMITTAL FORM

*(to be used for all correspondence after initial filing)*

Total Number of Pages in This Submission

| | |
|---|---|
| Application Number | 16/306,290 |
| Filing Date | November 30, 2018 |
| First Named Inventor | Michael AXELSON |
| Art Unit | 1647 |
| Examiner Name | LOCKARD, JOHN MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

## ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
- ☑ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☑ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☑ Other Enclosure(s) (please Identify below):

E-Payments: $1360 RCE Fee; $140 Petition Fee; Request for Continued Examination Transmittal; E-Petition for Withdraw from Issue; Copies NPL37-NPL46

**Remarks**

The Office may charge any fee deficiency for any submission made with this transmittal to Deposit Account 19-0036.

## SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

| | | | |
|---|---|---|---|
| Firm Name | Sterne, Kessler, Goldstein & Fox P.L.L.C. | | |
| Signature | /Brian M. Dudley/ | | |
| Printed name | Brian Dudley | | |
| Date | September 7, 2021 | Reg. No. | 77,546 |

## CERTIFICATE OF TRANSMISSION/MAILING

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| Doc Code: PET.AUTO<br>Document Description: Petition automatically granted by EFS-Web | PTO/SB/140<br>U.S. Patent and Trademark Office<br>Department of Commerce |
|---|---|

| | |
|---|---|
| Electronic Petition Request | **PETITION TO WITHDRAW AN APPLICATION FROM ISSUE AFTER PAYMENT OF THE ISSUE FEE UNDER 37 CFR 1.313(c)** |
| Application Number | 16306290 |
| Filing Date | 30-Nov-2018 |
| First Named Inventor | Michael AXELSON |
| Art Unit | 1647 |
| Examiner Name | |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |
| Title | METHODS OF TREATING COLORECTAL CANCER |

An application may be withdrawn from issue for further action upon petition by the applicant. To request that the Office withdraw an application from issue, applicant must file a petition under this section including the fee set forth in § 1.17(h) and a showing of good and sufficient reasons why withdrawal of the application from issue is necessary.

APPLICANT HEREBY PETITIONS TO WITHDRAW THIS APPLICATION FROM ISSUE UNDER 37 CFR 1.313(c).

A grantable petition requires the following items:
(1) Petition fee; and
(2) One of the following reasons:
(a) Unpatentability of one or more claims, which must be accompanied by an unequivocal statement that one or more claims are unpatentable, an amendment to such claim or claims, and an explanation as to how the amendment causes such claim or claims to be patentable;
(b) Consideration of a request for continued examination in compliance with § 1.114 (for a utility or plant application only); or
(c) Express abandonment of the application. Such express abandonment may be in favor of a continuing application, but not a CPA under 37 CFR 1.53(d).

Petition Fee

| | |
|---|---|
| ○ | Small Entity |
| ○ | Micro Entity |
| ◉ | Regular Undiscounted |

Reason for withdrawal from issue

| |
|---|
| ◯ One or more claims are unpatentable<br><br>◉ Consideration of a request for continued examination (RCE) (List of Required Documents and Fees)<br><br>◯ Applicant hereby expressly abandons the instant application (any attorney/agent signing for this reason must have power of attorney pursuant to 37 CFR 1.32(b)). |
| RCE request,submission, and fee.<br><br>☐ I certify, in accordance with 37 CFR 1.4(d)(4) that :<br>The RCE request ,submission, and fee have already been filed in the above-identified application on<br><br>☒ Are attached. |
| THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES<br><br>I certify, in accordance with 37 CFR 1.4(d)(4) that I am:<br><br>◉ An attorney or agent registered to practice before the Patent and Trademark Office who has been given power of attorney in this application.<br><br>◯ An attorney or agent registered to practice before the Patent and Trademark Office, acting in a representative capacity.<br><br>◯ A sole inventor<br><br>◯ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application<br><br>◯ A joint inventor; all of whom are signing this e-petition |

| | |
|---|---|
| Signature | /Brian M. Dudley/ |
| Name | Brian M. Dudley |
| Registration Number | 77546 |

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 16306290 |
| **Filing Date:** | 30-Nov-2018 |
| **Title of Invention:** | METHODS OF TREATING COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |

Filed as Large Entity

**Filing Fees for U.S. National Stage under 35 USC 371**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| PETITION FEE- 37 CFR 1.17(H) (GROUP III) | 1464 | 1 | 140 | 140 |
| RCE- 1ST REQUEST | 1801 | 1 | 1360 | 1360 |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Post-Allowance-and-Post-Issuance:** | | | | |
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **1500** |

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Decision Date : September 7, 2021

In re Application of :

Michael AXELSON

DECISION ON PETITION
UNDER CFR 1.313(c)(2)

Application No : 16306290

Filed : 30-Nov-2018

Attorney Docket No : 3338.0550001/ELE/C-K/BMD

This is an electronic decision on the petition under 37 CFR 1.313(c)(2), filed September 7, 2021, to withdraw the above-identified application from issue after payment of the issue fee.

The petition is **GRANTED.**

The above-identified application is withdrawn from issue for consideration of a submission under 37 CFR 1.114 (request for continued examination). See 37 CFR 1.313(c)(2).

**Petitioner is advised that the issue fee paid in this application cannot be refunded. If, however, this application is again allowed, petitioner may request that it be applied towards the issue fee required by the new Notice of Allowance.**

Telephone inquiries concerning this decision should be directed to the Patent Electronic Business Center (EBC) at 866-217-9197.

This application file is being referred to Technology Center AU 1647 for processing of the request for continuing examination under 37 CFR 1.114 .

Office of Petitions

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 43700662 |
| **Application Number:** | 16306290 |
| **International Application Number:** | |
| **Confirmation Number:** | 7097 |
| **Title of Invention:** | METHODS OF TREATING COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Customer Number:** | 115018 |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Filer Authorized By:** | Brian Mason Dudley |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |
| **Receipt Date:** | 07-SEP-2021 |
| **Filing Date:** | 30-NOV-2018 |
| **Time Stamp:** | 18:08:34 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $1500 |
| RAM confirmation Number | E202197I08272692 |
| Deposit Account | |
| Authorized User | |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Request for Continued Examination (RCE) | 2021-09-01-RCE-3338-0550001.PDF | 1350073<br>21c1d319090be924cd445319b1afc795e8c041c4 | no | 3 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Quick Path Information Disclosure Statement | 2021-09-07-2nd-Supp-IDS-Pleading-3338-0550001.PDF | 108172<br>19b9398356fec2d210bdd7c9e328d07e31c9d1b7 | no | 3 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Quick Path Information Disclosure Statement | 2021-09-07-2nd-Supp-IDS-SB08A-B-3338-0550001.PDF | 127708<br>b0e03aa48bd171f181ce2c5bf3d1c2457eef9178 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Transmittal Letter | 2021-09-07-Transmittal-3338-0550001.PDF | 188788<br>9b14af4e337bfa8d4360e23d9e70c95513831e94 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | ePetition Request Form | petition-request.pdf | 39930<br>f30991f302cb19cbb3fe9fe50b25946a3b2add94 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Fee Worksheet (SB06) | fee-info.pdf | 40478<br>fb4995fb801d1c820531d1cccaa55e163690090f | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |

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**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

Disease Markers 20 (2004) 237–250
IOS Press



# Development of a fluorescent multiplex assay for detection of MSI-High tumors

Jeffery W. Bacher[a,*], Laura A. Flanagan[a], Regenia L. Smalley[b], Nadine A. Nassif[a], Lawrence J. Burgart[b], Richard B. Halberg[a], Wael M. Abdel Megid[a] and Stephen N. Thibodeau[b]
[a]*Promega Corporation, Madison, WI, USA*
[b]*Mayo Clinic, College of Medicine, Rochester, MN, USA*

**Abstract**. Determining whether a tumor exhibits microsatellite instability (MSI) is useful in identifying patients with hereditary non-polyposis colorectal cancer and sporadic gastrointestinal cancers with defective DNA mismatch repair (MMR). The assessment of MSI status aids in establishing a clinical prognosis and may be predictive of tumor response to chemotherapy. A reference panel of five markers was suggested for MSI analysis by a National Cancer Institute (NCI) workshop in 1997 that has helped to standardize testing. But this panel of markers has limitations resulting from the inclusion of dinucleotide markers, which are less sensitive and specific for detection of tumors with MMR deficiencies compared to other types of markers that are currently available. This study demonstrates that mononucleotides are the most sensitive and specific markers for detection of tumors with defects in MMR and identifies an optimal panel of markers for detection of MSI-H tumors. A set of 266 mono-, di-, tetra- and penta-nucleotide repeat microsatellite markers were used to screen for MSI in colorectal tumors. The best markers for detection of MSI-H tumors were selected for a *MSI Multiplex System*, which included five mononucleotide markers: *BAT-25*, *BAT-26*, *NR-21*, *NR-24* and *MONO-27*. In addition, two pentanucleotide markers were added to identify sample mix-ups and/or contamination. We classified 153 colorectal tumors using the new MSI Multiplex System and compared the results to those obtained with a panel of 10 microsatellite markers combined with immunohistochemical (IHC) analysis. We observed 99% concordance between the two methods with nearly 100% accuracy in detection of MSI-H tumors. Approximately 5% of the MSI-H tumors had normal levels of four MMR proteins and as a result would have been misclassified based solely on IHC analysis, emphasizing the importance of performing MSI testing. The new *MSI Multiplex System* offers several distinct advantages over other methods of MSI testing in that it is both extremely sensitive and specific and amenable to high-throughput analysis. The *MSI Multiplex System* meets the new recommendations proposed at the recent 2002 NCI workshop on HNPCC and MSI testing and overcomes problems inherent to the original five-marker panel. The use of a single multiplex fluorescent MSI assay reduces the time and costs involved in MSI testing with increased reliability and accuracy and thus should facilitate widespread screening for microsatellite instability in tumors of patients with gastrointestinal cancers.

Keywords: Microsatellite instability, MSI, hereditary non-polyposis colorectal cancer, HNPCC and mononucleotide

## 1. Introduction

The majority of tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC also known as Lynch syndrome) and approximately 15% of sporadic colorectal tumors exhibit microsatellite instability (MSI), which is characterized by alterations in the length of short tandem repeats in tumor DNA [1,16,44]. Microsatellite instability is caused by DNA mismatch repair (MMR) deficiency and subsequent failure to repair errors introduced during replication of DNA [6,11, 22]. Repetitive DNA sequences like microsatellites are especially prone to errors during DNA replication. The analysis of their stability combined with the analysis of DNA MMR proteins' levels by immunohistochemistry (IHC) provides a sensitive and specific method for identifying tumors with defective DNA mismatch re-

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pair. The MSI status of a tumor is predictive of clinical outcome and may also be predictive of tumor response to adjuvant therapies [9,13,28–30,35,37,39,47,49].

In 1997, a National Cancer Institute sponsored workshop recommended a panel of five microsatellite markers for detection of MSI consisting of two mononucleotide markers (*BAT-25* and *BAT-26*) and three dinucleotide repeats (*D2S123, D5S346* and *D17S250*). This panel of markers is referred to as the Bethesda panel [5]. Tumors with instability in two or more of these markers are defined as MSI-High (MSI-H), whereas those with one unstable marker are designated as MSI-Low (MSI-L). Tumors with no detectible alterations are stable (MSS). The Bethesda panel of markers provides a uniform set of markers and criteria for tumor classification that has helped standardize MSI analysis. This panel of markers does, however, have some limitations that were detailed at a 2002 NCI workshop that revised recommendations for MSI testing (46). These limitations are a result of including dinucleotide repeats in the panel, as they are less sensitive and less specific for detection of tumors with MMR deficiencies than mononucleotides and can result in misclassification of MSI-L tumors as MSI-H. In addition, interpretation of allelic profiles using dinucleotide repeats is complicated by the presence of stutter, which is a PCR artifact produced by repeat slippage during amplification.

Current evidence suggests that only the MSI-H phenotype is associated with HNPCC and sporadic tumors with MMR defects and that there may be molecular and clinopathological differences between MSI-H and MSS/MSI-L colorectal cancers [references]. As compared to MSS tumors, MSI-H tumors are more likely to occur in individuals with a positive family history of colorectal cancer, to arise in the right colon, to occur in women, and to be associated with a favorable prognosis [25,26,28,32,39,40,44,49]. MSI-H tumors are more likely to have mutations in genes with short repetitive tracts including*TGF-βRII, BAX, IGF2R* genes [27,30, 32,34,42,50] and less likely to have loss of *APC* [1,15, 31,38] or have mutations in p53 [1,7,24,31,38,41] and K-*ras* genes [1,24,31,38]. Tumors with MSI-H phenotype are usually diploid or nearly diploid as opposed to MSS/MSI-L tumors that are typically aneuploid [18]. At the morphological level, MSI-H tumors are characteristically mucinous, poorly differentiated and show lymphocytic infiltration [17,18,20,36].

While the distinction between MSI-H and MSI-L tumors is generally accepted, the separation between MSI-L and MSS groups remains controversial [14, 21,23,45]. Most non-MSI-H colorectal tumors exhibit some low level of instability if enough markers are used. Halford and colleagues reported that 68% of sporadic non-MSI-H tumors displayed instability in at least one marker when 44 microsatellite markers were tested [14]. Similarly, Laiho and colleagues found 79% of *BAT-26* stable tumors were MSI-L using 377 dinucleotide repeats [21]. Further, MSI analysis with different sub-sets of microsatellite markers resulted in identification of almost totally different MSI-L groups. While it was suggested that MSI-L is a real phenomenon and a quantitative trait, no evidence was found of a discrete MSI-L group or association between MSI-L and any clinopathological feature. It remains unclear whether MSI-L is caused by some specific underlying defect or results from the process of carcinogenesis in general, such as random microsatellite slippage accompanied by clonal expansion.

Therefore, we focused our efforts on distinguishing between tumors with the MSI-H phenotype, a well-established subgroup of colorectal cancers with a characterized underlying cause, and non-MSI-H tumors. Our goals were to improve on the existing panel of MSI markers described by Boland and colleagues to significantly increase the throughput and accuracy of MSI tests [5]. We identified an optimal set of markers that provided maximal sensitivity and specificity for MSI-H tumors lacking expression of one or more MMR genes and incorporated them into a multiplex fluorescent assay for simple, rapid and accurate detection of MSI-H tumor phenotype.

## 2. Material and methods

### 2.1. DNA samples

The three different sets of colorectal tumor and matching normal samples used in this study were derived from samples collected at the Mayo Clinic (Rochester, MN), with the exception of sample set A that also contained blood and tissue samples obtained through the Cooperative Human Tissue Network (Columbus, OH). Sample set A consisted of 35 MSI-H and 30 non-MSI-H pairs, sample set B consisted of 49 MSI-H and 114 non-MSI-H pairs and sample set C consisted of 72 MSI-H and 81 non MSI-H pairs. DNA from tumor samples was isolated from paraffin-embedded material and corresponding normal DNA was derived from peripheral blood as described elsewhere [23].

Genomic DNA from 177 Caucasian-American, 236 African-American and 127 Asian-American healthy in-

dividuals was obtained from the BODE Technologies Group (Springfield, VA) and genotyped to determine allelic size ranges and allele frequencies for *BAT-25, BAT-26, NR-21, NR-24* and *MONO-27* markers. The frequency of germline mutations in a selected group of microsatellite markers was estimated by analyzing 200–300 healthy parent-child kindreds also obtained from BODE Technologies Group.

### 2.2. MSI and IHC analysis

The MSI status of all tumor DNA samples analyzed in this study had been previously determined with a panel of 10 microsatellite markers including mononucleotides *BAT-25, BAT-26, BAT-40, BAT34c4*, dinucleotides *D5S346, D17S250, ACTC, D18S55, D10S197* and a complex penta-tetra-mononucleotide repeat marker *MYCL1*. In addition, the status of mismatch repair proteins was determined by immunohistochemistry assays either for MLH1 and MSH2 in sample set A, or for MLH1, MSH2 and MSH6 in samples sets B and C. IHC assay for PMS2 was performed on sample set C in cases where loss of the other MMR proteins was not observed. Immunohistochemical analysis revealed a lack of MLH1, MSH2, MSH6 or PMS2 proteins in 63%, 25%, 9% and 2% of MSI-H tumor samples tested, respectively. MSI assays and IHC analysis were carried out as described by Lindor *et al.* [23].

We evaluated a total of 266 mono-, di-, tetra- and penta-nucleotide repeat microsatellite loci for sensitivity to MSI. Microsatellite markers screened for MSI included: (a) 7 mononucleotide repeats including, *BAT-25, BAT-26, BAT-34c4, BAT-40, NR-21, NR-24* and *MONO-27* [8,43], (b) 6 dinucleotide markers from the Bethesda panel of markers including, *D2S123, D5S346, D17S250, ACTC, D18S55* and *D10S197* [5, 23], (c) 245 tetranucleotide repeat markers from the Research Genetics CHLC/Weber Human Screening Set Version 9.0 (Research Genetics, Huntsville, AL) plus *MYCL1*, and (d) 8 pentanucleotide repeats including, *TP53-penta* and *Penta A, B, C, D, E, F*, and *G* markers [2,3,8]. All markers have been described previously except, *MONO-27* (Genbank accession #AC007684), which was identified by screening Genbank DNA sequence database. PCR and gel electrophoresis of markers from the CHLC/Weber Human Screening Set Version 9.0 were amplified following the manufacturer's protocol. All other markers were amplified in 25 $\mu$l PCR reactions using 2.5 $\mu$l GoldST★R 10X Buffer (Promega, Madison, WI), 0.1-1 $\mu$M each primers, 0.05 $\mu$l AmpliTaq Gold DNA Polymerase (5Units/$\mu$l) (Perkin Elmer, Wellesley, MA) per locus and 1-2ng DNA. PCR was performed on a PE 9600 Thermal Cycler using cycling profile: 1 cycle 95°C for 11 minutes; 1 cycle 96°C for 1 minute; 10 cycles 94°C for 30 seconds, ramp 68 seconds to 56°C, hold for 30 seconds, ramp 50 seconds to 70°C, hold for 45 seconds; 20 cycles at 90°C for 30 seconds, ramp 60 seconds to 56°C, hold for 30 seconds, ramp 50 seconds to 70°C, hold for 45 seconds; 60°C for 30 minutes final extension; 4°C hold.

Separation and detection of amplified fragments was performed on an ABI PRISM® 310 or 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Data was analyzed with GeneScan Analysis and Genotyper Software packages from Applied Biosystems to identify predominate allele size for each locus. Allelic patterns or genotypes for normal and tumor pairs were compared and scored as MSI positive if one or more different alleles were present in the tumor DNA samples that were not found in normal samples from the same individual.

The classification of microsatellite instability was based on guidelines suggested by a National Cancer Institute workshop [5]. Using the 5-locus Bethesda panel of microsatellite markers, tumor samples with 40% MSI were classified as MSI-high (MSI-H), less than 40% as MSI-low (MSI-L), and no alterations were classified as microsatellite-stable (MSS). If more than 5 markers are to be used, MSI-H group was defined as tumors having MSI at 30% or more of the markers tested, whereas the MSI-L tumors exhibit MSI in 1–29% of the markers.

### 2.3. Multiplex analysis

MSI multiplex analysis was performed using 1–2ng of template DNA in a 25 $\mu$l PCR reaction using the following protocol: 17 $\mu$l nuclease free water, 2.5 $\mu$l GoldST★R 10X Buffer (Promega), 2.5 $\mu$l 10X multiplex primer mix, 0.5 $\mu$l AmpliTaq Gold DNA Polymerase at 5Units/$\mu$l and 2.5 $\mu$l DNA at 0.4–0.8ng/$\mu$l. PCR was performed on a PE 9600 Thermal Cycler using cycling profile: 1 cycle 95°C for 11 minutes; 1 cycle 96°C for 1 minute; 10 cycles 94°C for 30 seconds, ramp for 68 seconds to 56°C, hold for 30 seconds, ramp 50 seconds to 70°C, hold for 45 seconds; 20 cycles at 90°C for 30 seconds, ramp for 60 seconds to 56°C, hold for 30 seconds, ramp 50 seconds to 70°C, hold for 45 seconds; 60°C for 30 minutes final extension; 4°C hold.

Table 1
Sensitivity and specificity of microsatellite markers for MSI-H tumors

| Locus | Repeat type | Genbank accession number | | Sensitivity | Specificity | Sample set |
|---|---|---|---|---|---|---|
| *BAT-40* | Mono | M38180 | | 100% (118/118) | 95% (188/198) | B & C |
| *MONO-27* | Mono | AC007684 | | 98% (107/109) | 100% (177/177) | B & C |
| *NR-21* | Mono | XM_033393 | | 99% (64/65) | 100% (74/74) | C |
| *BAT-25* | Mono | U63834 | | 96% (115/120) | 99.5% (187/188) | B & C |
| *BAT-26* | Mono | U41210 | | 93% (111/120) | 100% (194/194) | B & C |
| *NR-24* | Mono | X60152 | | 92% (56/61) | 100% (73/73) | C |
| *BAT-34c4* | Mono | – | | 92% (102/111) | 99.5% (188/189) | B & C |
| | | | Mean | 96% | 99% | |
| *D2S123* | Di | Z16551 | | 91% (31/34) | 86% (25/29) | A |
| *D18S55* | Di | Z16621 | | 90% (88/98) | 97% (182/188) | B & C |
| *D10S197* | Di | Z16611 | | 89% (88/99) | 96% (171/179) | B & C |
| *D17S250* | Di | X54562 | | 88% (93/106) | 93% (170/182) | B & C |
| *ACTC* | Di | NM_005159 | | 84% (93/111) | 95% (184/193) | B & C |
| *D5S346* | Di | NM_005669 | | 81% (91/112) | 98% (188/192) | B & C |
| | | | Mean | 87% | 94% | |
| *D3S2432* | Tetra | G08240 | | 76% (32/42) | 98% (80/82) | B |
| *D7S3070* | Tetra | G27340 | | 74% (35/47) | 95% (105/111) | B |
| *D7S3046* | Tetra | G10353 | | 68% (27/40) | 98% (92/94) | B |
| *D7S1808* | Tetra | G08643 | | 67% (31/46) | 98% (112/114) | B |
| *D10S1426* | Tetra | G08812 | | 61% (28/46) | 95% (105/111) | B |
| | | | Mean | 69% | 97% | |
| *Penta-E* | Penta | – | | 57% (16/28) | 100% (29/29) | A |
| *Penta-B* | Penta | – | | 43% (12/28) | 96% (27/28) | A |
| *Penta-D* | Penta | AC000014 | | 35% (30/87) | 100% (104/104) | A & C |
| *Penta-A* | Penta | – | | 31% (9/29) | 100% (29/29) | A |
| *Penta-F* | Penta | – | | 21% (6/29) | 100% (29/29) | A |
| *Penta-G* | Penta | Z83847 | | 21% (6/29) | 97% (28/29) | A |
| *Penta-C* | Penta | AL138752 | | 14% (12/87) | 98% (100/102) | A & C |
| *TP53-penta* | Penta | – | | 14% (4/29) | 97% (28/29) | A |
| | | | Mean | 27% | 99% | |
| *MYCL1* | Complex | | | 80% (94/117) | 85% (164/192) | B & C |

Sensitivity is defined as the percent of MSI-H samples with MSI
Specificity is defined as the percent non-MSI-H samples in which that marker was stable
Data Set A = MSI-H ($N = 35$), non MSI-H ($n = 30$)
Data Set B = MSI-H ($N = 49$), non MSI-H ($n = 114$)
Data Set C = MSI-H ($N = 74$), non MSI-H ($n = 81$).

Small pool PCR was also performed with the MSI multiplex using 6pg genomic DNA (roughly 1 genome equivalent), as determined by PicoGreen dsDNA Quantitative Kit (Molecular Probes, Eugene OR) following the manufacturer's protocol. The approximate number of genome equivalents were estimated by amplifying increasing amounts of (0.1–1 $\mu$l) of a 10pg/$\mu$l DNA dilution in a total of 10 PCR reactions, followed by Poisson analysis of the number of reactions positive and negative for a given marker [51]. Small pool PCR was carried out in 10 $\mu$l reactions by proportionally reducing the PCR reaction components described above for the MSI Multiplex and by increasing the number of PCR cycles to 35. Capillary electrophoresis and sample analysis was as describe above except, GeneScan (Applied Biosystems) data was imported into a Genotyper software (Applied Biosystems) macro developed for the MSI Multiplex System to automate genotyping of samples, including assigning locus names, allele sizes and to compare matching normal and tumor sample pairs for shifts in allele sizes.

### 2.4. Allele frequency determinations

Allele size ranges for all loci included in MSI multiplex were determined by genotyping at least 540 normal DNA samples from individuals from three different racial groups (African-American, Asian-American, and Caucasian-American) using the MSI Multiplex System. Genotypes were determined for mononucleotide repeat loci *BAT-25, BAT-26, MONO-27, NR-21* and *NR-24* to calculate allele frequencies and percent heterozygosity.

Table 2
Germline and somatic mutation frequencies

| | | Germline mutation frequency | | | Somatic mutation frequency in non-MSI-H tumors | | |
|---|---|---|---|---|---|---|---|
| Locus | Repeat | Number alleles | Number mutants | Mutation rate | Number alleles | Number mutants | Mutation rate |
| *BAT-25* | Mono | 560 | 0 | $< 1.8 \times 10^{-3}$ | 576 | 2 | $3.4 \times 10^{-3}$ |
| *BAT-26* | Mono | 560 | 0 | $< 1.8 \times 10^{-3}$ | 606 | 0 | $< 1.7 \times 10^{-3}$ |
| *MONO-27* | Mono | 560 | 0 | $< 1.8 \times 10^{-3}$ | 420 | 0 | $< 2.4 \times 10^{-3}$ |
| *BAT-40* | Mono | 534 | 2 | $3.7 \times 10^{-3}$ | 436 | 10 | $2.3 \times 10^{-2}$ |
| *D2S123* | Di | 534 | 0 | $< 1.9 \times 10^{-3}$ | 60 | 4 | $6.7 \times 10^{-2}$ |
| *D5S346* | Di | 534 | 0 | $< 1.9 \times 10^{-3}$ | 525 | 4 | $7.6 \times 10^{-3}$ |
| *D17S250* | Di | 534 | 0 | $< 1.9 \times 10^{-3}$ | 426 | 13 | $3.1 \times 10^{-2}$ |
| *D7S1808* | Tetra | 560 | 0 | $< 1.8 \times 10^{-3}$ | 290 | 2 | $6.9 \times 10^{-3}$ |
| *D7S3046* | Tetra | 560 | 0 | $< 1.8 \times 10^{-3}$ | 250 | 4 | $1.6 \times 10^{-2}$ |
| *D7S3070* | Tetra | 560 | 1 | $1.8 \times 10^{-3}$ | 284 | 7 | $2.5 \times 10^{-2}$ |
| *D10S1426* | Tetra | 560 | 0 | $< 1.8 \times 10^{-3}$ | 284 | 9 | $3.2 \times 10^{-2}$ |
| *MYCL1* | Tetra | 534 | 0 | $< 1.9 \times 10^{-3}$ | 386 | 36 | $9.3 \times 10^{-2}$ |

### 2.5. Mutation studies

Germline mutation frequency was determined for selected mononucleotides (*BAT-25, BAT-26, BAT-40, MONO-27*), dinucleotides (*D2S123, D5S346, D17S250*), tetranucleotides (*D3S2432, D7S1808, D7S3046, D7S3070, D10S1426* and *MYCL1*) by pedigree analysis of up to 280 healthy parent-child pairs. Parentage of all parent-child kindreds was confirmed by analysis with PowerPlex®16 (Promega) which provided paternity/maternity indices exceeding a million to one in favor of parentage for each kindred. Somatic mutations frequencies were estimated by MSI analysis of non-MSI-H high tumor DNA samples with normal mismatch repair protein expression.

## 3. Results

### 3.1. Screening microsatellite markers

Several criteria were used to select the best markers for MSI analysis, including sensitivity, specificity, ease of interpretation and percent stutter. Sensitivity of a marker was defined as the percent of MSI-H samples in which that marker was unstable, whereas specificity of a marker was defined as the percent of non-MSI-H samples (MSS and MSI-L) in which that marker was stable. Stutter products are PCR artifacts that are generated by polymerase slippage during amplification of short tandem repeat sequences that differ in size from the actual allele by one or more repeat units.

Markers showing the highest sensitivity and specificity for each of the different repeat types are summarized in Table 1. By far the most sensitive and specific markers were the mononucleotides, showing between 92 and 100% sensitivity and 99.5 to 100% specificity for MSI-H samples. The order of sensitivity observed was mononucleotides, dinucleotides, tetranucleotides then pentanucleotides with an average of 96%, 87%, 69% and 27% of MSI-H tumors exhibiting instability, respectively. The ranking for specificity was different, with mononucleotide markers showing an average of 99% specificity (excluding *BAT-40*), followed by pentanucleotide markers at slightly less than 99%, tetranucleotides at 97% and dinucleotides at 94% specificity. Dinucleotide markers *D2S123* and *D17S250* and tetranucleotide marker *MYCL1* exhibited lowest specificities for MSI-H. This observation was also reported by Whitehall *et al.* (2001) who found that *MYCL1* and *D2S123* were the most commonly mutated microsatellite loci in MSI-L cancers, showing MSI in 67% (28/42) and 24% (10/42) of MSI-L tumors, respectively [48].

Two qualities of microsatellite markers that affect the ease of interpreting allelic profiles are the amount of stutter product generated during PCR amplification and heterozygosity. In general, loci with shorter repeat units have a higher incidence of stutter (Fig. 1). For example, mononucleotide markers *BAT-25* and *BAT-26* had stutter peaks at the N-1 position (1bp or one repeat unit less that actual allele peak) that were on average 79% and 72% as high as the actual allele peak. Dinucleotide markers *D5S346, D17S250* and *D2S123* had stutter peaks at N-2 position that were on average 35%, 49% and 53% as high as the actual allele peaks. In comparison, tetranucleotide repeat loci *D10S1426, D7S1808* and *D7S3046* had N-4 stutter peaks averaging of 6–7%, and pentanucleotide loci *Penta-D, Penta-C and Penta-E* had N-5 stutter peaks all averaging less than 1%. The amount of stutter clearly affects ease of scoring as demonstrated by MSI analysis with high stutter dinucleotide and low stutter tetranucleotides (Fig. 2). But unfortunately the more stable tetra- and

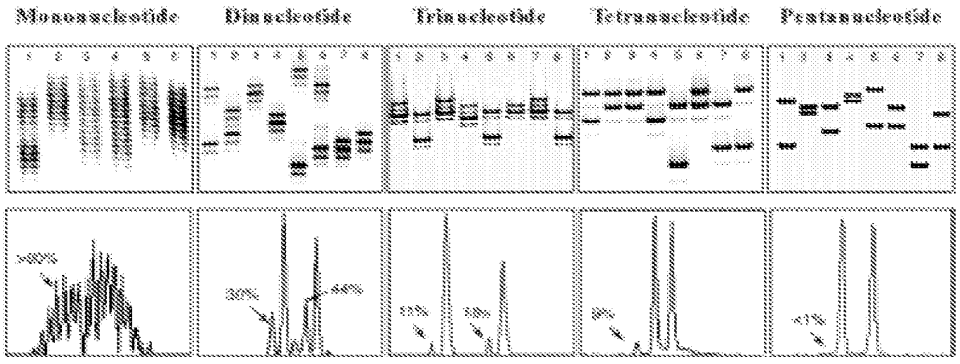



Fig. 1. Effect of microsatellite repeat unit length on stutter (repeat slippage). DNA samples were amplified using fluorescently labeled primers for mono- (*BAT-40*), di- (*D5S346*), tri- (*TBP*), tetra- (*D5S818*) and pentanucleotide microsatellite repeat loci (*Penta D*). Stutter is observed more frequently with shorter repeat unit length.

pentanucleotide repeats generally displayed lower sensitivity for MSI. Another factor that affects ease of genotyping is heterozygosity. Genotyping DNA from homozygous individuals is easier than from a heterozygous individual. Dinucleotide repeats exhibited both high levels of stutter and heterozygosity. By contrast, mononucleotides were nearly monomorphic and allelic shifts in MSI-H tumors were generally large, making MSI analysis relatively straightforward.

### *3.2. Somatic and germline mutation rates*

Dinucleotide and tetranucleotide repeats were frequently altered in non-MSI-H tumor samples exhibiting normal levels of MMR protein staining in IHC assays. We estimated the frequency of somatic mutations in up to 303 non-MSI-H tumor samples with normal IHC (Table 2). Elevated somatic mutation rates were observed for most di- and tetranucleotide repeats tested and for mononucleotide marker *BAT-40*. The greatest increase in mutation rates were observed in *MYCL1* and *D2S123* markers, with 19% and 13% non-MSI-H tumors showing mutations, respectively.

We speculated the reduced specificity of some microsatellite repeats reflects higher spontaneous mutation rates of these markers. We tested this hypothesis by genotyping 208 parent-child kindreds for germline mutations in mononucleotide markers (*BAT-25, BAT-26, BAT-40* and *MONO-27*), dinucleotide markers (*D2S123, D5S346* and *D17S250*), tetranucleotide markers (*D3S2432, D7S1808, D7S3046, D7S307* and *D10S1426*) and one complex penta/tetra/mono marker (*MYCL1*). One germline mutation was observed out of 560 alleles at tetranucleotide marker *D7S3046* and two mutations out of 534 alleles were observed at mononucleotide marker *BAT-40* (Table 2). This data does not support an association between high somatic mutation frequency in non-MSI-H tumors and higher germline mutations frequencies, except for *BAT-40*. Germline hypermutability of the *BAT-40* poly-A tract was also observed by Bacon and colleagues (2001) who reported mutations in 7% of germline allele transmissions and suggested that stable markers may be too sensitive to provide the specificity needed for MSI testing [4].

We predict that MSI-L tumors could be misclassified as MSI-H if microsatellite markers with lower specificity are used. In our study the average percent of dinucleotide markers found mutated in non-MSI-H tumors with normal IHC was 6% (Table 1). Thus, the estimated background mutation frequency for any two of these dinucleotide markers in all colorectal tumors is approximately 0.4%. At this background mutation frequency, around 3% of MSI-H tumors could be misclassified using a panel of five markers containing two dinucleotide markers with 94% specificity. To test this hypothesis, we analyzed a set of colorectal tumors (sample set A) using the Bethesda panel and observed 1 out of 30 (3%) non-MSI-H tumor samples with mutations in both *D2S123* and *D17S250*. This sample would have been incorrectly misclassified as MSI-H using the Bethesda guidelines as indicated by subsequent analysis showing stability at nine other markers and normal IHC. By contrast, the chance of misclassifying MSI-L samples as MSI-H using the five mononucleotide markers in the MSI Multiplex System based on our specificity data is virtually nonexistent, since only *BAT-25* was altered in non-MSI-H samples and this occurred in only 0.5% (1/188) of the samples.

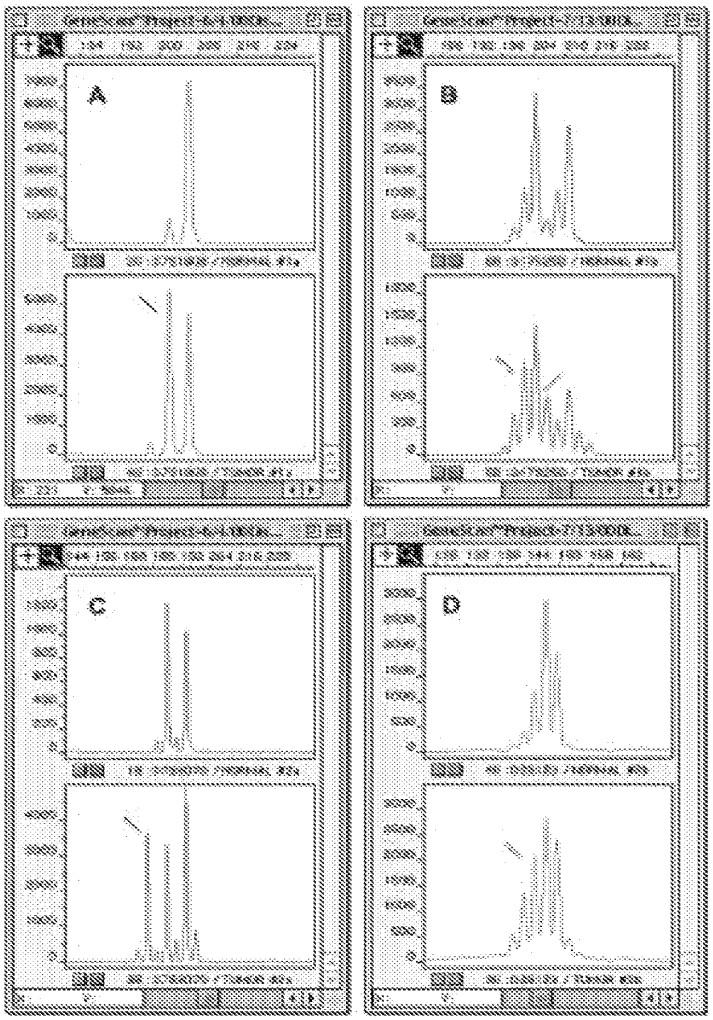



Fig. 2. MSI analysis with dinucleotide and tetranucleotide markers. Allelic profiles resulting from amplification of tetranucleotide repeat markers were simpler to interpret than were those of dinucleotide markers because of lower stutter. Matching normal and MSI-H tumor samples were amplified with tetranucleotide marker *D7S1808* (A) and dinucleotide marker *D17S250* (B) loci. A second matching pair of normal and MSI-H tumor samples were amplified with tetranucleotide *D7S3070* (C) and dinucleotide *D2S123* (D) marker. New alleles present in MSI-H sample (bottom panels) are indicated with an arrow. High stutter in dinucleotide markers complicated MSI analysis, especially when shifted alleles were the same size as stutter peaks.

### 3.3. *Construction and evaluation of the fluorescent MSI Multiplex System*

Five mononucleotide repeat markers were selected for multiplexing based upon the characteristics of the different classes of repeat markers and the variation in the sensitivity and specificity of particular markers. Information about the mononucleotide markers *BAT-25, BAT-26, NR-21, NR-24* and *MONO-27* included in the *MSI Multiplex System* is given in Table 3. These five mononucleotide markers were found to be the most sensitive and specific for detection of MSI-H tumors. In addition, these markers were found to be nearly monomorphic, which simplifies interpretation

of allelic patterns when only a single allele is present in normal DNA and offers the possibility of performing MSI assays without the need for matching normal samples [43]. Two polymorphic pentanucleotide repeats, *Penta-C* and *Penta-D*, were included in multiplex to detect potential sample mix-ups and/or contamination problems that might not be apparent when using only the nearly monomorphic markers. A matching probability between 1:10 and 1:16 was calculated for *Penta-C*, and between 1:18 and 1:33 for *Penta-D* based upon allele frequency data [2]. Matching probability is the average number of people you would have to survey before you would find the same allelic pattern as a randomly selected individual. Using both pentanucleotide markers increases the matching probability such that only 0.2 to 0.6% of individuals will have the same allelic profile (depending upon ethnic background). Polymorphic pentanucleotide markers were selected instead of di- and tetranucleotides because pentanucleotides are the most stable repeat type in mismatch repair deficient cells, making sample identification less ambiguous. The pentanucleotide repeat markers were not used in determining MSI status.

PCR primers for the multiplex were designed to allow robust co-amplification all markers in a single PCR reaction without generation of non-specific products. Fluorescently labeled PCR products were resolved by size and color by fluorescent capillary electrophoresis with AB 310 or 3100 Genetic Analyzers. An example of a GeneScan (Applied Biosystems) electropherogram showing a typical allelic pattern resulting from amplification of normal DNA or MSS tumors with the MSI Multiplex System is shown in Fig. 3. All PCR products observed for the mononucleotide markers were less than 160bp, allowing robust amplification of fragmented DNA extracted from most paraffin-embedded tissues.

### *3.4. Allele size ranges and frequencies*

Genomic DNA from 177 Caucasian-American, 236 African-American and 127 Asian-American healthy individuals was genotyped to determine allelic size ranges to confirm there was no overlap between markers (Table 3). The common allele size for *NR-21, BAT-26, BAT-25, NR-24* and *MONO-27* markers was 98, 113, 120, 130 and 150bp, respectively. PCR products ranged from 98 to 160bp for all mononucleotide markers, and between 137 and 209bp for all pentanucleotide markers. There was sufficient size difference between markers to prevent overlap even with shortened alleles observed in MSI-H samples.

We examined allele frequencies and heterozygosity levels for MSI multiplex makers in 540 individuals to determine whether they were sufficiently monomorphic to distinguish between mutations and polymorphisms if only tumor samples were used in MSI analysis (Table 4). The most polymorphic population examined was the African-American group and, therefore, this ethnic background would have the highest likelihood of being heterozygous for the mononucleotide markers [33,43]. The percent heterozygosity for *BAT-25, BAT-26, MONO-27, NR-21* and *NR-24* markers in the African-American population ($n = 236$) was 16.1%, 10.9%, 2.5%, 2.2% and 0.9%, respectively. Based on these data, the highest proportion of African-American individuals that are heterozygous for two mononucleotide markers, *BAT-25* and *BAT-26*, would be 1.8%. The highest proportion of individuals heterozygous for the three markers, *BAT-25, BAT-26* and *MONO-27*, would be 0.04%. When we only considered heterozygotes that differed by $\leqslant$ 3bp, the percent heterozygosity values lowered to 11.5%, 9.6%, 0.9%, 1.3% and 0.9% for *BAT-25, BAT-26, MONO-27, NR-21* and *NR-24*, respectively. This reduced the predicted frequency of individuals heterozygous for two markers, *BAT-25* and *BAT-26*, to 1.1%, and for three markers, *BAT-25, BAT-26* and *NR-24*, to 0.014%.

The majority of allelic variation in mononucleotide markers was within 3bp of the most common allele. To test whether this variation reflected real differences is allele sizes, or was due to run-to-run differences in sizing, we looked at consistency of sizing between multiple ABI 3100 runs of the same PCR product. Allele sizes were uniform across runs with the exception of a small number of alleles that had two major peaks of similar height differing in size by 1bp. Small pool PCR analysis of the ambiguously sized alleles revealed that small variations in sizing of mononucleotide markers can occur in individuals heterozygous for alleles that differ in size by a few base pairs and we hypothesize that random events could result in unequal amplification and subsequent variation in the prominent allele. Thus, only shifts of 3 bp or more should be considered mutants to avoid confusion with natural low-level polymorphic variation in this type of marker.

### *3.5. Determination of MSI tumor phenotype with the MSI Multiplex System*

Multiplex MSI analysis was performed on a set of 72 MSI-H and 81 non MSI-H matching tumor/normal

Table 3
PCR product sizes and respective fluorescent dyes for loci contained in the MSI Multiplex System

| Locus name | Genebank number | Gene | Repeat motif | Size range (bp) | Fluorescent label |
|---|---|---|---|---|---|
| *NR-21* | XM_033393 | SLC7A8 | $(A)_{21}$ | 94–101 | JOE |
| *BAT-26* | U41210 | MSH2 | $(A)_{26}$ | 103–115 | FL |
| *BAT-25* | L04143 | c-kit | $(A)_{25}$ | 113–124 | JOE |
| *NR-24* | X60152 | ZNF-2 | $(A)_{24}$ | 130–133 | TMR |
| *MONO-27* | AC007684 | MAP4K3 | $(A)_{27}$ | 142–154 | JOE |
| *Penta C* | AL138752 | – | $(AAAAG)_{3-15}$ | 143–194 | TMR |
| *Penta D* | AC000014 | – | $(AAAAG)_{2-17}$ | 135–201 | FL |

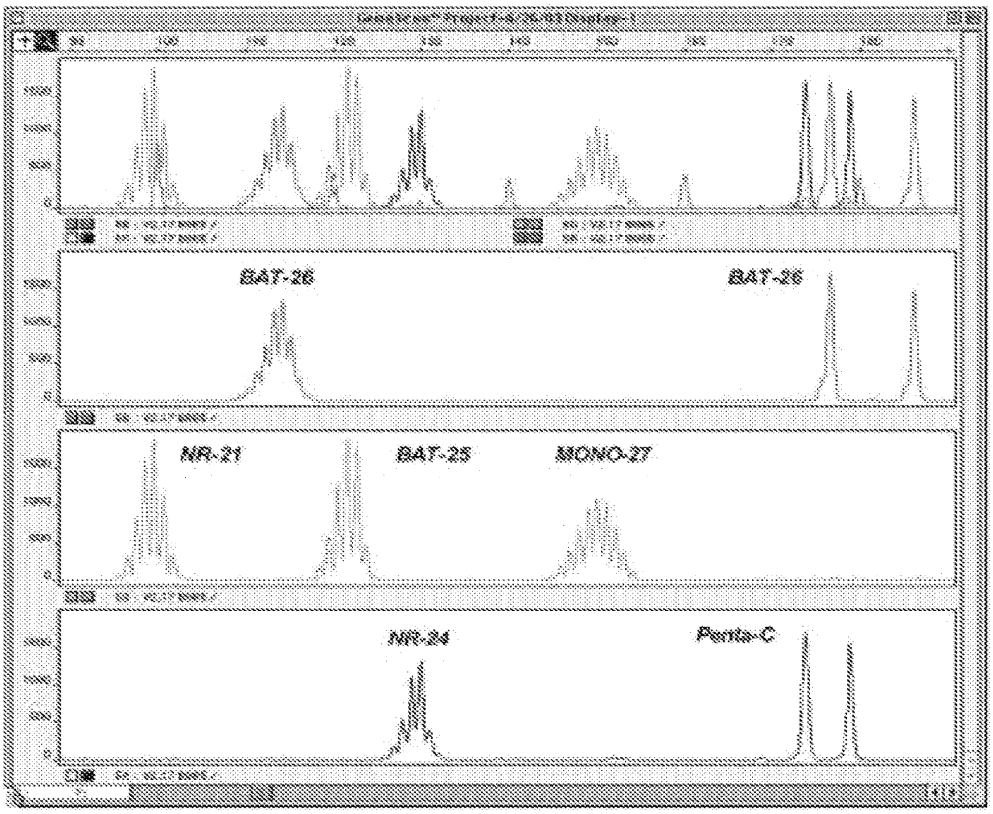



Fig. 3. MSI analysis of normal DNA samples with the *MSI Multiplex System*. The fluorescent *MSI Multiplex System* contains five nearly monomorphic mononucleotide repeat loci (*BAT-25, BAT-26, MONO-27, NR-21* and *NR-24*) that are very sensitive and specific for instability in MSI-H tumors. In addition, two polymorphic pentanucleotide repeats (*Penta-C* and *Penta-D*) are included as internal controls to identify sample mix-ups and/or contamination problems. One nanogram of template DNA was amplified using fluorescently labeled primers in a single PCR reaction and products analyzed on an ABI 3100 capillary electrophoresis instrument.

pairs (sample set C) that had been previously characterized for MSI status using a panel of 10 microsatellite markers and by IHC analysis for MLH1, MSH2, MSH6 and PMS2 A typical example of shifts in allele sizes observed in MSI-H samples is shown in Fig. 4. There was 97% (70/72) concordance between the MSI classification using the *MSI Multiplex System* and the 10-marker panel for the MSI-H group of specimens. One of these samples was unstable for 4 of 8 informative markers with the 10-marker panel (3 dinucleotide markers and *BAT-40*) and exhibited no instability with the *MSI Multiplex System*. This sample had normal

Table 4
Allele Frequencies for mononucleotide markers in the MSI Multiplex System

| *NR-21* | | | |
|---|---|---|---|
| Size (bp) | Caucasian | African | Asian |
| 94 | 1 | 2 | 8 |
| 95 | 1 | 0 | 0 |
| 96 | 0 | 3 | 0 |
| 97 | 0 | 60 | 0 |
| 98 | 27 | 100 | 14 |
| 99 | 134 | 62 | 89 |
| 100 | 14 | 9 | 23 |
| 101 | 1 | 0 | 1 |
| Total | 178 | 236 | 135 |

| *BAT-26* | | | |
|---|---|---|---|
| Size (bp) | Caucasian | African | Asian |
| 103 | 0 | 23 | 0 |
| 104 | 0 | 0 | 0 |
| 105 | 0 | 0 | 0 |
| 106 | 0 | 2 | 1 |
| 107 | 1 | 0 | 0 |
| 108 | 0 | 0 | 0 |
| 109 | 0 | 0 | 0 |
| 110 | 0 | 0 | 0 |
| 111 | 0 | 4 | 0 |
| 112 | 14 | 38 | 3 |
| 113 | 140 | 131 | 115 |
| 114 | 22 | 57 | 8 |
| 115 | 0 | 0 | 0 |
| Total | 177 | 255 | 127 |

| *NR-24* | | | |
|---|---|---|---|
| Size (bp) | Caucasian | African | Asian |
| 130 | 60 | 197 | 49 |
| 131 | 113 | 34 | 77 |
| 132 | 0 | 0 | 0 |
| 133 | 0 | 2 | 2 |
| Total | 173 | 233 | 128 |

| *MONO-27* | | | |
|---|---|---|---|
| Size (bp) | Caucasian | African | Asian |
| 142 | 0 | 1 | 0 |
| 143 | 0 | 0 | 0 |
| 144 | 0 | 0 | 0 |
| 145 | 0 | 0 | 0 |
| 146 | 0 | 0 | 0 |
| 147 | 0 | 0 | 0 |
| 148 | 0 | 3 | 0 |
| 149 | 59 | 88 | 42 |
| 150 | 107 | 141 | 83 |
| 151 | 5 | 4 | 1 |
| 152 | 1 | 1 | 0 |
| 153 | 0 | 0 | 0 |
| 154 | 0 | 1 | 0 |
| 155 | 0 | 0 | 1 |
| Total | 172 | 239 | 127 |

| *BAT-25* | | | |
|---|---|---|---|
| Size (bp) | Caucasian | African | Asian |
| 113 | 1 | 0 | 0 |
| 114 | 0 | 1 | 0 |
| 115 | 0 | 12 | 0 |
| 116 | 0 | 7 | 0 |
| 117 | 0 | 1 | 0 |
| 118 | 0 | 8 | 0 |
| 119 | 0 | 14 | 0 |
| 120 | 7 | 111 | 4 |
| 121 | 108 | 103 | 93 |
| 122 | 58 | 12 | 30 |
| 123 | 8 | 5 | 0 |
| 124 | 0 | 0 | 0 |
| Total | 182 | 274 | 127 |

IHC for all four MMR proteins analyzed. The second discordant sample demonstrated instability in 3 of 10 informative markers with the 10-marker panel (two dinucleotide markers and *BAT-40*) but did not show instability at any of the markers in the MSI Multiplex System panel. Repeat analysis of the MSI testing confirmed the original results. Additionally, this sample was negative for MLH1, but the IHC could not be repeated because tissue sections were no longer available. A sub-group of the MSI-H samples from sample set C showed normal IHC for all MMR proteins tested, but exhibited high levels of MSI with the MSI Multiplex System (100% of markers were altered in all samples) and the 10-marker panel (over 50% markers altered). This data indicates that approximately 5% (4/72) of these samples would have been misclassified if only IHC analysis had been performed. All samples classified as MSI-L with the 10-marker panel were stable when analyzed with the *MSI Multiplex System* and all exhibited normal MMR protein staining with IHC. In fact, no samples were classified as MSI-L using the *MSI Multiplex System*. All of the MSS tumor/normal pairs were correctly classified. Thus, the *MSI Multiplex System* accurately distinguishes between MSI-H and MSS/MSI-L tumors.

## 4. Conclusion

This study confirms the observation that mononucleotide repeats are most sensitive and specific markers for MSI analysis [8]. Overall, the order of sensitivity we observed was mononucleotides, dinucleotides, tetranucleotides then pentanucleotides with an average of 96%, 87%, 69% and 27% of MSI-H samples showing instability, respectively. The ranking for specificity was mononucleotide (99%), pentanucleotide (99%), tetranucleotides (97%) and then dinucleotides (94%).

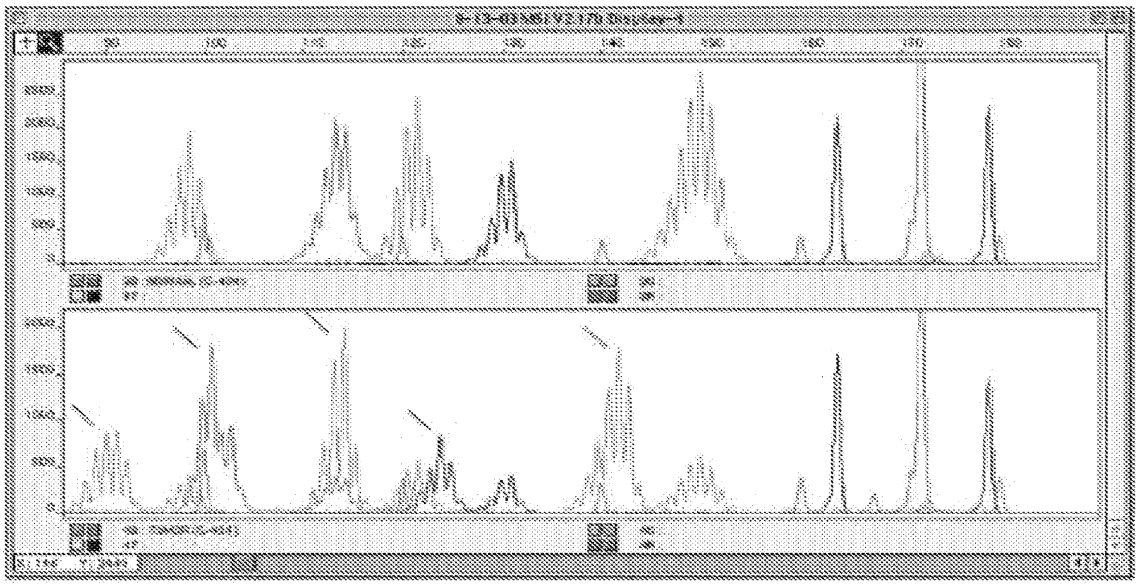


Fig. 4. MSI analysis of colorectal tumor and corresponding normal DNA with the *MSI Multiplex System*. Electropherogram showing an allelic profile generated from a normal sample (top panel) or from a matching MLH1-deficient tumor sample (bottom panel) using the *MSI Multiplex System*. New alleles present in tumor sample that are not found in matching normal sample indicate MSI (new allele peaks indicated by arrows). Notice that 100% of the mononucleotide repeats were unstable while the pentanucleotide repeats remained unchanged. The pentanucleotide repeats are not used for MSI classifications, only for the detection of sample mix-ups or contamination problems.

Markers exhibiting the lowest specificity for MSI-H tumors were mononucleotide *BAT-40*, dinucleotide markers *D2S123* and *D17S250* and tetranucleotide marker *MYCL1*. Our data also supports the conclusion by Dietmaier et al. (1997) that dinucleotides are most difficult, and that mononucleotides are the easiest type of marker with which to interpret allelic changes in MSI-H tumors [8].

The five most sensitive and specific markers for detection of MSI-H tumors were selected from a panel of 266 mono-, di-, tetra- and penta-nucleotide repeat microsatellite markers. They included mononucleotide markers *BAT-25, BAT-26, NR-21, NR-24* and *MONO-27*, which were combined into a single fluorescent multiplex assay for rapid and accurate determination of tumor MSI tumor phenotype. The sensitivity for the individual markers ranged from 92% to 98% and specificity from 99.5% to 100% (Table 1). We classified 153 colorectal tumors using the new MSI Multiplex System and compared the results to those obtained with a panel of 10 microsatellite markers and immunohistochemical (IHC) analysis. Over 97% (70/72) of MSI-H samples were correctly identified and overall there was 99% (151/153) concordance in MSI classification between the two methods. The reasons for the possible discordant cases are unclear at this time. Approximately 5% of the tumors that were classified as MSI-H using the new MSI Multiplex System exhibited no loss of any MMR proteins by IHC. This value is in close agreement with larger studies conducted at the Mayo Clinic Rochester, where 4 to 5% of MSI-H samples were found to exhibit normal IHC results for MLH1, MSH2, MSH6 and PMS2 (S. Thibodeau, unpublished data). These observations indicate MSI testing may identify MSI-H tumors that are misclassified based only on IHC. The most striking difference between the two marker panels was in classification of the MSI-L group. All of the 43 samples classified as MSI-L using the 10-marker panel (unstable at dinucleotide or *BAT-40* markers only) were scored as MSS with the *MSI Multiplex System*, indicating the high specificity of the MSI multiplex markers for instability in MSI-H tumors only.

Use of mononucleotide markers for MSI testing overcomes one of the disadvantages of the Bethesda panel, that is, the misclassification of MSI-L as MSI-H in cases where only dinucleotide markers are altered. This misclassification can occur because instability of some

of the dinucleotide markers included in the Bethesda panel is not specific to tumors with MMR deficiencies. We found that around 6% of non-MSI-H tumors with normal IHC carried a mutation in a single dinucleotide repeat. Based on our data we estimate that as much as 3% of MSI-H tumors could be misclassified using the Bethesda panel. In fact, we did find that 1 out of 30 non-MSI-high tumors (sample set A) exhibited mutations in dinucleotide markers *D2S123* and *D17S250* from the Bethesda panel. This potential problem was recognized at the 2002 NCI workshop and it was suggested that mononucleotide markers could be used instead of dinucleotide markers to increase sensitivity and specificity [46].

Suraweera and colleagues have proposed that MSI status of a tumor can be assessed without reference to matching normal DNA if a panel of five nearly monomorphic mononucleotide markers is used [43]. Any deviation of 3 or more base pairs in the tumor allele size from the commonly occurring alleles found in the larger population was considered MSI. MSI in 0 to 2 markers was scored as MSS and in 3 or more markers as MSI-H. In general, our allele frequency data for African-American populations supports the conclusion that instability in 3 out of 5 markers in MSS tumors would only occur at very low frequencies (=0.014% false positives) using the makers in our *MSI Multiplex System*. But, the problem of dismissing an actual MSI-H case by defining MSS as less or equal to 2 out of 5 markers unstable was observed. In 74 MSI-H cases, we found one MSI-H case (1.4% false negatives) where only 2 out of 5 mononucleotide makers were unstable. False negatives could be avoided, however, in the small number of cases with only two unstable markers by testing matching normal sample to determine if changes in allele sizes were due to polymorphism rather than mutations. The use of a panel of five nearly monomorphic mononucleotide markers for MSI testing of tumor samples without comparison to matching normal samples has been included into the updated NCI workshop recommendations for MSI testing [46].

Determination of MSI status has become a useful tool for screening for HNPCC and sporadic MSI-H tumors, and defines a subset of colorectal cancers with distinctive molecular and clinopathological features. This study provides strong evidence that mononucleotides are the most sensitive and specific markers for detection of MSI-H and identifies an optimal panel of markers that detects MSI-H tumors with nearly 100% accuracy. The *MSI Multiplex System* was designed to analyze small amounts of DNA isolated from paraffin-embedded tumor samples alone or as matching tumor/normal pairs. The selected panel of markers includes five nearly monomorphic mononucleotides for MSI determinations and two polymorphic pentanucleotide for detection of sample mix-ups and/or contamination problems. This configuration of markers in a single multiplex assay offers several distinct advantages over other methods of MSI analysis in that it provides a method that offers both extremely high sensitivity and specificity while still being amenable to automated high-throughput analysis using existing fluorescent capillary electrophoresis technology. The *MSI Multiplex System* meets the new recommendations proposed at the recent 2002 NCI workshop on HNPCC and MSI testing and overcomes problems inherent to the original five-marker panel. This approach should facilitate widespread screening for microsatellite instability in tumors of patients with gastrointestinal cancers.

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## History of Changes for Study: NCT01876511

## Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors

Latest version (submitted February 4, 2020) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare. One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of th
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in ~~red~~.
- Study additions are displayed in *green*.

### Study Record Versions

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 1 | ◉ | ○ | June 10, 2013 | None (earliest Version on record) |
| 2 | ○ | ○ | June 12, 2013 | Outcome Measures and Study Status |
| 3 | ○ | ◉ | September 20, 2013 | Recruitment Status, Study Status, Contacts/Locations and Oversight |
| 4 | ○ | ○ | May 21, 2014 | Study Status and Conditions |
| 5 | ○ | ○ | June 25, 2014 | Study Status, Contacts/Locations and Outcome Measures |
| 6 | ○ | ○ | September 15, 2014 | Study Status and Contacts/Locations |
| 7 | ○ | ○ | December 26, 2014 | Contacts/Locations and Study Status |
| 8 | ○ | ○ | January 29, 2015 | Study Status and Contacts/Locations |
| 9 | ○ | ○ | February 26, 2015 | Study Status and Contacts/Locations |
| 10 | ○ | ○ | April 15, 2015 | Study Status, Contacts/Locations and Eligibility |
| 11 | ○ | ○ | June 9, 2015 | Study Status, Eligibility and Study Design |
| 12 | ○ | ○ | January 4, 2016 | Study Status and Eligibility |
| 13 | ○ | ○ | June 28, 2016 | Study Status |
| 14 | ○ | ○ | November 10, 2016 | Study Status |
| 15 | ○ | ○ | May 3, 2017 | Study Status and Contacts/Locations |
| 16 | ○ | ○ | August 28, 2017 | Study Status |
| 17 | ○ | ○ | October 5, 2017 | Contacts/Locations, Arms and Interventions, Study Status, Eligibility, Outcome Measures, Conditions and Study Description |
| 18 | ○ | ○ | December 13, 2017 | Study Status and Conditions |
| 19 | ○ | ○ | February 27, 2018 | Contacts/Locations and Study Status |
| 20 | ○ | ○ | March 27, 2018 | References, Study Status, Eligibility and Conditions |
| 21 | ○ | ○ | January 8, 2019 | Study Status, Contacts/Locations and Eligibility |
| 22 | ○ | ○ | February 18, 2019 | Contacts/Locations, Study Status, Eligibility and Conditions |
| 23 | ○ | ○ | February 18, 2019 | Contacts/Locations and Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 24 | ○ | ○ | March 4, 2019 | Conditions, Study Status |
| | | | ▶ Results Submission Events | |
| 25 | ○ | ○ | December 20, 2019 | Recruitment Status, Study Status, Outcome Measures, Contacts/Locations, Arms and Interventions, Study Design, Conditions, Oversight, Stud Identification, Documents, Results, IPDSharing, Eligibility and Study Description |
| 26 | ○ | ○ | January 17, 2020 | Study Status and Reported Adverse Events |
| 27 | ○ | ○ | February 3, 2020 | Study Status |
| 28 | ○ | ○ | February 4, 2020 | Study Status |

Compare  Comparison Format: ◉ Merged ○ Side-by-Side

Scroll up to access the controls

**Changes (Merged) for Study: NCT01876511**
**June 10, 2013 (v1) -- September 20, 2013 (v3)**

**Changes in:** Study Status, Oversight, Outcome Measures **and** Contacts/Locations

☐ **Show only changed modules**

**Study Identification**

Unique Protocol ID: J1365

Brief Title: Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors

Official Title: Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors

Secondary IDs: MK-3475-016 [Merck]
NA_00085756 [JHMIRB]

**Study Status**

Record Verification: ~~June 2013~~ *September 2013*

Overall Status: ~~Not yet~~ Recruiting

Study Start: ~~July 2013~~ *September 2013*

Primary Completion: June 2017 [Anticipated]

Study Completion:

First Submitted: June 10, 2013

First Submitted that Met QC Criteria: June 10, 2013

First Posted: June 12, 2013 [Estimate]

Last Update Submitted that Met QC Criteria: ~~June 10, 2013~~ *September 20, 2013*

Last Update Posted: ~~June 12~~ *September 24*, 2013 [Estimate]

**Sponsor/Collaborators**

Sponsor: Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Responsible Party: Sponsor

Collaborators: Merck Sharp & Dohme Corp.

**Oversight**

U.S. FDA-regulated Drug:

U.S. FDA-regulated Device:

Data Monitoring: Yes

## Study Description

**Brief Summary:** This study will be looking at whether MK-3475 (an antibody that blocks negative signals to T cells) is effective (anti-tumor activity safe in three different patient populations. These include: 1. patients with MSI positive colon cancer, 2. patients with MSI negative colon cancer, and 3. patients with other MSI positive cancers.

**Detailed Description:**

## Conditions

**Conditions:** MSI Positive Colorectal Cancer
MSI Negative Colorectal Cancer
MSI Positive Non-Colorectal Cancers

**Keywords:**

## Study Design

**Study Type:** Interventional

**Primary Purpose:** Treatment

**Study Phase:** Phase 2

**Interventional Study Model:** Parallel Assignment

**Number of Arms:** 3

**Masking:** None (Open Label)

**Allocation:** Non-Randomized

**Enrollment:** 71 [Anticipated]

## Arms and Interventions

| Arms | Assigned Interventions |
|---|---|
| Experimental: MSI Positive Colorectal Cancer | Drug: MK-3475<br>MK-3475 10 mg/kg every 14 days |
| Experimental: MSI Negative Colorectal Cancer | Drug: MK-3475<br>MK-3475 10 mg/kg every 14 days |
| Experimental: MSI Positive Non-Colorectal Cancer | Drug: MK-3475<br>MK-3475 10 mg/kg every 14 days |

**Outcome Measures**

Primary Outcome Measures:

1. Immune-related progression free survival (irPFS) rate at 20 weeks in patients with MSI positive and negative colorectal adenocarcinoma using immune re response criteria (irRC)

   [Time Frame: 4 years]

2. Objective response rate (irORR) at 20 weeks in patients with MSI positive and negative colorectal adenocarcinoma using immune related response criteri (irRC)

   [Time Frame: 4 years]

3. Immune-related progression free survival (irPFS) rate in patients with MSI positive non-colorectal adenocarcinoma using immune related response criteria (irRC) at 20 weeks

   [Time Frame: 4 years]

Secondary Outcome Measures:

4. Overall survival

   [Time Frame: 4 years]

5. irPFS and PFS in patients with MSI positive and negative tumors at 28 weeks using irRC and RECIST 1.1

   [Time Frame: 4 years]

6. Best overall response rate and disease control rate in patients with MSI positive and negative tumors

   [Time Frame: 4 years]

7. Number of participants experiencing immune-related toxicities (IRAEs)

   [Time Frame: ~~Continuous~~ *4 years*]

8. Does MSI as a Marker Predict Treatment Response

   [Time Frame: 4 years]

## Eligibility

Minimum Age: 18 Years

Maximum Age:

Sex: All

Gender Based:

Accepts Healthy Volunteers: No

Criteria: Inclusion Criteria:

1. Arm 1 only: Patients with MSI positive colorectal cancer
2. Arm 2 only: Patients with MSI negative colorectal cancer
3. Arm 3 only: Patients with MSI positive non-colorectal cancer
4. Have measurable disease
5. ECOG Performance Status of 0 to 1
6. Adequate organ function as defined by study-specified laboratory tests
7. Must use acceptable form of birth control through the study and for 28 days after final dose of study drug
8. Signed informed consent form
9. Willing and able to comply with study procedures
10. Agree to have a biopsy of their cancer

Exclusion Criteria:

1. Currently have or have history of certain study-specified heart, liver, kidney, lung, neurological, immune or other medical conditions
2. Systemically active steroid use
3. Patients on home oxygen
4. Patient who have had prior treatment with anti-PD-1, anti-PD-L1, anti-PD-L2, anti-CD137, anti-OX-40, anti-CD40, or anti-C 4 antibodies
5. Another investigational product within 28 days prior to receiving study drug
6. Pregnant or lactating
7. Conditions, including alcohol or drug dependence, or intercurrent illness that would affect the patient's ability to comply witl study visits and procedures

## Contacts/Locations

Study Officials: Dung Le, MD
Principal Investigator
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Locations: **United States, Maryland**

Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
*[Recruiting]*
Baltimore, Maryland, United States, 21231
Contact: Contact: Holly Kemberling, RN 443-287-5013 hkember1@jhmi.edu
Contact: Principal Investigator: Dung Le, MD

## IPDSharing

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CANCERS OF THE COLON AND RECTUM

# Retrospective review of colorectal cancer specimens in individuals younger than age 50 for microsatellite instability testing and DNA mismatch repair enzyme expression.

N. Durie , H. Staszewski , T. Palaia , L. Ragolia , W. Nugent

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## Abstract

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**Background:** Data from studies performed at tertiary referral centers suggest that the yield of patients with the underlying defect in hereditary non-polyposis colon cancer (HNPCC) could be improved by screening every colon cancer for the phenotypic expression of the MMR defect by immunohistochemistry (IHC) and/or an assay for microsatellite instability (MSI). We propose to determine the incidence of microsatellite unstable colon cancers and the incidence of absence of expression of DNA MMR enzymes in de-identified colorectal cancer specimens from patients under the age of 50 who have presented to Winthrop University Hospital over the past ten years. This incidence will be compared with those suggested by recent large retrospective studies in tertiary care centers.

**Methods:** Immunoperoxidase staining for MLH1, MSH2, MSH6 and PMS2 were performed on formalin-fixed tissue. MSI assays were performed on microdissected DNA from paraffin-embedded tissue blocks. All cases were tested with five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D). Tumor samples in which two or more altered monoclonal repeat markers were found out of five were classified as MSI-H.

**Results**: Screening for expression of DNA mismatch repair enzymes and MSI in an enriched selected (by age <50) population of colorectal cancer patients resulted in detection of 7/51 (14%) specimens that were MSI-H and 7/61 (12%) that were “positive” for lack of expression of at least one DNA mismatch repair enzyme.

**Conclusions:** Our results are similar to those reported in the literature in unselected series of patients with colorectal cancer. IHC staining or MSI analysis alone may be insufficient in selecting those colorectal cancer patients that should be referred for genetic testing for HNPCC. However, screening of an unselected population with both these modalities should have a low but clinically

significant yield. Since other research have shown that traditional criteria such as the Bethesda guidelines are inadequate, we support the use of both measures prospectively in all colorectal cancer patients.

No significant financial relationships to disclose.

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# Checkpoint Inhibition of PD-1: The Promise of Pembrolizumab (MK-3475) and Beyond

Zeynep Eroglu, MD

Immunotherapy

The idea of harnessing a patient's own defenses and actively augmenting a deficient immune response to help fight disease has been implemented since the 18th century. Adoption of immunotherapy into clinical practice has been difficult until recently, when many late-stage clinical trials demonstrating an overall survival (OS) advantage in melanoma and prostate cancer have brought immunotherapy to the forefront. Immunotherapy is not a new concept for physicians treating malignant melanoma. The approval of interferons as adjuvant therapy, with relapse-free survival and OS advantage, and high-dose interleukin-2 (IL-2) in the metastatic setting has allowed patients to achieve durable responses. However, translation into clinical practice has been difficult due to toxicity and restricted access to therapy.

The advent of new immunotherapeutic strategies targeting immune checkpoint pathways, leading to enhanced immune response to tumors, has provided renewed promise in immunotherapy that extends beyond malignant melanoma. Success with the anti–CTLA-4 (cytotoxic T-lymphocyte antigen-4) antibody, ipilimumab, in metastatic melanoma led to approval by the FDA in 2011. This was based on 2 randomized phase 3 studies demonstrating improved survival in the ipilimumab treatment arms. In a phase 3 trial comparing ipilimumab to the gp100 vaccine in previously treated advanced melanoma, an overall response rate (ORR) of 10.9% and a median OS of 10.1 months was observed with the ipilimumab arm, with 46.6% of patients alive at 12 months.[1] This benefit

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has led to extended survival at 10 years.[2] As the field of immune checkpoint inhibitor therapy has grown, a number of other antibodies that block inhibitory pathways or stimulate agonist pathways have appeared on the horizon. Initial trials have shown promising results of the ability to overcome tumor defenses (Table 1).

**Table 1** Checkpoint Inhibitors in Clinical Trials

| Name | Class | Efficacy in Advanced Melanoma | Efficacy in Advanced NSCLC |
|---|---|---|---|
| Ipilimumab | Anti-CTLA-4 | ORR 10%, median OS approximately 10 months (n=4846) | ORR of 32%, median OS 12.2 months (n=68, phased regimen) |
| Tremelimumab | Anti-CTLA-4 | ORR 10.7%, median OS 12.6 months (n=328) | Ongoing phase 1 in combination with MEDI4736 (NCT02000947); with gefitinib (NCT02040064) |
| Pembrolizumab | Anti-PD-1 | ORR 41% (n=116) | ORR 21%, median OS 11.7 months (n=33) |
| Nivolumab | Anti-PD-1 | ORR 31%, median OS 16.8 months (n=107) | ORR of 17%, median OS 9.9 months (n=129) |
| Pidilizumab (CT-011) | Anti-PD-1 | Ongoing phase 2 trial as single agent (NCT01435369) | |
| MPDL3280A | Anti-PD-L1 | ORR of 28% (n=43) | ORR of 23% (n=53) |
| BMS-936559 | Anti-PD-L1 | ORR of 17% (n=52) | ORR of 10% (n=49) |
| MEDI4736 | Anti-PD-L1 | Ongoing phase 1 trial combination with trametinib ± dabrafenib (NCT02027961) | ORR of 50% (n=6) |
| MSB0010718C | Anti-PD-L1 | Ongoing phase 1 trials in solid tumors (NCT01772004, NCT01943461) | |
| Lirilumab (BMS-986015) | Anti-KIR antibody | Ongoing phase 1 trials in combination with nivolumab (NCT01714739) or ipilimumab (NCT01750580) in solid tumors | |
| BMS-986016 | Anti-LAG3 antibody | Ongoing phase 1 trial in combination with nivolumab in solid tumors (NCT01968109) | |
| MGA271 | Anti-B7-H3 antibody | Ongoing phase 1 trial in solid tumors (NCT01391143) | |

CTLA-4 indicates cytotoxic T-lymphocyte antigen-4; KIR, killer cell immunoglobulin-like receptor; LAG3, lymphocyte activation gene; NSCLC, non–small cell lung cancer; ORR, overall response rate; OS, overall survival; PD-1, programmed death–1; PD-L1, programmed death-1 ligand 1.

Pembrolizumab is a humanized monoclonal antibody against the immune checkpoint receptor programmed death—1 (PD-1). PD-1 and its ligands, PD-L1 and PD-L2, inhibit T-cell response and consequently suppress antitumor immunity. Recognition of a tumor by the T cell through major histocompatibility complex/antigen interaction mediates PD-L1/2 upregulation on the tumor, which enables cancer cells to evade T-cell–mediated death through immune suppression. While PD-1 expression is induced when a T cell is activated, it is also induced on other non–T lymphocytes including B cells and natural killer cells.3 PD-L1 expression has been shown on a number of solid tumors, including melanoma, lung, colon, breast, and other malignancies.[4-7] PD-1 expression is also upregulated in tumor-infiltrating lymphocytes (TILs) in many tumor types, which may contribute to tumor immunosuppression.

PD-L1 expression correlates with unfavorable prognosis in several cancers.[4,8] In a study of melanoma patients, Breslow tumor thickness in the high-expression PD-L1 group was significantly higher than in the low-expression group, and the OS rate of the high-expression group was significantly lower than that of the low-expression group. In all patients with stage IV disease examined as part of the study, tumor-infiltrating T cells expressed high levels of PD-1, and this

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expression was elevated further during the clinical course.[9] PD-1 blockade enhances the activity of T cells in the tumor microenvironment, restoring antitumor T-cell activity, and may also increase natural killer cell activity in tumors and antibody production of PD-1–positive B cells.[3] In addition, chronic antigen exposure seen in cancer can lead to persistent PD-1 expression, inducing anergy in antigen-specific T cells, which may also be reversible by PD-1 blockade.[3] Preclinical studies of cancer in mouse models have demonstrated enhanced antitumor immunity through antibody blockade of PD-1 or its ligands.[6,10] Preclinically, pembrolizumab has shown antitumor activity in multiple tumor types and has high affinity for the PD-1 receptor, strongly inhibiting binding of both PD-L1 (IC50 ? 0.1-0.3 nM) and PD-L2 (IC50 ? 0.5-0.9 nM).[11]

## Clinical Experience

The first phase 1 trial of pembrolizumab was conducted to determine its safety, pharmacokinetics, and response rates in several advanced cancers refractory to prior therapies.[12] Doses of 1, 3, and 10 mg/kg were given on the first day of a 28-day cycle and then continued with every 2-week dosing. Enrolled patients included 3 with non–small cell lung cancer (NSCLC), 2 each with melanoma and rectal cancer, and 1 each with sarcoma and carcinoid. No dose-limiting toxicities were observed in the 3 dose levels. One drug-related grade 2 adverse event (AE) of pruritus was reported. No drug-related grade ?3 AEs were observed. Using RECIST 1.1 response criteria with every 8-week imaging, 1 patient with melanoma had a partial response, and tumor size reduction was seen in 3 other patients.[12]

A phase 1 expansion study was performed in 135 advanced melanoma patients with an ECOG score of 0 or 1 who had received ?2 prior systemic therapies if they were ipilimumab naive, with no restriction on lines of therapy if they were ipilimumab pretreated.[11,13] Patients with active and untreated central nervous system metastases were excluded. Pembrolizumab was administered in a 10-mg/kg dose every 2 or 3 weeks, or 2 mg/kg every 3 weeks, with response assessments performed every 12 weeks via RECIST criteria. Most frequent treatment-related AEs included fatigue (37%), pruritus (26%), rash (22%), and diarrhea (21%). Two patients each had grade 3/4 treatment-related AEs (aspartate transaminase increase, fatigue, rash, and renal failure). Most treatment-related AEs were successfully managed with treatment discontinuation, supportive care, and occasionally, glucocorticoids. Potentially immune-related AEs are discussed further below.

In an update of the results presented, an ORR of 41% was observed across all doses, with a 51% rate seen in the 10-mg/kg-every-2-week cohort along with a 14% complete response (CR) rate in this group.[11] Overall, a reduction in tumor size was seen in 74% of evaluable patients. Patients with a confirmed CR who had been on therapy for at least 6 months were able to discontinue therapy after receiving at least 2 doses beyond initial determination of CR, with the ability to resume therapy in the case of progression. Overall, a 9% CR rate and a 41% response rate were seen among all patients treated. In contrast to earlier checkpoint inhibitor therapies, evidence of early

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tumor size reduction was seen that continued throughout therapy. Late responses were seen as far as 48 weeks from initial therapy, with response rates for all cohorts improving over time.

Similar to previous trials with immune checkpoint inhibitor therapy, patients had significant disease burden, with 77% of patients having M1c disease (visceral, brain, high lactate dehydrogenase), and 36% having received prior ipilimumab. Median time to CR was 32 weeks. Subgroup evaluation of the ipilimumab-naive and ipilimumab-pretreated cohorts showed equal benefit and response. This mimics the benefits seen in the initial ipilimumab trials, as patients pretreated with IL-2 have shown no detriment in chance of clinical response. Additionally, an 18% response rate was seen in patients with previously treated, stable brain metastases. While an 81% OS rate was observed at 12 months, median OS was not yet reached for any dosing cohort. The 81% 1-year survival, although preliminary, compares favorably with the historical 25% and 46% 1-year survivals quoted for ipilimumab. It is hoped that this high percentage of response and its durability will ultimately translate into survival benefit.

Anti–PD-1 therapy holds the promise of improved benefits for more patients in the field of immune checkpoint blockade. Initial reported response rates are nearly triple those previously observed in ipilimumab clinical trials prior to its approval in metastatic melanoma. An international phase 3 study of pembrolizumab compared with ipilimumab as first- or second-line therapy in advanced melanoma is ongoing (NCT01866319). In this study, 645 patients are randomized 1:1:1 into 1 of 2 dosing cohorts of pembrolizumab 10 mg/kg every 2 or 3 weeks or ipilimumab 3 mg/kg. Coprimary end points include progression-free survival (PFS) or OS, with ORR as a secondary end point.

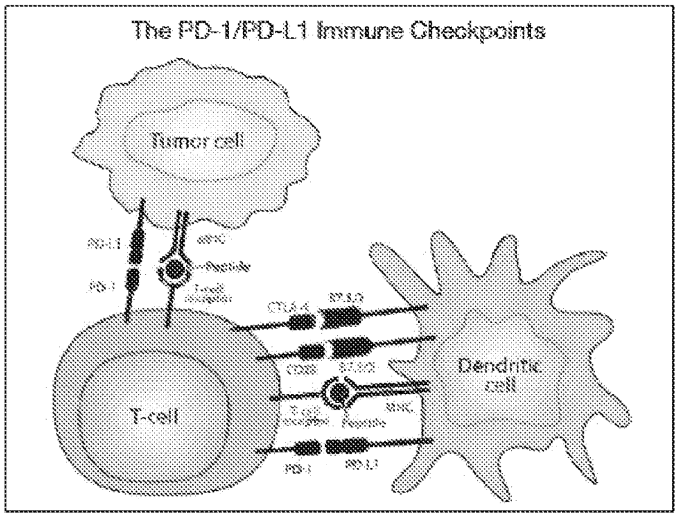
Other anti–PD-1 antibodies are also currently in clinical trials in advanced melanoma, with response rates similar to or slightly lower than those observed with pembrolizumab. One such anti–PD-1 antibody is nivolumab, which also has high affinity for PD-1 and blocks binding to both PD-L1 and PD-L2. In a phase 1 study, nivolumab was administered every 2 weeks and showed an acceptable safety profile.[14] In a cohort expansion for 107 heavily pretreated patients with advanced melanoma, ORR was 31%, with a median PFS of 3.7 months.[15] Median duration of response was 24 months, and median OS was 16.8 months. The 1-year survival rate was 62%.

Nivolumab was combined with a multipeptide vaccine in another phase 1 trial in patients with advanced melanoma.[16] In both ipilimumab-refractory and -naive patients, a RECIST response rate of 25% was seen, while the median duration of response was not yet reached at a median follow-up of 8.2 months. Using immunohistochemistry (IHC) staining for PD-L1, the ORR was 67% (8 of 12 patients) in the 5% membranous staining positive group, while ORR was 19% (6 of 32 patients) for those with negative staining.

MPDL3280A is an antibody that targets PD-L1 and prevents binding to PD-1 receptors. In an expansion of a phase 1 study, metastatic melanoma patients received MPDL3280A every 3 weeks

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for up to 1 year.[17] In 43 evaluable patients, an ORR of 28% by RECIST was observed, with a 24-week PFS of 41%. Patients with tumor samples that were IHC 2 and IHC 3 had ?5% tumor-infiltrating immune cells positive for PD-L1, whereas IHC 0 and IHC 1 had <5% positive cells for PD-L1. Although ORRs were similar, tumor sample analysis showed that PD-L1–positive patients had a higher rate of disease control (CR + progressive disease + stable disease; 16 of 20 patients, 80%) versus PD-L1–negative patients (5 of 19, 60%). Serial biopsy examinations revealed enhanced immune cell infiltrates, PD-L1 induction, and increased markers of T helper cell type 1 activation (including granzymes, interferon gamma [IFN-?], and TNF-?) in the tumors of responding patients relative to baseline. Grade 3/4 treatment-related AEs included elevated AST/alanine amminotrans ferase, increased amylase, fatigue, increased gamma-glutamyl transferase, increased bilirubin, increased lipase, and decreased lymphocytes. There was no grade 3-5 pneumonitis or colitis observed.




**Pembrolizumab in NSCLC**

The expression rate of PD-L1 in NSCLC has also been associated with OS. Patients with either an adenocarcinoma subtype or less than a 3-year survival after surgery showed a higher expression rate of PD-L1.[18] In a phase 1b trial, pembrolizumab was administered at 10 mg/kg every 3 weeks to patients with NSCLC previously treated with 2 systemic regimens.[19] Imaging was done every 9 weeks to check for response. PD-L1 expression on the pretreatment tumor sample was determined by IHC. Thirty-eight patients were enrolled, 16% with squamous histology. Ten percent had previously treated, stable brain metastases. The median OS was 51 weeks. Using RECIST criteria, the ORR was 21%.

Higher levels of PD-L1 expression appeared to be associated with increased clinical activity. An ORR of 57% (4 of 7) by RECIST was seen in patients whose tumors had high levels of PD-L1

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results of an additional cohort of 44 NSCLC patients with nonsquamous histology and PD-L1 expression >0 demonstrated similar response rates, with an ORR of 27%. The most common AEs were rash (21%), pruritus (18%), fatigue (16%), and diarrhea (13%). Only 1 patient had a drug-related grade 3/4 AE, grade 3 pulmonary edema. A phase 2/3 trial is now ongoing comparing pembrolizumab at 2 dose levels versus docetaxel in NSCLC patients who have received at least 1 prior treatment regimen (NCT01905657). There is also a phase 1 trial in patients with NSCLC of pembrolizumab in combination with cisplatin/pemetrexed or carboplatin/paclitaxel (NCT01840579), and a phase 1b trial in PD-L1–positive NSCLC (NCT02007070) with a primary end point of ORR.

Ipilimumab and anti–PD-1/PD-L1 antibodies have also been studied in NSCLC. A randomized phase 2 trial of paclitaxel plus carboplatin alone versus with ipilimumab on 2 different schedules (phased or concurrent) was conducted in stage IIIB/IV NSCLC. The trial included 204 chemotherapy-naive patients with advanced NSCLC. The median OS was 12.2 months for the phased schedule, 9.7 months for the concurrent schedule, and 8.3 months for the chemotherapy only group, with ORR by World Health Organization criteria of 32%, 21%, and 14%, respectively.[20]

An expansion of a phase 1 trial with nivolumab included heavily pretreated NSCLC patients.[14] In an update of the trial results of the 129 patients with NSCLC evaluable for response, objective responses were seen in 17% of patients by RECIST, including both squamous and nonsquamous histology. Median duration of response was 17 months, with a median OS of 9.6 months and a 1-year survival rate of 42%. Patients without tumor expression of PD-L1 had no documented tumor responses, but 36% of patients with PD-L1 expression were objective responders.

A phase 1 trial of an anti PD-L1 monoclonal antibody (BMS-936559) also included a dose expansion cohort of patients with advanced NSCLC.[21] Objective response was seen in 5 of 49 NSCLC patients evaluable for response. Tumor response was seen in both squamous (1 of 13 patients, 8%) and nonsquamous (4 of 36 patients, 11%) histologies. Another PD-L1 antibody, MPDL3280A, was tested in NSCLC patients in a phase 1 expansion study.[22] Grade 3/4 AEs regardless of attribution were 34%, with no grade 3-5 pneumonitis seen. In 53 NSCLC patients, an ORR of 23% (12 of 53) was demonstrated. The objective response rate was 46% in patients with intermediate PD-L1 expression (IHC 2 and IHC 3) and 83% (5 of 6) in patients with the highest level of PD-L1 expression (IHC 3).

### Toxicity

The toxicity spectrum of lambrolizumab and other PD-1/PD-L1 antibodies differs significantly from other immunotherapies. Similar to other checkpoint inhibitors, treatment with pembrolizumab is associated with immune-related AEs (irAEs). Surprisingly, the spectrum and severity of these irAEs differ from toxicity seen with anti–CTLA-4 therapy. Multiple irAEs, particularly pneumonitis, have

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been associated with PD-1 blockade. In the phase 1 expansion trial of pembrolizumab in advanced melanoma, 7 patients (5%) had grade 1/2 pneumonitis, and 1 patient had grade 2 interstitial lung disease. The incidence of drug-related AEs appears to be lower compared with ipilimumab, with 10% to 15% of patients experiencing grade 3/4 AEs,[1] including 1 patient with grade 3 hyperthyroidism, 2 patients with grade 3/4 transaminase elevations, 2 patients with grade 3 renal insufficiency, and 1 patient with grade 3/4 colitis.[11] In an updated analysis of the nivolumab phase 1 trial, 12 of 306 patients (4%) had pneumonitis, including 4 patients (1%) with grade 3/4 disease,[23] and 3 patients died of pneumonitis. In the nivolumab and ipilimumab phase 1 trial, there was a 5% (4 of 86 patients) incidence of pneumonitis, including 1 patient (1%) with grade 3/4 severity.[24] Nivolumab in combination with a multipeptide vaccine showed a 3% (3 of 90) incidence of pneumonitis, with 2 patients having grade 3/4 severity.[16] This has led to increased precaution for early detection and management of pneumonitis in clinical trial patients, with dose delays and discontinuation if needed. The severity of pneumonitis appears to be less with anti–PD-L1 antibodies; with BMS-936559, only 3 of 284 patients (1%) experienced this AE, and none were grade 3/4. All AEs can be successfully managed with early identification and intervention.

### Biomarkers

Given the role of PD-L1 in immune inhibition within the tumor microenvironment, PD-L1 expression patterns were reviewed in an attempt to identify its role as a predictive marker. Preclinical data suggest that expression patterns of PD-1 ligands may be crucial for determining the suitability of therapeutic blockade of this pathway.[3] Several clinical trials with anti–PD-1 and anti–PD-L1 antibodies have attempted to determine whether tumor PD-L1 expression is correlated with improved prognosis and likelihood of benefit from PD-1–targeted therapy. Initial data presented indicated no benefit in patients (0 of 17 patients) with solid tumors without PD-L1 expression.[14,19] Recent trials show that patients with negative staining for PD-L1 have response to treatment,[16] with a proportion of patients achieving responses despite negative PD-L1 expression by IHC. This response rate in PD-L1–negative tumors is higher than that seen with previous melanoma therapies. This finding points out the need to improve our understanding of PD-L1 expression as a marker of response. Different trials have used different methods for PD-L1 staining or cutoff points for PD-L1 expression positivity. Tumor expression of PD-L1 may also be dynamic, depending upon changes in the tumor microenvironment. In an evaluation of 150 melanoma tumor samples, tumor PD-L1 expression was highly concordant with immune infiltrates and the presence of IFN-? in the tumor microenvironment.[25] Also, as soluble cytokines such as IFN-? upregulate PD-L1 expression, fresh tumor samples may have better predictive value than the archived tumor biopsy tissues that are frequently used in clinical trials.[26] The utility of PD-L1 staining as a predictive biomarker will need to be further explored in large randomized studies. Current phase 3 trials with pembrolizumab are evaluating PD-L1 expression in tumor tissue from metastatic sites with prospective randomization based on expression.

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In combination therapies with anti–PD-1 and anti–CTLA-4 antibodies, TILs were evaluated to elucidate the role of other coinhibitory ligand receptors that could suppress the tumor microenvironment.[27] Interestingly, TIL and peripheral blood lymphocyte CTLA-4 expression increased in response to nivolumab treatment,[28] and PD-1 expression was induced in T cells after CTLA-4 blockade. This finding indicates a probable benefit of combination therapy or sequential therapy with immune checkpoint inhibition. When nivolumab was administered with ipilimumab in a concurrent or sequenced regimen, a 53% ORR was seen.[24] IHC testing was used to measure PD-L1 expression of tumor samples, with a sample defined as positive if at least 5% of tumor cells expressed PD-L1 staining. With the concurrent regimen, objective responses were observed in patients with either PD-L1–positive tumor samples (6 of 13 patients) or PD-L1–negative tumor samples (9 of 22 patients). In the sequenced regimen cohorts, a higher number of overall responses were seen among patients with PD-L1–positive tumor samples (4 of 8 patients) than among patients with PD-L1–negative tumor samples (1 of 13 patients).

### Upcoming Studies

While most of the current clinical trials have been focused on advanced melanoma and NSCLC patients, pembrolizumab is also undergoing further testing in other solid tumors (**Table 2**). In high-risk renal cell carcinoma (RCC) patients, higher levels of immune cells expressing PD-1 have been observed.[29] An expansion cohort of 34 patients investigated nivolumab in RCC and demonstrated an ORR of 29%, with a median PFS of 7.3 months. A median OS of more than 22 months was observed along with a 1-year survival rate of 70%.[23] A phase 1 trial of pembrolizumab in combination with pazopanib in RCC patients has recently opened (NCT02014636).

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Table 1 Ongoing Clinical Trials With Pembrolizumab

| Identifier | Disease | N | Estimated Completion | Study Description | Objectives |
|---|---|---|---|---|---|
| NCT01866319 | Unresectable stage III or metastatic melanoma, with 0-1 prior systemic therapies | 645 | 2015 | Randomized phase 3: 3 arms, 2 dosing schedules of pembrolizumab vs ipilimumab | 1. PFS, OS<br>2. ORR |
| NCT01704287 | Unresectable stage III or metastatic melanoma, refractory to ipilimumab | 510 | 2015 | Randomized phase 2: 2 dosing schedules of pembrolizumab vs investigator's choice of chemo | 1. PFS, OS<br>2. ORR, response duration |
| NCT01840579 | NSCLC (stage IIIB/IV), first line | 30 | 2015 | Phase 1b: cisplatin/pemetrexed or carboplatin/paclitaxel | 1. Safety/toxicity |
| NCT02007070 | NSCLC (stage IIIB/IV), PD-L1+, refractory to platinum-containing doublet | 24 | 2016 | Phase 1b | 1. ORR, safety, toxicity<br>2. PFS, OS, response duration |
| NCT01905657 | NSCLC (stage IIIB/IV), PD-L1+, refractory to platinum-containing doublet | 920 | 2015 | Randomized phase 2/3: docetaxel vs 2 dosing schedules of pembrolizumab | 1. PFS, OS, safety, toxicity<br>2. ORR, response duration |
| NCT02014636 | Locally advanced or metastatic RCC, no prior systemic therapy | 228 | 2020 | Phase 1/2<br>Phase 1: MTD of pazopanib + pembrolizumab<br>Randomized phase 2: 3 arms, pembrolizumab and pazopanib alone and in combination | (For phase 2)<br>1. PFS<br>2. ORR, OS, time to response, toxicity, PK parameters |
| NCT01876511 | MSI-positive CRC, MSI-negative CRC, MSI-positive non-CRC | 71 | 2017 | Nonrandomized phase 2 in 3 cohorts | 1. PFS, ORR<br>2. OS, safety, toxicity, PFS, ORR, whether MSI predicts treatment response |
| NCT01848834 | Advanced triple-negative breast cancer, head and neck cancer, urothelial cancer, or gastric cancer, any number of prior treatments | 114 | 2014 | Phase 1b in 4 cohorts | 1. Safety, toxicity, ORR<br>2. Change from baseline in cytokines, ORR for HPV+ head/neck cancer patients |
| NCT01953692 | Refractory MDS, high-risk smoldering multiple myeloma, relapsed/refractory HL or NHL | 106 | 2015 | Phase 1b in 4 cohorts | 1 Safety, toxicity, ORR |

CRC indicates colorectal cancer; HL, Hodgkin lymphoma; HPV, human papilloma virus; MDS, myelodysplastic syndrome; MSI, microsatellite instability; MTD, maximum tolerated dose; NHL, non-Hodgkin lymphoma; NSCLC, non–small cell lung cancer; ORR, overall response rate; OS, overall survival; PD-L1, programmed death–1 ligand 1; PFS, progression-free survival; PK, pharmacokinetic; RCC, renal cell carcinoma.

An evaluation of tissue samples from colorectal cancer (CRC) patients revealed that PD-L1 and PD-L2 status may be a predictor of prognosis for these patients.[30] In the nivolumab phase 1 trial, no responses were seen in CRC or castrate-resistant prostate cancer patients. Pembrolizumab is also undergoing evaluation in a phase 2 trial with 3 arms including microsatellite instability (MSI)-positive colon cancer, MSI-negative colon cancer, and patients with other MSI-positive cancers (NCT01876511). PD-L1 immunodetection was shown to correlate to tumor size, invasion, lymph node metastasis, and survival time of patients with gastric cancer, as enhanced PD-L1 immunolabeling was seen when the tumor infiltrated into the deep muscular layers.[31] Patients with esophageal PD-L1–positive cancer were also found to have a significantly poorer prognosis than PD-L1–negative patients.[32] An ongoing phase 1b trial is examining pembrolizumab in 4 cohorts of advanced gastric, head and neck, urothelial, and triple-negative breast cancer patients (NCT01848834).

PD-1 and PD-L1 are also expressed in multiple myeloma cells, some primary T-cell leukemias and lymphomas, and Hodgkin lymphoma.[33-35] There is an ongoing phase 1b trial of pembrolizumab in several hematologic malignancies (NCT01953692). Other anti–PD-1 and PD-L1 drugs are also

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pidilizumab in combination with rituximab was tested in patients with relapsed follicular lymphoma. No autoimmune or treatment-related grade 3/4 AEs were seen. Of the 29 evaluable patients, 19 (66%) had objective responses, including a CR in 15 (52%), and measurable tumor regression occurred in 86% of patients.[36]

These ongoing trials with pembrolizumab will further help define its role in advanced melanoma, including whether it is appropriate for use in the first-line setting or in ipilimumab-refractory patients; there will also be trials of pembrolizumab in combination with other immunotherapeutics, including ipilimumab and other immune checkpoint inhibitors, to determine if improved response rates can be achieved while maintaining acceptable toxicity. The recent approval of dual targeted therapy (BRAF + MEK inhibition) in melanoma has led to a proposed triple combination trial with pembrolizumab. Results of ongoing studies of pembrolizumab in NSCLC may also help to determine not only where it fits into the current armamentarium of therapeutics available for these patients but also further delineate the role of PD-L1 expression as a biomarker. Improved understanding of predictive and prognostic markers will increase the efficacy of PD-1 antibody therapy with pembrolizumab.

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Cancer Epidemiology, Biomarkers & Prevention

*Short Communication*

# Programmed Cell Death 1 (PD-1) and Its Ligand (PD-L1) in Common Cancers and Their Correlation with Molecular Cancer Type

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## Abstract

Cancer cells expressing PD-1 ligands (PD-L1/PD-L2) inhibit immune-modulatory T-cell activation facilitating disease progression. Preliminary clinical trials exploring interruption of PD-1/PD-L1 signaling showed benefit in several cancer types. We analyzed the distribution of PD-1–positive tumor-infiltrating lymphocytes (TIL) and cancer cells' expression of PD-L1 in a molecularly profiled cohort of 437 malignancies (380 carcinomas, 33 sarcomas, and 24 melanomas). We showed that the presence of PD-1$^+$ TILs significantly varied among cancer types (from 0% in extraskeletal myxoid chondrosarcomas to 93% in ovarian cancer), and was generally associated with the increased number of mutations in tumor cells ($P = 0.029$). Cancer cell expression of PD-L1 varied from absent (in Merkel cell carcinomas) to 100% (in chondro- and liposarcomas), but showed the inverse association with the number of detected mutations ($P = 0.004$). Both PD-1 and PD-L1 expression were significantly higher in triple-negative breast cancers (TNBC) than in non-TNBC ($P < 0.001$ and 0.017, respectively). Similarly, MSI-H colon cancers had higher PD-1 and PD-L1 expression than the microsatellite stable tumors ($P = 0.002$ and 0.02, respectively). *TP53*-mutated breast cancers had significantly higher PD-1 positivity than those harboring other driver mutations (e.g., *PIK3CA*; $P = 0.002$). In non–small cell lung cancer, PD-1/PD-L1 coexpression was identified in 8 cases (19%), which lacked any other targetable alterations (e.g., *EGFR*, *ALK*, or *ROS1*). Our study demonstrated the utility of exploring the expression of two potentially targetable immune checkpoint proteins (PD-1/PD-L1) in a substantial proportion of solid tumors, including some aggressive subtypes that lack other targeted treatment modalities. *Cancer Epidemiol Biomarkers Prev; 23(12); 2965–70. ©2014 AACR.*

## Introduction

Programmed death-1 (PD-1, CD279) is an immune-suppressive molecule that is upregulated on activated T cells and other immune cells. It is activated by binding to its ligand PD-L1 (B7-H1, CD274), which results in intracellular responses that reduce T-cell activation. Aberrant PD-L1 expression had been observed on cancer cells, leading to the development of PD-1/PD-L1–directed cancer therapies, which have shown promising results in late-phase clinical trials. Blockade of the PD-1 and PD-L1 interaction led to good clinical responses in several, but not all cancer types, and the heterogeneous cellular expression of PD-1/PD-L1 may underlie these selective responses (1–6).

PD-1/PD-L1 expression has been studied by various methods in different cancer subtypes (7). Most of the published articles focused on prognostic relevance of PD-1/PD-L1, whereas little is known about their predictive value as well as their relationship with molecular genetic alterations in solid tumors (1). In the present study, we analyzed the distribution of PD-1$^+$ tumor-infiltrating lymphocytes (TIL) and PD-L1 expression in the most common solid cancers and further correlated these biomarkers with genotypic and phenotypic characteristics of tumors.

## Materials and Methods

### Tumor samples

The study cohort consisted of 437 tumor samples (both primary and metastatic) representing both major and some rare solid cancer types: 380 carcinomas [breast, colon, lung, pancreas, prostate, Merkel cell, ovary, liver,

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endometrial, bladder, kidney, and cancers of unknown primary (CUP)], 33 soft tissue sarcomas (liposarcomas, chondrosarcomas, extraskeletal myxoid chondrosarcomas, and uterine sarcomas) and 24 malignant cutaneous melanomas.

### Molecular methods

Tumor samples were evaluated using a commercial multiplatform approach consisting of protein analysis (immunohistochemistry), gene copy-number analysis (in situ hybridization) and gene sequencing (Next-Generation Sequencing; Caris Molecular Intelligence, Caris Life Sciences) as previously described (8).

The presence of PD-1$^+$ lymphocytes was evaluated with monoclonal antibody NAT105 (Cell Marque), whereas the expression of PD-L1 was analyzed with B7-H1 antibody (R&D Systems), using automated immunohistochemical methods.

Because of the biopsy size–related dependence on the detection of PD-1 TILs (9, 10), we evaluated their density using a hotspot approach, analogous to the previously described method for measuring neoangiogenesis (11). The whole tumor sample was reviewed at a low power (4× objective) and the area of highest density of TILs in direct contact with malignant cells of the tumor at 400× visual field (40× objective × 10× ocular) was enumerated (number of PD-1$^+$ TIL/HPF). The intensity of the cancer cells' expression of PD-L1 was recorded on a semiquantitative scale (0–3+): 0 for no staining, 1+ for weak cytoplasmic staining, 2+ moderate membranous and cytoplasmic staining, and 3+ strong membranous and cytoplasmic staining. Percentage of tumor cells expressing PD-L1 at the highest intensity was recorded.

### Statistical methods

The two-tailed Fisher exact test and $\chi^2$ test were applied for the correlation between the variables ($P \leq 0.05$).

## Results

### PD-1 and PD-L1 expression in solid tumors

PD-1 and PD-L1 expression in various solid tumors and their subtypes are summarized in Tables 1–4.

PD-1$^+$ lymphocytes were consistently identified in reactive, peritumoral lymphoid follicles, which served as an internal positive control. PD-1$^+$ TILs in direct contact with cancer cells were distinctly uncommon in some cancer types (e.g., 0% in extraskeletal myxoid chondrosarcoma), whereas triple-negative breast cancer (TNBC), bladder cancer, microsatellite instability high (MSI-H) colon cancer, non–small cell lung cancer (NSCLC), endometrial, and ovarian cancer were frequently (70%–100%) infiltrated with PD-1$^+$ TILs. When present, PD-1$^+$ TILs' density varied from 1 to >20/hpf (Table 1).

PD-L1 was consistently expressed in the tumor microenvironment, including endothelial cells, macrophages, and dendritic cells, at strong (2+/3+) intensity and was used as internal positive control. In contrast, the cancer cells expressed PD-L1 at widely varying levels and proportions. Consistent, strong membranous staining was a feature of only a few, specific cancer types, including endometrial carcinomas (Fig. 1) and malignant melanomas (88% and 92%, respectively), metaplastic breast carcinomas, chondrosarcomas and liposarcomas (both 100%; Tables 1 and 4).

Simultaneous expression of PD-L1 in tumor cells and presence of PD-1$^+$ TILs was frequently observed in kidney cancer (33%), ovarian cancer (36%), NSCLC (43%), TNBC

Table 1. Overview of PD-1 and PD-L1 expression in various types of solid tumors

| Tumor types ($n$ = 437 total) | PD-1 expression (% and range) | PD-L1 (tumor cells; %) | Concurrent PD-1 and PD-L1 expression (%) |
|---|---|---|---|
| Carcinomas ($n$ = 380 total) | | | |
| Breast ($n$ = 116) | 51% (1–20) | 45% | 29% |
| Colon ($n$ = 87) | 50% (1–>20) | 21% | 12% |
| NSCLC ($n$ = 44) | 75% (1–20) | 50% | 43% |
| Pancreas ($n$ = 23) | 43% (1–16) | 23% | 9% |
| Prostate ($n$ = 20) | 35% (1–6) | 25% | 5% |
| Merkel cell carcinoma ($n$ = 19) | 17% (1–4) | 0% | 0% |
| Endometrium ($n$ = 16) | 86% (1–13) | 88% | 79% |
| Ovary ($n$ = 14) | 93% (1–16) | 43% | 36% |
| Liver ($n$ = 13) | 38% (1–5) | 8% | 0% |
| Bladder ($n$ = 11) | 73% (1–10) | 55% | 55% |
| Kidney ($n$ = 11) | 36% (1–3) | 67% | 33% |
| CUP ($n$ = 6) | 50% (1–4) | 33% | 33% |
| Sarcomas ($n$ = 33 total) | 30% (1–>10) | 97% | 30% |
| Melanoma ($n$ = 24 total) | 58% (1–15) | 92% | 58% |



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Table 2. PD-1 and PD-L1 expression in breast cancers, according to the molecular subtype

| Breast cancer subtypes ($n$ = 116) | PD-1 expression/hpf (TILs; % and range) | PD-L1 (tumor cells; %) | Concurrent PD-1 and PD-L1 expression (%) |
|---|---|---|---|
| Luminal tumors ($n$ = 58) | | | |
| Luminal A ($n$ = 33) | 25% (1–>10) | 33% | 13% |
| Luminal B ($n$ = 25) | 44% (1–20) | 33% | 17% |
| HER2 positive ($n$ = 5) | 60% (1–9) | 20% | 20% |
| Triple-negative ($n$ = 53) | 70% (1–20)[a] | 59%[a] | 45%[a] |

Abbreviation: hpf, high-power fields.
[a]Significantly higher than in luminal tumors.

(45%), dedifferentiated liposarcomas (50%), bladder cancer (55%), malignant melanomas (58%), endometrial cancer (79%), but was infrequent in other cancer types [e.g., 0% in liver cancer and Merkel cell carcinoma, 4% microsatellite-stable (MSS) colon cancer, 5% prostate cancer, 8% liver cancer, 9% pancreatic cancer, and 13% in luminal A breast cancer (Table 1)].

### Association of PD-1 and PD-L1 expression with genotypic and phenotypic characteristics of the tumors

In the entire study set, expression of PD-1$^+$ TILs was associated with an increasing number of mutations in tumor cells ($P$ = 0.029, Fisher exact test), whereas PD-L1 status showed the opposite association ($P$ = 0.004, Fisher exact test). Consequently, copresence of PD-1$^+$ TILs and cancer cells expressing PD-L1 showed no association with overall mutational status ($P$ = 0.67, Fisher exact test).

In breast cancer PD-1$^+$ TILs were significantly more common in TNBC than in luminal-type tumors (70% vs. 25%–44%, $P$ < 0.001, $\chi^2$ test; Table 2). Similarly, PD-L1 expression was the highest in TNBC as compared with other subtypes (59% vs. 33% in luminal tumors, $P$ = 0.017). Among TNBC, 9 cases were metaplastic breast carcinomas and all were positive for PD-L1. Consequently, coexpression of PD-1$^+$TIL/cancer cells PD-L1$^+$ was the highest in the TNBC subgroup (45% vs. 13%–17% non-TNBC, $P$ = 0.001, $\chi^2$ test). Similarly, *TP53*-mutated breast cancers exhibited significantly higher PD-1 TIL positivity compared with breast cancers that harbored other mutations (e.g., *PIK3CA* mutations) or breast tumors without mutations (42% vs. 10%, $P$ = 0.002, $\chi^2$ test). In contrast, PD-L1$^+$ did not correlate with any of the detected mutations in breast cancer.

In the colon cancer cohort, MSI-H tumors exhibited a significantly higher rate of positivity for PD-1$^+$ TILs than MSS colon cancers (77% vs. 39%, $P$ = 0.002, Fisher exact test; Table 3). Also, the proportion of PD-L1$^+$ cancers was significantly higher in MSI-H than in the MSS colon cancers (38% vs. 13%, $P$ = 0.02, Fisher exact test). Of note, MSI-H cases were predominantly stage I and II (75%), whereas the majority of the MSS cases were at advanced stage (III and IV, 93%; $P$ < 0.001). Both PD-1 and PD-L1 positivity significantly decreased with the tumor stage in colorectal cancer ($P$ = 0.021 and 0.031, respectively).

In NSCLC, PD-1$^+$ TILs and PD-L1–expressing tumor cells were seen in 18 of 42 cases (43%) of which 8 cases lacked other biologic targets (such as activating *EGFR* mutations, *HER2*, *cMET*, *ALK*, or *ROS1* rearrangements).

## Discussion

Recent clinical trials have demonstrated that blocking of the PD-1/PD-L1 pathway induces an objective and durable remission in patients with advanced solid tumors (2–6). The efficacy of these agents has been primarily linked to the expression of PD-L1 in the tumor cells and PD-1 on activated T lymphocytes (12–14). Expression of both markers has already been explored in several human malignancies, particularly in renal cell carcinomas, malignant melanoma, and NSCLC (13–15). Our PD-L1 results for these three cancer types are comparable with the data provided by Taube and colleagues (13). Consistent with a

Table 3. PD-1 and PD-L1 expression in colorectal carcinomas in relationship to the microsatellite instability status

| Colon cancer subtypes ($n$ = 87) | PD-1 expression/hpf (TILs; % and range) | PD-L1 (tumor cells; %) | Concurrent PD-1/ PD-L1 expression (%) |
|---|---|---|---|
| MSS colon cancers ($n$ = 60) | 39% (1–11) | 13% | 4% |
| MSI-H colon cancers ($n$ = 27) | 77% (1–>20)[a] | 38%[a] | 32%[a] |

Abbreviation: hpf, high-power fields.
[a]Significantly higher.



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Table 4. Overview of PD-1 and PD-L1 expression in sarcoma subtypes

| Sarcoma subtypes ($n$ = 33) | PD-1 expression/hpf (TILs; % and range) | PD-L1 (tumor cells; %) | Concurrent PD-1 and PD-L1 expression (%) |
|---|---|---|---|
| Liposarcoma ($n$ = 20) | 45% (1–>10) | 100% | 45% |
| Chondrosarcoma ($n$ = 8) | 12% (0–1) | 100% | 12% |
| Extraskeletal myxoid chondrosarcoma ($n$ = 3) | 0% | 67% | 0% |
| Uterine sarcoma ($n$ = 2) | 0% | 100% | 0% |

previous report by Vanderstraeten and colleagues (16), endometrial cancer appears to be abundantly enriched with both PD-1 and PD-L1.

The broad array of tumors screened for this study also allowed the assessment of PD-1/PD-L1 expression in several less common cancer types. Our study revealed a low expression of both PD-1 and PD-L1 in several highly aggressive tumors, including Merkel cell carcinoma, hepatocellular, and pancreatic carcinoma. In contrast, PD-L1 expression was particularly high in dedifferentiated liposarcomas, which is in line with a recent report by Kim and colleagues (17). We also report for the first time the PD-L1 positivity in chondrosarcomas and extraskeletal myxoid chondrosarcomas. Furthermore, PD-1 and PD-L1 positivity was observed in cancers of unknown primary, a group of cancers with particularly difficult treatment decisions.

Marked variations in PD-1/PD-L1 positivity have also been observed within general histologic types, but subtype analysis revealed significant correlations. For example, PD-1/PD-L1 were differently expressed in molecular subtypes of breast (TNBC vs. non-TNBC)

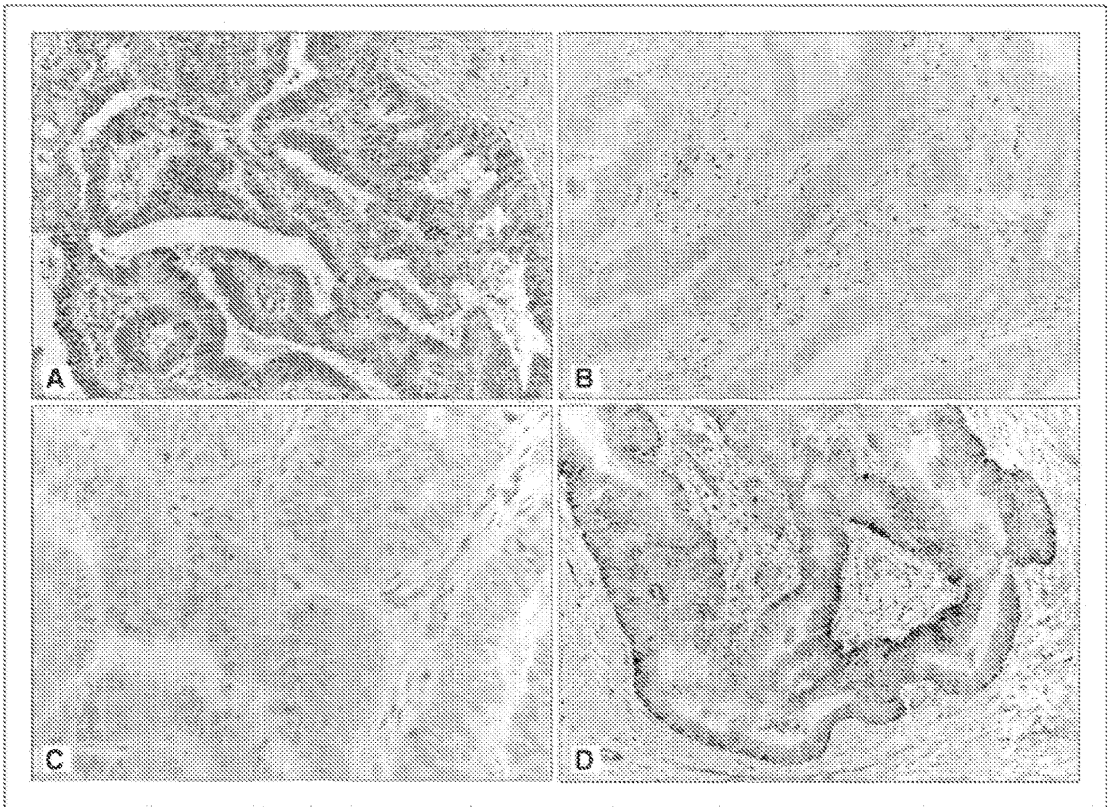



Figure 1. A case of endometrial adenocarcinoma (A, hematoxylin and eosin stained section) exhibiting microsatellite instability caused by the loss of MLH-1 protein (note retained MLH-1 protein expression in the nuclei of the tumor-infiltrating lymphocytes); B, immunohistochemical stain; PD-1$^+$ TILs (C, immunohistochemical stain); and aberrant expression of PD-L1 in the tumor cells' basolateral membranes (D, immunohistochemical stain).



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and colon cancer (MSI-H vs. MSS cases) providing an indication for potential benefit of targeted immunotherapy in aggressive subtypes of breast and colon cancers for which no targeted therapy is currently available. We found PD-L1 expression to be the highest in TNBC (59%) in contrast with a recent study that reported the highest frequency (34%) in HER2-positive breast cancers (18). However, our cohort was enriched (8%) for rare metaplastic TNBC, which were all PD-L1 positive, whereas we analyzed only 5 HER2-positive breast cases. Of note, *TP53*-mutated breast carcinomas exhibited significantly higher PD-1 expression in comparison with breast carcinomas harboring other types of mutations. High PD-1$^+$ TILs had been recently associated with a more aggressive phenotype and poorer outcome in operable breast cancers (19).

Some immunogenic tumors (e.g., MSI-H CRC) attract TILs, which produce IFNγ and could upregulate PD-L1 on tumor epithelial cells. Modulation of the IFNγ receptor (IFNγRα) expression on the tumor epithelial cells may play a critical role in tumor immunoediting (20), including acquisition of stem cell–like phenotype (21) and resistance to granule-mediated cytotoxic T-lymphocyte killing (22).

Our data for colon cancer also appear to differ from those reported by Droeser and colleagues who reported more frequent expression of PD-L1 in the MSS than in MSI-H colon cancers (23). The discrepancy may be caused by the fact that tested MSI-H and MSS cases differed significantly in regard to the tumor stage as the majority of MSI-H was at stage I and II, whereas MSS tumors were predominantly stage III and IV. Overall, the expression of both PD-1 and PD-L1 in colon cancer inversely correlated with the tumor stage.

Another relevant finding in our study is that a substantial proportion of NSCLCs with PD-1/PD-L1 positivity were devoid of the most common and targetable alterations (e.g., *EGFR, HER2, cMET, ALK, ROS1*). In contrast with previous studies, we did not find any association between PD-1/PD-L1 expression and *EGFR* alterations in lung cancer (24, 25).

In summary, our survey demonstrated expression of two potentially targetable immune checkpoint proteins (PD-1/PD-L1) in a substantial proportion of solid tumors including some aggressive subtypes that lack targeted treatment modalities.

## Disclosure of Potential Conflicts of Interest

T. Maney has ownership interest (including patents) in the stocks of Caris Life Sciences. S. Vranic is a consultant/advisory board member for Caris Life Sciences. D.D. Von Hoff has ownership interest (including patents) in and is a consultant/advisory board member for Caris Life Sciences. O. Hamid is a consultant/advisory board member for Caris Life Sciences. Z. Gatalica, A. Ghazalpour, D. Holterman, N. Xiao, T. Maney, I. Rose, P. Overberg, and G. Basu are employees of Caris Life Sciences.

## Authors' Contributions

**Conception and design:** Z. Gatalica, C. Snyder, D.A. Holterman, D.D. Von Hoff

**Development of methodology:** Z. Gatalica, C. Snyder, D.A. Holterman, O. Hamid

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Z. Gatalica, C. Snyder, I. Rose, H.T. Lynch, O. Hamid

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Z. Gatalica, A. Ghazalpour, D.A. Holterman, N. Xiao, S. Vranic, O. Hamid

**Writing, review, and/or revision of the manuscript:** Z. Gatalica, C. Snyder, T. Maney, A. Ghazalpour, G.D. Basu, S. Vranic, H.T. Lynch, D.D. Von Hoff, O. Hamid

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** P. Overberg, I. Rose, S. Vranic

**Study supervision:** Z. Gatalica, T. Maney, O. Hamid

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Cancer Epidemiology, Biomarkers & Prevention

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# Programmed Cell Death 1 (PD-1) and Its Ligand (PD-L1) in Common Cancers and Their Correlation with Molecular Cancer Type

Zoran Gatalica, Carrie Snyder, Todd Maney, et al.

*Cancer Epidemiol Biomarkers Prev* 2014;23:2965-2970. Published OnlineFirst November 12, 2014.

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DEVELOPMENTAL THERAPEUTICS—IMMUNOTHERAPY

# Phase 2 study of programmed death-1 antibody (anti-PD-1, MK-3475) in patients with microsatellite unstable (MSI) tumors.

Dung T. Le , Nilofer Saba Azad , Dan Laheru , Ilene S. Browner , Hao Wang , Jennifer N. Uram...

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individual tumors and the response to immune checkpoint inhibitors since each protein-coding mutation has the potential to serve as a novel antigen for an immune response. This may explain why lung cancers and melanomas, which harbor hundreds of somatic tumors, respond so well to these agents. Microsatellite unstable (MSI) tumors are deficient in DNA mismatch repair which leads to hundreds to thousands of spontaneous mutations. Furthermore, similar to melanoma, MSI positive colon cancers, there is often prominent lymphocyte infiltration. PD-1 is upregulated on activated T cells and provides inhibitory signals to T cells undergoing activation. MK-3475 is a humanized monoclonal IgG4 antibody against PD-1 and is showing activity in multiple tumor types. This study aims to establish the treatment benefit and evaluate MSI as a predictive marker of response to anti-PD-1 blockade. **Methods:** This is an open-label, 2-stage, phase 2 study to evaluate the clinical activity of MK-3475 in 3 cohorts of patients: MSI positive colorectal cancer

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include disease control rate, progression-free survival, overall survival and safety. Each MSI positive and MSI negative CRC cohort will evaluate up to 25 patients, and the cohort with MSI positive solid tumors but not CRC will enroll up to 21 patients. Results from exome sequencing and PD-L1 expression using IHC on tumor tissue will be correlated with response. Clinical trial information: NCT01876511.

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Clinical Cancer Research

# Durable Cancer Regression Off-Treatment and Effective Reinduction Therapy with an Anti-PD-1 Antibody

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## Abstract

**Purpose:** Results from the first-in-human phase I trial of the anti–programmed death-1 (PD-1) antibody BMS-936558 in patients with treatment-refractory solid tumors, including safety, tolerability, pharmacodynamics, and immunologic correlates, have been previously reported. Here, we provide long-term follow-up on three patients from that trial who sustained objective tumor regressions off therapy, and test the hypothesis that reinduction therapy for late tumor recurrence can be effective.

**Experimental Design:** Three patients with colorectal cancer, renal cell cancer, and melanoma achieved objective responses on an intermittent dosing regimen of BMS-936558. Following cessation of therapy, patients were followed for more than 3 years. A patient with melanoma who experienced a prolonged partial regression followed by tumor recurrence received reinduction therapy.

**Results:** A patient with colorectal cancer experienced a complete response, which is ongoing after 3 years. A patient with renal cell cancer experienced a partial response lasting 3 years off therapy, which converted to a complete response, which is ongoing at 12 months. A patient with melanoma achieved a partial response that was stable for 16 months off therapy; recurrent disease was successfully treated with reinduction anti-PD-1 therapy.

**Conclusion:** These data represent the most prolonged observation to date of patients with solid tumors responding to anti-PD-1 immunotherapy and the first report of successful reinduction therapy following delayed tumor progression. They underscore the potential for immune checkpoint blockade with anti-PD-1 to reset the equilibrium between tumor and the host immune system. *Clin Cancer Res; 19(2); 462–8. ©2012 AACR.*

## Introduction

The specific or selective expression of antigens by cancer cells creates an opportunity for endogenous cell-mediated and serologic immune attack and for immunotherapeutic interventions. The development of effective cancer immunotherapies depends on reversing inhibitory and tolerogenic signals in the tumor microenvironment, thereby enhancing the visibility of tumor cells to the immune system. In the last decade, it has become clear that the immune regulatory pathway composed of programmed death-1 (PD-1, CD279), a receptor expressed on activated T and B cells and its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), plays an integral role in the down-modulation of antitumor immunity. Inhibition of this pathway using blocking monoclonal antibodies (mAb) against PD-1 or PD-L1 is emerging as an effective method for reversing cancer immunosuppression and thereby causing tumor regression in patients with advanced disease (1). Although such therapies are still in development, they have shown promising clinical results not only for tumors classically considered to be immunogenic such as melanoma and renal cell carcinoma (RCC), but also for common epithelial malignancies such as non–small cell lung cancer (NSCLC), historically resistant to immunotherapy.

BMS-936558 (MDX-1106/ONO-4538) is a blocking mAb specific for human PD-1. The first-in-human single-dose, dose-escalation trial of this agent reported by Brahmer and colleagues (2) enrolled 39 patients with treatment-refractory metastatic solid tumors including melanoma, RCC, colorectal cancer (CRC), NSCLC, and castration-resistant prostate cancer (CRPC). A favorable safety profile and preliminary evidence of clinical activity in all histologies except CRPC led to the design of a multidose trial of

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**Translational Relevance**

This report provides long-term follow-up on patients from the first-in-human trial of the anti-PD-1 antibody BMS-936558 (MDX-1106) who achieved objective tumor regressions. They include patients with metastatic melanoma, colorectal, and renal cancer. Remarkably, durable responses including continued tumor regression off therapy were observed. Furthermore, a patient with melanoma, who experienced tumor recurrence following a prolonged partial response, responded to reinduction with anti-PD-1 therapy. These findings of durable treatment effect underscore the potential for immune-based therapies such as antibodies blocking the immunosuppressive PD-1/PD-L1 pathway to reset the equilibrium between tumor and the host immune system, distinguishing them from conventional chemotherapies as well as small-molecule inhibitors of oncogenic driver pathways. They represent the most prolonged observation of patients with solid tumors responding to anti-PD-1 immunotherapy to date, and the first evidence that reinduction therapy with this agent after late tumor recurrence may be effective.

BMS-936558 in 296 patients that was recently reported (3), confirming antitumor efficacy in melanoma, RCC, and NSCLC.

The adaptive immune system has a vast capacity for specific antigen recognition and holds the potential to adjust to ongoing tumor evolution, including genetic and epigenetic changes that typically cause resistance to small-molecule inhibitors. This adaptability, coupled with an immune memory component, implies that immunotherapy can "reorient" endogenous antitumor immunity with durable effects even after the cessation of therapy. Here, we provide long-term follow-up on patients from the first-in-human trial of BMS-936558 who achieved objective tumor regressions, including a patient with CRC who maintained a complete response (CR) off therapy; a patient with RCC who sustained a partial response (PR) that converted to a CR off therapy; and a patient with melanoma who experienced a prolonged PR off therapy followed by tumor progression and then response to reinduction therapy with the same anti-PD-1 antibody. These data represent the most prolonged observation of patients with solid tumors responding to anti-PD-1 therapy and the first evidence that reinduction therapy with this agent after late tumor progression may be effective. The observed durability of responses underscores the potential for immune checkpoint blockade with anti-PD-1 to reset the equilibrium between tumor and host and provide durable tumor control after cessation of therapy.

## Materials and Methods

### Treatments administered and response evaluation

BMS-936558, a fully human immunoglobulin G4 (IgG4) blocking mAb against PD-1, was administered in a multi-institutional, first-in-human, phase I dose-escalation study (2). The protocol was approved by local Institutional Review Boards (IRB), and all participating subjects signed informed consent. The antibody was given as a single intravenous infusion at 0.3 to 10 mg/kg per dose, followed by radiologic restaging at 8 and 12 weeks. Responses were evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) 1.0. Patients who tolerated the drug well (no adverse events $\geq$ grade 3 and no development of human antihuman antibodies) and showed stable disease or any evidence of tumor regression were eligible for retreatment at weeks 12 and 16. Patients who achieved an objective response (PR or CR) discontinued dosing and were observed with periodic radiologic restaging.

A patient with melanoma who experienced a prolonged PR followed by tumor progression received reinduction therapy with BMS-936558 10 mg/kg on days 1, 29, and 86, repeated every 120 days, on a single patient use clinical trial following IRB approval and informed consent. In contrast to the original dosing schema, treatment on this trial may continue until the patient develops a confirmed CR, confirmed progressive disease, or unacceptable toxicity.

### Tumor biopsies and immunohistochemistry

Histologic slides were reviewed to confirm the cancer diagnosis, and one representative formalin fixed, paraffin embedded (FFPE) block of tumor was chosen for immunohistochemical (IHC) analysis to characterize the tumor and its microenvironment. PD-L1 immunostaining was conducted with the mAb 5H1 as previously described (4). The method for PD-1 immunostaining parallels that reported for PD-L1 with minor modifications. The murine antihuman PD-1 mAb, clone M3, was used at a concentration of 1.0 μg/mL. Citrate buffer (pH 6.0) was used for antigen retrieval, and a CSA System (DAKO) was used for signal amplification, followed by development with diaminobenzidine chromagen that results in brown staining. In the heavily pigmented melanoma, a Giemsa counterstain was used for the PD-1 antibody. This results in green coloration of the melanin pigment and facilitates interpretation of the brown signal (5). CD3, CD4, CD8, CD68, and T-cell intracytoplasmic antigen-1 (TIA-1) immunostains were conducted according to routine automated methods.

## Results

Among 39 patients with treatment–refractory solid tumors receiving BMS-936558 on an intermittent dosing regimen (2), 3 objective tumor regressions were observed. Following are the clinical and immunologic assessments of these patients during prolonged observation.

### Durable complete response in colorectal cancer

A 71-year-old male with CRC underwent a right hemicolectomy in October 2003, revealing a moderately differentiated adenocarcinoma with metastases to 4 of 16 pericolonic lymph nodes and vascular and perineural invasion [$G_2$, pT3N2; microsatellite instability (MSI)-high genotype]. He received adjuvant 5-fluorouracil (5-FU) and



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leucovorin; however, a CT scan the following year revealed metastatic disease. Over the subsequent 3 years, the patient received multiple chemotherapeutic regimens with temporary response but then progression at multiple lymph node sites (gastrohepatic, portacaval, and peripancreatic); therapies included FOLFOX, irinotecan, bevacizumab, and cetuximab. Chemotherapy was last administered in April 2007. The patient began therapy with anti-PD-1 at 3 mg/kg per dose in July 2007 after documentation of disease progression, and received 5 doses over the next 9 months. CT scans conducted 8 and 12 weeks after a single dose of anti-PD-1 showed a partial response (Fig. 1A). A CR was achieved in January 2008, and periodic CT and PET scans have revealed no evidence of recurrence since then. The patient was most recently evaluated in April 2011, at which time he had not received any antineoplastic therapy for 3 years and had no evidence of disease recurrence.

IHC studies of the primary colon tumor, conducted on FFPE tissues archived 4 years before the initiation of anti-PD-1 therapy, revealed cell surface ("membranous") expression of PD-L1 by infiltrating macrophages and lymphocytes and by rare tumor cells, associated with infiltrating PD-1+ and CD3+ T cells (Fig. 1B). We have previously reported preliminary evidence for a correlation between tumor cell membranous PD-L1 expression and the likelihood of response to anti-PD-1 therapy (2). Potential relationships between PD-L1 expression on nontumor cells and clinical outcomes require further study (4).

### Evolution of complete tumor regression off therapy in renal cell carcinoma

A 76-year-old male underwent a right nephrectomy and vena caval thrombectomy in 2001, showing clear cell renal carcinoma. He developed bilateral pulmonary metastases in 2003 and received systemic therapies including the histone deacetylase inhibitor mocetinostat, sorafenib, and sunitinib, experiencing disease progression with new metastatic sites in mediastinal lymph nodes, bone, and paraspinal musculature. In January 2008, he began treatment with anti-PD-1 at 10 mg/kg per dose. CT scans conducted in March 2008, 8 weeks after a single dose of anti-PD-1, showed a mixed response: pulmonary, lymph node, and intramuscular metastases were regressing, but lesions were enlarging in the pancreas and bone. The patient also developed hypothyroidism, which was assessed as possibly treatment related. He received 2 more doses of anti-PD-1 in April

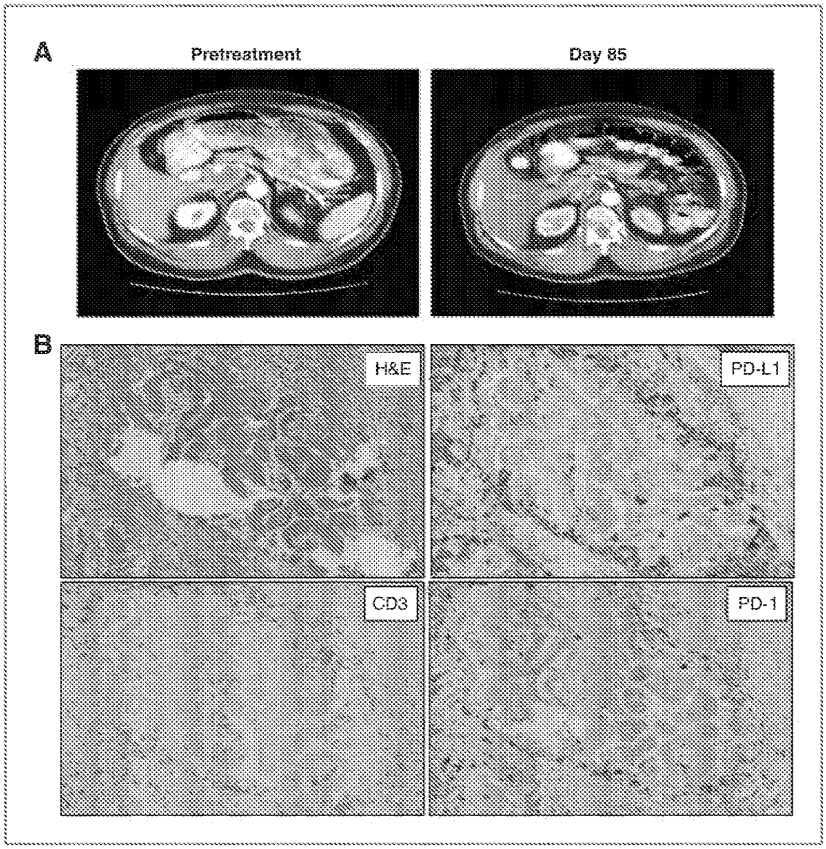



Figure 1. Response of metastatic colorectal cancer to anti-PD-1 therapy. A, computed tomographic scan showing partial regression of a representative lymph node metastasis in a patient with CRC, after receiving a single dose of anti-PD-1. B, hematoxylin and eosin (H&E) staining and IHC analysis of primary colon tumor from this patient, showing PD-L1+ lymphohistiocytic infiltrates surrounding tumor cells, and rare PD-L1+ tumor cells, interposed with CD3+ and PD-1+ infiltrating T cells. Arrow denotes tumor cells. Original magnification, ×200.



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and May 2008; a CT scan in July 2008 showed a PR compared with baseline tumor measurements, including disappearance of the pancreatic lesion (Fig. 2A); the bone metastasis slowly resolved (2). PR status was maintained off therapy until February 2009, when a restaging brain CT and subsequent brain MRI showed a new enhancing 1.4-cm lesion in the inferior right frontal lobe, with intralesional hemorrhage and surrounding edema, consistent with metastasis (Fig. 2B). Resection of this asymptomatic mass revealed fibrovascular tissue with macrophages and chronic

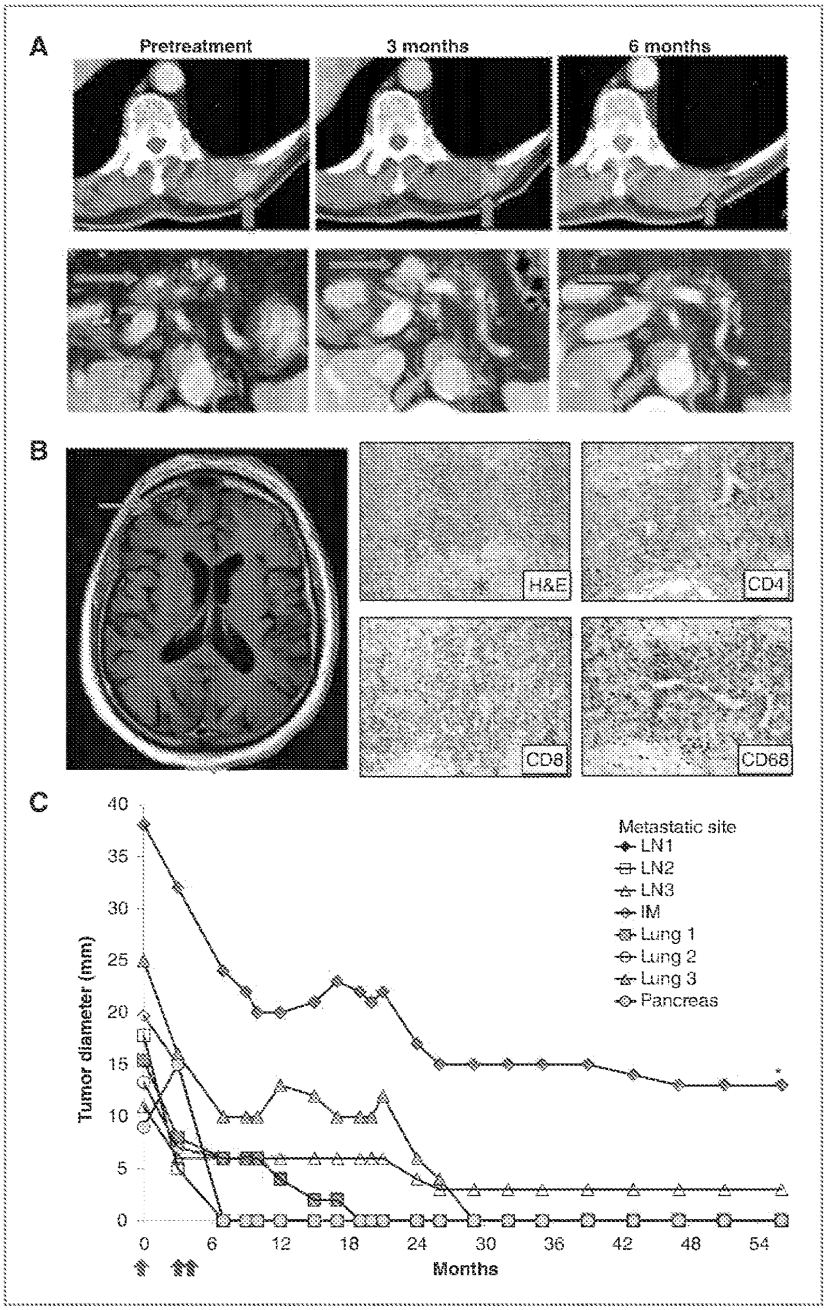



Figure 2. Regression of metastatic RCC following anti-PD-1 therapy, with "immune-related" response characteristics. A, contrast-enhanced CT scans showing regression of a biopsy-proven paraspinal intramuscular metastasis (top row, arrows) and concomitant progression followed by regression of a pancreatic lesion (bottom row, arrows) in a patient who received 3 doses of anti-PD-1. B, newly appearing lesion radiologically compatible with brain metastasis, as shown on T1-weighted contrast-enhanced magnetic resonance imaging, with intralesional hemorrhage and surrounding edema (left). Pathologic evaluation of the resected lesion on H&E staining revealed fibrosis (asterisk), a chronic inflammatory cell infiltrate, and old hemorrhage consistent with a resolving process. There was no morphologic or immunohistochemical evidence of RCC (no expression of PAX-8, EMA, or AE1/AE3; data not shown). Immunostaining to characterize the inflammatory infiltrate shows a lymphocytic infiltrate with CD8 predominance when compared with CD4. The CD8 cells were also TIA-1 positive, supporting a cytotoxic phenotype (not shown). Staining for CD68 highlighted numerous hemosiderin-laden macrophages in the inflammatory infiltrate. Original magnification, ×200. C, continued regression of multiple metastatic sites over time, following cessation of anti-PD-1 therapy. Drug administration is indicated by arrows. The lesion marked with an asterisk became calcified on CT scan and showed no FDG activity on PET scan. LN, lymph node; IM, intramuscular.



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inflammation but no evidence of tumor; this was consistent with a resolving lesion of undetermined origin. No further antineoplastic therapy was administered and metastatic lesions continued to regress off therapy (Fig. 2C). A CR was documented with CT and PET scans in August 2011. This patient remains in remission as of radiologic evaluation in August 2012, more than 4 years after discontinuation of anti-PD-1 therapy.

**Reinduction therapy for recurrent melanoma**

A 55-year-old female was diagnosed with metastatic melanoma following biopsy of a left axillary mass in 2006; the primary site of origin was undetermined. Further workup revealed liver metastases. Disease progression occurred despite therapy with high-dose interleukin-2 and temozolomide. Eight weeks after receiving one dose of anti-PD-1 at 10 mg/kg in October 2007, CT scans revealed a mixed picture: lesions in the liver and left axilla were stable or regressing, whereas a left subpectoral lymph node had enlarged (Fig. 3A). Two more doses of anti-PD-1 were administered in January and February 2008. CT scans conducted in April and May 2008 again showed a mixed response with interval development of central necrosis in some lesions. The patient continued to receive anti-PD-1 therapy through June 2009, and a PR compared with baseline measurements was documented in August 2009. Per protocol, treatment was discontinued. A stable PR persisted until December 2010, when a restaging PET/CT scan revealed newly enlarged, FDG-avid precarinal and subcarinal lymph nodes. A transbronchial biopsy of the subcarinal lesion confirmed a diagnosis of melanoma (Fig. 3B). Similar to prior IHC studies of an axillary lymph

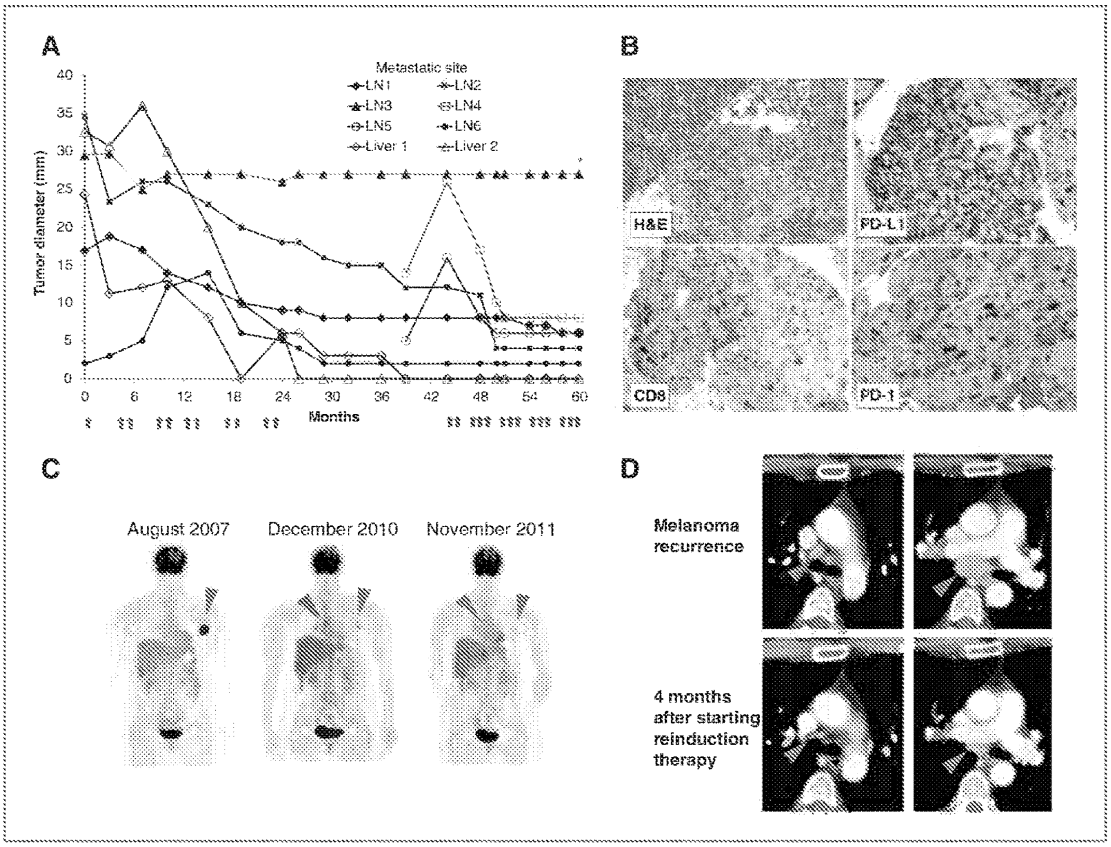



Figure 3. Response of metastatic melanoma to reinduction anti-PD-1 therapy. A, initial partial regression of liver and lymph node (LN) metastases in a melanoma patient treated with anti-PD-1. Drug administration is indicated by arrows. Recurrent disease in mediastinal lymph nodes while off therapy was successfully treated with reinduction therapy. The lesion marked with an asterisk developed peripheral calcification and showed minimal PET activity. B, membranous (cell surface) PD-L1 expression by metastatic melanoma cells in a transbronchial biopsy of a subcarinal lymph node mass, before reinduction therapy. Tumor infiltration by CD8+ and PD-1+ T cells is evident (arrows). Original magnification, ×200. C, PET scans before first treatment with anti-PD-1 (August 2007), before reinduction therapy (December 2010), and following reinduction (November 2011). Resolution of a left axillary lymph node metastasis that was present before anti-PD-1 therapy, and response of a new subcarinal lymph node metastasis to reinduction therapy, are shown (arrows). D, regression of new mediastinal lymph node metastasis following reinduction therapy with anti-PD-1, shown in contrast-enhanced CT scans.



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node metastasis in this patient (2), there was intense membranous tumor cell expression of PD-L1 and infiltration by CD8+ and PD-1+ T cells. A patient-specific protocol providing for reinduction therapy with BMS-936558 was approved by the Johns Hopkins IRB (Baltimore, MD), and treatment was reinitiated in May 2011. After the patient received 2 doses of anti-PD-1, a PET/CT scan in November 2011 showed near complete resolution of the abnormal metabolic activity in the subcarinal lymph node and no new lesions (Fig. 3C). CT scan showed a 40% tumor regression of the new mediastinal adenopathy compared with reinduction baseline measurements (Fig. 3D). The patient continues on anti-PD-1 at the time of this report, with a sustained PR documented 16 months following initiation of reinduction therapy.

## Discussion

The successful application of PD-1/PD-L1 pathway blockade in treating a diversity of solid tumors underscores the power of immunotherapy in combating not only cancers traditionally considered to be immunogenic, such as melanoma and RCC, but also epithelial malignancies such as NSCLC and CRC which are commonly viewed as refractory to immunotherapy. The activity of agents blocking this pathway is currently being explored in multiple new indications in oncology, including ovarian, breast, gastric, and pancreatic cancer. Preclinical evidence suggests that continuous antigen exposure of cytolytic T cells may induce a tolerant or "exhausted" state in which T-cell effector functions and transition to memory T cells are impaired. PD-1 pathway blockade may restore the functions of exhausted T cells with long-term clinical benefit generating powerful memory T cells that may provide an ongoing antitumor dynamic and keep tumors in check for months to years, even in the absence of continued therapy (6). This hypothesis is consistent with the results of the current study and is highlighted by the RCC case, in which partially responsive metastatic deposits continued to regress off therapy and eventuated in a durable CR. The new brain lesion resected from this patient in the interim, while not independently diagnostic, was consistent with a healing lesion such as might occur at a site of tumor regression. Similar pathologic features, including tumor necrosis in the presence of complex immune cell infiltrates, have been described in post-treatment tumor biopsies from patients receiving anti-CTLA-4 immunotherapy (7). These findings suggest the potential of PD-1 pathway blockade to reset the immune equilibrium between tumor and host (8). It follows that tumor recurrence after a response may reflect a disturbance in this equilibrium, as illustrated by the melanoma patient described herein. Repeat application of PD-1 blockade in this patient, whose recurrent tumor strongly expressed PD-L1 and contained PD-1+ infiltrating lymphocytes, reinduced a rapid partial tumor regression. Unlike tumors exposed to tyrosine kinase inhibitors that may rapidly acquire resistance and exhibit progression during continued drug exposure, due to the emergence of new genetic or epigenetic alterations (9), tumor progression in this patient occurred only after therapy was discontinued. These findings suggest a potential role for continued administration of anti-PD-1 on an intermittent "maintenance" schedule after achieving an objective tumor regression to preserve an immune equilibrium and protect the host from tumor regrowth.

The findings described herein also show the importance of establishing new immune-related RECIST (iRECIST) criteria to evaluate patients undergoing therapy with agents blocking immune checkpoints such as anti-PD-1. These agents are not directly tumoricidal but work indirectly by activating antitumor immunity and thus may be associated with delayed response kinetics (10). As exemplified by the patients with melanoma and RCC reported here, metastatic lesions may appear anew or enlarge before showing evidence of regression (11). "Mixed" responses characterized by the simultaneous growth of some lesions and regression of others have also been observed. These unconventional response patterns may provide survival benefits, which remain to be determined in future randomized trials of PD-1 blockade. Indeed, these principles are illustrated by randomized trials of immune checkpoint blockade with ipilimumab (anti-CTLA-4) in patients with advanced melanoma, where overall survival rates exceeded objective response rates (CR+PR; refs. 12, 13), suggesting the possibility of a new paradigm for evaluating clinical benefit that does not rely solely on traditional RECIST response criteria. Development of iRECIST criteria in combination with biomarkers that reliably predict clinical response will be vital as an increasing number of immunotherapies become commercially available (14).

Since the publication of the first-in-human phase I trial of BMS-936558 using an intermittent dosing regimen (2), this drug has been tested in a follow-up phase I trial with cohort expansion, using biweekly administration to patients with various treatment-refractory solid malignancies (3). Objective responses (PR or CR) were observed in 18% of patients with NSCLC, 28% with melanoma, and 27% with RCC. Grade 3 to 4 treatment-related adverse events occurred in 14% of patients, and there were 3 deaths from pulmonary toxicity. Similar to observations from the first-in-human trial, responses were durable: among 31 responding patients with 1 year or more of follow-up, 20 responses lasted 1 year or more. Data from this and trials of other agents blocking the PD-1 pathway (15–17) should inform future studies involving combinatorial immune-based approaches, which have shown preclinical evidence of synergistic antitumor activity (18, 19).

The colocalization of tumor-infiltrating T cells and PD-L1+ tumor and stromal cells seen in biopsies from the patients with CRC and melanoma reported here represents a more general phenomenon, and is consistent with observations suggesting that IFN-γ secretion by intratumoral lymphohistiocytic infiltrates fosters an immunosuppressive microenvironment by promoting PD-L1 expression ("adaptive immune resistance"; ref. 4). PD-1 pathway blockade may be particularly effective in these



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cases, as illustrated by the recent report from Topalian and colleagues showing preliminary evidence for a correlation between tumor cell surface expression of PD-L1 in pretreatment tumor specimens and objective response to anti-PD-1 therapy (3). PD-L1 expression as a predictive tumor marker of response to reinduction therapy with PD-1 pathway blockade, as shown in the patient with melanoma described in this report, remains to be explored in larger studies.

## Disclosure of Potential Conflicts of Interest

W.H. Sharfman has honoraria from speakers' bureau from Prometheus and is a consultant/advisory board member of Merck and Genentech. C.G. Drake is a consultant/advisory board member of Bristol Myers Squibb, Amplimmune Inc., and Dendreon Inc. R.A. Anders has a commercial research grant from BMS. S. Yao is employed (other than primary affiliation; e.g., consulting) in Amplimmune, Inc. as a scientist. D.M. Pardoll is a consultant/advisory board member of BMS. J.R. Brahmer is consultant/advisory board member of Bristol-Myers Squibb. S.L. Topalian has a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member of Bristol-Myers Squibb and Amplimmune Inc. S.L. Topalian also has royalties via institution from Bristol-Myers Squibb and Amplimmune Inc. No potential conflicts of interest were disclosed by the other authors.

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**Writing, review, and/or revision of the manuscript:** E.J. Lipson, W.H. Sharfman, C.G. Drake, I. Wollner, J.M. Taube, H. Xu, S. Yao, L. Chen, D.M. Pardoll, J.R Brahmer, S.L. Topalian
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E.J. Lipson, H. Xu, A. Pons
**Study supervision:** E.J. Lipson, W.H. Sharfman, J.R Brahmer, S.L. Topalian

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# Durable Cancer Regression Off-Treatment and Effective Reinduction Therapy with an Anti-PD-1 Antibody

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# Immune checkpoints expression in MSI versus MSS colorectal cancers and their potential therapeutic implications.

Nicolas Jose Llosa , Franck Housseau , Elizabeth C. Wick , Lizzy Hechenbleikner , Michael Cruise ,Robert Anders...

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Abstract Disclosures

## Abstract

**3620**

**Background**: Colorectal cancer (CRC) is divided into 2 groups based on genomic differences: chromosomal instability (80%), and microsatellite instability (MSI) (20%). MSI CRC is associated with a favorable prognosis independent of classic clinical prognostic factors. MSI CRC has been characterized by an intense immune infiltration which was assumed to be related to a high density of mutations creating numerous neoantigens. We compared the nature of inflammation in MSS and MSI CRC specimens to identify biomarkers which could guide therapeutic interventions.**Methods:** Pairs of CRC and normal tissues were enzymatically dissociated to isolate leukocytes by density gradient and their phenotypic characterization was done via multi-parameter flow cytometry (MFC). Immune infiltration was scored using immunohistochemistry (IHC) and *in situ* molecular analysis was performed via laser capture microdissection (LCM) and TaqMan quantitative RT-PCR. **Results:**IHC analysis of immune infiltrates in 15 CRC specimens delineated 3 distinct areas denominated intraepithelial lymphocytes (TIL), tumor stroma (TS) and invasive margin (IM). Scoring of $CD3^+$, $CD4^+$ and $CD8^+$ cells demonstrated that MSI specimens (n=8) were characterized by a higher infiltration of all three populations compared to MSS samples (n=7). Molecular analysis of the TIL (3 MSI versus 3 MSS) indicated that the tumor microenvironment of MSI CRC was characterized by a high expression of IFNg and genes associated with cytotoxic T cells (CTL) including *CD8A*, *TBX21*, *PERF*, *GZM*. Strikingly, MSI samples had a greater expression of IFN-g-driven immune checkpoints such as PD-1, PD-L1, IDO-1 and INOS suggesting that the high density of infiltrating CTL and IFNg stimulate mechanisms of adaptive immune resistance in MSI tumors. MFC analysis of TIL performed on fresh tumors confirmed the higher presence of IFNg-producing $PD1^{hi}$ $CD8^+$CTL in MSI samples (n=8). **Conclusions:** The genetic origin of CRC dictates the nature and intensity of inflammation. A strong IFNg -driven inflammatory response in MSI CRC patients triggered the expression of PD-L1 and IDO-1 which could be targeted to enhance clinical benefit and be predictive of response to immune checkpoint inhibitors.

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**NCS 3 National referral criteria for Ehlers–Danlos Syndrome National Diagnostic Service**

Referrals are invited from consultants in secondary and/or tertiary care for patients in whom the diagnosis of EDS is suspected but not confirmed for one of the following reasons:

- Diagnostic criteria according to Villefranche classification are not met
- Diagnostic testing does not confirm the diagnosis suspected
- Diagnostic criteria of more than one type of EDS are identified
- There are significant additional findings aside from the diagnostic criteria

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# Mainstreaming genetic testing of cancer predisposition genes

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Cancer predisposition genes (CPGs) describe genes in which germline mutations result in increased risk of cancer. Over 100 CPGs have already been discovered and transformative advances in DNA analysis are leading to many new CPG discoveries. These fast, affordable methods for analysing the DNA sequence can also be utilised in diagnostics to substantially increase clinical testing of CPGs. In turn, this has potential to provide substantial cost-effective health benefits with respect to cancer treatment in people with the disease and cancer prevention in healthy individuals. In this review, I outline the clinical benefits of testing for CPGs and how increased testing can be achieved.

## Cancer predisposition genes

There are two ways in which gene mutations contribute to cancer. Oncogenic mutations that occur after birth, within a specific cell, are a hallmark of cancer and are called 'somatic cancer mutations'.[1] Mutations that are present in every cell, either because they have been inherited or occur at conception, are called 'germline mutations'. Genes in which germline mutations lead to increased risks of cancer are called CPGs.[2] It is currently estimated that, overall, approximately 3% of cancers are the result of germline mutations in CPGs. However, the contribution to individual cancers is variable. A high proportion of childhood embryonal cancers, such as retinoblastoma and pleuropulmonary blastoma, are caused by germline mutations in *RB1* and *DICER1*, respectively.[3,4] By contrast, CPG mutations make a small contribution to some adult cancers, such as prostate and lung cancer. However, germline CPG mutations in multiple genes predispose to other adult cancers, such as breast, colorectal and ovarian cancer (Table 1). For some cancers, the overall contribution of CPGs is sizeable, with approximately 15% of ovarian cancer, approximately 20% of medullary thyroid cancer and >30% of phaeochromocytoma resulting from CPG mutations.[5–7]

## Clinical utility of cancer predisposition genes

Identifying whether a cancer is the result of an underlying CPG mutation has significant impact for the cancer patient and, potentially, their relatives. As such, CPG testing has become standard for many genes, although typically only in highly selected cases. From the patient perspective, simply having a better understanding of why their cancer occurred is usually highly valued.

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**Key points**

A proportion of cancer cases are caused by mutations in cancer predisposition genes (CPGs)

Identification of a CPG mutation provides information that can be used to optimise the management of individuals with cancer

Identification of a CPG mutation provides opportunities to reduce cancer incidence in healthy individuals

New sequencing technologies can be used to deliver fast, affordable mutation testing that will enable testing of more genes in more individuals

Flexible testing processes that allow increased, simplified access to gene testing for patients with cancer are being developed

KEYWORDS: Cancer predisposition genes, gene testing, next-generation sequencing, *BRCA1*, *BRCA2* ※

**Table 1** Contribution of cancer predisposition genes to selected cancers

| Cancer | Gene symbol | Approximate proportion caused by known genes | Approximate annual number of cases caused by known genes (in the UK) |
|---|---|---|---|
| Breast | *BRCA1* | 3–5% | ~2,000 |
| | *BRCA2* | | |
| | *ATM* | | |
| | *BRIP1* | | |
| | *CHEK2* | | |
| | *CDH1* | | |
| | *PALB2* | | |
| | *PTEN* | | |
| | *STK11* | | |
| | *TP53* | | |
| Colorectal | *APC* | 3–5% | ~2,000 |
| | *BMPR1A* | | |
| | *MLH1* | | |
| | *MSH2* | | |
| | *MSH6* | | |
| | *MUTYH* | | |
| | *PMS2* | | |
| | *POLD1* | | |
| | *POLE* | | |
| | *PTEN* | | |
| | *SMAD4* | | |
| | *STK11* | | |
| Ovarian | *BRCA1* | ~15% | ~1,000 |
| | *BRCA2* | | |
| | *BRIP1* | | |
| | *MLH1* | | |
| | *MSH2* | | |
| | *MSH6* | | |
| | *PMS2* | | |
| | *RAD51C* | | |
| | *RAD51D* | | |

## Cancer diagnosis and management

Identifying a CPG mutation provides important information that can aid diagnosis and management. The surgical management might be altered; for instance, more radical surgery might be appropriate in CPG mutation carriers, who are at increased risk of further cancer. Radiation treatment might be modified or excluded because some CPGs are associated with increased radiation sensitivity. Chemotherapy might be changed because some treatments are more effective and others less effective in CPG mutation carriers. For example, platinum-based therapies are not standard treatment for breast cancer but can have utility in *BRCA1/2* carriers, who are particularly sensitive to platinum-based drugs.[8,9] Newer, personalised therapies that either target the CPG mutation directly or pathways that become vulnerable because of the CPG mutation, are increasingly being developed. For example, some gastrointestinal tumours result from germline gain-of-function mutations in *KIT* or *PDGFRA* that could be inhibited by imantinib.[10] Similarly, poly (ADP-ribose) polymerase (PARP) inhibitors that target DNA repair pathways that become vulnerable in women with *BRCA1* or *BRCA2* mutations are currently in phase III trials.[11]

Identifying an underlying CPG mutation can also provide prognostic information. For instance, survival is significantly better for patients with *BRCA2* mutation-positive ovarian cancer, but significantly worse for patients with *BRCA2* mutation-positive prostate cancer.[12,13] The likelihood of recurrence, a new primary and/or a second malignancy can all be increased in CPG mutation carriers, who require ongoing review and consideration of tailored surveillance and/or risk-reducing interventions. Management of noncancer-associated problems can also be important; for example, certain *WT1* mutations result in insidious renal dysfunction that requires monitoring and early intervention.

## Cancer screening and prevention

CPGs are unusual because they can serve as a biomarker of future disease. Identifying a CPG mutation provides a window of opportunity to implement surveillance and/or risk-reducing measures that mitigate or prevent cancer. The type of screening is naturally determined by the type of cancer, but usually involves imaging to detect a lesion before it presents clinically. Prevention usually involves surgical removal of the at-risk tissue and is necessarily reserved for nonessential organs in individuals at very high-risk, such as the stomach in *CDH1* mutation carriers, the thyroid in *RET* mutation carriers

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and the breast and ovaries in *BRCA1* mutation carriers.[14–16] Chemoprevention is an attractive strategy, but to date there have been few applications. A notable exception is individuals with increased risk of colorectal cancer in whom the cancer risk is significantly reduced by daily aspirin.[17] Although it is often overlooked, there is high patient and economic value in using CPG mutation testing to identify relatives without the familial mutation. Such individuals are released from anxiety for themselves and their offspring, and do not require costly screening and interventions.

## Cancer predisposition gene testing methods

DNA sequencing technology has evolved rapidly over recent years. Traditionally, gene testing relied on development of individual tests for each CPG using costly, time-consuming methods. Now it is possible to sequence multiple genes in parallel, faster and more affordably than a single gene test with traditional methods, using a technique known as next-generation sequencing (NGS).[18] In turn, this means many more genes and many more people can have gene testing, and the approach is of particular value if the cancer predisposition is heterogeneous (ie multiple different genes predispose to the cancer). Gene panels of 5–100 CPGs are currently available. In time, it is likely that these will be superseded by whole-genome sequencing, which would also enable genetic information about other medical conditions or drug responses to be harvested.

## Increasing cancer predisposition gene testing – an 'oncogenetic' model

The laborious, expensive nature of gene testing previously meant that: (i) strict eligibility criteria to limit testing were required, and (ii) testing infrastructure was developed primarily to serve the complex needs of unaffected individuals who had time to wait for the results. Faster, more affordable testing now enables eligibility criteria to be relaxed and for results to be delivered within the time frame required to impact cancer management. However, changes to the testing process are required to achieve this, particularly in relation to providing greater flexibility for cancer patients to access testing. In the UK, almost all CPG testing is currently performed through genetics services irrespective of cancer status, although gene testing in patients with some diseases is already performed by nongeneticists. An 'oncogenetic' model of CPG testing, whereby testing in patients with cancer can be performed through the cancer team, with support as required from genetics, is being piloted by several services, including the Mainstreaming Cancer Genetics programme (www.mcgprogramme.com). If the test is positive, the patient is automatically sent an appointment by the genetics service to address future issues for the patient and implications for the family. The cancer team use the information to tailor cancer management as appropriate. If the test is negative, it is still useful information for the cancer team, but typically does not require input from genetics, unless there is an extensive or complex family history. Any patient that requires more detailed discussions can be referred to genetics in the usual fashion. This model enables more streamlined, more flexible and more efficient testing for people who already have cancer. All gene tests in healthy individuals without cancer are undertaken by genetics services, because detailed discussions about whether and when to have the test are typically required (Fig 1).

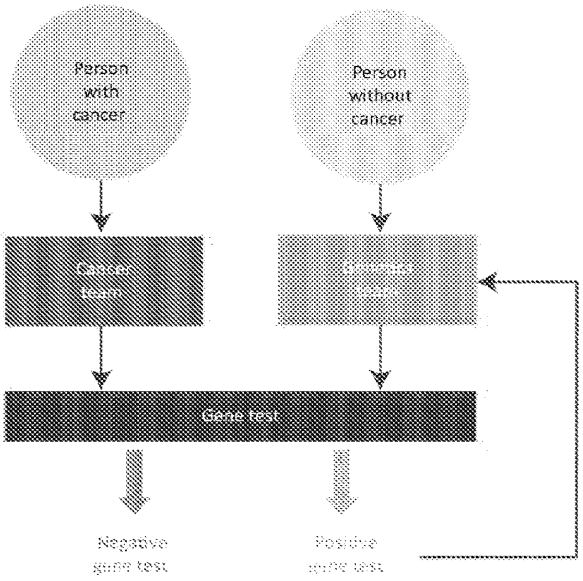



**Fig 1. Proposed new pathways to deliver cancer predisposition gene testing.**

## Interpreting and utilising gene information

Gene variants and/or mutations occur frequently in our genomes and are usually innocuous. Distinguishing which are benign and which are pathogenic can be challenging even for genes that were discovered many years ago and have been extensively studied, such as *BRCA1*. Current gene test reports are often complex, provide unclear information (eg a variant of unknown significance was discovered) and are not linked to clear management guidance. As such, inconsistent, ad hoc and inappropriate interventions are implemented. For mainstreaming to be successful, expert genetic analysis and triage to clear management protocols is essential. Although it is appropriate and anticipated that all clinicians will become genome aware, it is neither feasible nor necessary for all clinicians to be genome experts. Rather, the role and training of clinical geneticists will need to be reformed such that they can provide expert interpretation of the clinical implications of complex gene tests, with automated pipelines producing routine results.

## Conclusion

Mainstreaming gene testing offers unprecedented opportunities to improve the equity and quality of care provided to patients with cancer and the wider population. The paradigm shift of providing genetic testing within mainstream medical services will require education of physicians, allied health practitioners, patients and the general public. Remodelling of existing processes and additional infrastructure, including specialised


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laboratories with bioinformatics and clinical interpretive expertise, will be required. However, if correctly implemented, the potential for improvement in cancer treatment and cancer prevention is high. ■

## Acknowledgments

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U.S. National Library of Medicine
ClinicalTrials.gov archive

## History of Changes for Study: NCT02060188

## A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)

Latest version (submitted November 4, 2019) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare. One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of th
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in ~~red~~.
- Study additions are displayed in *green*.

### Study Record Versions

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 1 | ○ | ○ | February 10, 2014 | None (earliest Version on record) |
| 2 | ○ | ○ | March 25, 2014 | Recruitment Status, Study Status, Contacts/Locations and Study Description |
| 3 | ○ | ○ | March 28, 2014 | Contacts/Locations and Study Status |
| 4 | ○ | ○ | April 29, 2014 | Contacts/Locations and Study Status |
| 5 | ○ | ○ | May 2, 2014 | Study Status |
| 6 | ○ | ○ | May 7, 2014 | Contacts/Locations, References and Study Status |
| 7 | ○ | ○ | May 27, 2014 | Contacts/Locations and Study Status |
| 8 | ○ | ○ | May 30, 2014 | Study Status |
| 9 | ○ | ○ | June 12, 2014 | Contacts/Locations and Study Status |
| 10 | ○ | ○ | June 25, 2014 | Contacts/Locations and Study Status |
| 11 | ○ | ○ | July 9, 2014 | Contacts/Locations and Study Status |
| 12 | ○ | ○ | July 23, 2014 | Contacts/Locations and Study Status |
| 13 | ○ | ○ | August 11, 2014 | Contacts/Locations and Study Status |
| 14 | ○ | ○ | August 28, 2014 | Contacts/Locations and Study Status |
| 15 | ○ | ○ | September 15, 2014 | Contacts/Locations and Study Status |
| 16 | ○ | ○ | October 13, 2014 | Contacts/Locations and Study Status |
| 17 | ◉ | ◉ | October 30, 2014 | Contacts/Locations and Study Status |
| 18 | ○ | ○ | November 13, 2014 | Contacts/Locations and Study Status |
| 19 | ○ | ○ | November 20, 2014 | Contacts/Locations and Study Status |
| 20 | ○ | ○ | December 5, 2014 | Contacts/Locations and Study Status |
| 21 | ○ | ○ | December 31, 2014 | Study Status |
| 22 | ○ | ○ | January 27, 2015 | Contacts/Locations and Study Status |
| 23 | ○ | ○ | February 6, 2015 | Contacts/Locations and Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 24 | ○ | ○ | February 19, 2015 | Study Status |
| 25 | ○ | ○ | March 5, 2015 | Study Status |
| 26 | ○ | ○ | March 23, 2015 | Study Status |
| 27 | ○ | ○ | April 13, 2015 | Study Status and Contacts/Locations |
| 28 | ○ | ○ | April 20, 2015 | Contacts/Locations and Study Status |
| 29 | ○ | ○ | June 3, 2015 | Study Status |
| 30 | ○ | ○ | June 4, 2015 | Study Status and Contacts/Locations |
| 31 | ○ | ○ | June 22, 2015 | Study Status |
| 32 | ○ | ○ | July 3, 2015 | Study Status |
| 33 | ○ | ○ | July 17, 2015 | Contacts/Locations and Study Status |
| 34 | ○ | ○ | August 6, 2015 | Contacts/Locations and Study Status |
| 35 | ○ | ○ | September 3, 2015 | Contacts/Locations and Study Status |
| 36 | ○ | ○ | September 16, 2015 | Study Status |
| 37 | ○ | ○ | October 15, 2015 | Study Status |
| 38 | ○ | ○ | November 4, 2015 | Study Status |
| 39 | ○ | ○ | December 8, 2015 | Study Status |
| 40 | ○ | ○ | December 21, 2015 | Study Status |
| 41 | ○ | ○ | January 6, 2016 | Study Status |
| 42 | ○ | ○ | January 21, 2016 | Contacts/Locations and Study Status |
| 43 | ○ | ○ | January 26, 2016 | Contacts/Locations and Study Status |
| 44 | ○ | ○ | February 19, 2016 | Study Status |
| 45 | ○ | ○ | February 25, 2016 | Study Status |
| 46 | ○ | ○ | March 11, 2016 | Study Status |
| 47 | ○ | ○ | March 28, 2016 | Study Status |
| 48 | ○ | ○ | April 12, 2016 | Study Status |
| 49 | ○ | ○ | May 10, 2016 | Study Status |
| 50 | ○ | ○ | May 25, 2016 | Contacts/Locations and Study Status |
| 51 | ○ | ○ | June 24, 2016 | Study Status |
| 52 | ○ | ○ | June 28, 2016 | Study Status |
| 53 | ○ | ○ | July 11, 2016 | Study Status |
| 54 | ○ | ○ | July 29, 2016 | Study Status |
| 55 | ○ | ○ | August 8, 2016 | Study Status and Study Design |
| 56 | ○ | ○ | August 23, 2016 | Study Status |
| 57 | ○ | ○ | September 7, 2016 | Study Status |
| 58 | ○ | ○ | September 23, 2016 | Study Status |
| 59 | ○ | ○ | October 14, 2016 | Arms and Interventions, Study Description, Study Status, Contacts/Locations and Eligibility |
| 60 | ○ | ○ | October 25, 2016 | Contacts/Locations, Study Design and Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 61 | ○ | ○ | December 6, 2016 | Contacts/Locations and Study Status |
| 62 | ○ | ○ | December 13, 2016 | Study Status and Contacts/Locations |
| 63 | ○ | ○ | December 23, 2016 | Contacts/Locations and Study Status |
| 64 | ○ | ○ | January 5, 2017 | Arms and Interventions, Outcome Measures, Study Status, Study Identification, Eligibility, Study Design and Study Description |
| 65 | ○ | ○ | January 27, 2017 | Study Status |
| 66 | ○ | ○ | February 16, 2017 | Contacts/Locations and Study Status |
| 67 | ○ | ○ | February 28, 2017 | Contacts/Locations, Study Status and References |
| 68 | ○ | ○ | March 3, 2017 | Study Status, References and Contacts/Locations |
| 69 | ○ | ○ | March 21, 2017 | Study Status, Oversight, Study Identification, References, Contacts/Locations and Study Description |
| 70 | ○ | ○ | March 29, 2017 | Study Status, References and Contacts/Locations |
| 71 | ○ | ○ | April 17, 2017 | Study Status, Contacts/Locations and Study Design |
| 72 | ○ | ○ | May 15, 2017 | Study Status, References, Contacts/Locations, Eligibility, Conditions and Study Description |
| 73 | ○ | ○ | June 1, 2017 | Conditions and Study Status |
| 74 | ○ | ○ | June 8, 2017 | References, Contacts/Locations and Study Status |
| 75 | ○ | ○ | June 26, 2017 | Contacts/Locations, References and Study Status |
| 76 | ○ | ○ | July 24, 2017 | Study Status |
| 77 | ○ | ○ | August 2, 2017 | Study Status |
| 78 | ○ | ○ | September 1, 2017 | Study Status and Contacts/Locations |
| 79 | ○ | ○ | September 14, 2017 | Contacts/Locations and Study Status |
| 80 | ○ | ○ | October 3, 2017 | Study Status |
| 81 | ○ | ○ | November 3, 2017 | Study Status |
| 82 | ○ | ○ | December 11, 2017 | Contacts/Locations and Study Status |
| 83 | ○ | ○ | January 9, 2018 | Study Status and Contacts/Locations |
| 84 | ○ | ○ | January 18, 2018 | Contacts/Locations and Study Status |
| 85 | ○ | ○ | January 26, 2018 | Contacts/Locations and Study Status |
| 86 | ○ | ○ | February 22, 2018 | Study Status and Contacts/Locations |
| 87 | ○ | ○ | March 26, 2018 | Study Status and Contacts/Locations |
| 88 | ○ | ○ | April 27, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 89 | ○ | ○ | November 6, 2018 | Study Status and References |
| 90 | ○ | ○ | November 19, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 91 | ○ | ○ | December 3, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 92 | ○ | ○ | June 5, 2019 | Study Status |
| 93 | ○ | ○ | November 4, 2019 | Study Status |

Compare

Comparison Format: ◉ Merged ○ Side-by-Side

Scroll up to access the controls

**Study NCT02060188**
**Submitted Date: October 30, 2014 (v17)**

## Study Identification

Unique Protocol ID: CA209-142

Brief Title: A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)

Official Title: A Phase 2 Clinical Trial of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Microsatellite High (MSI-H) Cc Cancer

Secondary IDs: 2013-003939-30 [EudraCT Number]

## Study Status

Record Verification: May 2014

Overall Status: Recruiting

Study Start: March 2014

Primary Completion: December 2016 [Anticipated]

Study Completion: July 2017 [Anticipated]

First Submitted: December 18, 2013

First Submitted that Met QC Criteria: February 10, 2014

First Posted: February 11, 2014 [Estimate]

Last Update Submitted that Met QC Criteria: October 30, 2014

Last Update Posted: October 31, 2014 [Estimate]

## Sponsor/Collaborators

Sponsor: Bristol-Myers Squibb

Responsible Party: Sponsor

Collaborators:

## Oversight

U.S. FDA-regulated Drug:

U.S. FDA-regulated Device:

Data Monitoring: No

## Study Description

Brief Summary: The purpose of this study is to examine if Nivolumab alone or in combination with Ipilimumab will demonstrate a meaningful obje response rate in patients with recurrent and metastatic colon cancer who also have a specific biomarker in their tumors.

Detailed Description: Allocation: The Microsatellite Instability High (MSI-High) Part of the trial is Non-randomized, The Non-MSI high part of the trial contains a randomized portion

## Conditions

Conditions: MSI Positive Colorectal Cancer
MSI Negative Colorectal Cancer

Keywords:

## Study Design

Study Type: Interventional

Primary Purpose: Treatment

Study Phase: Phase 1/Phase 2

Interventional Study Model: Parallel Assignment

Number of Arms: 2

Masking: None (Open Label)

Allocation: Non-Randomized

Enrollment: 96 [Anticipated]

## Arms and Interventions

| Arms | Assigned Interventions |
|---|---|
| Experimental: Nivolumab Monotherapy<br>Nivolumab administered as IV infusion at a dose of 3mg/kg every 2 weeks until disease progression | Drug: Nivolumab<br>Other Names:<br>• BMS-936558 |
| Experimental: Nivolumab + Ipilimumab<br>Dose Escalation Phase:<br>Dose Level -1: Nivolumab 0.3mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 1: Nivolumab 1mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 2a: Nivolumab 1mg/Kg IV combined with Ipilimumab 3 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 2b: Nivolumab 3mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression | Drug: IpilimumabDrug: Nivolumab<br>Other Names:<br>• BMS-936558 |

## Outcome Measures

Primary Outcome Measures:

1. Objective response rate (ORR) in all MSI-High subjects as determined by Investigators
   (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter u
   disease progression or treatment is discontinued (whichever occurs later)) (Tumor imaging assessments will occur every 6 weeks from the date of first do
   (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later))

   [Time Frame: The final analysis of the primary endpoint will occur at least 6 months after the last enrolled subject's first dose of study therapy (Approximat
   up to 34 months)]

Secondary Outcome Measures:

2. ORR in all MSI-H subjects based on IRRC determination
   Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/- wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter unt
   disease progression or treatment is discontinued(whichever occurs later)

   [Time Frame: The final analysis of the secondary endpoint will occur the time of the primary endpoint analysis (Approximately up to 34 months)]

## Eligibility

Minimum Age: 18 Years

Maximum Age:

Sex: All

Gender Based:

Accepts Healthy Volunteers: No

Criteria: For more information regarding BMS clinical trial participation, please visit www.BMSStudyConnect.com

Inclusion Criteria:

- Men and women ≥ 18 years of age
- Eastern Cooperative Oncology Group (ECOG) performance status 0 to 1
- Histologically confirmed colorectal cancer
- Measurable disease by CT or MRI
- Testing for MSI Status
- Adequate organ function as defined by study-specific laboratory tests
- Must use acceptable form of birth control throughout the study. After the final dose of study drug, an acceptable form of birt control must be used for 23 weeks for women of childbearing potential (WOCBP) and 31 weeks for men who are sexually a with WOCBP
- Signed informed consent
- Willing and able to comply with study procedures

Exclusion Criteria:

- Active brain metastases or leptomeningeal metastases are not allowed.
- Prior treatment with an anti-Programmed Death Receptor (PD)-1, anti-PD-L1, anti-PD-L2, anti-Cytotoxic T-Cell Lymphoma Antigen (CTLA-4) antibody, or any other antibody or drug specifically targeting T-cell co-stimulation or immune checkpoint pathways
- Prior malignancy active within the previous 3 years except for locally curable cancers
- Subjects with active, known or suspected autoimmune disease
- Subjects with a condition requiring systemic treatment with either corticosteroids or other immunosuppressive medications within 14 days of study drug administration

## Contacts/Locations

Central Contact Person: Recruiting sites have contact information. Please contact the sites directly. If there is no contact information, please email:
Email: Clinical.Trials@bms.com

Central Contact Backup: First line of the email MUST contain NCT# and Site #.

Study Officials: Bristol-Myers Squibb
Study Director
Bristol-Myers Squibb

Locations: **United States, Arizona**

Local Institution
[Not yet recruiting]
Gilbert, Arizona, United States, 85234
Contact: Contact: Site 0028

**United States, California**

USC Norris Comprehensive Cancer Center
[Recruiting]
Los Angeles, California, United States, 90033
Contact: Contact: Heinz-Josef Lenz, Site 0004 323-865-0820

Pacific Hematology Oncology Associates
[Recruiting]
San Francisco, California, United States, 94115
Contact: Contact: Ari Baron, Site 0001 415-600-1775

**United States, Georgia**

Emory University
[Recruiting]
Atlanta, Georgia, United States, 30322
Contact: Contact: Bassel El-Rayes, Site 0008 404-778-1900

**United States, Massachusetts**

Massachusetts General Hospital
[Recruiting]
Boston, Massachusetts, United States, 02114
Contact: Contact: Eunice Kwak, Site 0002 617-724-9347

Massachusetts General Hospital
[Recruiting]
Boston, Massachusetts, United States, 02114
Contact: Contact: Eunice Kwak, Site 0036

**United States, Minnesota**

Allina Health
[Recruiting]
Minneapolis, Minnesota, United States, 55407
Contact: Contact: Joseph Leach, Site 0034 612-863-5658

**United States, New York**

Local Institution
[Not yet recruiting]
New York, New York, United States, 11065
Contact: Contact: Site 0009

**United States, North Carolina**

Duke University Medical Center
[Recruiting]
Durham, North Carolina, United States, 27710
Contact: Contact: Michael Morse, Site 0024 919-668-1861

Novant Health Oncology Specialists
[Recruiting]
Winston-Salem, North Carolina, United States, 27103
Contact: Contact: Franklin L Chen, Site 0029 336-718-8461

**United States, Oregon**

Providence Cancer Center Oncology and Hematology Care- Eastside
[Recruiting]
Portland, Oregon, United States, 97213
Contact: Contact: Todd Crocenzi, Site 0005 503-215-2492

**United States, Pennsylvania**

Upmc Cancer Pavilion
[Recruiting]
Pittsburgh, Pennsylvania, United States, 15232
Contact: Contact: James Lee, Site 0013 412-647-8205

**United States, Tennessee**

Vanderbilt-Ingram Cancer Ctr
[Recruiting]
Nashville, Tennessee, United States, 37232
Contact: Contact: Emily Chan, Site 0006 615-936-5847

**United States, Texas**

The University Of Texas Md Anderson Cancer Center
[Recruiting]
Houston, Texas, United States, 77030
Contact: Contact: Michael Overman, Site 0003 713-563-7757

**Australia, New South Wales**

Local Institution
[Not yet recruiting]
Westmead, New South Wales, Australia, 2145
Contact: Contact: Site 0040

**Australia, Queensland**

Local Institution
[Recruiting]
Southport, Queensland, Australia, 4215
Contact: Contact: Site 0039

**Australia, Victoria**

Local Institution
[Not yet recruiting]
Parkville, Victoria, Australia, 3050
Contact: Contact: Site 0037

**Belgium**

Local Institution
[Active, not recruiting]
Brussels, Belgium, 1000

Local Institution
[Active, not recruiting]
Brussels, Belgium, 1090

Local Institution
[Active, not recruiting]
Leuven, Belgium, 3000

**Canada, Alberta**

Local Institution
[Not yet recruiting]
Edmonton, Alberta, Canada, T6G 1Z2
Contact: Contact: Site 0027

**Canada, Ontario**

Mount Sinai Hospital
[Recruiting]
Toronto, Ontario, Canada, M5G 1X5
Contact: Contact: Albiruni Razak, Site 0016 416-946-4501

**France**

Local Institution
[Recruiting]
Montpellier Cedex 05, France, 34298
Contact: Contact: Site 0026

Local Institution
[Recruiting]
Paris, France, 75012
Contact: Contact: Site 0025

**Ireland**

Local Institution
[Active, not recruiting]
Dublin 4, Ireland

Local Institution
[Completed]
Dublin 9, Ireland

Local Institution
[Recruiting]
Galway, Ireland
Contact: Contact: Site 0033

**Italy**

Local Institution
[Not yet recruiting]
Modena, Italy, 41124
Contact: Contact: Site 0035

Local Institution
[Recruiting]
Padova, Italy, Padova
Contact: Contact: Site 0032

**Italy, TO**

Local Institution
[Recruiting]
Candiolo, TO, Italy, 10060
Contact: Contact: Site 0030

**Spain**

Local Institution
[Recruiting]
Madrid, Spain, 28009
Contact: Contact: Site 0012

Local Institution
[Recruiting]
Madrid, Spain, 28050
Contact: Contact: Site 0010

Local Institution
[Recruiting]
Sevilla, Spain, 41013
Contact: Contact: Site 0011

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Links: URL: http://www.bms.com/studyconnect/Pages/home.aspx
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U.S. National Library of Medicine | U.S. National Institutes of Health | U.S. Department of Health & Human Services

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 43663990 |
| **Application Number:** | 16306290 |
| **International Application Number:** | |
| **Confirmation Number:** | 7097 |
| **Title of Invention:** | METHODS OF TREATING COLORECTAL CANCER |
| **First Named Inventor/Applicant Name:** | Michael AXELSON |
| **Customer Number:** | 115018 |
| **Filer:** | Brian Mason Dudley/Jeanette Yando |
| **Filer Authorized By:** | Brian Mason Dudley |
| **Attorney Docket Number:** | 3338.0550001/ELE/C-K/BMD |
| **Receipt Date:** | 07-SEP-2021 |
| **Filing Date:** | 30-NOV-2018 |
| **Time Stamp:** | 18:10:16 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| Submitted with Payment | no |
|---|---|

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Non Patent Literature | 39_Bacher.pdf | 173101<br>7e49c35f72f2d568d23d656420f84d59d7662417 | no | 14 |

**Warnings:**

| | | | | | |
|---|---|---|---|---|---|
| **Information:** | | | | | |
| 2 | Non Patent Literature | 38_NCT01876511.pdf | 72918<br>99d486b993243517eb0ea9e6fa5277f0125b5b52 | no | 5 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Non Patent Literature | 40_DURIE_.pdf | 24516<br>e034619538789db8f651916405d7d3f07410ab5b | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Non Patent Literature | 41_EROGLU_.pdf | 231407<br>0445f3fcf86ebae5265c45b7406c431f2309d541 | no | 13 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | Non Patent Literature | 42_GATALICA_.pdf | 214860<br>9540d8df5806c52f06998bf984b26139187e8d5a | no | 7 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Non Patent Literature | 43__Phase2studyofprogrammeddeath-1antibody_Journalof.pdf | 84067<br>78b0fa25f7099618de18bfffcdc394c04cbd8ea7 | no | 4 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 7 | Non Patent Literature | 44_LIPSON_.pdf | 295577<br>f8d6d24f0ddb7f5f7a7890aa5e31594f2a4f6361 | no | 8 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 8 | Non Patent Literature | 45_LLOSA_2014.pdf | 23743<br>eec78811aa2ffe1e6206f6beb5ca2e63430aa638 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| 9 | Non Patent Literature | 46_RAHMAN_2014.pdf | 98103<br>1bfedc9697d5c3dcfd6c1c8a4a3519010bbe58dd | no | 4 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 10 | Non Patent Literature | 37_NCT02060188.pdf | 107103<br>ebd95d85191ebd4e86f0ae17d0f04e3aaf71701e | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | | **Total Files Size (in bytes):** | 1325395 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

# NOTICE OF ALLOWANCE AND FEE(S) DUE

115018 7590 09/20/2021

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER |
|---|
| LOCKARD, JON MCCLELLAND |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1647 | |

DATE MAILED: 09/20/2021

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

TITLE OF INVENTION: METHODS OF TREATING COLORECTAL CANCER

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $1200.00 | $0 | 12/20/2021 |

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

**HOW TO REPLY TO THIS NOTICE:**

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

**IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.**



PTOL-85 (Rev. 02/11)

## PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

115018 7590 09/20/2021
Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

**Certificate of Mailing or Transmission**

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

| (Typed or printed name) |
|---|
| (Signature) |
| (Date) |

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

TITLE OF INVENTION: METHODS OF TREATING COLORECTAL CANCER

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $1200.00 | $0 | 12/20/2021 |

| EXAMINER | ART UNIT | CLASS-SUBCLASS |
|---|---|---|
| LOCKARD, JON MCCLELLAND | 1647 | 424-139100 |

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1_____
2_____
3_____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required) ❑Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/306,290 | 11/30/2018 | Michael AXELSON | 3338.0550001/ ELE/C-K/BMD | 7097 |

115018 7590 09/20/2021

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue NW
Washington, DC 20005

| EXAMINER | |
|---|---|
| LOCKARD, JON MCCLELLAND | |
| ART UNIT | PAPER NUMBER |
| 1647 | |

DATE MAILED: 09/20/2021

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

| Notice of Allowability | Application No. 16/306,290 | Applicant(s) AXELSON, Michael | |
|---|---|---|---|
| | Examiner JON M LOCKARD | Art Unit 1647 | AIA (FITF) Status Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--*

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the Request for Continued Examination filed 07 September 2021.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1,3-5,7-8,10-13 and 16-23 (renumbered as claims 1-18, respectively) . As a result of the allowed claim(s ), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   **Certified copies:**

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   **Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

| /J.L/<br>Examiner, Art Unit 1647 | /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 |
|---|---|

***Notice of Pre-AIA or AIA Status***

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

***Continued Examination Under 37 CFR 1.114***

2. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after allowance or after an Office action under *Ex Parte Quayle*, 25 USPQ 74, 453 O.G. 213 (Comm'r Pat. 1935). Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, prosecution in this application has been reopened pursuant to 37 CFR 1.114. Applicant's submission filed on 07 September 2021 has been entered.

***Information Disclosure Statement***

3. The information disclosure statement (IDS) submitted on 07 September 2021 has been considered by the examiner.

**REASONS FOR ALLOWANCE**

4. The following is an examiner's statement of reasons for allowance: The information disclosure statement (IDS) filed 07 September 2021 has been considered by the Examiner. After careful consideration, the Examiner has determined that none of the information contained therein raises new issues of patentability.

5. Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue

fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

***Summary***

6. Claims 1, 3-5, 7-8, 10-13 and 16-23 are allowed.

***Advisory Information***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to **Jon M. Lockard** whose telephone number is **(571) 272-2717**. The examiner can normally be reached on Monday through Friday, 8:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, **Joanne Hama**, can be reached on **(571) 272-2911**. The fax number for the organization where this application or proceeding is assigned is **571-273-8300**.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at **866-217-9197** (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/J. M. L./
Examiner, Art Unit 1647
September 10, 2021

/Christine J Saoud/
Primary Examiner, Art Unit 1647

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | Examiner | Art Unit |
| | JON M LOCKARD | 1647 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| NONE | | 12/19/2020 | JML |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| EAST (USPAT, US-PGPUB, EPO, DERWENT): See attached search history. | 12/19/2020 | JML |
| STN (MEDLINE, SCISEARCH, EMBASE, BIOSIS): See attached search history. | 12/19/2020 | JML |
| PALM: Inventor search. | 12/19/2020 | JML |

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | Examiner | Art Unit |
| | JON M LOCKARD | 1647 |

| Interference Search | | | |
|---|---|---|---|
| **US Class/CPC Symbol** | **US Subclass/CPC Group** | **Date** | **Examiner** |
| | EAST (USPAT): See attached search history. | 04/23/2021 | JML |
| | PALM: Inventor search. | 04/23/2021 | JML |
| | EAST (USPAT): See attached search history. | 09/10/2021 | JML |
| | PALM: Inventor search. | 09/10/2021 | JML |

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | **Examiner** | **Art Unit** |
| | JON M LOCKARD | 1647 |

| CPC | | | | | | |
|---|---|---|---|---|---|---|
| **Symbol** | | | | | **Type** | **Version** |
| C07K | / | 16 | / | 2818 | F | 2013-01-01 |
| A61P | / | 35 | / | 00 | I | 2018-01-01 |
| C07K | / | 16 | / | 2827 | I | 2013-01-01 |
| C07K | / | 16 | / | 2878 | I | 2013-01-01 |
| A61K | / | 2039 | / | 507 | A | 2013-01-01 |

| CPC Combination Sets | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Symbol** | | | | | **Type** | **Set** | **Ranking** | **Version** |
| | / | | / | | | | | |

| /JON M LOCKARD/<br>Examiner, Art Unit 1647<br>(Assistant Examiner) | 10 September 2021<br>(Date) | **Total Claims Allowed:**<br>18 | |
|---|---|---|---|
| /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647<br>(Primary Examiner) | 10 September 2021<br>(Date) | O.G. Print Claim(s)<br>1 | O.G. Print Figure<br>NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20210910

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | **Examiner** | **Art Unit** |
| | JON M LOCKARD | 1647 |

| INTERNATIONAL CLASSIFICATION | | | | |
|---|---|---|---|---|
| **CLAIMED** | | | | |
| A61K | / | 39 | / | 395 |
| C07K | / | 16 | / | 28 |

| NON-CLAIMED | | | | |
|---|---|---|---|---|
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| US ORIGINAL CLASSIFICATION | |
|---|---|
| **CLASS** | **SUBCLASS** |
| | |

| CROSS REFERENCES(S) | | | | | | |
|---|---|---|---|---|---|---|
| **CLASS** | **SUBCLASS (ONE SUBCLASS PER BLOCK)** | | | | | |
| | | | | | | |

| /JON M LOCKARD/<br>Examiner, Art Unit 1647 | 10 September 2021 | **Total Claims Allowed:** | |
|---|---|---|---|
| (Assistant Examiner) | (Date) | 18 | |
| /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 | 10 September 2021 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20210910

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/306,290 | AXELSON, Michael |
| | **Examiner** | **Art Unit** |
| | JON M LOCKARD | 1647 |

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

**CLAIMS**

| Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original |
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|---|---|---|---|
| /JON M LOCKARD/<br>Examiner, Art Unit 1647 | 10 September 2021 | **Total Claims Allowed:** | |
| (Assistant Examiner) | (Date) | 18 | |
| /CHRISTINE J SAOUD/<br>Primary Examiner, Art Unit 1647 | 10 September 2021 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | NONE |

U.S. Patent and Trademark Office — Part of Paper No.: 20210910

Receipt date: 09/07/2021

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

# SECOND SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD, JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

| NON-PATENT LITERATURE DOCUMENTS | | | |
|---|---|---|---|
| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
| | NPL37 | Anonymous: "A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)". Retrieved from the Internet: URL: https://clinicaltrials.gov/ct2/history/NCT02060188?V_17=View#StudyPageTop [retrieved on August 30, 2021], 9 pages. | ☐ |
| | NPL38 | Anonymous: "Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors - Full Text View Clinical Trials. gov " September 24, 2013 (2013-09-24). Retrieved from the Internet: URL:https://clinicaltrials.gov/ct2/show/NCT01876511 [retrieved on August 30, 2021], 4 pages. | ☐ |
| | NPL39 | BACHER, J.W., et al., "Development of a fluorescent multiplex assay for detection of MSI-High tumors," Dis Markers 20(4-5):237-250, IOS Press, United States (2004). | ☐ |
| | NPL40 | DURIE, N., et al., "Retrospective review of colorectal cancer specimens in individuals younger than age 50 for microsatellite instability testing and DNA mismatch repair enzyme expression," Journal of Clinical Oncology 29(4):392-392, American Society of Clinical Oncology, United States (2011). | ☐ |
| | NPL41 | EROGLU, Z., et al., "Checkpoint Inhibition of PD-1: The Promise of Pembrolizumab (MK-3475) and Beyond," Perzonalied Medicine in Oncology: 13 pages, The Lynx Group, United States (2014). | ☐ |
| | NPL42 | GATALICA, Z., et al., "Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type," Cancer Epidemiol Biomarkers Prev 23(12):2965-2970, American Association of Cancer Research, United States (2014). | ☐ |
| | NPL43 | LE, D., et al., "Phase 2 study of programmed death-1 antibody (anti-PD-1, MK-3475) in patients with microsatellite unstable (MSI) tumors," Journal of Clinical Oncology, 32(15): 4 pages, American Society of Clinical Oncology Journal, United States (2014). | ☐ |

| Examiner Signature | | Date Considered | |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.*

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L/

Receipt date: 09/07/2021

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# SECOND SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

| Complete if Known | |
|---|---|
| Application Number | 16/306,290 |
| Int'l Filing Date | June 2, 2017 |
| First Named Inventor | AXELSON; Michael |
| Art Unit | 1647 |
| Examiner Name | LOCKARD, JON MCCLELLAND |
| Attorney Docket Number | 3338.0550001/ELE/C-K/BMD |

| NON-PATENT LITERATURE DOCUMENTS | | | |
|---|---|---|---|
| Examiner Initials* | Cite No.[1] | Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published. | T[2] |
| | NPL44 | LIPSON, E.J., et al., "Durable Cancer Regression off-treatment and effective Reintroduction Therapy with an Anti-PD-1 Antibody," Clinical Cancer Research 19(2):462-468, American Association for Cancer Research, United States (2013). | ☐ |
| | NPL45 | LLOSA, N.J., et al., "Immune checkpoints expression in MSI versus MSS colorectal cancers and their potential therapeutic implications," Journal of Clinical Oncology 32(15): 3620-3620, American Society of Clinical Oncology Journal, United States (2014). | ☐ |
| | NPL46 | RAHMAN, N., "Mainstreaming genetic testing of cancer predisposition genes," Clinical Medicine 14(4):436-439, Royal College of Physicians, United Kingdom (2014). | ☐ |

17255030_1

| Examiner Signature | /JON M LOCKARD/ | Date Considered | 09/10/2021 |
|---|---|---|---|

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
*If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.*

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.L/

# Inventor Information for 16/306290

/J.L./

| Inventor Name | City | State/Country |
|---|---|---|
| AXELSON, MICHAEL | PRINCETON | NEW JERSEY |

Appln Info | Contents | Petition Info | Atty/Agent Info | Continuity Data | Foreign Data | **Inventors** | Applicants | Address | Fees | Post Info | Pre Gr

**Search Another: Application #** Search **or Patent #** Search **or International Registration #** Search

**PCT /** / Search **or PG PUBS #** Search

**Attorney Docket #** Search

**Bar Code #** Search

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**EAST Search History**

**EAST Search History (Interference)** /J.L./

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | Time Stamp |
|---|---|---|---|---|---|---|
| L12 | 13,530 | PDCD1 CD279 PD-1 PD1 SLEB2 hPD-1 hPD-l hSLE1 "Programmed cell death 1" | USPAT | OR | ON | 2021/09/10 13:52 |
| L13 | 2,307 | L12 near5 (antibody or antibodies) | USPAT | OR | ON | 2021/09/10 13:52 |
| L14 | 11,053 | CTLA4 ALPS5 CD152 CELIAC3 CTLA-4 GRD4 IDDM12 "cytotoxic T-lymphocyte associated protein 4" | USPAT | OR | ON | 2021/09/10 13:52 |
| L15 | 3,810 | L14 near5 (antibody or antibodies) | USPAT | OR | ON | 2021/09/10 13:52 |
| L16 | 13,530 | PDCD1 CD279 PD-1 PD1 SLEB2 hPD-1 hPD-l hSLE1 (Programmed adj cell adj death adj "1") | USPAT | OR | ON | 2021/09/10 13:52 |
| L17 | 398 | (colorectal adj cancer) same ((microsatellite adj instability) or msi-h) | USPAT | OR | ON | 2021/09/10 13:52 |
| L18 | 728 | L13 with L15 | USPAT | OR | ON | 2021/09/10 13:52 |
| L19 | 8 | L17 and L18 | USPAT | OR | ON | 2021/09/10 13:52 |
| L20 | 0 | L17 same L18 | USPAT | OR | ON | 2021/09/10 13:52 |
| L21 | 5 | axelson-m$.in. | USPAT | OR | ON | 2021/09/10 13:52 |
| L22 | 0 | (L13 or L15) and L21 | USPAT | OR | ON | 2021/09/10 13:52 |

9/10/2021 2:06:32 PM
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**To:** ckim@sternekessler.com,e-office@sternekessler.com,pcalvo@sternekessler.com
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| Application | Document | Mailroom Date | Attorney Docket No. |
|---|---|---|---|
| 16306290 | NOA | 09/20/2021 | 3338.0550001/ELE/C-K/BMD |
| | 1449 | 09/20/2021 | 3338.0550001/ELE/C-K/BMD |

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Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (02-18)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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# REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
## (Submitted Only via EFS-Web)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Application Number | 16/306,290 | Filing Date | 2018-11-30 | Docket Number (if applicable) | 3338.0550001/ELE/C-K/BMD | Art Unit | 1647 |
| First Named Inventor | Michael AXELSON | | | Examiner Name | LOCKARD, JOHN MCCLELLAND | | |

**This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.**
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

### SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☐ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

### MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

### FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 190036

### SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

| Signature of Registered U.S. Patent Practitioner | | | |
|---|---|---|---|
| Signature | /Brian M. Dudley/ | Date (YYYY-MM-DD) | 2021-12-20 |
| Name | Brian M. Dudley | Registration Number | 77546 |

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

# Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

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ORIGINAL ARTICLE

# High microsatellite instability (MSI-H) colorectal carcinoma: a brief review of predictive biomarkers in the era of personalized medicine

**Zoran Gatalica[1] · Semir Vranic[2] · Joanne Xiu[1] · Jeffrey Swensen[1] · Sandeep Reddy[1]**

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**Abstract** Approximately 15 % of colorectal carcinomas (CRC) display high level microsatellite instability (MSI-H) due to either a germline mutation in one of the genes responsible for DNA mismatch repair (Lynch syndrome, 3 %) or somatic inactivation of the same pathway, most commonly through hypermethylation of the *MLH1* gene (sporadic MSI-H, 12 %). Although heterogeneous, MSI-H colorectal carcinomas as a group show some distinct biologic characteristics when compared to CRC with stable or low level microsatellite instability. In the present review we will highlight therapeutically relevant characteristics of MSI-H tumors which could lead to specific responses to some conventional chemotherapy or novel targeted therapy agents.

**Keywords** Colorectal cancer · Microsatellite instability · Lynch syndrome · Biomarkers · Conventional chemotherapy · Targeted therapy

## Introduction

Colorectal carcinoma (CRC) represents the third most common malignancy in the developed world and one of the leading causes of cancer-related death [1]. At the molecular level, CRC is a heterogeneous disease with several molecular subtypes that harbor distinct molecular genetic, pathologic and clinical characteristics [2]. Recently, the consensus molecular subtypes (CMS) of CRC have been defined [3]. According to the new classification, four CMS with distinguishing characteristics have been proposed: CMS1 (microsatellite instability immune subtype: hypermutated subtype of CRC, microsatellite unstable with a strong immune activation); CMS2 (canonical subtype of CRC: epithelial subtype with upregulation of the WNT and MYC signaling pathways); CMS3 (metabolic subtype of CRC: epithelial subtype with metabolic dysregulation); and CMS4 [mesenchymal subtype of CRC with prominent transforming growth factor-β (TGF-β) activation, stromal invasion and neoangiogenesis] [3].

MSI refers to the hypermutable state of cells caused by impaired DNA mismatch repair (MMR). It consists of insertion and deletion mutations in stretches of short tandem DNA repeats (microsatellites) as well as nucleotide substitutions throughout the genome [4]. In this review, we simplify classification of molecular subtypes of CRC based on MSI status into two broad subgroups: MSI-high (MSI-H) and MSI-negative (low or stable) CRCs, in an effort to highlight potential therapeutic differences between these easily separable groups.

### MSI-high (MSI-H) colorectal cancer

MSI-H CRC accounts for 15 % of all CRC and includes hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome (3 %) and sporadic MSI-H CRC (12 %). Lynch syndrome is a highly penetrant (80 % life time risk for CRC), autosomal-dominant disorder caused by germline mutations in one of the MMR genes: *MLH1, MSH2* (70 %), *MSH6* and *PMS2* (~30 %) [5, 6] (Fig. 1A, B). In addition, germline deletions of the last exon of the epithelial cell adhesion molecule [(*EPCAM*), a gene

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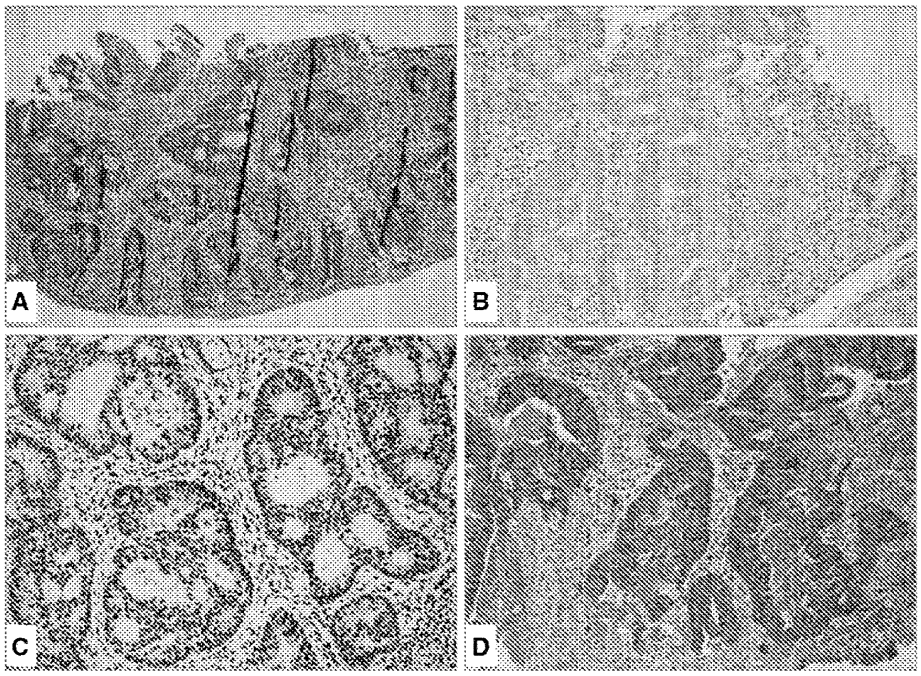



**Fig. 1** A colorectal carcinoma from a case of Lynch syndrome caused by an *MLH1* gene mutation: **A** hematoxylin and eosin (H&E) stained slide, **B** immunohistochemistry (IHC) showing concurrent loss of PMS2 in tumor cells, **C** tumor cells were diffusely positive (90–100 %) for topoisomerase 1 and **D** strongly positive (3+) for thymidylate synthase

located upstream of MSH2] cause Lynch syndrome via epigenetic inactivation of *MSH2* [7].

Sporadic MSI-H CRCs are typically caused by somatic methylation of the *MLH1* gene promoter [4] (Fig. 2A, B). It is worth noting that a small subset of MSI-H tumors harbor no alterations in the MMR genes, but overexpress various miRNAs that may silence the MMR genes. Thus, miRNA-155 downregulates MLH1, MSH2 and MSH6 mRNA, inducing MSI in CRC cell lines [8]. Similarly, miRNA-21, targeting MSH2 and MSH6 mRNA, has been found to be overexpressed in MSI-H CRC [9]. In addition, Li et al. [10, 11] found that cells lacking the SETD2 histone methyltransferase displayed microsatellite instability.

Regardless of the origin (hereditary or sporadic) or type of mutation, MSI-H CRCs share some distinct histologic cancer features (mucin-rich, signet ring and medullary types, often admixed) with increased numbers of tumor-infiltrating lymphocytes (TILs) and prominent Crohn's-like lymphoid reaction [6, 12]. In addition, patients with Lynch syndrome have an increased risk of synchronous or metachronous tumors that include extracolonic sites (small bowel, stomach, endometrium, skin, genitourinary tract) [5, 13]. Prognostically, patients with HNPCC have a more favorable outcome (overall survival) in comparison with stage-matched sporadic CRCs [14, 15].

Methylation of the *MLH1* promoter region that is typically seen in sporadic MSI-H CRC, but not in Lynch syndrome, is strongly associated with the *BRAF* V600E gene mutation [16, 17] (Fig. 2D). In fact, presence of the *BRAF* V600E mutation in CRC essentially excludes Lynch syndrome, with the exception of rare cases associated with *PMS2* germline mutation [18, 19].

## MSI-H colorectal cancers in the era of personalized medicine

CRC is the second leading cause of cancer-related death in the developed world, [20]. Although the response rate of metastatic CRC to the combined chemotherapy is around 50 %, progression of the disease is inevitable and less than 10 % of patients survive >2 years [20]. In adjunct to conventional chemotherapy (e.g. 5-FU, capecitabine, oxaliplatin, irinotecan), metastatic CRC is now treated with a number of drugs aimed at target-specific signaling pathways [e.g. anti-EGFR based therapy (panitumumab and cetuximab for *KRAS/NRAS* wild type CRC); bevacizumab (for inhibition of angiogenesis)] [20, 21]. There is an urgent need for more specific predictive markers that will tailor the CRC treatment modalities and improve overall survival in patients with locally advanced and/or metastatic disease.



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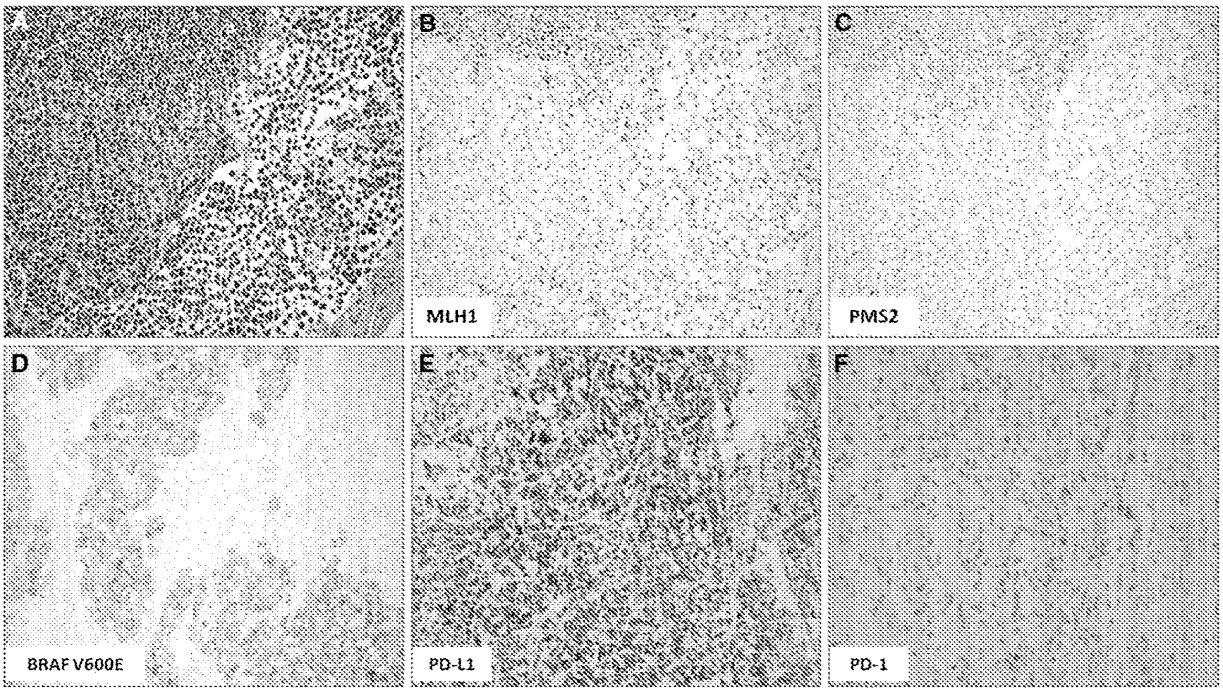



**Fig. 2** A poorly differentiated (signet ring) colorectal carcinoma with microsatellite instability-high status caused by the loss of MLH1: **A** H&E-stained slide, **B** loss of MLH1 in tumor cells by IHC, **C** concurrent loss of PMS2 in tumor cells by IHC; note retained expression of both MLH1 and PMS2 proteins in adjacent tumor-infiltrating lymphocytes, **D** IHC showing that the tumor also harbored the BRAF V600E mutation, **E** the tumor cells exhibited 2+ PD-L1 expression in ~85 % of the tumor cells (anti-PD-L1 clone SP142) and **F** while tumor infiltrating lymphocytes were positive for PD-1 protein

## Predictive biomarkers of conventional chemotherapy

MSI-H status due to loss of MMR gene function is not only a key player in the pathogenesis of CRC, but is also associated with a different response to classic chemotherapeutic treatment modalities [6].

A seminal clinical study by Ribic et al. [15] revealed the benefit of 5-FU-based adjuvant chemotherapy in patients with stage II and stage III MSI-negative CRC (HR = 0.72, $p = 0.04$) but not in those with MSI-H status (HR = 1.07, $p = 0.80$). Preclinical data also confirmed that tumor cells with MSI-H status are resistant to fluoropyrimidines [e.g. 5-fluorouracil (5-FU) and capecitabine], but may be sensitive to irinotecan and mitomycin C [4, 6, 21]. A meta-analysis by Guetz et al. [22] also highlighted MSI-H status as a strong predictive factor for non-response to 5-FU based chemotherapy. Several enzymes including dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP) and thymidylate synthase (TS) have been associated with the metabolism of the 5-FU pathway [21]. TS is a key enzyme involved in the synthesis of 2′-deoxythymidine-5′-monophosphate, which represents an essential precursor for DNA synthesis [23]. Although data from the available literature are not consistent [(different methodologies, cutoff values), reviewed in Koopman et al.], several studies have found significantly higher expression of TS in MSI-H CRCs, including Lynch syndrome cases, and an association with resistance to 5-FU chemotherapy regimen [24–26] (Fig. 1D). In contrast, MSI-H CRCs appear to be more sensitive to irinotecan which functions as an inhibitor of topoisomerase 1 (TOP1) [4]. Colorectal cancer cell lines exhibit sensitivity to irinotecan when harboring increased *TOP1* gene copy number or increased TOP1/CEP20 ratio [27]. Topoisomerase 1 protein overexpression has also been described in MSI-H CRC [28], although Søndenstrup et al. [29] recently reported an absence of *TOP1* gene copy number gain.

Our results, based on the analysis of both sporadic and hereditary MSI-H and MSI-negative CRCs support the reported differences in TS protein [30] (Fig. 1C; Table 1). TS expression was significantly higher in MSI-H tumors, both sporadic (86 %) and hereditary (100 %), compared to an MSI-negative cohort (31 %, $p < 0.0001$) (Table 1; Fig. 1D). Protein expression of TOP1 trends higher in the Lynch cohort, but is indistinguishable between the sporadic MSI-H and MSI-negative cohorts [30, 31] (Table 1; Fig. 1C).



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**Table 1** Differential protein expressions in colorectal tumors with different microsatellite instability status

| | MSI-H colorectal cancers | | | MSI-negative colorectal cancers | p-value (MSI-H vs. MSI-negative) | p-value (Somatic MSI-H vs. Lynch Syndrome) |
|---|---|---|---|---|---|---|
| | *BRAF*-Mutated | *BRAF*-Wild type | Confirmed LS | Percent (N/Total) | | |
| | Percent (N/Total) | Percent (N/Total) | Percent (N/Total) | | | |
| **TS expression** | 85% (93/105) | | | 31.1% (463/1488) | <0.0001 | 0.1518 |
| | 86% (31/36) | 80% (43/54) | 100% (19/19) | | | |
| **PD-1 expression** | 75% (68/91) | | | 44% (653/1485) | <0.0001 | 0.7082 |
| | 78% (28/36) | 72% (37/54) | 69% (9/13) | | | |
| **PD-L1 expression** | 14% (5/36) | | | 4% (22/551) | 0.0196 | n/a |
| | 14% (2/14) | 14% (3/21) | n/a | | | |
| **MGMT expression** | 46% (42/91) | | | 56% (832/1475) | 0.0643 | n/a |
| | 33% (12/36) | 54% (29/54) | n/a | | | |
| **TOPO1 expression** | 43% (37/87) | | | 48% (705/1456) | 0.3 | 0.15 |
| | 50% (18/36) | 36% (18/50) | 74% (14/19) | | | |

All biomarkers were evaluated using immunohistochemistry

*LS* Lynch syndrome, *MGMT* O6-methylguanine DNA methyltransferase, *MSI* microsatellite instability, *H* high, *Negative* low or stable, *n/a* not available, *PD-1* programmed cell death protein 1, *PD-L1* programmed-death ligand 1, *Topo1* topoisomerase 1, *TS* thymidylate synthase

*p* values were calculated using Fisher-Exact two tail tests

Another biomarker which has been associated with MSI-H CRC is $O^6$-methylguanine DNA methyltransferase (MGMT). MGMT is a DNA repair protein with the ability to remove various carcinogenic adducts from the $O^6$ position of guanine [32, 33]. Aberrant methylation of the *MGMT* gene promoter occurs in CRCs with the CpG island methylator phenotype (CIMP) [34], and this correlates with loss of *MGMT* expression [32]. In CRC, *MGMT* hypermethylation has been described in subsets of both sporadic and hereditary MSI-H tumors [32, 35, 36] (Fig. 3A, B).

In our cohort of CRC tumors, MGMT protein expression was much lower (33 %) in the sporadic MSI-H cohort compared to MSI-H tumors without *BRAF* mutation (54 %); this difference may be attributable to CIMP in the *BRAF* mutant tumors (Table 1).

MGMT has also been shown to serve as a predictor of response to alkylating agents (temozolomide and dacarbazine) that have been approved for the treatment of various cancers including brain tumors (astrocytoma and glioblastoma multiforme), melanoma, sarcoma, and Hodgkin lymphoma [26, 37]. In colorectal cancer, temozolomide showed limited clinical activity in unselected patient cohorts, but when patients were selected for low expression of MGMT, very promising results were seen [32, 37–40]. However, Karran in his comment [41] pointed out the importance of defects within the mismatch repair machinery in cancer and potential resistance to various chemotherapeutics, including alkylating drugs. In the context of MSI-H CRC and alkylating agents, it is worth noting a study by Hunter et al. [42] who confirmed that inactivating somatic mutations of the *MSH6* gene not only confer a resistance to alkylating agents in brain tumors (gliomas) but also promote tumor growth and progression.

## Next generation sequencing (NGS) profiling in MSI-H

Studies using the currently available NGS platforms allow for investigation into molecular pathways known to contribute to tumorigenesis and progression of CRC and their



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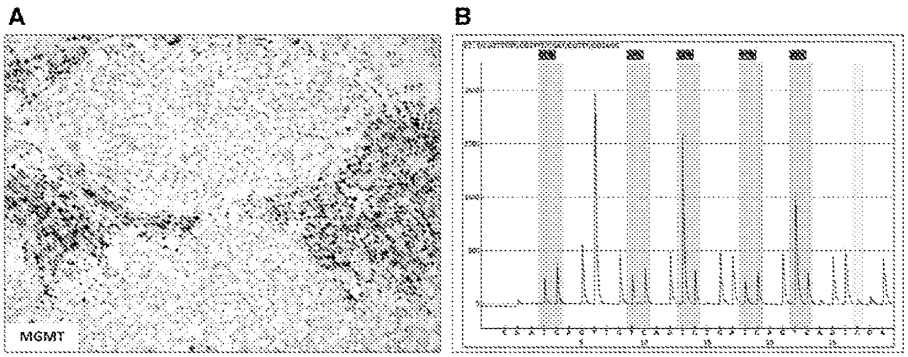



**Fig. 3** The case from Fig. 2: **A** showing loss of MGMT protein expression by IHC and **B** pyrosequencing results showing hypermethylation of the MGMT promoter (57–66 % methylation at five different sites)

differential contributions in the setting of sporadic and germline MSI-H CRCs. Several studies reported a frequent disruption of the WNT signaling pathway in Lynch syndrome, with mutations commonly affecting the *APC* and *CTNNB1* (beta-catenin) genes, which are less commonly mutated in sporadic MSI-H CRC [43]. Recent comprehensive molecular profiling of CRC using whole-genome sequencing revealed that the majority (75 %) of hypermutated CRCs exhibited MSI-H status, associated with hypermethylation of *MLH1*, while the remaining 25 % had somatic mismatch-repair gene and polymerase ε (*POLE*) mutations [44]. Along with the expected mutations of *APC, TP53, SMAD4, PIK3CA* and *KRAS*, the study revealed frequent mutations in *ARID1A*, *SOX9* and *FAM123B* genes, while copy-number alterations included amplification of the *ERBB2* and *IGF2* genes. Integrative analysis also indicated an important role for MYC-directed transcriptional activation and repression [44]. Timmermann et al. [45] also reported a significantly higher incidence of mutations in MSI-H than in MSI-negative CRCs.

Le et al. [46] used whole-exome sequencing and showed a mean of 1782 somatic mutations per tumor in patients with mismatch repair-deficient cancer (N = 9) as compared with 73 mutations per tumor in patients with mismatch repair-proficient cancer (N = 6) ($p = 0.007$). Similarly, we performed NGS using a more limited 591-gene panel (available here: http://www.carismolecularintelligence.com/) on 8 MSI-H CRCs, and observed an average of 130.25 mutations per tumor compared to an average of 55.17 mutations per tumor in 189 MSI-negative CRCs ($p = 0.0004$, Student's *t* test, unpublished data).

## Immune checkpoint proteins PD-1 and PD-L1 in MSI-H colorectal cancer

The PD-1 signaling pathway, composed of the immune cell co-receptor Programmed Death 1 (*PDCD1*, CD279) and its ligands PD-L1 (B7-H1, *CD274*) and PD-L2 (*PDCD1LG2*, B7-DC, CD273), is actively involved in local immunosuppression in human tumors [47]. PD-L1 expression in tumor and associated inflammatory cells has been described in different malignancies, correlating with poor clinical outcome but also with the likelihood of response to targeted immune check point inhibition therapy [48–52]. Several therapeutic monoclonal antibodies inhibiting either PD-1 (nivolumab, pembrolizumab) or PD-L1 (MPDL3280A, Medi4736, BMS-936559) have been developed and are now used for the treatment of various malignancies (e.g. metastatic melanoma, non-small cell lung carcinoma, renal cell carcinoma, bladder carcinoma and Hodgkin lymphoma) [52–54].

In contrast to MSI-negative CRCs, MSI-H CRCs exhibit an active immune microenvironment infiltrated by cytotoxic (CD8+) T-lymphocytes and activated Th1 cells characterized by interferon-γ production and the Th1 transcription factor TBET. This response likely results from the presence of numerous neoantigens (mutated proteins) resulting from the hyper-mutated state of the tumor cells [46, 55–57]. Despite such a "hostile" microenvironment, MSI-H tumor cells are not eliminated by the immune system due to the cancer specific upregulation of various immune inhibitory molecules (checkpoints) including PD-1, PD-L1, Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Lymphocyte-activation gene 3 (LAG-3), and Indoleamine (2,3)-dioxygenase (IDO) [31, 55, 58] (Fig. 2E, F; Table 1). These data indicate that MSI-H CRCs are good candidates for checkpoint immunotherapy as recently shown in a small phase 2 clinical trial which included 11 patients with MSI-H CRC. The study showed that the immune-related objective response and immune-related progression-free survival rates were 40 and 78 %, respectively for refractory/and metastatic MSI-H CRC in contrast to MSI-negative CRC (0 and 11 %, respectively) [46]. This led the US Food and Drug Administration to rapidly approve the anti-PD-1 drug pembrolizumab for the treatment of metastatic/refractory MSI-H CRC. Additional studies and clinical trials involving more samples/patients should define the optimal predictive biomarkers for



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immune checkpoint inhibitors as well as their therapeutic benefits for patients with MSI-H CRC [53].

## Conclusions

Although MSI-H colorectal cancers are heterogeneous (i.e. they can be caused by germline or somatic mutations in different genes), they have similar characteristics that allow them to be grouped together for treatment and clinical management. MSI-H CRCs respond poorly to 5-FU-based chemotherapy (based on thymidylate synthase overexpression), but they may be efficiently treated with camptothecin derivatives (based on topoisomerase 1 overexpression). Hypermethylation/loss of expression of MGMT, which differs between MSI-H cancers based on underlying pathogenic mechanism (i.e. sporadic CIMP MSI-H CRC will be expected to have significantly different MGMT expression from hereditary Lynch MSI-H CRC), may identify a patient subset for clinical investigation. These findings point to the need for individualized profiling of biomarkers and tailoring of therapy for CRCs. Due to an active immune microenvironment and high expression of various checkpoint molecules, MSI-H CRCs are good candidates for targeted immunotherapy with immune checkpoint inhibitors (e.g. pembrolizumab). These cancers also exhibit a distinct hyper-mutated profile that may be amenable to additional treatment options such as vaccination and adoptive-cell-transfer therapy.

#### Compliance with ethical standards

**Conflict of interest** Zoran Gatalica, Joanne Xiu, Jeffrey Swensen, and Sandeep Reddy are employees of Caris Life Sciences. Semir Vranic has received honoraria from Caris Life Sciences.

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IMMUNOTHERAPY OF MALIGNANCY (MA MORSE, SECTION EDITOR)

# Modulation of Immune System Inhibitory Checkpoints in Colorectal Cancer

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**Abstract** T cell infiltration of colorectal cancer is associated with improved clinical outcome, underlining the importance of the immune system in cancer control; however, immune checkpoints, including the inhibitory T cell molecules CTLA-4 and PD-1 that temper the native immune response, mitigating autoimmunity, are coopted by tumors to facilitate escape from immune surveillance. Blockade of CTLA-4 and PD-1 and its ligand PD-L1, expressed by many tumors, have shown impressive activity in melanoma, renal cell carcinoma, and lung cancer. Immune checkpoint inhibition has been less well studied in colorectal cancer, but preclinical and clinical investigations are in progress.

**Keywords** Colorectal cancer · Immune checkpoint · CTLA-4 · PD-1 · PD-L1 · mCRC · Immunomodulation · Vaccine · Colon cancer · Ipilimumab · Tremelimumab · Nivolumab · Lambrolizumab

## Introduction

Colorectal cancer remains the second most common cause of cancer-related death in the US and fourth worldwide amongst men and women combined [1]. The standard management of advanced colorectal cancer has historically focused on multiagent chemotherapy. More recently, recognition of the interactions of the tumor and host microenvironment has led to the development of monoclonal antibodies targeting VEGF and EGFR, and the small molecule drug regorafenib targeting angiogenesis and the MAPK pathway [2–4]. However, durable results have been modest and novel therapeutic approaches are warranted.

Although tumors such as melanoma and renal cell carcinoma have been traditionally considered immunologic targets, it is clear that other solid tumors are immunologically responsive also. The immune response to colorectal cancer is associated with clinical outcome [5]. Furthermore, colorectal cancer cells remain susceptible to T cell-mediated killing despite prior exposure to chemotherapy or immunotherapy [6]. Modulation of the anti-tumor immune response through antigen vaccination, exogenous cytokine treatment, adoptive T cell transfer, dendritic cell immunotherapy, and combinations of the aforementioned modalities have been tested in metastatic colorectal cancer and have been reviewed elsewhere [7–9]. This review will focus on the burgeoning area of immune checkpoint inhibitors.

The discovery of immune checkpoints facilitating tumor escape from immune surveillance has led to the development of multiple novel agents that are either FDA-approved (ipilimumab targeting CTLA-4) or are in advanced stages of clinical development (nivolumab and lambrolizumab targeting PD-1) [10–12]. In particular, melanoma, renal cell carcinoma (RCC), and non-small cell lung cancer (NSCLC) have shown responses to immune checkpoint inhibition [13••, 14••]. More importantly, responses to this form of immunotherapy are often long-term and can result in durable complete remission (CR). For example, in patients treated with ipilimumab for melanoma, 15/177 (8 %) had a complete response and 14/15 of those patients continue to have responses 54+ months since therapy completion [15]. Thus, immune checkpoint inhibition not only shows promise in achieving CR in refractory patients, but inducing durable, long-term remissions also.

Investigation into immune checkpoint inhibition in gastrointestinal malignancies such as colorectal cancer is in progress on the basis of promising preclinical and early phase clinical trials. The purpose of this review is to summarize the current

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state of research related to immune checkpoint inhibition in colorectal cancer. A brief overview of the biology of immune checkpoints will lead into a discussion of the preclinical data related to the immunosuppressive colorectal tumor microenvironment and the use of immune checkpoint inhibitors to restore T cell function and tumor control. The current state of clinical development of immune checkpoint inhibitors will be highlighted with a focus on colorectal cancer.

## Biology of Immune Checkpoints in Cancer

Regulation of activated T cells is important in maintaining self-tolerance and preventing autoimmunity. After T cell receptor (TCR) engagement of its cognate MHC–peptide complex on an antigen-presenting cell or tumor ("Signal 1"), costimulatory or coinhibitory molecules modulate the amplitude of T cell activation ("Signal 2") [16]. Costimulatory interactions such as CD28-CD80/CD86, B7-ICOS, CD27-CD70, and OX40 and CD137 with their respective ligands amplify T cell responses. Conversely, higher affinity inhibitory CTLA-4 competitive binding of CD80/CD86 diminishes T cell response. Other inhibitory T cell-APC interactions include PD1-PD-L1/PD-L2, HVEM/BTLA, GAL9/TIM3, and TCR-LAG3 [17•]. These interactions are summarized in Fig. 1. Of these, the CTLA-4 and PD-1 axes are furthest along in clinical development.

CTLA-4 is involved in tempering the central immune response during the T cell activation stage through inhibition of effector T cells (Teff) and helper T cells, and activation of immunosuppressive regulatory T cells (Treg) where CTLA-4 is expressed [18]. The inhibitory effect of CTLA-4 on native immune regulation is substantial, as CTLA-4 knockout mice develop a fatal lymphoproliferative disorder [19]. Therapeutic anti-CTLA-4 antibody in a mouse colorectal model (B7-51BLim10) demonstrated single agent activity with durable complete responses to 70+ days [20].

PD-1 is another immune checkpoint expressed on T cells that is related to control of autoimmunity in the peripheral effector phase of T cell activation leading to immune tolerance in the presence of PD-L1 and PD-L2 [21]. PD-1, similar to CTLA-4, is also preferentially expressed on Tregs which contribute to the immunosuppressive tumor microenvironment [22]. PD-1/PD-L1 activation induces peripheral immunologic tolerance in T cells, resulting in a milder autoimmune phenotype in PD-1 and PD-L1 knockout mice relative to CTLA-4 knockouts. This observation correlates well with observed toxicity of anti-PD-1/anti-PD-L1 and anti-CTLA-4 therapeutic monoclonal antibodies in clinical trials [23]. PD-L1 is expressed in a variety of cell types including vascular endothelium, muscle, hepatocytes, pancreatic islet cells, placenta, epithelium, and mesenchymal stem cells, and on immune cells such as T cells, B cells, dendritic cells, macrophages, and bone marrow-derived mast cells [24]. PD-L2, another ligand for PD-1, has a more restricted expression pattern, predominantly on immune cells such as dendritic cells, macrophages, and bone marrow derived mast cells [24]. Both PD-L1 and PD-L2 are upregulated in the setting of interferon-gamma and are an "immune shield" for native cells to protect against autoimmune attack in the setting of chronic inflammation or infection, ultimately resulting in T cell exhaustion [25]. Multiple solid tumor types—melanoma, RCC, NSCLC, glioma, thymoma, ovarian, and colorectal cancer among others—have coopted this defense mechanism and express PD-L1 to facilitate an immunosuppressive tumor microenvironment and avoid T cell cytolysis [26–29]. This

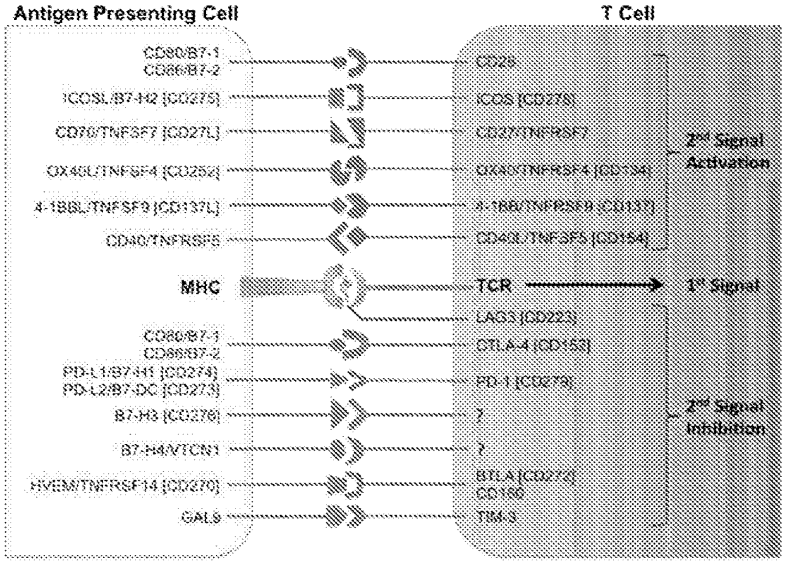



**Fig. 1** T cell immune checkpoint regulation. Overview of known T cell immune checkpoint modulation. Antigen presenting cell (potentially tumor cell) in *yellow*, T cell in *blue*. MHC-TCR interaction represents signal 1. *Green-colored* receptor interactions represent positive costimulatory signal 2 interactions that amplify T cell responses. *Red-colored* receptor interactions represent negative costimulatory signal 2 interactions that downregulate T cell responses



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process of coopting the innate immune system shield results in poorer prognosis in multiple solid tumor types and is thus an attractive target for novel therapeutic intervention [30–33].

## Preclinical Data on Immune Checkpoints in Colorectal Cancer

### Immune Microenvironment

The colorectal cancer microenvironment is populated by a myriad of immune cells whose constitution and function are incompletely understood. Clinically, it has been observed that patients with microsatellite unstable primary tumors, associated with a superior prognosis, have higher levels of tumor-infiltrating lymphocytes (TILs) [34]. Development in the primary tumor of a lymphocytic ("Crohn's-like") reaction is also associated with an improved prognosis, irrespective of lymph node involvement [35]. Furthermore, analysis of the immune infiltrate in human colorectal cancer specimens have resulted in multiple observations that have led to increased comprehension of the various immune compartments involved. The role of effector memory T cells (CD45RO+CD8+) T cells as a protective Th1 response to maintain local control of primary tumor has been reported [36, 37•, 38]. The effect of this T cell compartment on prognosis is substantial, and outperformed tumor stage in terms of prognosis for stage 1 and 2 patients. Paradoxically, FoxP3+ Treg infiltrate has been associated with improved prognosis in colorectal cancer, despite being associated with poorer prognosis in almost all other solid tumor types [39]. Whether this represents a functional Treg compartment (natural vs. innate Treg), a form of immunologic surveillance related to the gut microbiome, or other unknown factor remains to be seen. However, the importance of the immune infiltrate in tumor control in colorectal cancer is reflected by differences in prognosis related to the makeup of the T cell immune infiltrate.

### CTLA-4

There have been multiple preclinical colorectal cancer models of immune checkpoint inhibition with promising efficacy. Indeed, one of the first demonstrations of anti-CTLA-4 therapy was in the mouse colorectal model B7-51Blim10 in a BALB/c mouse by Allison and colleagues [20]. In a mouse MCA38 colon cancer model treated with an anti-CTLA-4 antibody, an abscopal effect was demonstrated with fractionated local radiation [40]. An abscopal effect occurs when local radiation leads to a systemic response to tumor, associated with systemic immune activation that is potentiated by immune checkpoint inhibition. Combinatorial immune checkpoint blockade with anti-CTLA-4 and anti-4-1BB has also been shown to enhance anticancer T cell activity in a MC38 mouse model [41].

### PD-1/PD-L1

The PD-1/PD-L1 axis has also been investigated in preclinical solid tumor and colorectal cancer models [42]. Overall, approximately 64 % of screened human cancer cell lines were positive for PD-L1 (with positive defined in general as >5 % expression on IHC utilizing the 5H1 anti-PD-L1 antibody). Early phase clinical data suggested that PD-L1 expression would be an important predictive marker for response to anti-PD-1 therapy [43]. For example, an analysis of 116 patients with colorectal cancer found that PD-L1 expression on tumors was inversely correlated with CD8+ T cells and that intratumoral PD-1+ T cells were associated with advanced tumor stage and poorer prognosis on retrospective review [44]. Upregulation of PD-1 in the immunosuppressive colon cancer microenvironment has been demonstrated in CT26 mouse models, where IL-18 upregulated PD-1 leading to increased colorectal metastasis [45]. In a CT26 colon cancer model in BALB/c mice, treatment with anti-PD-1 antibody reduced lung metastases [46]. Combinatorial therapy with immunostimulatory cytokine IL-15 with anti-PD-L1 resulted in increased CTL killing of tumors and improved survival in a metastatic CT26 colon cancer mouse model [47]. Additionally, dual immune checkpoint inhibition of PD-L1 and Tim-3 resulted in robust synergistic responses in another CT26 colon cancer model, demonstrating the potential for effective combinatorial immune checkpoint blockade [48, 49].

The interaction of conventional chemotherapeutics and targeted therapy utilized in colorectal cancer and their effect on the PD-1/PD-L1 axis is increasingly being recognized. Human plasmacytoid dendritic cells exposed to oxaliplatin upregulated PD-L1, possibly decreasing their immunostimulatory function [50]. Use of an anti-PD-L1 ligand antibody would therefore be of interest in combination with oxaliplatin-based therapy. Human monocyte-derived dendritic cells exposed to oxaliplatin downregulated PD-L1 and especially PD-L2, resulting in an increase in T cell recognition of tumor cells [51]. Thus, the effect of oxaliplatin and other platinum compounds on the tumor immune microenvironment is context specific and likely dependent on the presence and ratio of plasmacytoid versus monocyte-derived dendritic cell compartments, and likely several other immune cell types.

Therapeutic monoclonal antibodies seem to modulate the PD-1/PD-L1 axis also. Cetuximab has been found to activate immunosuppressive M2 macrophages, which are abundant in colorectal carcinomas, with an increase in PD-L1 expression on M2 macrophages and VEGF and IL-10 production [52]. M2 macrophage-mediated immunosuppressive activity was especially pronounced in high affinity FcγRIIIA which correlates with the clinical finding from the CAIRO-2 trial that patients with high affinity FcγRIIIA had poorer progression-free survival with dual cetuximab and bevacizumab therapy with chemotherapy [53]. A recent evaluation of patients with mCRC



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found that bevacizumab with chemotherapy reduced the proportion of circulating PD-1+ CD4+ T cells. Sunitinib, but not VEGF-sparing masitinib, resulted in a decrease in circulating PD1+ Tim-3+ T cells in a CT26 colorectal mouse model, suggesting that anti-VEGF directed therapy target exhausted T cell subsets, representing an attractive combinatorial approach for anti-VEGF/anti-PD-1 directed therapy [54].

### Other Immune Checkpoints in Colorectal Cancer

The OX40 (CD134)/OX40L axis is an immunostimulatory pathway that has been investigated in colorectal cancer because of the association between high levels of OX-40+ TIL and improved survival in colorectal cancer [55]. A combinatorial approach in a MCA26 hepatic colorectal metastasis model with an immunostimulatory OX40 and 4-1BB antibodies, and IL-12 resulted in a significant increase in memory CTL response with durable complete remission in mice, emphasizing the importance of the effector memory T cell compartment in colorectal cancer [56]. Similarly 4-1BB ligand plus IL-12 had similar results in a MCA26 mouse model at inducing long-term remissions of liver metastasis [57].

## Overview of Immune Checkpoint Inhibitors in Clinical Development for Colorectal Cancer

Ipilimumab is currently FDA-approved for treatment of metastatic melanoma, and additional studies in RCC and NSCLC are currently in progress [10, 58, 59]. In colorectal cancer, there is an active phase 2 trial assessing intratumoral CTLA-4 injection with local therapy, which is of particular interest given reports of a systemic abscopal response in other tumor types [60].

Tremelimumab, a monoclonal antagonistic antibody to CTLA-4, was initially developed for treatment of advanced solid tumors on the basis of promising preclinical and early phase clinical trials. A phase 2 trial of tremelimumab monotherapy in 47 patients with metastatic colon cancer was reported negative, with 43 patients not receiving the second dose of agent because of RECIST progression. Only one patient received more than one dose of tremelimumab, and that patient had a partial response for six months after receiving five doses of therapy [61]. Of note, immune-related response criteria, which require confirmation of progression on a subsequent scan performed four weeks later, were not used in this trial and it is unknown if utilization of traditional RECIST progression criteria resulted in premature discontinuation of therapy for patients who may otherwise have ultimately benefited from CTLA-4 blockade [62••, 63••]. After a negative phase 3 clinical trial in melanoma, and with the approval of ipilimumab, tremelimumab development has been halted at the current time [64].

Interest in PD-1 inhibition stemmed from a phase 1 trial looking at anti-PD-1 (nivolumab; BMS-936558; MDX-1106) in 39 patients, of which 14 had metastatic colorectal cancer (mCRC). One long-term complete response was observed amongst the 14 colorectal patients [65]. The larger phase 1 study of MDX-1106 with 296 patients had 19 mCRC patients, with no responses. Seven mCRC specimens were available for PD-L1 expression analysis on tumor, of which only one colorectal specimen was positive for PD-L1, but at a low level of 5 % in the one primary specimen (and a second primary specimen was negative). This observation is likely of some significance, as none of the 17 patients (among all tumor types evaluated) with PD-L1 negative tumors had a response to anti-PD-1 therapy, while of the 25 patients with positive PD-L1 tumors, 36 % had an objective response [13••]. Thus far, there are limited data on concordance of PD-L1 expression in primary versus metastatic tumor sites, and the clinical significance of discordant expression remains unclear.

In a large phase 1 trial of anti-PD-L1 (BMS-936559) evaluating 207 patients with refractory solid tumors, 18 had mCRC of

**Table 1** Active Clinical Trials with Immune Checkpoint Inhibitors. Overview of active clinical trials related to immune checkpoint modulation from clinicaltrials.gov

| Agent | Company | Stage of development |
|---|---|---|
| Ipilimumab (anti-CTLA4) | BMS | FDA-approved for metastatic melanoma |
| | | Phase 2 NSCLC with sequential carboplatin/paclitaxel |
| | | Phase 2 prostate with ADT |
| | | Phase 1/2 prostate |
| Nivolumab (BMS-936558; anti-PD1) | BMS | Phase 3 melanoma |
| BMS-936559 (anti-PD-L1) | BMS | Phase 1 solid tumors |
| Lambrolizumab (MK-3475; anti-PD-1) | Merck | Phase 1/2 refractory melanoma. |
| CT-011 (anti-PD-1) | CureTech | Recruiting Phase 1 in melanoma, lymphoma, myeloma, mCRC |
| MPDL3280A (anti-PD-L1) | Roche | Phase 1 melanoma, solid tumors |
| MEDI4736 (anti-PD-L1) | Medimmune | Phase 1 |

BMS, Bristol-Myers Squibb; NSCLC, non-small cell lung cancer; ADT, androgen deprivation therapy



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which none had any objective response [14••]. Other phase 1 studies of anti-PD-L1 agents (such as MPDL3280A) are in progress in advanced solid tumors. In addition, other inhibitory monoclonal antibodies to immune checkpoints such as LAG-3, TIM-3, B7-H3, B7-H4, and others are in development [16] (Table 1).

## Conclusion

Immune checkpoint inhibition is a powerful strategy in oncology with the potential for long-term remission on the basis of results for melanoma, RCC, and NSCLC. The utility of this strategy in GI malignancies, particularly colon cancer, remains unknown with promising preclinical data but with a paucity of impressive responses in early phase clinical trials to date. However, an improving understanding of the delayed nature of immunologic responses with immune checkpoint inhibition and predictive biomarkers, particularly related to anti-PD-1, should enable improved patient selection.

The colorectal cancer microenvironment is richly populated by tumor infiltrating lymphocytes and various suppressive elements including Tregs, MDSCs, and immunosuppressive tumor associated macrophages. Utilizing selective inhibition of CTLA-4, PD-1, PD-L1, and other immune checkpoints to alter these immune compartments to improve tumor control and tilt the immune microenvironment towards a more favorable prognosis is an important objective in this disease. The importance of a robust immune response in colorectal cancer is seen in patients with MSI-H tumors and those with a Crohn's-like lymphocytic reaction, who have a superior prognosis. Indeed, the nature of the immune microenvironment, with particular emphasis on the positive prognosis that CD8+ T cells and effector memory T cells confer, has a potent effect on prognosis on par with conventional tumor staging.

The benefits of a broad multidisciplinary approach, with initial cytoreduction with surgery and chemotherapy/targeted therapy, followed by combinations of immunomodulatory compounds (such as immune checkpoint inhibitors) and tumor vaccines to achieve durable remissions, are only beginning to be investigated. Preclinical studies suggest areas where there may be interactions between therapy. Use of a PD-1 or PD-L1 antibody would therefore be of interest in combination with oxaliplatin-and bevacizumab-based therapy. The success of immune checkpoint inhibition against a variety of solid tumors indicates the broad applicability of this strategy, and more importantly, the need for better predictive markers in immunotherapy. The effects of protective immunity in colorectal cancer are already seen by patients with an innate predisposition to cytotoxic and effector memory Th1-type responses—those patients with MSI-H tumors and Crohn's-like lymphocytic reaction. The objective of immune checkpoint inhibition and immunotherapy is to further modulate the tumor microenvironment to confer similar durable immune protection to all patients afflicted with colorectal cancer.

### Compliance with Ethics Guidelines

**Conflict of Interest** Sandip P. Patel declares that he has no conflict of interest. Takuya Osada declares that he has no conflict of interest. Koya Osada declares that he has no conflict of interest. Herbert Hurwitz has received compensation for service as a consultant and is supported by grants from Roche, Genentech, Bristol-Myers Squibb, Sanofi-Aventis, Amgen, Regeneron, Novartis, TRACON, and Threshold Pharmaceuticals. H. Kim Lyerly declares that he has no conflict of interest. Michael A. Morse is supported be a grant from Bristol-Myers Squibb, and has received payment for lectures including service on speakers bureaus from Genentech.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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Papers of particular interest, published recently, have been highlighted as:

- • Of importance
- •• Of major importance


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*establishment of PD-L1 on tumor as putative predictive biomarker for response.*

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ClinicalTrials.gov archive

# History of Changes for Study: NCT02060188

## A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142)

Latest version (submitted November 4, 2019) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare. One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of the study.
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in ~~red~~.
- Study additions are displayed in *green*.

## Study Record Versions

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 1 | ○ | ○ | February 10, 2014 | None (earliest Version on record) |
| 2 | ○ | ○ | March 25, 2014 | Recruitment Status, Study Status, Contacts/Locations and Study Description |
| 3 | ○ | ○ | March 28, 2014 | Contacts/Locations and Study Status |
| 4 | ○ | ○ | April 29, 2014 | Contacts/Locations and Study Status |
| 5 | ○ | ○ | May 2, 2014 | Study Status |
| 6 | ○ | ○ | May 7, 2014 | Contacts/Locations, References and Study Status |
| 7 | ○ | ○ | May 27, 2014 | Contacts/Locations and Study Status |
| 8 | ○ | ○ | May 30, 2014 | Study Status |
| 9 | ○ | ○ | June 12, 2014 | Contacts/Locations and Study Status |
| 10 | ○ | ○ | June 25, 2014 | Contacts/Locations and Study Status |
| 11 | ○ | ○ | July 9, 2014 | Contacts/Locations and Study Status |
| 12 | ○ | ○ | July 23, 2014 | Contacts/Locations and Study Status |
| 13 | ○ | ○ | August 11, 2014 | Contacts/Locations and Study Status |
| 14 | ○ | ○ | August 28, 2014 | Contacts/Locations and Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 15 | ○ | ○ | September 15, 2014 | Contacts/Locations and Study Status |
| 16 | ○ | ○ | October 13, 2014 | Contacts/Locations and Study Status |
| 17 | ○ | ○ | October 30, 2014 | Contacts/Locations and Study Status |
| 18 | ○ | ○ | November 13, 2014 | Contacts/Locations and Study Status |
| 19 | ○ | ○ | November 20, 2014 | Contacts/Locations and Study Status |
| 20 | ○ | ○ | December 5, 2014 | Contacts/Locations and Study Status |
| 21 | ○ | ○ | December 31, 2014 | Study Status |
| 22 | ○ | ○ | January 27, 2015 | Contacts/Locations and Study Status |
| 23 | ○ | ○ | February 6, 2015 | Contacts/Locations and Study Status |
| 24 | ○ | ○ | February 19, 2015 | Study Status |
| 25 | ○ | ○ | March 5, 2015 | Study Status |
| 26 | ○ | ○ | March 23, 2015 | Study Status |
| 27 | ○ | ○ | April 13, 2015 | Study Status and Contacts/Locations |
| 28 | ○ | ○ | April 20, 2015 | Contacts/Locations and Study Status |
| 29 | ○ | ○ | June 3, 2015 | Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 30 | | | June 4, 2015 | Study Status and Contacts/Locations |
| 31 | | | June 22, 2015 | Study Status |
| 32 | | | July 3, 2015 | Study Status |
| 33 | | | July 17, 2015 | Contacts/Locations and Study Status |
| 34 | | | August 6, 2015 | Contacts/Locations and Study Status |
| 35 | | | September 3, 2015 | Contacts/Locations and Study Status |
| 36 | | | September 16, 2015 | Study Status |
| 37 | | | October 15, 2015 | Study Status |
| 38 | | | November 4, 2015 | Study Status |
| 39 | | | December 8, 2015 | Study Status |
| 40 | | | December 21, 2015 | Study Status |
| 41 | | | January 6, 2016 | Study Status |
| 42 | | | January 21, 2016 | Contacts/Locations and Study Status |
| 43 | | | January 26, 2016 | Contacts/Locations and Study Status |
| 44 | | | February 10, 2016 | Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 45 | ○ | ○ | February 25, 2016 | Study Status |
| 46 | ○ | ○ | March 11, 2016 | Study Status |
| 47 | ○ | ○ | March 28, 2016 | Study Status |
| 48 | ○ | ○ | April 12, 2016 | Study Status |
| 49 | ● | ● | May 10, 2016 | Study Status |
| 50 | ○ | ○ | May 25, 2016 | Contacts/Locations and Study Status |
| 51 | ○ | ○ | June 24, 2016 | Study Status |
| 52 | ○ | ○ | June 28, 2016 | Study Status |
| 53 | ○ | ○ | July 11, 2016 | Study Status |
| 54 | ○ | ○ | July 26, 2016 | Study Status |
| 55 | ○ | ○ | August 8, 2016 | Study Status and Study Design |
| 56 | ○ | ○ | August 23, 2016 | Study Status |
| 57 | ○ | ○ | September 7, 2016 | Study Status |
| 58 | ○ | ○ | September 23, 2016 | Study Status |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 59 | | | October 14, 2016 | Arms and Interventions, Study Description, Study Status, Contacts/Locations and Eligibility |
| 60 | | | October 25, 2016 | Contacts/Locations, Study Design and Study Status |
| 61 | | | December 6, 2016 | Contacts/Locations and Study Status |
| 62 | | | December 13, 2016 | Study Status and Contacts/Locations |
| 63 | | | December 23, 2016 | Contacts/Locations and Study Status |
| 64 | | | January 6, 2017 | Arms and Interventions, Outcome Measures, Study Status, Study Identification, Eligibility, Study Design and Study Description |
| 65 | | | January 27, 2017 | Study Status |
| 66 | | | February 16, 2017 | Contacts/Locations and Study Status |
| 67 | | | February 26, 2017 | Contacts/Locations, Study Status and References |
| 68 | | | March 3, 2017 | Study Status, References and Contacts/Locations |
| 69 | | | March 21, 2017 | Study Status, Oversight, Study Identification, References, Contacts/Locations and Study Description |
| 70 | | | March 29, 2017 | Study Status, References and Contacts/Locations |
| 71 | | | April 17, 2017 | Study Status, Contacts/Locations and Study Design |
| 72 | | | May 18, 2017 | Study Status, References, Contacts/Locations, Eligibility, Conditions and Study |

| Version | | | Submitted Date | Description of Changes |
|---|---|---|---|---|
| 73 | | | June 1, 2017 | Conditions and Study Status |
| 74 | | | June 8, 2017 | References, Contacts/Locations and Study Status |
| 75 | | | June 26, 2017 | Contacts/Locations, References and Study Status |
| 76 | | | July 24, 2017 | Study Status |
| 77 | | | August 2, 2017 | Study Status |
| 78 | | | September 1, 2017 | Study Status and Contacts/Locations |
| 79 | | | September 14, 2017 | Contacts/Locations and Study Status |
| 80 | | | October 3, 2017 | Study Status |
| 81 | | | November 3, 2017 | Study Status |
| 82 | | | December 11, 2017 | Contacts/Locations and Study Status |
| 83 | | | January 9, 2018 | Study Status and Contacts/Locations |
| 84 | | | January 18, 2018 | Contacts/Locations and Study Status |
| 85 | | | January 26, 2018 | Contacts/Locations and Study Status |
| 86 | | | February 22, 2018 | Study Status and Contacts/Locations |

| Version | A | B | Submitted Date | Changes |
|---|---|---|---|---|
| 87 | | | March 26, 2018 | Study Status and Contacts/Locations |
| 88 | | | April 27, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 89 | | | November 6, 2018 | Study Status and References |
| 90 | | | November 19, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 91 | | | December 3, 2018 | Recruitment Status, Contacts/Locations and Study Status |
| 92 | | | June 5, 2019 | Study Status |
| 93 | | | November 4, 2019 | Study Status |

Compare

Comparison Format: Merged Side-by-Side

Scroll up to access the controls

**Study NCT02060188**

**Submitted Date: May 10, 2016 (v49)**

## Study Identification

| | |
|---|---|
| Unique Protocol ID: | CA209-142 |
| Brief Title: | A Study of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Colon Cancer (CheckMate 142) |
| Official Title: | A Phase 2 Clinical Trial of Nivolumab and Nivolumab Plus Ipilimumab in Recurrent and Metastatic Microsatellite High (MSI-H) Colon Cancer |
| Secondary IDs: | 2013-003939-30 [EudraCT Number] |

## Study Status

| | |
|---|---|
| Record Verification: | December 2015 |
| Overall Status: | Recruiting |
| Study Start: | March 2014 |
| Primary Completion: | September 2016 [Anticipated] |
| Study Completion: | August 2017 [Anticipated] |
| First Submitted: | December 18, 2013 |
| First Submitted that Met QC Criteria: | February 10, 2014 |
| First Posted: | February 11, 2014 [Estimate] |
| Last Update Submitted that Met QC Criteria: | May 10, 2016 |
| Last Update Posted: | May 12, 2016 [Estimate] |

## Sponsor/Collaborators

Sponsor: Bristol-Myers Squibb

Responsible Party: Sponsor

Collaborators:

## Oversight

U.S. FDA-regulated Drug:

U.S. FDA-regulated Device:

Data Monitoring: No

## Study Description

Brief Summary: The purpose of this study is to examine if Nivolumab alone or in combination with Ipilimumab will demonstrate a meaningful objective response rate in patients with recurrent and metastatic colon cancer who also have a specific biomarker in their tumors.

Detailed Description: Allocation: The Microsatellite Instability High (MSI-High) Part of the trial is Non-randomized, The Non-MSI high part of the trial contains a randomized portion

## Conditions

Conditions: MSI Positive Colorectal Cancer
MSI Negative Colorectal Cancer

Keywords:

## Study Design

| | |
|---|---|
| Study Type: | Interventional |
| Primary Purpose: | Treatment |
| Study Phase: | Phase 1/Phase 2 |
| Interventional Study Model: | Parallel Assignment |
| Number of Arms: | 2 |
| Masking: | None (Open Label) |
| Allocation: | Non-Randomized |
| Enrollment: | 96 [Anticipated] |

## Arms and Interventions

| Arms | Assigned Interventions |
|---|---|
| Experimental: Nivolumab Monotherapy<br>Nivolumab administered as IV infusion at a dose of 3mg/kg every 2 weeks until disease progression | Drug: Nivolumab<br>Other Names:<br>• BMS-936558 |

| Arms | Assigned Interventions |
| --- | --- |
| Experimental: Nivolumab + Ipilimumab<br>Dose Escalation Phase:<br>Dose Level -1: Nivolumab 0.3mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 1: Nivolumab 1mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 2a: Nivolumab 1mg/Kg IV combined with Ipilimumab 3 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression<br>Dose Level 2b: Nivolumab 3mg/Kg IV combined with Ipilimumab 1 mg/Kg IV every 3 weeks for 4 doses followed by Nivolumab 3mg/Kg IV every 2 weeks until disease progression | Drug: IpilimumabDrug: Nivolumab<br>Other Names:<br>• BMS-936558 |

## Outcome Measures

Primary Outcome Measures:

1. Objective response rate (ORR) in all MSI-High subjects as determined by Investigators
   (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later)) (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later))

   [Time Frame: The final analysis of the primary endpoint will occur at least 6 months after the last enrolled subject's first dose of study therapy (Approximately up to 34 months)]

Secondary Outcome Measures:

2. ORR in all MSI-H subjects based on IRRC determination
   Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/- wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued(whichever occurs later)

   [Time Frame: The final analysis of the secondary endpoint will occur the time of the primary endpoint analysis (Approximately up to 34 months)]

## Eligibility

Minimum Age: 18 Years

Maximum Age:

Sex: All

Gender Based:

Accepts Healthy Volunteers: No

Criteria: For more information regarding BMS clinical trial participation, please visit www.BMSStudyConnect.com

Inclusion Criteria:

- Men and women ≥ 18 years of age
- Eastern Cooperative Oncology Group (ECOG) performance status 0 to 1
- Histologically confirmed colorectal cancer
- Measurable disease by CT or MRI
- Testing for MSI Status
- Adequate organ function as defined by study-specific laboratory tests
- Must use acceptable form of birth control throughout the study. After the final dose of study drug, an acceptable form of birth control must be used for 23 weeks for women of childbearing potential (WOCBP) and 31 weeks for men who are sexually active with WOCBP
- Signed informed consent
- Willing and able to comply with study procedures

Exclusion Criteria:

- Active brain metastases or leptomeningeal metastases are not allowed.
- Prior treatment with an anti-Programmed Death Receptor (PD)-1, anti-PD-L1, anti-PD-L2, anti-Cytotoxic T-Cell Lymphoma-4 Antigen (CTLA-4) antibody, or any other antibody or drug specifically targeting T-cell co-stimulation or immune checkpoint pathways

- Prior malignancy active within the previous 3 years except for locally curable cancers
- Subjects with active, known or suspected autoimmune disease
- Subjects with a condition requiring systemic treatment with either corticosteroids or other immunosuppressive medications within 14 days of study drug administration

## Contacts/Locations

Central Contact Person: Recruiting sites have contact information. Please contact the sites directly. If there is no contact information, please email:
Email: Clinical.Trials@bms.com

Central Contact Backup: First line of the email MUST contain NCT# and Site #.

Study Officials: Bristol-Myers Squibb
Study Director
Bristol-Myers Squibb

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Citations:

Links: URL: http://www.bms.com/studyconnect/Pages/home.aspx
Description: BMS clinical trial educational resource

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Espacenet

# Bibliographic data: CN110505882 (A) — 2019-11-26

## COMPOSITIONS AND METHODS FOR TREATING CANCER WITH COMBINATION OF ANTAGONIST OF PD-1 AND ANTI-CTLA4 ANTIBODY

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**Applicant(s):** MERCK SHARP & DOHME ± (MERCK SHARP & DOHME)

**Classification:** **- international:** ***A61K39/395; A61P35/00; A61P35/04; C07K16/28; C07K16/30***

**- cooperative:** **A61P35/00 (EP, US); A61P35/04 (EP); C07K16/2818 (EP, US);** A61K2039/507 (EP, US); A61K2039/545 (EP, US); C07K2317/24 (EP, US); C07K2317/565 (US); C07K2317/76 (EP, US)

**Application number:** CN20188022473 20180328 Global Dossier

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**Also published as:** EP3600426 (A1) EP3600426 (A4) JP2020512354 (A) US2020115451 (A1) WO2018183408 (A1)

### Abstract of CN110505882 (A)

The present invention relates to methods for treating cancer comprising administering an antagonist of PD-1, e.g., anti-PD-1 antibody or antigen binding fragment thereof, and an anti-CTLA4 antibody orantigen binding fragment thereof, wherein the CTLA4 antibody or antigen binding fragment thereof is given at a fixed dose. Also provided are compositions and kits comprising a dosage of an anti-PD-1antibody and a dosage of an anti-CTLA4 antibody, and uses thereof.

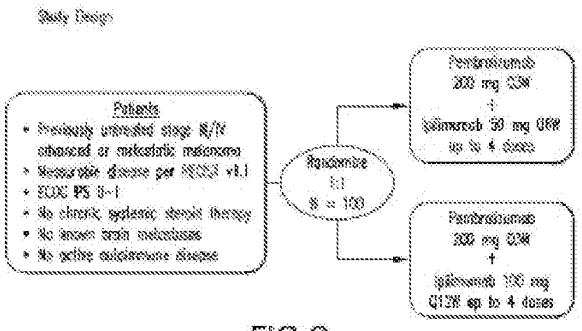



FIG.8

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*A61P 35/04*(2006.01)

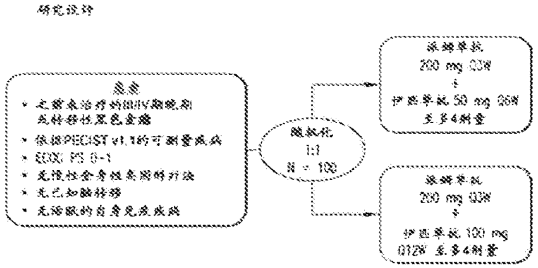
权利要求书5页 说明书41页
序列表92页 附图10页

(54)发明名称

用PD-1的拮抗剂和抗CTLA4抗体的组合治疗癌症的组合物和方法

(57)摘要

本发明涉及用于治疗癌症的方法，其包括施用PD-1的拮抗剂，例如抗PD-1抗体或其抗原结合片段，和抗CTLA4抗体或其抗原结合片段，其中CTLA4抗体或其抗原结合片段以固定剂量给予。还提供了包含抗PD-1抗体的剂量和抗CTLA4抗体的剂量的组合物和试剂盒，及其用途。



CN 110505882 A

1.一种治疗人类患者的癌症的方法,其包括施用:

(a)200mg或240mg抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段;或

(b)2mg/kg抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段,

其中所述抗CTLA4抗体或其抗原结合片段的量选自10mg、25mg、50mg和75mg,并且其中所述抗PD-1抗体或其抗原结合片段每三周施用一次,并且所述抗CTLA4抗体或其抗原结合片段每三周施用一次或每六周施用一次。

2.一种治疗人类患者的癌症的方法,其包括施用:

(a)200mg或240mg抗PD-1抗体或其抗原结合片段和75mg或100mg抗CTLA4抗体或其抗原结合片段;或

(b)2mg/kg抗PD-1抗体或其抗原结合片段和75mg或100mg抗CTLA4抗体或其抗原结合片段,

其中所述抗PD-1抗体或其抗原结合片段每三周施用一次,并且所述抗CTLA4抗体或其抗原结合片段每十二周施用一次。

3.根据权利要求1所述的方法,其包括施用200mg抗PD-1抗体和50mg抗CTLA4抗体。

4.根据权利要求2所述的方法,其包括施用200mg抗PD-1抗体和100mg抗CTLA4抗体。

5.根据权利要求1所述的方法,其包括施用200mg抗PD-1抗体和25mg抗CTLA4抗体。

6.根据权利要求1或权利要求2所述的方法,其包括施用200mg抗PD-1抗体和75mg抗CTLA4抗体。

7.根据权利要求1所述的方法,其包括施用2mg/kg抗PD-1和50mg抗CTLA4抗体。

8.根据权利要求2所述的方法,其包括施用2mg/kg抗PD-1抗体和100mg抗CTLA4抗体。

9.根据权利要求1所述的方法,其包括施用200mg抗PD-1抗体和25mg抗CTLA4抗体。

10.根据权利要求1或权利要求2所述的方法,其包括施用200mg抗PD-1抗体和75mg抗CTLA4抗体。

11.根据权利要求1、3、5-7、9或10中任一项所述的方法,其中所述抗CTLA4抗体或其抗原结合片段每六周施用一次,持续24周或更短。

12.根据权利要求2、4、6、8或10中任一项所述的方法,其中所述抗CTLA4抗体或其抗原结合片段每十二周施用一次,持续48周或更短。

13.根据权利要求1-12中任一项所述的方法,其还包括在停止施用所述抗CTLA4抗体后继续每三周一次施用所述抗PD-1抗体。

14.根据权利要求1-13中任一项所述的方法,其中所述癌症是黑色素瘤、非小细胞肺癌、头颈癌、尿路上皮癌、乳腺癌、胃肠癌、多发性骨髓瘤、肝细胞癌、非霍奇金淋巴瘤、肾癌、霍奇金淋巴瘤、间皮瘤、卵巢癌、小细胞肺癌、食道癌、肛门癌、胆道癌、结直肠癌、宫颈癌、甲状腺癌或唾液癌。

15.根据权利要求1-14中任一项所述的方法,其中所述抗PD-1抗体是派姆单抗、纳武单抗、或与派姆单抗或纳武单抗交叉竞争以结合人PD-1的抗体。

16.根据权利要求1-15中任一项所述的方法,其中所述抗CTLA4抗体选自:

(a)伊匹单抗,

(b)曲美木单抗,

(c)8D2/8D2(RE)或其变体,

(d) 8D2H1L1或其变体，

(e) 8D2H2L2或其变体，

(f) 8D3H3L3或其变体，

(g) 8D2H2L15或其变体，

(h) 8D2H2L17或其变体，

(i) 4G10H1L1，

(j) 4G10H3L3，

(k) 4G10H4L3，

(l) 4G10H5L3，和

(m) 与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体；

其中所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体和所述8D2H2L17变体的可变重链(VH)氨基酸序列的第18位处的甲硫氨酸独立地被选自亮氨酸、缬氨酸、异亮氨酸和丙氨酸的氨基酸置换。

17.根据权利要求16所述的方法，其中所述抗CTLA4抗体是8D2/8D2(RE)或其变体、8D2H1L1或其变体、8D2H2L2或其变体、8D3H3L3或其变体、8D2H2L15或其变体、或8D2H2L17或其变体。

18.根据权利要求17所述的方法，其中抗CTLA4抗体是8D2/8D2(RE)的变体、8D2H1L1的变体、8D2H2L2的变体、8D3H3L3的变体、8D2H2L15的变体、或8D2H2L17的变体。

19.根据权利要求18所述的方法，其中所述抗CTLA4抗体是8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

20.根据权利要求1-19中任一项所述的方法，其中所述癌症是非小细胞肺癌或黑色素瘤。

21.一种组合物，其包含抗PD-1抗体或其抗原结合片段的剂量和抗CTLA4抗体或其抗原结合片段的剂量，其中所述剂量选自：

(i) 200mg或240mg抗PD-1抗体或其抗原结合片段和10mg抗CTLA4抗体或其抗原结合片段；

(ii) 200mg或240mg抗PD-1抗体或其抗原结合片段和25mg抗CTLA4抗体或其抗原结合片段；

(iii) 200mg或240mg抗PD-1抗体或其抗原结合片段和50mg抗CTLA4抗体或其抗原结合片段；

(iv) 200mg或240mg抗PD-1抗体或其抗原结合片段和75mg抗CTLA4抗体或其抗原结合片段；

(v) 200mg或240mg抗PD-1抗体或其抗原结合片段和100mg抗CTLA4抗体或其抗原结合片段；

(vi) 2mg/kg抗PD-1抗体或其抗原结合片段和10mg抗CTLA4抗体或其抗原结合片段；

(vii) 2mg/kg抗PD-1抗体或其抗原结合片段和25mg抗CTLA4抗体或其抗原结合片段；

(viii) 2mg/kg抗PD-1抗体或其抗原结合片段和50mg抗CTLA4抗体或其抗原结合片段；

(ix) 2mg/kg抗PD-1抗体或其抗原结合片段和75mg抗CTLA4抗体或其抗原结合片段；和

(x) 2mg/kg抗PD-1抗体或其抗原结合片段和100mg抗CTLA4抗体或其抗原结合片段。

22.根据权利要求21所述的组合物，其中所述抗PD-1抗体是派姆单抗、纳武单抗、或与派姆单抗或纳武单抗交叉竞争以结合人PD-1的抗体。

23.根据权利要求21或权利要求22所述的组合物，其中所述抗CTLA4抗体选自：

(a)伊匹单抗，

(b)曲美木单抗，

(c)8D2/8D2(RE)或其变体，

(d)8D2H1L1或其变体，

(e)8D2H2L2或其变体，

(f)8D3H3L3或其变体，

(g)8D2H2L15或其变体，

(h)8D2H2L17或其变体，

(i)4G10H1L1，

(j)4G10H3L3，

(k)4G10H4L3，

(l)4G10H5L3，和

(m)与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体；

其中所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体和所述8D2H2L17变体的可变重链(VH)氨基酸序列的第18位处的甲硫氨酸独立地被选自亮氨酸、缬氨酸、异亮氨酸和丙氨酸的氨基酸置换。

24.根据权利要求21-23中任一项所述的组合物，其中所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是伊匹单抗。

25.根据权利要求21-23中任一项所述的组合物，其中所述抗PD-1抗体是派姆单抗和所述抗CTLA-4抗体是伊匹单抗。

26.根据权利要求21-23中任一项所述的组合物，其中所述抗PD-1抗体是纳武单抗和所述抗CTLA-4抗体选自8D2/8D2(RE)或其变体、8D2H1L1或其变体、8D2H2L2或其变体、8D3H3L3或其变体、8D2H2L15或其变体、或8D2H2L17或其变体，其中在所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体和所述8D2H2L17变体的所述可变重链(VH)氨基酸序列的第18位处的甲硫氨酸被异亮氨酸置换。

27.根据权利要求26所述的组合物，其中所述抗PD-1抗体是纳武单抗和所述抗CTLA-4抗体选自8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

28.根据权利要求21-23所述的组合物，其中所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体、或所述8D2H2L17变体，其中所述抗CTLA4抗体的所述可变重链(VH)氨基酸序列的第18位处的甲硫氨酸被异亮氨酸置换。

29.根据权利要求28所述的组合物，其中所述抗PD-1抗体是派姆单抗和所述抗CTLA-4抗体选自8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

30.一种用于治疗患有癌症的患者的试剂盒，所述试剂盒包括：



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(a)抗PD-1抗体或其抗原结合片段的剂量和抗CTLA4抗体或其抗原结合片段的剂量，其选自：

(i)200mg或240mg抗PD-1抗体或其抗原结合片段和10mg抗CTLA4抗体或其抗原结合片段；

(ii)200mg或240mg抗PD-1抗体或其抗原结合片段和25mg抗CTLA4抗体或其抗原结合片段；

(iii)200mg或240mg抗PD-1抗体或其抗原结合片段和50mg抗CTLA4抗体或其抗原结合片段；

(iv)200mg或240mg抗PD-1抗体或其抗原结合片段和75mg抗CTLA4抗体或其抗原结合片段；和

(v)200mg或240mg抗PD-1抗体或其抗原结合片段和100mg抗CTLA4抗体或其抗原结合片段；和

(b)在权利要求1-16中任一项的方法中使用所述抗PD-1抗体或其抗原结合片段和所述抗CTLA4抗体或其抗原结合片段的说明书。

31.根据权利要求30所述的试剂盒，其中所述抗PD-1抗体是派姆单抗、纳武单抗、或与派姆单抗或纳武单抗交叉竞争以结合人PD-1的抗体。

32.根据权利要求30或权利要求31所述的试剂盒，其中所述抗CTLA4抗体选自：

(a)伊匹单抗，

(b)曲美木单抗，

(c)8D2/8D2(RE)或其变体，

(d)8D2H1L1或其变体，

(e)8D2H2L2或其变体，

(f)8D3H3L3或其变体，

(g)8D2H2L15或其变体，

(h)8D2H2L17或其变体，

(i)4G10H1L1，

(j)4G10H3L3，

(k)4G10H4L3，

(l)4G10H5L3，和

(m)与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体，

其中所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体和所述8D2H2L17变体的可变重链(VH)氨基酸序列的第18位处的甲硫氨酸独立地被选自亮氨酸、缬氨酸、异亮氨酸和丙氨酸的氨基酸置换。

33.根据权利要求30-32中任一项所述的试剂盒，其中所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是伊匹单抗。

34.根据权利要求30-32中任一项所述的试剂盒，其中所述抗PD-1抗体是派姆单抗和所述抗CTLA-4抗体是伊匹单抗。

35.根据权利要求30-32中任一项所述的试剂盒，其中所述抗PD-1抗体是纳武单抗和所述抗CTLA-4抗体选自8D2/8D2(RE)或其变体、8D2H1L1或其变体、8D2H2L2或其变体、8D3H3L3

或其变体、8D2H2L15或其变体和8D2H2L17或其变体，其中所述抗CTLA4抗体的所述可变重链(VH)氨基酸序列的第18位处的甲硫氨酸被异亮氨酸置换。

36.根据权利要求35所述的试剂盒，其中所述抗PD-1抗体是纳武单抗和所述抗CTLA-4抗体选自8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

37.根据权利要求30-32中任一项所述的试剂盒，其中所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体选自所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体或所述8D2H2L17变体，其中所述抗CTLA4抗体的所述可变重链(VH)氨基酸序列的第18位处的甲硫氨酸被异亮氨酸置换。

38.根据权利要求35所述的试剂盒，其中所述抗PD-1抗体是派姆单抗和所述抗CTLA-4抗体选自8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

39.根据权利要求21-29中任一项所述的组合物或权利要求30-38中任一项所述的试剂盒用于治疗患有癌症的个体的用途。

40.根据权利要求39所述的用途，其中所述癌症是黑色素瘤、肺癌、头颈癌、膀胱癌、乳腺癌、胃肠癌、多发性骨髓瘤、肝细胞癌、淋巴瘤、肾癌、间皮瘤、卵巢癌、食道癌、肛门癌、胆道癌、结直肠癌、宫颈癌、甲状腺癌或唾液癌。

# 用PD-1的拮抗剂和抗CTLA4抗体的组合治疗癌症的组合物和方法

## 技术领域

[0001] 本发明涉及可用于治疗癌症的组合疗法。特别地，本发明涉及包含程序性死亡1蛋白(PD-1)的拮抗剂和固定剂量的抗CTLA4抗体或其抗原结合片段的组合疗法。

[0002] 相关申请的交叉引用

## 背景技术

[0003] PD-1被认为是免疫调节和外周耐受维持的重要参与者。PD-1在初始(naive)T、B和NKT细胞上中等表达并通过淋巴细胞、单核细胞和骨髓细胞上的T/B细胞受体信号通路上调(Sharpe等,The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection.Nature Immunology(2007);8:239-245)。

[0004] PD-1的两个已知配体PD-L1(B7-H1)和PD-L2(B7-DC)，在发生于不同组织的人类癌症中表达。在例如卵巢癌、肾癌、结直肠癌、胰腺癌、肝癌和黑色素瘤的大型样本集中，显示PD-L1表达与预后不良和降低的总体存活相关，无论随后的治疗如何(Dong等,Nat Med.8(8):793-800(2002);Yang等Invest Ophthalmol Vis Sci.49:2518-2525(2008);Ghebeh等Neoplasia 8:190-198(2006);Hamanishi等,Proc.Natl.Acad.Sci.USA 104:3360-3365(2007);Thompson等,Cancer 5:206-211(2006);Nomi等,Clin.Cancer Research 13:2151-2157(2007);Ohigashi等,Clin.Cancer Research 11:2947-2953;Inman等,Cancer 109:1499-1505(2007);Shimauchi等Int.J.Cancer 121:2585-2590(2007);Gao等Clin.Cancer Research 15:971-979(2009);Nakanishi J.Cancer Immunol Immunother.56:1173-1182(2007);和Hino等,Cancer 00:1-9(2010))。

[0005] 类似地，发现PD-1在肿瘤浸润淋巴细胞上的表达标志着乳腺癌和黑色素瘤的功能障碍性T细胞(Ghebeh等,BMC Cancer.20088:5714-15(2008);Ahmadzadeh等,Blood 114:1537-1544(2009))并且与肾癌的不良预后相关(Thompson等,Clinical Cancer Research 15:1757-1761(2007))。因此，已经提出表达PD-L1的肿瘤细胞与表达PD-1的T细胞相互作用以减弱T细胞的激活和逃避免疫监视，从而促进对肿瘤的免疫应答受损。

[0006] 靶向PD-1轴的免疫检查点疗法已在多种人类癌症的临床响应中取得了突破性的改进(Brahmer等,N Engl J Med 2012,366:2455-65;Garon等N Engl J Med 2015,372:2018-28;Hamid等,N Engl J Med 2013,369:134-44;Robert等,Lancet 2014,384:1109-17;Robert等,N Engl J Med 2015,372:2521-32;Robert等,N Engl J Med 2015,372:320-30;Topalian等,N Engl J Med 2012,366:2443-54;Topalian等,J Clin Oncol 2014,32:1020-30;Wolchok等,N Engl J Med 2013,369:122-33)。靶向PD-1轴的免疫疗法包括针对PD-1受体的单克隆抗体(Merck and Co.,Inc.,Kenilworth,NJ,USA的KEYTRUDA™(派姆单抗)和Bristol-Myers Squibb Company,Princeton,NJ,USA的OPDIVO™(纳武单抗)以及与PD-L1配体结合的那些(MPDL3280A;Genentech,San Francisco,CA,USA的TECENTRIQ™(阿特珠单抗))。两种治疗方法都已证明在许多癌症类型中具有抗肿瘤作用。

[0007] 已经提出，如果与其他已批准的或实验性的癌症疗法(例如，放射、手术、化学治疗剂、靶向疗法、抑制在肿瘤中失调的其他信号通路的药剂，和其他免疫增强剂)组合施用，可以增强这样的抗体的功效。一种已经测试与PD-1的拮抗剂组合的这样的药剂是细胞毒性T淋巴细胞相关抗原4(缩写为CTLA4)。

[0008] CTLA4与CD28分子在基因结构、染色体定位、序列同源性和基因表达方面具有非常密切的关系。它们两者均是共刺激分子B7的受体，主要在激活的T细胞的表面上表达。与B7结合后，CTLA4可抑制小鼠和人T细胞的激活，对T细胞的激活起负调节作用。

[0009] CTLA4 mAb或CTLA4配体可防止CTLA4结合至其天然配体，从而阻断通过CTLA4T的T细胞负调节信号的转导并且增强T细胞对各种抗原的响应性。在这方面，体内和体外研究的结果基本上一致。目前，有一些在临床试验中进行测试的CTLA4 mAb，用于治疗前列腺癌、膀胱癌、结直肠癌、胃肠道(gastrointestinal tract)的癌、肝癌、恶性黑色素瘤等(Grosso等，CTLA-4 blockade in tumor models:an overview of preclinical and translational research.Cancer Immun.13:5(2013))。

[0010] 作为影响T细胞功能的重要因素，CTLA4和CTLA4 mAb可通过干扰体内免疫微环境对疾病产生特定的治疗效果。它们具有很高的功效并且弥补传统药物的不足，开辟了一条基因治疗的新途径。CTLA4和CTLA4 mAb正在实验和临床试验的各个阶段进行测试。例如，在自身免疫性疾病中，它们在哮喘的动物模型中有效地抑制气道高响应、预防风湿性疾病的发展、介导对体内同种异体移植物的免疫耐受等。另一方面，尽管生物基因治疗在短期临床试验中未显示出任何不良影响，但应注意长期应用后的潜在影响。例如，通过CTLA4 mAb的对CTLA4-B7信号传导的过度阻断可能导致自身免疫疾病的发展。由于抗体可特异性地结合至其抗原，并且诱导靶细胞的裂解或阻断病理的进展，基于抗体特别是人源化抗体的药物的开发和利用在人类的恶性肿瘤和其他免疫疾病的临床治疗中具有重要意义。

## 发明内容

[0011] 本发明涉及用于治疗个体的癌症的方法，所述方法包括施用PD-1的拮抗剂(例如，抗PD-1抗体或其抗原结合片段)和抗CTLA4抗体或其抗原结合片段的组合，其中抗CTLA4抗体或其结合片段相对于作为单疗法施用时相同抗体或其抗原结合片段的量是减少的量。在优选的实施方式中，抗CTLA4抗体或其抗原结合片段的剂量是固定的，即剂量不依赖于个体的体重。本发明的方法和组合物相对于作为单疗法的任一药剂的施用提供了增强的功效(例如增加的总体响应比率)，并且可以提供相对于包含组合的治疗方案改进的安全性和耐受性，其中所述组合中的每种药剂均以与基于该药剂作为单疗法的相同剂量提供。

[0012] 因此，在一个实施方式中，本发明提供一种治疗人类患者的癌症的方法，其包括施用：(a)200mg或240mg抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段；或(b)2mg/kg抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段，其中所述抗CTLA4抗体或其抗原结合片段的量选自10mg、25mg、50mg和75mg，并且其中所述抗PD-1抗体或其抗原结合片段每三周施用一次，并且所述抗CTLA4抗体或其抗原结合片段每三周施用一次或每六周施用一次。

[0013] 在另一个实施方式中，本发明提供一种治疗人类患者的癌症的方法，其包括施用：(a)200mg或240mg抗PD-1抗体或其抗原结合片段和75mg或100mg抗CTLA4抗体或其抗原结合



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片段;或(b)2mg/kg抗PD-1抗体或其抗原结合片段和75mg或100mg抗CTLA4抗体或其抗原结合片段,其中所述抗PD-1抗体或其抗原结合片段每三周施用一次,并且所述抗CTLA4抗体或其抗原结合片段每十二周施用一次。

[0014] 本文还提供了一种组合物,其包含抗PD-1抗体的剂量和抗CTLA4抗体的剂量的组合物,其中所述剂量选自:(1)200mg或240mg抗PD-1抗体和10mg抗CTLA4抗体;(2)200mg或240mg抗PD-1抗体和25mg抗CTLA4抗体;(3)200mg或240mg抗PD-1抗体和50mg抗CTLA4抗体;(4)200mg或240mg抗PD-1抗体和75mg抗CTLA4抗体;(5)200mg或240mg抗PD-1抗体和100mg抗CTLA4抗体;(6)2mg/kg抗PD-1抗体和10mg抗CTLA4抗体;(7)2mg/kg抗PD-1抗体和25mg抗CTLA4抗体;(8)2mg/kg抗PD-1抗体和50mg抗CTLA4抗体;(9)2mg/kg抗PD-1抗体和75mg抗CTLA4抗体;和(10)2mg/kg抗PD-1抗体和100mg抗CTLA4抗体。

[0015] 本发明还提供了用于治疗患有癌症的患者的试剂盒,所述试剂盒包括:(a)抗PD-1抗体或其抗原结合片段的剂量和抗CTLA4抗体或其抗原结合片段的剂量,和(b)在本发明的方法中使用抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段的说明书。

[0016] 本发明还涉及所述组合物、组合和试剂盒用于治疗癌症的用途。

[0017] 在所有的上述治疗方法、组合物和用途中,PD-1拮抗剂抑制PD-L1与PD-1的结合,并且优选地还抑制PD-L2与PD-1的结合。在上述治疗方法、组合物和用途的一些优选的实施方式中,PD-1拮抗剂是单克隆抗体或其抗原结合片段,其特异性地结合至PD-1或至PD-L1,并且阻断PD-L1与PD-1的结合。在一个特别优选的实施方式中,PD-1拮抗剂是抗PD-1抗体,其包含重链和轻链,并且其中所重链和轻链包含如图6所示的氨基酸序列(SEQ ID NO:21和SEQ ID NO:22)或其中重链和轻链包含如图7所示的氨基酸序列(SEQ ID NO:23和SEQ ID NO:24)。

[0018] 此外,在任何上述治疗方法、组合物和用途的一些实施方式中,癌症表达PD-L1和PD-L2的一者或两者。在一些实施方式中,PD-L1表达在癌症中升高。

## 附图说明

[0019] 图1显示了可用于本发明的示例性抗PD-1单克隆抗体的轻链和重链CDR的氨基酸序列(SEQ ID NO:1-6)。

[0020] 图2显示了可用于本发明的方法的另一示例性抗PD-1单克隆抗体的轻链和重链CDR的氨基酸序列(SEQ ID NO:7-12)。

[0021] 图3显示了可用于本发明的方法的示例性抗PD-1单克隆抗体的重链可变区和全长重链的氨基酸序列(SEQ ID NO:13和SEQ ID NO:14)。

[0022] 图4显示了可用于本发明的方法的示例性抗PD-1单克隆抗体的替代轻链可变区的氨基酸序列(SEQ ID NO:15-17)。

[0023] 图5显示了可用于本发明的方法的示例性抗PD-1单克隆抗体的替代轻链的氨基酸序列(SEQ ID NO:18-20)。

[0024] 图6显示了派姆单抗的重链和轻链的氨基酸序列(分别为SEQ ID NO:21和22)。$V_H$和$V_L$链的氨基酸序列加下划线表示,CDR加框表示(重链CDR如SEQ ID NO:10、11和12所示,轻链CDR如SEQ ID NO:7、8和9所示)。

[0025] 图7显示了纳武单抗的重链和轻链的氨基酸序列(分别为SEQ ID NO:23和24)。$V_H$



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和$V_L$链的氨基酸序列加下划线表示，CDR加框表示(重链CDR如SEQ ID NO:28、29和30所示，轻链CDR如SEQ ID NO:31、32和33所示)。

[0026] 图8提供了如实施例2所述的根据本发明的用于治疗癌症的方法的研究设计。

[0027] 图9提供了用于如图8所示和实施例2所述的研究的患者适格标准。

[0028] 图10显示了伊匹单抗的重链和轻链的氨基酸序列(分别为SEQ ID NO:83和84)。

## 具体实施方式

[0029] 1.定义和缩写

[0030] 如在整个说明书和所附权利要求中所使用的，以下缩写适用：

[0031] AE 不良事件

[0032] CDR 互补决定区

[0033] CHO 中国仓鼠卵巢

[0034] CR 完全响应

[0035] CTLA4 细胞毒性T淋巴细胞相关抗原4

[0036] DOR 响应持续时间

[0037] FFPE 福尔马林固定，石蜡包埋

[0038] FR 框架区

[0039] IgG 免疫球蛋白G.

[0040] IHC 免疫组织化学或免疫组化

[0041] IPI 伊匹单抗

[0042] MEL 黑色素瘤

[0043] ORR 总体响应比率

[0044] OS 总体存活期

[0045] PD-1 程序性死亡1(又名程序性细胞死亡1和程序性死亡受体1)

[0046] PD-L1 程序性细胞死亡1受体1

[0047] PD-L2 程序性细胞死亡1受体2

[0048] PD 进行性疾病

[0049] PFS 无进展存活期

[0050] PR 部分响应

[0051] Q3W 每三周一次剂量

[0052] Q6W 每六周一次剂量

[0053] Q12W 每12周一次剂量

[0054] SD 稳定疾病

[0055] TPS 肿瘤比例评分

[0056] VGPR 非常好的部分响应

[0057] $V_H$ 免疫球蛋白重链可变区

[0058] $V_L$ 免疫球蛋白轻链可变区

[0059] 为了可以更容易地理解本发明，下面具体定义某些技术和科学术语。除非在本文件的其他地方特别定义，否则本文使用的所有其他技术和科学术语具有本发明所属领域的



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普通技术人员通常所理解的含义。

[0060] 除非上下文明确指出是所表明的可能性之一，否则提及“或”表示任一种或两种可能性。在一些情况下，“和/或”用于突出其中一种或两种可能性。

[0061] 如本文所用，包括所附权利要求中，除非上下文另有明确规定，否则词例如“一个/一种(a/an)”和“所述/该(the)”的单数形式包括它们相应的复数形式。

[0062] “施用”和“治疗”在适用于动物、人、实验对象、细胞、组织、器官或生物流体时意指外源药物、治疗剂、诊断剂或组合物与动物、人、受试者、细胞、组织、器官或生物流体的接触。如本文所用，“治疗(treat/treating)”癌症意指将PD-1拮抗剂和抗CTLA4抗体或其抗原结合片段的组合疗法施用至患有癌症或被诊断患有癌症的受试者，以实现至少一种积极的治疗效果，例如，减少癌细胞的数目、减小肿瘤大小、降低肿瘤细胞浸润周围器官的速率、或降低肿瘤转移或肿瘤生长的速率。“治疗”可以包括以下一种或多种：诱导/增加抗肿瘤免疫响应、减少一种或多种肿瘤标志物的数量、停止或延迟肿瘤或血癌的生长或与PD-1结合至其配体PD-L1和/或PD-L2相关的疾病(“PD-1相关疾病”)(例如癌症)的进展、稳定PD-1相关疾病、抑制肿瘤细胞的生长或存活、消除或减少一个或多个癌性病变或肿瘤的大小、降低一种或多种肿瘤标志物的水平、改善、消除PD-1相关疾病的临床表现、降低PD-1相关疾病(例如癌症)的临床症状的严重程度或持续时间、相对于类似的未治疗患者的预期存活延长患者的存活、和诱导癌性症状或其他PD-1相关疾病的完全或部分缓解。

[0063] 可以多种方式测量癌症中的积极治疗效果(参见，W.A.Weber，J Nucl.Med.50:1S-10S(2009))。例如，就肿瘤生长抑制而言，依据NCI标准，T/C≤42％是抗肿瘤活性的最低水平。T/C<10％被认为是高抗肿瘤活性水平，T/C(％)＝受治疗的肿瘤体积中值/对照的肿瘤体积中值×100。在一些实施方式中，通过治疗有效量所达到的治疗是无进展存活期(PFS)、无病存活期(DFS)或总体存活期(OS)中的任何一种。PFS也称为“至肿瘤进展的时间”，表示治疗期间和之后癌症不生长的时间长度，包括患者经历完全响应或部分响应的时间的量，以及患者经历稳定疾病的时间的量。DFS意指治疗期间和之后患者保持无疾病的时间长度。OS意指与初始(naive)的或未经治疗的个体或患者相比预期寿命的延长。尽管本发明的治疗方法、组合物和用途的实施方式可能无法在每一个患者中均有效地实现积极的治疗效果，但它应当在统计学显著数量的受试者中如此，如通过本领域已知的任何统计测试所确定的，例如学生t-检验、卡方检验、根据Mann和Whitney的U-检验、Kruskal-Wallis检验(H-检验)，Jonckheere-Terpstra检验和Wilcoxon检验。

[0064] 术语“患者”(在本文中可替代地称为“受试者”或“个体”)意指能够用本发明的制剂治疗的哺乳动物(例如，大鼠、小鼠、狗、猫、兔)，最优选地是人。在一些实施方式中，患者是成年患者。在其他实施方式中，患者是儿科患者。

[0065] 术语“抗体”意指表现出期望的生物学或结合活性的任何形式的抗体。因此，它以最广泛的含义使用，并且具体包括但不限于：单克隆抗体(包括全长单克隆抗体)、多克隆抗体、人源化、完全人抗体和嵌合抗体。“亲本抗体”是通过在为预期用途修饰抗体(例如用作人治疗剂的抗体的人源化)之前将免疫系统暴露于抗原而获得的抗体。

[0066] 通常，基本的抗体结构单元包含四聚体。每个四聚体包含两对相同的多肽链，每对具有一条“轻”链(约25kDa)和一条“重”链(约50-70kDa)。每条链的氨基末端部分包含主要负责抗原识别的约100至110个或更多个氨基酸的可变区。重链的羧基末端部分可定义主要



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负责效应子功能的恒定区。通常，人轻链被分类为κ和λ轻链。此外，人重链通常分类为μ、δ、γ、α或ε，并且分别将抗体的亚型定义为IgM、IgD、IgG、IgA和IgE。在轻链和重链内，可变区和恒定区通过约12个或更多个氨基酸的"J"区连接，重链还包含约10个以上氨基酸的"D"区。一般参见Fundamental Immunology Ch.7(Paul,W.,ed.,2nd ed.Raven Press,NY(1989))。

[0067] 每个轻链/重链对的可变区形成抗体结合位点。因此，通常，完整抗体具有两个结合位点。除了双功能或双特异性抗体外，两个结合位点通常是相同的。

[0068] 通常，重链和轻链两者的可变结构域包含三个高变区，也称为互补决定区(CDR)，其位于相对保守的框架区(FR)内。CDR通常由框架区对齐，使得能够结合特定表位。通常，从N末端到C末端，轻链可变结构域和重链可变结构域两者均包含FR1、CDR1、FR2、CDR2、FR3、CDR3和FR4。通常，每个结构域的氨基酸分配符合Sequences of Proteins of Immunological Interest,Kabat,等;National Institutes of Health,Bethesda,Md.;5th ed.;NIH Publ.No.91-3242(1991);Kabat(1978)Adv.Prot.Chem.32:1-75;Kabat,等,(1977)J Biol.Chem.252:6609-6616;Chothia,etal.,(1987)J Mol.Biol.196:901-917或Chothia,等,(1989)Nature 342:878-883中的定义。

[0069] 术语"高变区"意指负责抗原结合的抗体的氨基酸残基。高变区包含来自"互补决定区"或"CDR"(即轻链可变结构域中的CDRL1、CDRL2和CDRL3以及重链可变结构域中的CDRH1,CDRH2和CDRH3)的氨基酸残基。参见Kabat等(1991)Sequences of Proteins of Immunological Interest,5th Ed.Public Health Service,National Institutes of Health,Bethesda,Md.(通过序列定义抗体的CDR区)；还参见Chothia和Lesk(1987)J.Mol.Biol.196:901-917(通过结构定义抗体的CDR区)。术语"框架"或"FR"残基指除了本文定义为CDR残基的高变区残基之外的那些可变结构域残基。

[0070] 除非另外指明，否则"抗体片段"或"抗原结合片段"意指抗体的抗原结合片段，即保留了特异性结合至被全长抗体结合的抗原的能力的抗体片段，例如，保留了一个或多个CDR区的片段。抗体结合片段的实例包括但不限于Fab、Fab'、$F(ab')_2$和Fv片段。

[0071] "特异性结合至"特定靶蛋白的抗体是表现出与其他蛋白质相比优先与该靶标结合的抗体，但该特异性不需要绝对的结合特异性。如果抗体的结合决定了样品中靶蛋白的存在，则该抗体被认为对其预期靶标具有"特异性"，例如，没有产生非期望的结果，例如假阳性。可用于本发明的抗体或其结合片段将以比非靶蛋白的亲和力至少高2倍，优选至少高10倍，更优选至少高20倍，最优选至少高100倍的亲和力结合至靶蛋白。如本文所用，抗体被描述成特异性结合至包含给定氨基酸序列的多肽，例如成熟的人PD-1或人PD-L1分子的氨基酸序列、或成熟的人CTLA-4分子的氨基酸序列，如果它结合包含该序列的多肽但不结合缺乏该序列的蛋白质。

[0072] "嵌合抗体"意指一种抗体，以及这样的抗体的片段，只要它们表现出所需的生物活性，其中一部分重链和/或轻链与来源于特定物种(例如人)或属于特定抗体类或亚类的抗体中的相应序列相同或同源，而所述一条或多条链的其余部分与来源于另一物种(例如，小鼠)或属于另一抗体类别或亚类的抗体中的相应序列相同或同源。

[0073] "人抗体"意指仅包含人免疫球蛋白序列的抗体。如果在小鼠、小鼠细胞或衍生自小鼠细胞的杂交瘤中产生，人抗体可能含有鼠源碳水化合物链。类似地，"小鼠抗体"或"大



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鼠抗体”分别指仅包含小鼠或大鼠免疫球蛋白序列的抗体。

[0074] “人源化抗体”意指含有来自非人(例如鼠)抗体以及人抗体的序列的抗体形式。这样的抗体含有衍生自非人免疫球蛋白的最小序列。通常,人源化抗体将包含基本上所有的至少一个、通常是两个可变结构域,其中所有或基本上所有的高变环对应于非人免疫球蛋白的那些,并且所有或基本上所有的FR区是人免疫球蛋白序列的那些。所述人源化抗体任选地还包含至少一部分免疫球蛋白恒定区(Fc),通常是人免疫球蛋白的恒定区。当需要区分人源化抗体与亲本啮齿动物抗体时,将前缀“hum”、“hu”或“h”添加到抗体克隆命名中。啮齿动物抗体的所述人源化形式通常包含与亲本啮齿动物抗体的相同CDR序列,尽管可包含某些氨基酸置换以增加亲和力、增加人源化抗体的稳定性,或出于其他原因。

[0075] “抗CTLA-4抗体”意指抗体或其抗原结合片段,其结合至人CTLA-4以破坏CTLA-4的与人B7受体的相互作用。与B7结合后,CTLA4可抑制小鼠和人T细胞的激活,对T细胞的激活起负调节作用。如本文所用,除非特别说明,否则所述B7意指B7-1和/或B7-2;它们的特定蛋白质序列意指本领域已知的序列。可以参考文献或GenBank中公开的序列,例如B7-1(CD80,NCBI Gene ID:941)、B7-2(CD86,NCBI Gene ID:942)。

[0076] 术语“癌症”、“癌性”或“恶性”意指或描述哺乳动物中通常以不受调节的细胞生长为特征的生理状况。癌症的实例包括但不限于癌、淋巴瘤、白血病、胚细胞瘤和肉瘤。这样的癌症的更具体的实例包括鳞状细胞癌、骨髓瘤、小细胞肺癌、非小型细胞肺癌、神经胶质瘤、霍奇金淋巴瘤、非霍奇金淋巴瘤、急性髓性白血病(AML)、多发性骨髓瘤、胃肠(道)癌症、肾癌、卵巢癌、肝癌、成淋巴细胞性白血病、淋巴细胞性白血病、结直肠癌、子宫内膜癌、肾癌、前列腺癌、甲状腺癌、黑色素瘤、软骨肉瘤、神经母细胞瘤、胰腺癌、多形性成胶质细胞瘤、宫颈癌、脑癌、胃癌、膀胱癌、肝癌、乳腺癌、结肠癌和头颈癌。可根据本发明治疗的特别优选的癌症包括那些以测试组织样品中PD-L1和PD-L2之一或两者的表达升高为特征的癌症。

[0077] “生物治疗剂”意指生物分子,例如抗体或融合蛋白,其阻断支持肿瘤维持和/或生长或抑制抗肿瘤免疫应答的任何生物通路中的配体/受体信号传导。

[0078] 除非另有说明,否则“一个或多个CDR”表示使用Kabat编号系统定义的免疫球蛋白可变区中的互补决定区。

[0079] “化学治疗剂”是可用于治疗癌症的化合物。化学治疗剂的类别包括但不限于:烷化剂、抗代谢物、激酶抑制剂、紫杉烷毒素植物生物碱、细胞毒性/抗肿瘤抗生素、拓扑异构酶抑制剂、光敏剂、抗雌激素和选择性雌激素受体调节剂(SERMs)、抗孕酮、雌激素受体下调剂(ERD)、雌激素受体拮抗剂、促黄体(leutinizing)激素释放激素激动剂、抗雄性激素、芳香酶抑制剂、EGFR抑制剂、VEGF抑制剂、抑制与异常细胞增殖或肿瘤生长有关的基因表达的反义寡核苷酸。可用于本发明治疗方法的化学治疗剂包括细胞抑制剂和/或细胞毒性药剂。

[0080] “Chothia”意指Al-Lazikani等,JMB 273:927-948(1997)中描述的抗体编号系统。

[0081] “保守修饰的变体”或“保守置换”意指用具有相似特征的其他氨基酸(例如电荷、侧链大小、疏水性/亲水性、骨架构象和刚性等)置换蛋白质中的氨基酸,使得可以经常进行变化而不改变蛋白质的生物活性或其他期望特性,例如抗原亲和力和/或特异性。本领域技术人员认识到,通常,多肽的非必需区域中的单个氨基酸置换基本上不改变生物活性(参见,例如,Watson等(1987)Molecular Biology of the Gene,The Benjamin/Cummings Pub.Co.,p.224(4th Ed.))。此外,结构上或功能上相似的氨基酸的置换不太可能破坏生物



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活性。示例性的保守置换如表1所示。

[0082] 表1.示例性保守氨基酸置换

[0083]

| 原始残基 | 保守置换 |
|---|---|
| Ala(A) | Gly;Ser |
| Arg(R) | Lys;His |
| Asn(N) | Gln;His |
| Asp(D) | Glu;Asn |
| Cys(C) | Ser;Ala |
| Gln(Q) | Asn |
| Glu(E) | Asp;Gln |
| Gly(G) | Ala |
| His(H) | Asn;Gln |
| Ile(I) | Leu;Val |
| Leu(L) | Ile;Val |
| Lys(K) | Arg;His |
| Met(M) | Leu;Ile;Tyr |
| Phe(F) | Tyr;Met;Leu |
| Pro(P) | Ala |
| Ser(S) | Thr |
| Thr(T) | Ser |
| Trp(W) | Tyr;Phe |
| Tyr(Y) | Trp;Phe |
| Val(V) | Ile;Leu |

[0084] 如整个说明书和权利要求书中所用,“基本上由……组成(consists essentially of/consist essentially of/consisting essentially of)”表示包括任何列举的元素或元素组,以及任选地包括其他元素,所述其他元素具有与所列举的元素相似或不同的性质,不会实质上改变特定剂量方案、方法或组合物的基本或新的性质。作为非限制性实例,基本上由所列举的氨基酸酸序列组成的PD-1的拮抗剂还可以包含一个或多个氨基酸,包含一个或多个氨基酸残基的置换,其不会实质上影响结合化合物的性质。

[0085] 除非上下文因明确的语言或必要的含义而另外要求,否则“包含(comprising/comprise/comprises/comprised of)”在整个说明书和权利要求中以包含的含义使用,即,指定所述特征的存在但不排除存在或增加可以实质上增强本发明的任何实施方式的操作或实用性的其他特征。

[0086] “诊断性抗PD-L单克隆抗体”意指特异性结合至在某些哺乳动物细胞的表面上表达的所指定的PD-L(PD-L1或PDL2)的成熟形式的mAb。成熟的PD-L缺乏前分泌前导序列,也称为前导肽。术语“PD-L”和“成熟PD-L”在本文中可互换使用,并且应理解为表示相同的分子,除非另有说明或从上下文中显而易见。

[0087] 如本文所用,诊断性抗人PD-L1 mAb或抗hPD-L1 mAb意指特异性结合至成熟人PD-



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L1的单克隆抗体。成熟的人PD-L1分子由以下序列的第19至290位氨基酸组成：

[0088] MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFV HGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVV DPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENH TAELVIPELPLAHPPNERTHLVILGAILLCLGVALTFIFRLRKGRMMDVKKCGIQDTNSKKQSDTHLEET(SEQ ID NO:25)。

[0089] 用于免疫组织化学(IHC)检测福尔马林固定、石蜡包埋(FFPE)的肿瘤组织切片中PD-L1表达的诊断性mAb的诊断性抗人PD-L1mAb的具体实例是抗体20C3和抗体22C3，其描述于WO2014/100079中。这些抗体包含下表2中所示的轻链和重链可变区氨基酸序列：

[0090]

| 表2. 单克隆抗体20C3和22C3 | |
|---|---|
| 20C3轻链成熟可变区 | |
| DIVMSQSPSSLAVSAGEKVTMSCKSSQSLLNSRTRKNYLAWYQQ KPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQAEDLA VYYCQQSYDVVTFGAGTKLELK | SEQ ID NO:103 |
| 20C3重链成熟可变区 | |
| QVQVQQSGAELAEPGASVKMSCKASGYIFTSYWMHWLKQRPGQ GLEWIGYINPSSDYNEYSEKFMDKATLTADKASITAYMQLISLTS EDSAVYYCARSGWLVHGDYYFDYWGQGTTLTVSS | SEQ ID NO:104 |
| 22C3轻链成熟可变区 | |
| DIVMSQSPSSLAVSAGEKVTMTCKSSQSLLHTSTRKNYLAWYQQ KPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVQAEDLA VYYCKQSYDVVTFGAGTKLELK | SEQ ID NO:105 |
| 22C3重链成熟可变区 | |
| QVHLQQSGAELAKPGASVKMSCKASGYTFTSYWIHWIKQRPGQG LEWIGYINPSSGYHEYNQKFIDKATLTADRSSSTAYMHLTSLTSED SAVYYCARSGWLIHGDYYFDFWGQGTTLTVSS | SEQ ID NO:106 |

[0091] 已报道可用于IHC检测FFPE组织切片中PD-L1表达的另一抗人PD-L1 mAb(Chen, B.J.等,Clin Cancer Res 19:3462-3473(2013))是兔抗人PD-L1 mAb，可从Sino Biological,Inc.(北京，中国；目录号10084-R015)公开获得。

[0092] 如本文所用，“框架区”或“FR”意指除CDR区之外的免疫球蛋白可变区。

[0093] “分离的抗体”和“分离的抗体片段”意指纯化状态并且在这种情况下意味着命名的分子基本上不含其他生物分子，例如核酸、蛋白质、脂质、碳水化合物或其他材料，例如细胞碎片和生长培养基。通常，术语“分离的”无意指代完全不存在这样的物质或不存在水、缓冲液或盐，除非它们以基本上干扰本文所述的结合化合物的实验或治疗性用途的量存在。

[0094] 如本文所用，“Kabat”意指由Elvin A.Kabat((1991)Sequences of Proteins of Immunological Interest,5th Ed.Public Health Service,National Institutes of Health,Bethesda,Md.)开创的免疫球蛋白比对和编号系统。



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[0095] 如本文所用，“单克隆抗体”或“mAb”或“Mab”意指基本上同质的抗体群，即，群体所包含的抗体分子的氨基酸序列相同，除了可能少量存在的天然发生的突变。相反，常规(多克隆)抗体制剂通常包含在其可变结构域(特别是通常对不同表位具有特异性的CDR)中具有不同氨基酸序列的多种不同抗体。修饰语“单克隆”表示抗体的特征是从基本上同质的抗体群体获得的，并且不应解释为需要通过任何特定方法产生抗体。例如，根据本发明使用的单克隆抗体可以通过Kohler等(1975)Nature 256:495中首次描述的杂交瘤方法制备，或可以通过重组DNA方法制备(参见，例如，美国专利号4,816,567)。例如，还可以使用Clackson等(1991)Nature 352:624-628和Marks等(1991)J.Mol.Biol.222:581-597中描述的技术从噬菌体抗体文库中分离“单克隆抗体”。还参见Presta(2005)J.Allergy Clin.Immunol.116:731。

[0096] “PD-1拮抗剂”意指阻断癌细胞上表达的PD-L1与免疫细胞(T细胞、B细胞或NKT细胞)上表达的PD-1结合、并且优选地还阻断在癌细胞上表达的PD-L2与免疫细胞上表达的PD-1结合的任何化合物或生物分子。PD-1及其配体的替代名称或同义词包括：用于PD-1的PDCD1、PD1、CD279和SLEB2；用于PD-L1的PDCD1L1、PDL1、B7H1、B7-4、CD274和B7-H；和用于PD-L2的PDCD1L2、PDL2、B7-DC、Btdc和CD273。在本发明的用于治疗人类个体的任何治疗方法、药物和用途中，PD-1拮抗剂阻断人PD-L1与人PD-1的结合，并且优选地阻断人PD-L1和PD-L2与人PD-1的结合。人PD-1的氨基酸序列可以在NCBI基因座号NP_005009中找到。人PD-L1和PD-L2氨基酸序列可分别在NCBI基因座号NP_054862和NP_079515中找到。

[0097] 在本发明的任何的治疗方法、组合物和用途中有用的PD-1拮抗剂包括“抗PD-1抗体”和“抗PD-L1抗体”，两者均包括单克隆抗体(单抗)或其抗原结合片段，其分别特异性地结合至人PD-1和人PD-L1。抗PD-1抗体和抗PD-L1抗体可以是人抗体、人源化抗体或嵌合抗体，并且可以包括人恒定区。在一些实施方式中，人恒定区选自IgG1、IgG2、IgG3和IgG4恒定区，并且在优选的实施方式中，人恒定区是IgG1或IgG4恒定区。在一些实施方式中，抗原结合片段选自Fab、Fab'-SH、$F(ab')_2$、scFv和Fv片段。

[0098] 如本文所用，“PD-L1”或“PD-L2”表达意指细胞表面上指定的PD-L蛋白或细胞或组织内指定的PD-L mRNA的任何可检测水平的表达。可以在肿瘤组织切片的IHC测定或通过流式细胞术用诊断性PD-L抗体检测PD-L蛋白表达。或者，可以通过PET成像，使用特异性结合期望的PD-L靶标的结合剂(例如，抗体片段、亲和体等)，例如PD-L1或PD-L2，检测肿瘤细胞的PD-L蛋白质表达。用于检测和测量PD-L mRNA表达的技术包括RT-PCR和实时定量RT-PCR。

[0099] 已经描述了几种用于在肿瘤组织切片的IHC测定中定量PD-L1蛋白表达的方法。参见，例如，Thompson等，PNAS 101(49):17174-17179(2004)；Thompson等，Cancer Res.66:3381-3385(2006)；Gadiot等，Cancer 117:2192-2201(2011)；Taube等，Sci Transl Med 4,127ra37(2012)；和Toplian等，New Eng.J Med.366(26):2443-2454(2012)。

[0100] 一种方法采用PD-L1表达的阳性或阴性的简单二元终点，阳性结果以显示细胞表面膜染色的组织学证据的肿瘤细胞的百分比来定义。被视为PD-L1表达阳性的肿瘤组织切片至少是总肿瘤细胞的1%，优选地是5%。

[0101] 在另一种方法中，肿瘤组织切片中PD-L1的表达在肿瘤细胞以及浸润性免疫细胞(主要包含淋巴细胞)中定量。显示膜染色的肿瘤细胞和浸润性免疫细胞的百分比分别定量为<5%、5至9%，然后以10%增量至100%。对于肿瘤细胞，如果评分<5%评分，则PD-L1的表



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达被视为阴性，如果评分≥5％则视之为阳性。免疫浸润中PD-L1的表达报告为半定量测量值，称为经调整的炎症评分(AIS)，它是通过将膜染色细胞的百分比乘以浸润强度来确定的，所述浸润强度分级为无(0)、轻度(评分为1，罕见淋巴细胞)、中度(评分为2，由淋巴组织细胞聚集的肿瘤局灶性浸润)或严重(评分为3，弥漫性浸润)。如果AIS≥5，则肿瘤组织切片被视为通过免疫浸润的PD-L1表达呈阳性。

[0102] 已用诊断PD-L1抗体通过IHC染色的来自肿瘤的组织切片也可以通过使用评分方法评估组织切片中肿瘤细胞和浸润免疫细胞两者中PD-L1的表达来评分PD-L1。参见WO2014/165422。PD-L1评分方法包括检查组织切片中各个肿瘤巢的染色情况，并且将修改的H评分(MHS)和修改的比例评分(MPS)中的一个或两个分配给组织切片。为分配MHS，在所有检查的肿瘤巢中估算所有活肿瘤细胞和染色的单核炎性细胞的四个单独的百分比：(a)没有染色的细胞(强度＝0)，(b)弱染色(强度＝1+)，(c)中度染色(强度＝2+)和(d)强染色(强度＝3+)。细胞必须具有至少部分膜染色才被包括在弱、中度或强染色百分比中。然后将估算的百分比(其总和为100％)输入1×(弱染色细胞的百分比)+2×(中度染色细胞的百分比)+3×(强染色细胞的百分比)的公式中，并且结果作为MHS分配给所述组织切片。通过在所有检查的肿瘤巢中估算所有活肿瘤细胞和染色的单核炎性细胞中具有任何强度的至少部分膜染色的细胞的百分比来分配MPS，并且将得到的百分比作为MPS指定给组织切片。在一些实施方式中，如果MHS或MPS是阳性，则将肿瘤指定为PD-L1表达呈阳性。

[0103] 可以将PD-L mRNA表达的水平与经常用于定量RT-PCR的一种或多种参照基因的mRNA表达水平进行比较，例如泛素蛋白C。

[0104] 在一些实施方式中，通过恶性细胞和/或通过肿瘤内浸润免疫细胞的PD-L1表达(蛋白质和/或mRNA)的水平基于与通过适当对照的PD-L1表达(蛋白质和/或mRNA)的水平的比较确定为“过表达”或“提高的”。例如，对照PD-L1蛋白或mRNA表达水平可以是在相同类型的非恶性细胞中或在来自匹配的正常组织的切片中定量的水平。在一些优选的实施方式中，如果肿瘤样品中的PD-L1蛋白(和/或PD-L1 mRNA)比对照样品中高至少10％、20％或30％，则确定所述肿瘤样品中的PD-L1表达升高。

[0105] “持续响应”意指在用本文所述的治疗剂或组合疗法的治疗停止后的持续治疗效果。在一些实施方式中，持续响应的持续时间至少与治疗持续时间相同，或者比治疗持续时间长至少1.5、2.0、2.5或3倍。

[0106] “组织切片”意指组织样品的单个部分或片，例如，从正常组织或肿瘤的样品切下的组织薄切片。

[0107] “肿瘤”在用于被诊断患有或疑似患有的受试者时意指任何大小的恶性或潜在恶性肿瘤或组织块，并且包括原发性肿瘤和继发性肿瘤。实体瘤是通常不包含囊肿或液体区域的组织异常生长或团块。不同类型的实体瘤以形成它们的细胞类型命名。实体瘤的实例是肉瘤、癌和淋巴瘤。白血病(血液癌症)通常不形成实体瘤(National Cancer Institute, Dictionary of Cancer Terms)。

[0108] “肿瘤负担”(tumor burden)，也称为“肿瘤负荷”(/tumor load)意指分布在全身的肿瘤物质的总量。肿瘤负担意指在整个身体(包括淋巴结和骨髓)中的癌细胞的总数量或肿瘤的总大小。肿瘤负担可以通过本领域已知的多种方法确定，例如通过在从受试者移除时测量肿瘤的尺寸，例如使用卡尺，或者在体内使用成像技术测量，例如，超声、骨扫描、计



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算机断层扫描(CT)或磁共振成像(MRI)扫描。

[0109] 术语“肿瘤大小”意指肿瘤的总大小，其可以通过肿瘤的长度和宽度来测量。肿瘤大小可通过本领域已知的多种方法确定，例如通过在从受试者移除时测量肿瘤的尺寸，例如使用卡尺，或者在体内使用成像技术测量，例如，骨扫描、超声、CT或MRI扫描。

[0110] 如本文所用，“可变区”或“V区”意指在不同抗体之间的序列可变的IgG链的区段。它延伸至轻链中的Kabat残基109和重链中的113。

[0111] II.可用于本发明的PD-1拮抗剂、抗体和抗原结合片段，以及CTLA4抗体和抗原结合片段

[0112] 结合至人PD-1并且在本发明的治疗方法、组合物、试剂盒和用途中有用的mAb的实例描述于美国专利申请US 7521051、US 8008449和US 8354509中。在本发明的治疗方法、组合物、试剂盒和用途中作为PD-1拮抗剂的特异性抗人PD-1 mAb包括：派姆单抗(以前称为MK-3475、SCH 900475和lambrolizumab)，具有如WHO Drug Information,Vol.27,No.2,pages 161-162(2013)中所述的结构并且其包含图6中所示的重链和轻链氨基酸序列的人源化的IgG4 mAb，纳武单抗(BMS-936558)，具有如WHO Drug Information,Vol.27,No.1,pages 68-69(2013)中所述的结构并且其包含图7中所示的重链和轻链氨基酸序列的人IgG4 mAb；pidilizumab(CT-011，也称为hBAT或hBAT-1)；和人源化抗体h409A11、h409A16和h409A17，其描述于WO2008/156712中。

[0113] 结合人PD-L1并且在本发明的治疗方法、组合物、试剂盒和用途中有用的mAb的实例描述于WO2013/019906、WO2010/077634A1和US8383796中。在本发明的治疗方法、药物和用途中用作PD-1拮抗剂的特异性抗人PD-L1 mAb包括TECENTRIQ™(阿特珠单抗，Genentech，San Francisco,CA,USA，以前称为MPDL3280A)、BMS-936559、MEDI4736、MSB0010718C和分别包含WO2013/019906的SEQ ID NO:24和SEQ ID NO:21的重链和轻链可变区的抗体。

[0114] 在本发明的任何治疗方法、组合物、试剂盒和用途中有用的其他PD-1拮抗剂包括特异性结合PD-1或PD-L1的免疫粘附素，优选地特异性结合人PD-1或人PD-L1，例如，融合蛋白，其含有与恒定区(例如免疫球蛋白分子的Fc区)融合的PD-L1或PD-L2的细胞外或PD-1结合部分。特异性结合PD-1的免疫粘附分子的实例描述于WO2010/027827和WO2011/066342中。在本发明的治疗方法、药物和用途中用作PD-1拮抗剂的特异性融合蛋白包括AMP-224(也称为B7-DCIg)，其是PD-L2-FC融合蛋白并且结合至人PD-1。

[0115] 在本发明的治疗方法、组合物和用途的一些实施方式中，PD-1拮抗剂是单克隆抗体或其抗原结合片段，其包含：(a)包含如SEQ ID NO:1、2和3所示氨基酸序列的轻链CDR和包含如SEQ ID NO:4、5和6所示氨基酸序列的重链CDR；或(b)包含如SEQ ID NO:7、8和9所示氨基酸序列的轻链CDR和包含如SEQ ID NO:10、11和12所示氨基酸序列的重链CDR。

[0116] 在本发明的治疗方法、组合物和用途的进一步实施方式中，PD-1拮抗剂是单克隆抗体或其抗原结合片段，其包含：(a)包含如SEQ ID NO:31、32和33所示氨基酸序列的轻链CDR和包含如SEQ ID NO:28、29和30所示氨基酸序列的重链CDR。

[0117] 在本发明的治疗方法、组合物和用途的其他实施方式中，PD-1拮抗剂是单克隆抗体或其抗原结合片段，其特异性结合至人PD-1并且包含(a)包含如SEQ ID NO:13所示氨基酸序列或其变体的重链可变区，和(b)包含如SEQ ID NO:15所示氨基酸序列或其变体；如SEQ ID NO:16所示氨基酸序列或其变体；如SEQ ID NO:17所示氨基酸序列或其变体的轻链



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可变区。在本发明的治疗方法、组合物和用途的另外的实施方式中，PD-1拮抗剂是单克隆抗体或其抗原结合片段，其特异性结合至人PD-1并且包含(a)包含如SEQ ID NO:26所示氨基酸序列或其变体的重链可变区，和(b)包含如SEQ ID NO:27所示氨基酸序列或其变体的轻链可变区。

[0118] 重链可变区序列的变体与参考序列相同，除了在框架区(即在CDR以外)中具有至多17个保守氨基酸置换，并且优选地在框架区中具有少于10个、9个、8个、7个、6个或5个保守氨基酸置换。轻链可变区序列的变体与参考序列相同，除了在框架区(即CDR以外)具有至多5个保守氨基酸置换，并且优选在框架区中具有少于4个、3个或2个保守氨基酸置换。

[0119] 在本发明的治疗方法、组合物、试剂盒和用途的另一实施方式中，PD-1拮抗剂是单克隆抗体，其特异性结合至人PD-1并且包含(a)包含如SEQ ID NO:14所示氨基酸序列或由其组成的重链和(b)包含如SEQ ID NO:18、SEQ ID NO:19或SEQ ID NO:20所示氨基酸序列或由其组成的轻链。

[0120] 在本发明的治疗方法、组合物、试剂盒和用途的又一实施方式中，PD-1拮抗剂是单克隆抗体，其特异性结合至人PD-1并且包含(a)包含如SEQ ID NO:14所示氨基酸序列或由其组成的重链和(b)包含如SEQ ID NO:18所示氨基酸序列或由其组成的轻链。

[0121] 在本发明的治疗方法、组合物、试剂盒和用途的其他实施方式中，PD-1拮抗剂是单克隆抗体，其特异性结合至人PD-1并且包含(a)包含如SEQ ID NO:23所示氨基酸序列或由其组成的重链和(b)包含如SEQ ID NO:24所示氨基酸序列或由其组成的轻链。

[0122] 以下表3提供了用于本发明的治疗方法、组合物、试剂盒和用途的示例性抗PD-1mAb的氨基酸序列的列表。所述序列提供于图1至5中。



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[0123]

| 表 3. 示例性抗人 PD-1 抗体 | |
|---|---|
| A. 包含 WO2008/156712 中 hPD-1.08A 的轻链和重链 CDR | |
| CDRL1 | SEQ ID NO:1 |
| CDRL2 | SEQ ID NO:2 |
| CDRL3 | SEQ ID NO:3 |
| CDRH1 | SEQ ID NO:4 |
| CDRH2 | SEQ ID NO:5 |
| CDRH3 | SEQ ID NO:6 |
| B. 包含 WO2008/156712 中 hPD-1.09A 的轻链和重链 CDR | |
| CDRL1 | SEQ ID NO:7 |
| CDRL2 | SEQ ID NO:8 |
| CDRL3 | SEQ ID NO:9 |
| CDRH1 | SEQ ID NO:10 |
| CDRH2 | SEQ ID NO:11 |
| CDRH3 | SEQ ID NO:12 |
| C. 包含 WO 2008/156712 中成熟 h109A 重链可变区和一个成熟 K09A 轻链可变区 | |
| 重链 VR | SEQ ID NO:13 |
| 轻链 VR | SEQ ID NO:15 或 SEQ ID NO:16 或 SEQ ID NO:17 |
| D.包含 WO 2008/156712 中成熟 409 重链和一个成熟 K09A 轻链 | |
| 重链 | SEQ ID NO:14 |
| 轻链 | SEQ ID NO:18 或 SEQ ID NO:19 或 SEQ ID NO:20 |

[0124] 在本发明的治疗方法、组合物、试剂盒和用途的一个实施方式中，抗CTLA-4抗体是人单克隆抗体10D1，现在被称为伊匹单抗并且作为Yervoy™上市，其公开于美国专利号6,984,720和WHO Drug Information 19(4):61(2005)。在另一个实施方式中，抗CTLA-4抗体是曲美木单抗，也称为CP-675,206，其是IgG2单克隆抗体，其描述于美国专利申请公开号2012/263677或PCT国际申请公开号WO2012/122444或2007/113648 A2中。

[0125] 在本发明的治疗方法、组合物和用途的进一步实施方式中，抗CTLA4抗体或其抗原结合片段包含：(a)包含如SEQ ID NO:88、89和90所示氨基酸序列的轻链CDR和包含如SEQ ID NO:85、86和87所示氨基酸序列的重链CDR。

[0126] 在本发明的治疗方法、组合物和用途的其他实施方式中，抗CTLA4抗体是单克隆抗体或其抗原结合片段，其结合至人CTLA4并且包含(a)包含如SEQ ID NO:91所示氨基酸的重链可变区和(b)包含如SEQ ID NO:92所示氨基酸序列的轻链可变区。



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[0127]

| 表4. 示例性抗人CTLA4 抗体 | |
|---|---|
| A. 包含伊匹单抗的轻链和重链CDR | |
| CDRL1 | RASQSVGSSYLA (SEQ ID NO: 88) |
| CDRL2 | GAFSRAT (SEQ ID NO: 89) |
| CDRL3 | QQYGSSPWT (SEQ ID NO: 90) |
| CDRH1 | SYTMH (SEQ ID NO: 85) |
| CDRH2 | FISYDGNNKYYADSVKG (SEQ ID NO: 86) |
| CDRH3 | TGWLGPFDY (SEQ ID NO: 87) |
| C. 包含伊匹单抗的成熟重链可变区和成熟轻链可变区 | |
| 重链 VR | QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQA PGKGLEWVTFISYDGNNKYYADSVKGRFTISRDNSKNTLY LQMNSLRAEDTAIYYCARTGWLGPFDYWGQGTLVTVSS (SEQ ID NO: 91) |
| 轻链 VR | EIVLTQSPGT LSLSPGERATLSCRASQSVGSSYLAWYQQK PGQAPRLLIYGAFSRATGIPDRFSGSGSGTDFTLTISRLE PEDFAVYYCQQYGSSPWTFGQGTKVEIK (SEQ ID NO: 92) |
| D. 包含伊匹单抗的成熟重链和成熟轻链 | |

[0128]

| 重链 | SEQ ID NO:83 |
|---|---|
| 轻链 | SEQ ID NO:84 |

[0129] 在本发明的治疗方法、组合物、试剂盒和用途的一个实施方式中，抗CTLA-4抗体是单克隆抗体，其包含具有如SEQ ID NO:83所示氨基酸序列的重链和包含如SEQ ID NO:84所示氨基酸序列的轻链。在一些实施方式中，CTLA4抗体是SEQ ID NO:83和/或SEQ ID NO:84的抗原结合片段，其中抗原结合片段特异性结合至CTLA4。

[0130] 在本发明的治疗方法、组合物、试剂盒和用途的一个实施方式中，抗CTLA-4抗体是任何的抗CTLA-4抗体或其抗原结合片段，其公开于国际申请公开号WO 2016/015675 A1。在一个实施方式中，抗CTLA4抗体是单克隆抗体，其包含以下CDR：

[0131] CDRH1，其包含氨基酸序列GFTFSDNW(SEQ ID NO:34)

[0132] CDRH2，其包含氨基酸序列IRNKPYNYET(SEQ ID NO:35)

[0133] CDRH3，其包含氨基酸序列TAQFAY(SEQ ID NO:36)

[0134] 和/或

[0135] CDRL1，其包含氨基酸序列ENIYGG(SEQ ID NO:37)

[0136] CDRL2，其包含氨基酸序列GAT(SEQ ID NO:38)

[0137] CDRL3，其包含选自以下的氨基酸序列：QNVLRSPFT(SEQ ID NO:39)；QNVLSRHPG(SEQ ID NO:40)；或QNVLSSRPG(SEQ ID NO:41)

[0138] 在本发明的治疗方法、组合物、试剂盒和用途的一个实施方式中，抗CTLA-4抗体是



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8D2/8D2(RE)或其变体、8D2H1L1或其变体、8D2H2L2或其变体、8D3H3L3或其变体、8D2H2L15或其变体、或8D2H2L17或其变体。

[0139]

| 抗体 | $V_H$ | $V_L$ |
|---|---|---|
| 8D2/8D2（RE） | EVKLDETGGGLVQPGRPMKLSCVAS GFTFSDNWMNWVRQSPEKGLEWLA QIRNKPYNYETYYSDSVKGRFTISRD DSKSSVYLQMNNLRGEDMGIYYCTA QFAYWGQGTLVTVSA（SEQ ID | DIQMTQSPASLSASVGETVTITCGT SENIYGGLNWYQRKQGKSPQLLIF GATNLADGMSSRFSGSGSGRQYSL KISSLHPDDVATYYCQNVLRSPFTF GSGTKLEI（SEQ ID NO:43） |

[0140]

| | | |
|---|---|---|
| | NO:42） | |
| 8D2/8D2 RE 变体 1 | EVKLDETGGGLVQPGRPIKLSCVASG FTFSDNWMNWVRQSPEKGLEWLAQI RNKPYNYETYYSDSVKGRFTISRDDS KSSVYLQMNNLRGEDMGIYYCTAQF AYWGQGTLVTVSA（SEQ ID NO:94） | DIQMTQSPASLSASVGETVTITCGT SENIYGGLNWYQRKQGKSPQLLIF GATNLADGMSSRFSGSGSGRQYSL KISSLHPDDVATYYCQNVLRSPFTF GSGTKLEI（SEQ ID NO:43） |
| 8D2H1L1 | EVQLVESGGGLVQPGGSMRLSCAAS GFTFSDNWMNWVRQAPGKGLEWLA QIRNKPYNYETYYSDSVKGRFTISRD DSKNSVYLQMNSLKTEDTGVYYCTA QFAYWGQGTLVTVSS（SEQ ID NO:44） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKQGKSPKLLIY GATNLASGMSSRFSGSGSGTDYTL KISSLHPDDVATYYCQNVLRSPFTF GSGTKLEIK（SEQ ID NO:45） |
| 8D2H1L1 变体 1 | EVQLVESGGGLVQPGGSIRLSCAASG FTFSDNWMNWVRQAPGKGLEWLAQ IRNKPYNYETYYSDSVKGRFTISRDD SKNSVYLQMNSLKTEDTGVYYCTAQ FAYWGQGTLVTVSS（SEQ ID NO: 96） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKQGKSPKLLIY GATNLASGMSSRFSGSGSGTDYTL KISSLHPDDVATYYCQNVLRSPFTF GSGTKLEIK（SEQ ID NO:45） |
| 8D2H2L2 | EVQLVESGGGLVQPGGSMRLSCAAS GFTFSDNWMNWVRQAPGKGLEWLA QIRNKPYNYETYYSASVKGRFTISRD DSKNSVYLQMNSLKTEDTGVYYCTA QFAYWGQGTLVTVSS（SEQ ID NO:46） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKPGKSPKLLIY GATNLASGVSSRFSGSGSGTDYTL TISSLQPEDVATYYCQNVLRSPFTF GSGTKLEIK（SEQ ID NO:47） |
| 8D2H2L2 变体 1 | EVQLVESGGGLVQPGGSIRLSCAASG FTFSDNWMNWVRQAPGKGLEWLAQ IRNKPYNYETYYSASVKGRFTISRDD SKNSVYLQMNSLKTEDTGVYYCTAQ FAYWGQGTLVTVSS（SEQ ID NO: 98） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKPGKSPKLLIY GATNLASGVSSRFSGSGSGTDYTL TISSLQPEDVATYYCQNVLRSPFTF GSGTKLEIK（SEQ ID NO:47） |
| 8D3H3L3 | EVQLVESGGGLVQPGGSLRLSCAAS GFTFSDNWMNWVRQAPGKGLEWVA QIRNKPYNYETEYAASVKGRFTISRD DSKNSAYLQMNSLKTEDTAVYYCTA QFAYWGQGTLVTVSS（SEQ ID NO:48） | DIQMTQSPSSLSASVGDRVTITCRA SENIYGGLNWYQQKPGKAPKLLIY GATSLASGVPSRFSGSGSGTDYTLT ISSLQPEDFATYYCQNVLRSPFTFG SGTKLEIK（SEQ ID NO:49） |
| 8D2H2L15 | EVQLVESGGGLVQPGGSMRLSCAAS GFTFSDNWMNWVRQAPGKGLEWLA QIRNKPYNYETYYSASVKGRFTISRD | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKPGKSPKLLIY GATNLASGVSSRFSGSGSGTDYTL |



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[0141]

| | | |
|---|---|---|
| | DSKNSVYLQMNSLKTEDTGVYYCTA QFAYWGQGTLVTVSS（SEQ ID NO:50） | TISSLQPEDVATYYCQNVLSRHPGF GSGTKLEIK（SEQ ID NO:51） |
| 8D2H2L15<br><br>变体1 | EVQLVESGGGLVQPGGSIRLSCAASG FTFSDNWMNWVRQAPGKGLEWLAQ IRNKPYNYETYYSASVKGRFTISRDD SKNSVYLQMNSLKTEDTGVYYCTAQ FAYWGQGTLVTVSS（SEQ ID NO: 100） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKPGKSPKLLIY GATNLASGVSSRFSGSGSGTDYTL TISSLQPEDVATYYCQNVLSRHPGF GSGTKLEIK（SEQ ID NO:51） |
| 8D2H2L17 | EVQLVESGGGLVQPGGSMRLSCAAS GFTFSDNWMNWVRQAPGKGLEWLA QIRNKPYNYETYYSASVKGRFTISRD DSKNSVYLQMNSLKTEDTGVYYCTA QFAYWGQGTLVTVSS（SEQ ID NO:52） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKPGKSPKLLIY GATNLASGVSSRFSGSGSGTDYTL TISSLQPEDVATYYCQNVLSSRPGF GSGTKLEIK（SEQ ID NO:53） |
| 8D2H2L17<br><br>变体1 | EVQLVESGGGLVQPGGSIRLSCAASG FTFSDNWMNWVRQAPGKGLEWLAQ IRNKPYNYETYYSASVKGRFTISRDD SKNSVYLQMNSLKTEDTGVYYCTAQ FAYWGQGTLVTVSS（SEQ ID NO: 102） | DIQMTQSPSSLSASVGDRVTITCRT SENIYGGLNWYQRKPGKSPKLLIY GATNLASGVSSRFSGSGSGTDYTL TISSLQPEDVATYYCQNVLSSRPGF GSGTKLEIK（SEQ ID NO:53） |

[0142] 在本发明的治疗方法、组合物、试剂盒和用途的另一实施方式中，抗CTLA-4抗体是8D2/8D2(RE)的变体、8D2H1L1的变体、8D2H2L2的变体、8D2H2L15的变体或8D2H2L17的变体，其中VH链氨基酸序列的第18位处的甲硫氨酸(Met)独立地被选自以下的氨基酸置换：亮氨酸(Leu)、缬氨酸(Val)、异亮氨酸(Ile)或丙氨酸(Ala)。在本发明的实施方式中，抗CTLA-4抗体是如上表中所示的8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1或8D2H2L17变体1。在另一个实施方式中，8D2/8D2(RE)、8D2H1L1、8D2H2L2、8D2H2L15和8D2H2L17的变体具有VH链，其包含如SEQ ID NO:93、94、95、96、97、98、99、100、101和102中任何一个所示的氨基酸序列。

[0143]

| 抗体 | $V_H$（共有序列） |
|---|---|



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[0144]

| | |
|---|---|
| 8D2/8D2（RE） | EVKLDETGGGLVQPGRPXKLSCVASGFTFSDNWMNWVRQSPEKGLEWLAQIRNKPYNYETYYSDSVKGRFTISRDDSKSSVYLQMNNLRGEDMGIYYCTAQFAYWGQGTLVTVSA<br><br>其中 X＝L（leu）、V（val）、I（Ile）或 A（ala）<br><br>（SEQ ID NO:93） |
| 8D2H1L1 | EVQLVESGGGLVQPGGSXRLSCAASGFTFSDNWMNWVRQAPGKGLEWLAQIRNKPYNYETYYSDSVKGRFTISRDDSKNSVYLQMNSLKTEDTGVYYCTAQFAYWGQGTLVTVSS<br><br>其中 X＝L（leu）、V（val）、I（Ile）或 A（ala）<br><br>（SEQ ID NO:95） |
| 8D2H2L2 | EVQLVESGGGLVQPGGSXRLSCAASGFTFSDNWMNWVRQAPGKGLEWLAQIRNKPYNYETYYSASVKGRFTISRDDSKNSVYLQMNSLKTEDTGVYYCTAQFAYWGQGTLVTVSS<br><br>其中 X＝L（leu）、V（val）、I（Ile）或 A（ala）<br><br>（SEQ ID NO:97） |
| 8D2H2L15 | EVQLVESGGGLVQPGGSXRLSCAASGFTFSDNWMNWVRQAPGKGLEWLAQIRNKPYNYETYYSASVKGRFTISRDDSKNSVYLQMNSLKTEDTGVYYCTAQFAYWGQGTLVTVSS<br><br>其中 X＝L（leu）、V（val）、I（Ile）或 A（ala）<br><br>（SEQ ID NO:99） |
| 8D2H2L17 | EVQLVESGGGLVQPGGSXRLSCAASGFTFSDNWMNWVRQAPGKGLEWLAQIRNKPYNYETYYSASVKGRFTISRDDSKNSVYLQMNSLKTEDTGVYYCTAQFAYWGQGTLVTVSS<br><br>其中 X＝L（leu）、V（val）、I（Ile）或 A（ala）<br><br>（SEQ ID NO:101） |

[0145] 在本发明的治疗方法、组合物、试剂盒和用途的另一个实施方式中，抗CTLA-4抗体

是任何的抗CTLA4抗体或其抗原结合片段，其描述于国际申请公开号WO 2018/035710 A1（发布于2018年3月1日）。在一个实施方式中，抗CTLA4抗体是小鼠抗体4G10，其包含以下VH链和VL链氨基酸序列，以及该抗体的人源化形式。

[0146]

| 抗体 | $V_H$ | $V_L$ |
|---|---|---|
| 4G10<br>鼠 | QVKLQESGPELVKPGASMKISCKASG<br>YSFTGYTMNWVKQSHGKNLEWIGLI<br>NPYNNITNYNQKFMGKATFTVDKSS<br>STAYMELLRLTSEDSGVYFCARLDY<br>RSYWGQGTLVTVSA（SEQ ID NO: 54） | QAVVTQESALTTSPGETVTLTCRSS<br>TGAVTTSNFANWVQEKPDHLFTSL<br>IGGTNNRAPGVPARFSGSLIGDKA<br>ALTITGAQTEDEAIYFCALWYSNH<br>WVFGGGTKLTVLGQPKSSPSVTLF<br>QGQFC（SEQ ID NO:55） |

[0147] 在本发明的治疗方法、组合物、试剂盒和用途的另一个实施方式中，抗CTLA-4抗体是单克隆抗体，其包含下列CDR：

[0148] CDRH1，其包含选自GYSFTGYT（SEQ ID NO:56）或GYTX$_1$ N（SEQ ID NO:57）的氨基酸序列，其中X$_1$是M、V、L、I、G、A、S、T。

[0149] CDRH2，其包含选自INPYNX$_1$IX$_2$（SEQ ID NO:58）的氨基酸序列，其中X$_1$是N、D或E，X$_2$是T、D、E、G或A；或者选自LINPYNX$_1$IX$_2$NYX$_3$QKFX$_4$G（SEQ ID NO:59）的氨基酸序列，其中X$_1$是N、D；X$_2$是T、D、E、G或A；X$_3$是A或N；和X$_4$是Q或M。

[0150] CDRH3，其包含选自LDYRSY（SEQ ID NO:60）或ARLDYRSY（SEQ ID NO:61）的氨基酸序列。

[0151] 和/或

[0152] CDRL1，其包含选自TGAVTTSNF（SEQ ID NO:62）或GSSTGAVTTSNFX$_1$N（SEQ ID NO:63）的氨基酸序列，其中X$_1$是P或A；

[0153] CDRL2，其包含选自GTN或GTNNX$_1$AX$_2$（SEQ ID NO:64）的氨基酸序列，其中X$_1$是K、R或除M或C以外的任何氨基酸；X$_2$是S或P；

[0154] CDRL3，其包含选自ALX$_1$YSNHX$_2$（SEQ ID NO:65）的氨基酸序列，其中X$_1$是W或除M或C以外的任何氨基酸，和X$_2$是W或除M或C外的任何氨基酸；或者选自ALX$_1$YSNHX$_2$V（SEQ ID NO:66）的氨基酸序列，其中X$_1$是W或除M或C以外的任何氨基酸，和X$_2$是W或除M或C外的任何氨基酸。

[0155] 在另一个实施方式中，4G10抗体的人源化VH序列包含任何以下VH序列：



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[0156]

| 抗体 | $V_H$ |
|---|---|
| 4G10H1<br>人源化 | QVQLVESGAELVKPGASMKISCKASGYSFTGYTMNWVKQAPGQGLEWIG<br>LINPYNNITNYNQKFMGKATFTVDKSISTAYMELSRLTSDDSGVYFCARLD<br>YRSYWGQGTLVTVSA（SEQ ID NO:67） |
| 4G10H3<br>人源化 | QVQLVESGAEVKKPGASVKVSCKASGYSFTGYTMNWVRQAPGQGLEWI<br>GLINPYNNITNYAQKFQGRVTFTVDTSISTAYMELSRLRSDDTGVYFCARL<br>DYRSYWGQGTLVTVSA（SEQ ID NO:68） |
| 4G10H4<br>人源化 | QVQLVESGAEVKKPGASVKVSCKASGYSFTGYTMNWVRQAPGQGLEWI<br>GLINPYNDITNYAQKFQGRVTFTVDTSISTAYMELSRLRSDDTGVYFCARL<br>DYRSYWGQGTLVTVSA（SEQ ID NO:69） |
| 4G10H5<br>人源化 | QVQLVESGAEVKKPGASVKVSCKASGYSFTGYTMNWVRQAPGQGLEWI<br>GLINPYNNIDNYAQKFQGRVTFTVDTSISTAYMELSRLRSDDTGVYFCARL<br>DYRSYWGQGTLVTVSA（SEQ ID NO:70） |
| 4G10H<br>共有序列<br>人源化 | QVQLVESGAE$X_1$KKPGAS$X_2$K$X_3$SCKASGYSFTGYT$X_4$NWV$X_5$<br>QAPGQGLEWIGLINPYN$X_6$I$X_7$NY$X_8$QKF$X_9$G$X_{10}$$X_{11}$TFTVD$X_{12}$SISTAYMEL<br>SRL$X_{13}$SDD$X_{14}$GVYFCARLDYRSYWGQGTLVTVSA（SEQ ID NO:71）<br><br>$X_1$ = V 或 L<br>$X_2$ = V 或 M<br>$X_3$ = V 或 I<br>$X_4$ = M、V、L、I、G、A、S、T<br>$X_5$ = R 或 K |

[0157]

| | |
|---|---|
| | $X_6$ = N 或 D 或 E<br>$X_7$ = T 或 D 或 E 或 G 或 A<br>$X_8$ = A 或 N<br>$X_9$ = Q 或 M<br>$X_{10}$ = R 或 K<br>$X_{11}$ = V 或 A<br>$X_{12}$ = T 或 K<br>$X_{13}$ = R 或 T<br>$X_{14}$ = T 或 S |

[0158] 在本发明的治疗方法、组合物、试剂盒和用途的其他实施方式中，4G10抗体的人源化VL序列包含任何以下VL序列：



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[0159]

| 抗体 | $V_L$ |
|---|---|
| 4G10L1<br>人源化 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNFANWVQEKPGQAFR<br>SLIGGTNNRASWVPARFSGSLLGGKAALTISGAQPEDEAEYFCALWY<br>SNHWVFGGGTKLTVL（SEQ ID NO:72） |
| 4G10L3<br>人源化 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNFPNWVQQKPGQAPR<br>SLIGGTNNKASWTPARFSGSLLGGKAALTISGAQPEDEAEYYCALWY<br>SNHWVFGGGTKLTVL（SEQ ID NO:73） |
| 4G10L 共有序列<br>人源化 | QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNF$X_1$NWVQ<br>$X_2$KPGQA$X_3$RSLIGGTNN$X_4$A$X_5$W$X_6$PARFSGSLLGGKAALTISGAQPE<br>DEAEY$X_7$CAL$X_8$YSNH$X_9$VFGGGTKLTVL（SEQ ID NO:74）<br>$X_1$ = P 或 A<br>$X_2$ = Q 或 E<br>$X_3$ = P 或 F<br>$X_4$ = K 或 R 或除 M 或 C 以外的任何氨基酸<br>$X_5$= S 或 P<br>$X_6$ = T 或 V<br>$X_7$ = Y 或 F<br>$X_8$ =W 或除 M 或 C 以外的任何氨基酸<br>$X_9$ =W 或除 M 或 C 以外的任何氨基酸 |

[0160] 在一些实施方式中，抗CTLA4抗体是4G10H1L1、4G10H3L3、4G10H3L3和4G10H5L3，各自描述如下。

[0161]

| 抗体 | $V_H$ | $V_L$ |
|---|---|---|

[0162]

| | | |
|---|---|---|
| 4G10H1L1 | QVQLVESGAELVKPGASMKISCKA SGYSFTGYTMNWVKQAPGQGLE WIGLINPYNNITNYNQKFMGKATF TVDKSISTAYMELSRLTSDDSGVY FCARLDYRSYWGQGTLVTVSA （SEQ ID NO:75） | QAVVTQEPSLTVSPGGTVTLTCGS STGAVTTSNFANWVQEKPGQAFR SLIGGTNNRASWVPARFSGSLLGG KAALTISGAQPEDEAEYFCALWYS NHWVFGGGTKLTVL （SEQ ID NO:76） |
| 4G10H3L3 | QVQLVESGAEVKKPGASVKVSCK ASGYSFTGYTMNWVRQAPGQGLE WIGLINPYNNITNYAQKFQGRVTF TVDTSISTAYMELSRLRSDDTGVY FCARLDYRSYWGQGTLVTVSA （SEQ ID NO:77） | QAVVTQEPSLTVSPGGTVTLTCGS STGAVTTSNFPNWVQQKPGQAPRS LIGGTNNKASWTPARFSGSLLGGK AALTISGAQPEDEAEYYCALWYSN HWVFGGGTKLTVL （SEQ ID NO:78） |
| 4G10H4L3 | QVQLVESGAEVKKPGASVKVSCK ASGYSFTGYTMNWVRQAPGQGLE WIGLINPYNDITNYAQKFQGRVTF TVDTSISTAYMELSRLRSDDTGVY FCARLDYRSYWGQGTLVTVSA （SEQ ID NO:79） | QAVVTQEPSLTVSPGGTVTLTCGS STGAVTTSNFPNWVQQKPGQAPRS LIGGTNNKASWTPARFSGSLLGGK AALTISGAQPEDEAEYYCALWYSN HWVFGGGTKLTVL （SEQ ID NO:80） |
| 4G10H5L3 | QVQLVESGAEVKKPGASVKVSCK ASGYSFTGYTMNWVRQAPGQGLE WIGLINPYNNIDNYAQKFQGRVTF TVDTSISTAYMELSRLRSDDTGVY FCARLDYRSYWGQGTLVTVSA （SEQ ID NO:81） | QAVVTQEPSLTVSPGGTVTLTCGS STGAVTTSNFPNWVQQKPGQAPRS LIGGTNNKASWTPARFSGSLLGGK AALTISGAQPEDEAEYYCALWYSN HWVFGGGTKLTVL （SEQ ID NO:82） |

[0163] 在本发明的治疗方法、组合物、试剂盒和用途的另一个实施方式中，抗CTLA-4抗体是抗体或其抗原结合片段，其与伊匹单抗、曲美木单抗或任何上述抗体(包括8D2/8D2(RE)或其变体、8D2H1L1或其变体、8D2H2L2或其变体、8D3H3L3或其变体、8D2H2L15或其变体、8D2H2L17或其变体、4G10H1L1或其变体、4G10H3L3或其变体、4G10H3L3或其变体和4G10H5L3或其变体)交叉竞争以结合人CTLA-4、或结合至人CTLA-4的相同的表位区域。

[0164] III.本发明的方法和用途

[0165] 本发明提供一种治疗人类患者的癌症的方法，其包括施用：(a)200mg或240mg抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段；或(b)2mg/kg抗PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段，其中所述抗CTLA4抗体或其抗原结合片段的量选自10mg、25mg、50mg和75mg，并且其中所述抗PD-1抗体或其抗原结合片段每三周施用一次，并且所述抗CTLA4抗体或其抗原结合片段每三周施用一次或每六周施用一次(方法



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1)。

[0166] 在一个实施方式中,所述方法包括施用200mg抗PD-1抗体和10mg抗CTLA4抗体。

[0167] 在另一个实施方式中,所述方法包括施用240mg抗PD-1抗体和10mg抗CTLA4抗体。

[0168] 在其他的实施方式中,所述方法包括施用2mg/kg抗PD-1和10mg抗CTLA4抗体。

[0169] 在一个实施方式中,所述方法包括施用200mg抗PD-1抗体和25mg抗CTLA4抗体。

[0170] 在另一个实施方式中,所述方法包括施用240mg抗PD-1抗体和25mg抗CTLA4抗体。

[0171] 在其他的实施方式中,所述方法包括施用2mg/kg抗PD-1和25mg抗CTLA4抗体。

[0172] 在一个实施方式中,所述方法包括施用200mg抗PD-1抗体和50mg抗CTLA4抗体。

[0173] 在另一个实施方式中,所述方法包括施用240mg抗PD-1抗体和50mg抗CTLA4抗体。

[0174] 在其他的实施方式中,所述方法包括施用2mg/kg抗PD-1和50mg抗CTLA4抗体。

[0175] 在一个实施方式中,所述方法包括施用200mg抗PD-1抗体和75mg抗CTLA4抗体。

[0176] 在另一个实施方式中,所述方法包括施用240mg抗PD-1抗体和75mg抗CTLA4抗体。

[0177] 在其他的实施方式中,所述方法包括施用2mg/kg抗PD-1和75mg抗CTLA4抗体。

[0178] 还提供了如上所述用替代剂量治疗癌症的方法。在所述方法中,所述抗PD-1抗体或其抗原结合片段的剂量为约150mg至约250mg、约175mg至约250mg、约200mg至约250mg、约150mg至约240mg、约175mg至约240mg、约200mg至约240mg。在一些实施方式中,所述抗PD-1抗体或其抗原结合片段的剂量为150mg、175mg、200mg、225mg、240mg或250mg。

[0179] 在上述任何方法的一个实施方式中,所述抗CTLA4抗体或其抗原结合片段的剂量为10mg、25mg、50mg或75mg。

[0180] 在上述任何方法的一些实施方式中,所述抗CTLA4抗体或其抗原结合片段每六周施用一次,持续24周或更短时间。

[0181] 在上述任何方法的替代实施方式中,抗CTLA4抗体或其抗原结合片段每三周施用一次,持续24周或更短时间。

[0182] 本文还提供了如任何上述实施方式中所述的方法,所述方法还包括在停止施用抗CTLA4抗体后继续每三周一次施用抗PD-1抗体。

[0183] 本发明还提供了一种治疗人类患者的癌症的方法,其包括施用:(a)200mg或240mg抗PD-1抗体或其抗原结合片段和75mg或100mg抗CTLA4抗体或其抗原结合片段;或(b)2mg/kg抗PD-1抗体或其抗原结合片段和75mg或100mg抗CTLA4抗体或其抗原结合片段,其中所述抗PD-1抗体或其抗原结合片段每三周施用一次,并且所述抗CTLA4抗体或其抗原结合片段每十二周施用一次(方法2)。

[0184] 方法2的一个实施方式包括施用200mg抗PD-1抗体和100mg抗CTLA4抗体。

[0185] 另一个实施方式包括施用240mg抗PD-1抗体和100mg抗CTLA4抗体。

[0186] 一个实施方式包括施用2mg/kg抗PD-1抗体和100mg抗CTLA4抗体。

[0187] 在方法2的子实施方式中,所述抗CTLA抗体或其抗原结合片段每12周施用一次,持续48周或更短时间。

[0188] 本文还提供了如上述任何实施方式中所述的方法,所述方法还包括在停止施用所述抗CTLA4抗体后继续每三周一次施用所述抗PD-1抗体。

[0189] 在如上所述的本发明的任何方法(方法1或方法2)中,所述PD-1抗体或其抗原结合片段和所述CTLA4抗体或其抗原结合片段可以是如本文中具体实施方式第II部分“可用于



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本发明的PD-1拮抗剂、抗体和抗原结合片段，以及CTLA4抗体和抗原结合片段”中所描述的任何抗体或其抗原结合片段。

[0190] 在一些实施方式中，所述抗PD-1抗体是派姆单抗或其抗原结合片段、纳武单抗或其抗原结合片段、或是与派姆单抗或纳武单抗交叉竞争以结合人PD-1的抗体。

[0191] 在一些实施方式中，所述抗CTLA4抗体选自：

[0192] (a)伊匹单抗，

[0193] (b)曲美木单抗，

[0194] (c)8D2/8D2(RE)、8D2/8D2(RE)变体1或其其他变体，

[0195] (d)8D2H1L1、8D2H1L1变体1、或其其他变体，

[0196] (e)8D2H2L2、8D2H2L2变体1、或其其他变体，

[0197] (f)8D3H3L3或其变体，

[0198] (g)8D2H2L15、8D2H2L15变体1、或其其他变体，

[0199] (h)8D2H2L17、8D2H2L17变体1、或其其他变体，

[0200] (i)4G10H1L1，

[0201] (j)4G10H3L3，

[0202] (k)4G10H3L3，

[0203] (l)4G10H5L3，和

[0204] (m)与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体；其中8D2/8D2(RE)变体、8D2H1L1变体、8D2H2L2变体、8D3H3L3变体、8D2H2L15变体和8D2H2L17变体的VH链氨基酸序列的第18位处的甲硫氨酸独立地被选自亮氨酸、缬氨酸、异亮氨酸和丙氨酸的氨基酸置换。

[0205] 在具体的实施方式中，所述抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体；8D2H1L1、8D2H1L1变体1、或8D2H1L1的其它变体；8D2H2L2、8D2H2L2变体1、或8D2H2L2的其他变体；8D3H3L3或其变体；8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体；或8D2H2L17、8D2H2L17变体1、或8D2H2L17的其它变体。

[0206] 在一些具体的实施方式中，所述抗CTLA4抗体是8D2/8D2(RE)的变体、8D2H1L1的变体、8D2H2L2的变体、8D2H2L15的变体、或8D2H2L17的变体，其中所述抗CTLA4抗体的VH序列的第18位处的氨基酸是异亮氨酸。在其他实施方式中，所述抗CTLA4抗体是8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1或8D2H2L17变体1。

[0207] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是伊匹单抗。

[0208] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是曲美木单抗。

[0209] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1或8D2/8D2(RE)的其他变体。

[0210] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是8D2H1L1、8D2H1L1变体1或8D2H1L1的其他变体。

[0211] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是8D2H2L2、8D2H2L2变体1或8D2H2L2的其他变体。

[0212] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是8D3H3L3或其变体。

[0213] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是8D2H2L15、8D2H2L15变体1或8D2H2L15的其他变体。

[0214] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是8D2H2L17、8D2H2L17变体1或8D2H2L17的其他变体。

[0215] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是4G10H1L1。

[0216] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是4G10H3L3。

[0217] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是4G10H4L3。

[0218] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是4G10H5L3。

[0219] 在本发明方法的一些实施方式中，所述抗PD-1抗体是派姆单抗和所述抗CTLA4抗体是与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体。

[0220] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是伊匹单抗。

[0221] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是曲美木单抗。

[0222] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1或8D2/8D2(RE)的其他变体。

[0223] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D2H1L1变体1、或8D2H1L1的其他变体。

[0224] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D2H2L2、8D2H2L2变体1、或8D2H2L2其他的变体。

[0225] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D3H3L3或其变体。

[0226] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体。

[0227] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体。

[0228] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是4G10H1L1。

[0229] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是4G10H3L3。

[0230] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是4G10H4L3。

[0231] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗



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体是4G10H5L3。

[0232] 在本发明方法的一些实施方式中，所述抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体。

[0233] 在本发明的任何方法中，所述癌症选自：黑色素瘤、肺癌、头颈癌、膀胱癌、乳腺癌、胃肠癌、多发性骨髓瘤、肝细胞癌、淋巴瘤、肾癌、间皮瘤、卵巢癌、食道癌、肛门癌、胆道癌、结直肠癌、宫颈癌、甲状腺癌和唾液癌。

[0234] 在一些实施方式中，肺癌是非小细胞肺癌。

[0235] 在替代的实施方式中，肺癌是小细胞肺癌。

[0236] 在一些实施方式中，淋巴瘤是霍奇金淋巴瘤。

[0237] 在其他实施方式中，淋巴瘤是非霍奇金淋巴瘤。在特定的实施方式中，淋巴瘤是纵隔的大B细胞淋巴瘤。

[0238] 在一些实施方式中，乳腺癌是三阴性乳腺癌。

[0239] 在其他的实施方式中，乳腺癌是ER+/HER2-乳腺癌。

[0240] 在一些实施方式中，膀胱癌是尿路上皮癌。

[0241] 在一些实施方式中，头颈癌是鼻咽癌。在一些实施方式中，癌症是甲状腺癌。在其它实施方式中，癌症是唾液癌。

[0242] 在一些实施方式中，癌症是具有高水平微卫星不稳定性(MSI-H)的转移性结直肠癌。

[0243] 在一些实施方式中，癌症选自：黑色素瘤、非小细胞肺癌、复发或难治性经典霍奇金淋巴瘤、头颈部鳞状细胞癌、尿路上皮癌、食道癌、胃癌和肝细胞癌。

[0244] 在上述治疗方法的其他实施方式中，癌症是血红素恶性肿瘤。在某些实施方式中，血红素恶性肿瘤是急性成淋巴细胞性白血病(ALL)、急性髓性白血病(AML)、慢性淋巴细胞性白血病(CLL)、慢性髓性白血病(CML)、弥漫性大B细胞淋巴瘤(DLBCL)、EBV阳性DLBCL、原发性纵隔大B细胞淋巴瘤、富含T细胞/组织细胞的大B细胞淋巴瘤、滤泡性淋巴瘤、霍奇金淋巴瘤(HL)、套细胞淋巴瘤(MCL)、多发性骨髓瘤(MM)、髓样细胞白血病-1蛋白质(Mcl-1)、骨髓增生异常综合症(MDS)、非霍奇金淋巴瘤(NHL)或小淋巴细胞淋巴瘤(SLL)。

[0245] 在本文任何方法的实施方式中，向受试者静脉内(IV)输注包含本文所述的任何PD-1拮抗剂的药物和包含任何抗CTLA4抗体或其抗原结合片段的药物。

[0246] 所述组合疗法还可包含一种或多种“另外的治疗剂”(如本文所用，“另外的治疗剂”意指相对于PD-1拮抗剂和抗CTLA4抗体或其抗原结合片段的另外的药剂)。另外的治疗剂可以是，例如，除了抗PD-1抗体或抗CTLA4抗体以外的化学治疗剂、生物治疗剂(包括但不限于针对VEGF、EGFR、HER2/neu、VEGF受体、其他生长因子受体、CD20、CD40、CD-40L、OX-40、4-1BB和ICOS的抗体)、免疫原性因子(例如，减毒的癌细胞、肿瘤抗原、抗原呈递细胞例如用肿瘤衍生的抗原或核酸脉冲的树突状细胞、免疫刺激细胞因子(例如，IL-2、IFNα2、GM-CSF)和转染编码免疫刺激细胞因子(例如但不限于GM-CSF)的基因的细胞)。

[0247] 如上所述，在本发明方法的一些实施方式中，方法还包括施用另外的治疗剂。在特定的实施方式中，另外的治疗剂是抗LAG3抗体或其抗原结合片段、抗GITR抗体或其抗原结合片段、抗TIGIT抗体或其抗原结合片段、抗CD27抗体或抗原结合片段。在一个实施方式中，另外的治疗剂是表达IL-12的新城疫病毒载体。在其他的实施方式中，另外的治疗剂是



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dinaciclib。

[0248] 化学治疗剂的实例包括烷化剂，例如噻替哌和环磷酰胺；氨基磺酸盐，例如白消安、英丙舒凡(improsulfan)和哌泊舒凡(piposulfan)；氮杂环丙烷，如苯并二唑、卡波醌、meturedopa和uredopa；乙烯亚胺(ethylenimine)和methylamelamine，包括六甲蜜胺、三亚乙基三聚氰胺、三乙烯基磷酰胺、三亚乙基硫代磷酰胺和三羟甲基胺；acetogenins(特别是布拉他辛和bullatacinone)；喜树碱(包括合成类似物拓扑替康)；抑素；callystatin；CC-1065(包括其阿多来新、卡折来新和比折来新合成类似物)；念珠藻素(cryptophycins)(特别是念珠藻素1和念珠藻素8)；多拉司他汀；duocarmycin(包括其合成类似物，KW-2189和CBI-TMI)；五加素；水鬼蕉碱；sarcodictyin；软海绵素；氮芥，如苯丁酸氮芥、氯苯哒嗪、氯膦胺、雌莫司汀、异环磷酰胺、二氯甲基二乙胺、盐酸甲氧氮芥、美法仑、新恩比兴、苯芥胆甾醇、泼尼莫司汀、曲洛磷胺、乌拉莫司汀；亚硝基脲类，如卡莫司汀、氯嘧菌素、福莫司汀、洛莫司汀、尼莫司汀、马来酰汀；抗生素如烯二炔抗生素(例如加利车霉素，特别是加利车霉素γ1I和加利车霉素φI1，参见例如Agnew，Chem.Intl.Ed.Engl.，33:183-186(1994)；dynemicin，包括dynemicin A；双膦酸盐，例如氯膦酸盐；拉霉素；以及新抑癌菌素生色团和相关的色蛋白二炔类抗生素生色团)、aclacinomysins、放线菌素、authramycin、重氮丝氨酸、仙人掌霉素、cactinomycin、carabicin、caminomycin、嗜癌菌素、色霉素、更生霉素、道诺霉素、地托比辛、6-重氮-5-氧-L-正亮氨酸、阿霉素(包括吗啉代阿霉素、cyanomorpholino-doxorubicin、2-pyrrolino-doxorubicin和deoxydoxororicicin)、表柔比星、依索比星、伊达比星、麻西罗霉素、丝裂霉素例如丝裂霉素C、药酚酸、诺加霉素、橄榄霉素、派来霉素、potfiromycin、嘌呤霉素、三铁阿霉素、罗多比星、链黑霉素、链脲菌素、杀结核菌素、ubenimex、新制癌菌素、佐柔比星；抗代谢物例如甲氨蝶呤和5-氟尿嘧啶(5-FU)；叶酸类似物例如二甲叶酸、甲氨蝶呤、蝶罗呤、三甲曲沙；嘌呤类似物例如氟达拉滨、6-巯基嘌呤、硫胺素、硫鸟嘌呤；嘧啶类似物例如安西他滨、阿扎胞苷、6-氮杂尿苷、卡莫氟、阿糖胞苷、双脱氧尿苷、多西氟尿苷、依那西班、氟尿苷；calusterone、dromostanolone propionate、epitiostanol、mepitiostane、testolactone等雄性激素；抗肾上腺素例如氨基乙酰亚胺、米托坦、trilostane；叶酸补充剂例如氟喹啉酸；醋葡醛内酯；醛磷酰胺糖苷；氨基乙酰丙酸；恩尿嘧啶；安吖啶；bestrabucil；比生群；edatraxate；defofamine；美可辛；diaziquone；elformithine；醋酸乙烯酯；埃博霉素；etoglucid；硝酸镓；羟基脲；香菇多糖；氯尼达明；美登素类、如美登素和安沙霉素；mitoguazone；米itoxantrone；mopidamol；nitracrine；喷司他丁；phenamet；吡柔比星；洛索；鬼臼毒素；2-ethylhydrazide；甲基苄肼；razoxane；根霉素；sizofuran；螺环锗；tenuazonic acid；三亚胺；2,2',2"-三氯三乙胺；trichoth ecenes(尤其是T-2毒素、verracurin A、roridin A和anguidine)；氨基甲酸乙酯；长春地辛；达卡巴嗪；甘露醇氮芥；二溴甘露醇；卫矛醇；哌泊溴烷；gacytosine；阿拉伯糖苷("Ara-C")；环磷酰胺；塞替派；紫杉烷类例如紫杉醇和多西紫杉醇；苯丁酸氮芥；吉西他滨；6-硫鸟嘌呤；巯基嘌呤；甲氨蝶呤；铂类似物例如顺铂和卡铂；长春碱；铂；依托泊苷(VP-16)；异环磷酰胺；米托蒽醌；长春新碱；长春瑞滨；减瘤；替尼泊苷；依达曲沙；柔红霉素；氨基；希罗达；伊班膦酸钠；CPT-11；拓扑异构酶抑制剂RFS 2000；二氟甲基鸟氨酸(DMFO)；类维生素A、如维甲酸；卡培他滨；和任何上述的药学上可接受的盐、酸或衍生物。还包括用于调节或抑制激素对肿瘤的作用的抗激素剂，例如抗雌激素和选择性雌激素受体调



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节剂(SERM)，包括例如他莫昔芬、雷洛昔芬、屈洛昔芬、4-羟基三苯氧胺、曲奥西芬、keoxifene、LY117018、奥那司酮和toremifene(法乐通)；抑制酶芳香酶的芳香酶抑制剂，其调节肾上腺中的雌激素产生，例如，4(5)-咪唑、氨基乙酰亚胺、醋酸甲地孕酮、依西美坦、福美坦、法倔唑、伏唑唑、来曲唑和阿那曲唑；和抗雄性激素例如氟他胺、尼鲁米特、比卡鲁胺、亮丙瑞林和戈舍瑞林；以及任何上述的药学上可接受的盐、酸或衍生物。

[0249] 在包括施用另外的治疗剂(即除了PD-1拮抗剂和抗CTLA4抗体或其结合片段之外)的步骤的一些实施方式中，组合疗法中的另外的治疗剂可使用与当药剂作为单疗法用于治疗相同癌症时通常使用的相同的剂量方案(剂量、频率和治疗持续时间)施用。在其它实施方式中，患者接受组合疗法中相比于当另外的治疗剂作为单疗法使用时更低总量的另外治疗剂，例如，较小的剂量、较少频繁的剂量、和/或较短的治疗持续时间。

[0250] 组合疗法中另外的治疗剂可以口服或肠胃外施用，包括静脉内、肌内、腹膜内、皮下、直肠、局部和经皮施用途径。例如，组合治疗可包含抗PD-1抗体或其抗原结合片段，以及抗CTLA抗体或其抗原结合片段，其两者均可以静脉内施用，以及化学治疗剂，其可以是口服施用。

[0251] 可以在手术之前或之后使用本发明的组合疗法来移除肿瘤，并且可以在放射疗法之前、期间或之后使用。

[0252] 在一些实施方式中，将本发明的组合疗法施用于之前未用生物治疗剂或化学治疗剂治疗的患者，即未治疗的患者。在其他的实施方式中，将组合疗法施用于在用生物治疗剂或化学治疗剂预先治疗后未能实现持续响应的患者，即已经历治疗。

[0253] 本发明的组合疗法可用于治疗足够大以通过触诊或通过本领域熟知的成像技术(例如MRI、超声或CAT扫描)发现的肿瘤。在一些实施方式中，本发明的组合疗法被用于治疗具有至少约200mm$^3$、300mm$^3$、400mm$^3$、500mm$^3$、750mm$^3$或至多1000mm$^3$的尺寸的晚期肿瘤。

[0254] 在一些实施方式中，将本发明的组合疗法施用于患有对PD-L1表达测试呈阳性的癌症的人类患者。在一些实施方式中，PD-L1表达使用诊断性抗人PD-L1抗体或抗原结合片段在对从患者移除的肿瘤样品的FFPE或冷冻组织切片进行的IHC测定中检测。患者的医生可订购诊断性测试，以在开始用PD-1拮抗剂和抗CTLA-4抗体进行治疗之前确定从患者移除肿瘤组织样品中的PD-L1表达，但可以想到的是，医生可以在治疗开始后的任何时间，例如在完成治疗周期之后订购第一次或随后的诊断测试。

[0255] 选择另外的治疗剂的剂量可以包括依赖于几个因素，包括实体的血清或组织周转率、症状的水平、实体的免疫原性，和正治疗的个体中靶细胞、组织或器官的可接近性。另外的治疗剂的剂量应该是提供可接受水平的副作用的量。因此，每个另外的治疗剂(例如生物治疗或化学治疗剂)的剂量和给药频率将部分取决于特定的治疗剂、被治疗的癌症的严重程度，和病人的特征。可以获得选择适当抗体的剂量、细胞因子和小分子的指导。参见，例如，Wawrzynczak(1996)Antibody Therapy,Bios Scientific Pub.Ltd,Oxfordshire,UK;Kresina(ed.)(1991)Monoclonal Antibodies,Cytokines and Arthritis,Marcel Dekker,New York,NY;Bach(ed.)(1993)Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases,Marcel Dekker,New York,NY;Baert等(2003)New Engl.J.Med.348:601-608;Milgrom等(1999)New Engl.J.Med.341:1966-1973;Slamon等(2001)New Engl.J.Med.344:783-792;Beniaminovitz等(2000)New Engl.J.Med.342:613-



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619;Ghosh等(2003)New Engl.J.Med.348:24-32;Lipsky等(2000)New Engl.J.Med.343:1594-1602;Physicians'Desk Reference 2003(Physicians'Desk Reference,57th Ed);Medical Economics Company;ISBN:1563634457;57th edition(November 2002)。临床医生可以确定合适的剂量方案,例如,使用本领域已知或疑似的影响治疗或预期可影响治疗的参数或因素,并且将取决于例如患者的临床病史(例如,先前的治疗)、待治疗癌症的类型和阶段和对组合疗法中一种或多种治疗剂响应的生物标志物。

[0256] IV.组合物和试剂盒

[0257] 本发明还涉及包含一种组合物,其包含PD-1拮抗剂(例如抗PD-1抗体或其抗原结合片段)的剂量和抗CTLA4抗体或其抗原结合片段的的剂量,其中所述剂量选自:

[0258] (i)200mg或240mg抗PD-1抗体或其抗原结合片段和10mg抗CTLA4抗体或其抗原结合片段;

[0259] (ii)200mg或240mg抗PD-1抗体或其抗原结合片段和25mg抗CTLA4抗体或其抗原结合片段;

[0260] (iii)200mg或240mg抗PD-1抗体或其抗原结合片段和50mg抗CTLA4抗体或其抗原结合片段;

[0261] (iv)200mg或240mg抗PD-1抗体或其抗原结合片段和75mg抗CTLA4抗体或其抗原结合片段;

[0262] (v)200mg或240mg抗PD-1抗体或其抗原结合片段和100mg抗CTLA4抗体或其抗原结合片段;

[0263] (vi)2mg/kg抗PD-1抗体或其抗原结合片段和10mg抗CTLA4抗体或其抗原结合片段;

[0264] (vii)2mg/kg抗PD-1抗体或其抗原结合片段和25mg抗CTLA4抗体或其抗原结合片段;

[0265] (viii)2mg/kg抗PD-1抗体或其抗原结合片段和50mg抗CTLA4抗体或其抗原结合片段;

[0266] (ix)2mg/kg抗PD-1抗体或其抗原结合片段和75mg抗CTLA4抗体或其抗原结合片段;和

[0267] (x)2mg/kg抗PD-1抗体或其抗原结合片段和100mg抗CTLA4抗体或其抗原结合片段。

[0268] 本发明还提供了一种组合物,其包含如上所述的PD-1拮抗剂和药学上可接受的赋形剂。当PD-1拮抗剂是生物治疗剂(例如mAb)时,可以使用常规细胞培养和回收/纯化技术在CHO细胞中产生拮抗剂。

[0269] 在一个实施方式中,本发明提供了一种组合物,其包含抗PD-1抗体的剂量和抗CTLA4抗体的剂量,其中所述剂量选自:

[0270] (i)200mg抗PD-1抗体和10mg抗CTLA4抗体;

[0271] (ii)200mg抗PD-1抗体和25mg抗CTLA4抗体;

[0272] (iii)200mg抗PD-1抗体和50mg抗CTLA4抗体;

[0273] (iv)200mg抗PD-1抗体和75mg抗CTLA4抗体;和

[0274] (v)200mg抗PD-1抗体和100mg抗CTLA4抗体。

[0275] 在本发明的组合物中，PD-1抗体或其抗原结合片段和抗CTLA4抗体或其抗原结合片段可以是本文所述的任何抗体和抗原结合片段，即如本发明的具体实施方式的第II部分“可用于本发明的PD-1拮抗剂、抗体和抗原结合片段”中所描述的。

[0276] 在一些实施方式中，包含抗PD-1抗体作为PD-1拮抗剂的组合物可以作为液体制剂提供，或者通过在使用前用无菌注射用水重构冻干粉末来制备。WO2012/135408描述了包含派姆单抗的液体和冻干药物的制备，其适用于本发明。在一些优选的实施方式中，在含有约50mg的派姆单抗的玻璃小瓶中提供包含派姆单抗的药物。

[0277] 在本文组合物的一些实施方式中，抗PD-1抗体是派姆单抗、纳武单抗、或与派姆单抗或纳武单抗交叉竞争以结合人PD-1的抗体。

[0278] 在一些实施方式中，抗CTLA4抗体是伊匹单抗。

[0279] 在本发明的组合物的其他实施方式中，抗CTLA4抗体选自：

[0280] (a)伊匹单抗，

[0281] (b)曲美木单抗，

[0282] (c)8D2/8D2(RE)、8D2/8D2(RE)变体1、或其其他变体，

[0283] (d)8D2H1L1、或其其他变体，

[0284] (e)8D2H2L2、8D2H2L2、或其其他变体，

[0285] (f)8D3H3L3或其变体，

[0286] (g)8D2H2L15、8D2H2L15、或其其他变体，

[0287] (h)8D2H2L17、8D2H2L17、或其其他变体，

[0288] (i)4G10H1L1，

[0289] (j)4G10H3L3，

[0290] (k)4G10H4L3，

[0291] (l)4G10H5L3，和

[0292] (m)与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体；

[0293] 其中所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体和所述8D2H2L17变体的所述可变重链(VH)氨基酸序列的第18位处的甲硫氨酸独立地被选自亮氨酸、缬氨酸、异亮氨酸和丙氨酸的氨基酸置换。

[0294] 在本发明组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是伊匹单抗。

[0295] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是曲美木单抗。

[0296] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体。

[0297] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体。

[0298] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H2L2、8D2H2L2变体1、或8D2H2L2的其他变体。

[0299] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D3H3L3或其变体。



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[0300] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体。

[0301] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体。

[0302] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H1L1。

[0303] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H3L3。

[0304] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H4L3。

[0305] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H5L3。

[0306] 在本发明的组合物的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体。

[0307] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是伊匹单抗。

[0308] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是曲美木单抗。

[0309] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体。

[0310] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体。

[0311] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H2L2、8D2H2L2变体1、或8D2H2L2的其他变体。

[0312] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D3H3L3或其变体。

[0313] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体。

[0314] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体。

[0315] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H1L1。

[0316] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H3L3。

[0317] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H4L3。

[0318] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H5L3。

[0319] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是



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与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体。

[0320] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA-4抗体选自8D2/8D2(RE)、8D2/8D2(RE)变体1或8D2/8D2(RE)的其他变体；8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体；8D2H2L2、8D2H2L2变体1或8D2H2L2的其他变体；8D3H3L3或其变体；8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体；或8D2H2L17、8D2H2L17变体1或8D2H2L17的其他变体。

[0321] 在本发明的组合物的其他实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

[0322] 本发明还涉及一种用于治疗患有癌症的患者的试剂盒，所述试剂盒包括：(a)抗PD-1抗体或其抗原结合片段的剂量和抗CTLA4抗体或抗原结合片段的剂量，其选自：(i)200mg抗PD-1抗体或其抗原结合片段和10mg抗CTLA4抗体或其抗原结合片段；(ii)200mg抗PD-1抗体或其抗原结合片段和25mg抗CTLA4抗体或其抗原结合片段；(iii)200mg抗PD-1抗体或其抗原结合片段和50mg抗CTLA4抗体或其抗原结合片段；(iv)200mg抗PD-1抗体或其抗原结合片段和75mg抗CTLA4抗体或其抗原结合片段；和(v)200mg抗PD-1抗体或其抗原结合片段和100mg抗CTLA4抗体或其抗原结合片段；和(b)在权利要求1-16中任一项所述的方法中使用所述抗PD-1抗体或其抗原结合片段和所述抗CTLA-4抗体或其抗原结合片段的说明书。

[0323] 在本发明的任何试剂盒中，PD-1抗体或抗原结合片段和CTLA4抗体或抗原结合片段可以是本发明具体实施方式第II部分“可用于本发明的PD-1拮抗剂、抗体和抗原结合片段”中所描述的任何抗体或抗原结合片段。

[0324] 在一些实施方式中，抗PD-1抗体是派姆单抗、纳武单抗、或与派姆单抗或纳武单抗交叉竞争以结合人PD-1的抗体。

[0325] 在一些实施方式中，抗CTLA4抗体选自：伊匹单抗、曲美木单抗、8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体；8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体；8D2H2L2、8D2H2L2、或8D2H2L2的其他变体、8D3H3L3或其变体；8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体；8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体、4G10H1L1、4G10H3L3、4G10H3L3、4G10H5L3、和与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体，其中所述8D2/8D2(RE)变体、所述8D2H1L1变体、所述8D2H2L2变体、所述8D3H3L3变体、所述8D2H2L15变体和所述8D2H2L17变体的所述可变重链(VH)氨基酸序列的第18位处的甲硫氨酸独立地被选自亮氨酸、缬氨酸、异亮氨酸和丙氨酸的氨基酸置换。

[0326] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是伊匹单抗。

[0327] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是曲美木单抗。

[0328] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体。

[0329] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体。

[0330] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是



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8D2H2L2、8D2H2L2变体1、或8D2H2L2的其他变体。

[0331] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D3H3L3或其变体。

[0332] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体。

[0333] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体。

[0334] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H1L1。

[0335] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H3L3。

[0336] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H4L3。

[0337] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是4G10H5L3。

[0338] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体。

[0339] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是伊匹单抗。

[0340] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是曲美木单抗。

[0341] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体。

[0342] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体。

[0343] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和所述抗CTLA4抗体是8D2H2L2、8D2H2L2变体1、或8D2H2L2的其他变体。

[0344] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D3H3L3或其变体。

[0345] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体。

[0346] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体。

[0347] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H1L1。

[0348] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H3L3。

[0349] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H4L3。



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[0350] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是4G10H5L3。

[0351] 在本发明的试剂盒的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA4抗体是与伊匹单抗或曲美木单抗交叉竞争以结合人CTLA-4的抗体。

[0352] 在本发明的组合物的一些实施方式中，抗PD-1抗体是纳武单抗和抗CTLA-4抗体选自8D2/8D2(RE)、8D2/8D2(RE)变体1、或8D2/8D2(RE)的其他变体；8D2H1L1、8D2H1L1变体1、或8D2H1L1的其他变体；8D2H2L2、8D2H2L2变体1、或8D2H2L2的其他变体；8D3H3L3或其变体；8D2H2L15、8D2H2L15变体1、或8D2H2L15的其他变体；或是8D2H2L17、8D2H2L17变体1、或8D2H2L17的其他变体。

[0353] 在本发明的组合物的其他实施方式中，抗PD-1抗体是派姆单抗和抗CTLA4抗体是8D2/8D2(RE)变体1、8D2H1L1变体1、8D2H2L2变体1、8D2H2L15变体1、或8D2H2L17变体1。

[0354] 本发明的试剂盒可以提供在第一容器和第二容器中的抗PD-1和抗CTLA4抗体或其抗原结合片段和包装插入物。第一容器含有至少一个剂量的含有抗PD-1拮抗剂的药物，第二容器含有至少一个剂量的含有抗CTLA4抗体或其抗原结合片段的药物和包装插入物或者标记，其包含使用药物治疗患有癌症的患者的说明书。第一容器和第二容器可以包含相同或不同的形状(例如，小瓶、注射器和瓶子)和/或材料(例如，塑料或玻璃)。试剂盒还可以包括可能在施用药物时有用的其他材料(例如稀释剂、过滤器、输液袋和线、针头和注射器)。在试剂盒的一些优选实施方式中，抗PD-1的拮抗剂是抗PD-1抗体并且说明书指出药物旨在治疗患有肿瘤的患者的用途，其中通过例如IHC测定肿瘤表达PD-L1。在一些实施方式中，肿瘤具有≥1%PD-L1的肿瘤比例评分(TPS)。在另一实施方式中，肿瘤具有>50%PD-L1的TPS。PD-L1 TPS是表达PD-L1的样品中肿瘤细胞的数量。在其他的实施方式中，肿瘤具有≥5%PD-L1、≥10PD-L1、≥15%PD-L1、≥20%PD-L1、≥25%PD-L1、≥30%PD-L1、≥35%PD-L1、≥40%PD-L1或≥45%PD-L1的TPS。

[0355] 根据本文包含的指导，本发明的这些和其他方面，包括下面列出的示例性特定实施方式，将是显而易见的。

[0356] 通用方法

[0357] 分子生物学的标准方法描述于Maniatis(1982&1989 2nd Edition,2001 3rd Edition)Molecular Cloning,A Laboratory Manual,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,NY;Sambrook和Russell(2001)Molecular Cloning,3rd ed.,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,NY;Wu(1993)Recombinant DNA,Vol.217,Academic Press,San Diego,CA)。标准方法也出现于Ausbel,等(2001)Current Protocols in Molecular Biology,Vols.1-4,John Wiley and Sons,Inc.New York,NY,其描述了在细菌细胞中的克隆和DNA诱变(第1卷)、在哺乳动物细胞和酵母中的克隆(第2卷)、糖缀合物和蛋白质表达(第3卷)和生物信息学(第4卷)。

[0358] 蛋白质纯化的方法(包括免疫沉淀、色谱、电泳、离心和结晶)描述于(Coligan,等(2000)Current Protocols in Protein Science,Vol.1,John Wiley and Sons,Inc.,New York)。描述了化学分析、化学修饰、翻译后修饰、融合蛋白的产生、蛋白质的糖基化(参见，例如，Coligan,等(2000)Current Protocols in Protein Science,Vol.2,John Wiley and Sons,Inc.,New York;Ausubel,等(2001)Current Protocols in Molecular



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Biology,Vol.3,John Wiley and Sons,Inc.,NY,NY,pp.16.0.5-16.22.17;Sigma-Aldrich,Co.(2001)Products for Life Science Research,St.Louis,MO;pp.45-89;Amersham Pharmacia Biotech(2001)BioDirectory,Piscataway,N.J.,pp.384-391)。描述了多克隆和单克隆抗体的产生、纯化和片段化(Coligan,等(2001)Current Protocols in Immunology,Vol.1,John Wiley and Sons,Inc.,New York;Harlow and Lane(1999)Using Antibodies,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,NY;Harlow and Lane,supra)。用于表征配体/受体相互作用的标准技术是可用的(参见,例如,Coligan,等(2001)Current Protocols in Immunology,Vol.4,John Wiley,Inc.,New York)。

[0359] 可以制备单克隆、多克隆和人源化抗体(参见,例如,Sheperd和Dean(eds.)(2000)Monoclonal Antibodies,Oxford Univ.Press,New York,NY;Kontermann和Dubel(eds.)(2001)Antibody Engineering,Springer-Verlag,New York;Harlow和Lane(1988)Antibodies A Laboratory Manual,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,NY,pp.139-243;Carpenter,等(2000)J.Immunol.165:6205;He,等(1998)J.Immunol.160:1029;Tang等(1999)J.Biol.Chem.274:27371-27378;Baca等(1997)J.Biol.Chem.272:10678-10684;Chothia等(1989)Nature 342:877-883;Foote和Winter(1992)J.Mol.Biol.224:487-499;U.S.Pat.No.6,329,511)。

[0360] 人源化的替代方案是使用展示于转基因小鼠中的噬菌体或人抗体文库上的人抗体文库(Vaughan等(1996)Nature Biotechnol.14:309-314;Barbas(1995)Nature Medicine 1:837-839;Mendez等(1997)Nature Genetics 15:146-156;Hoogenboom和Chames(2000)Immunol.Today 21:371-377;Barbas等(2001)Phage Display:A Laboratory Manual,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,New York;Kay等(1996)Phage Display of Peptides and Proteins:A Laboratory Manual,Academic Press,San Diego,CA;de Bruin等(1999)Nature Biotechnol.17:397-399)。

[0361] 抗原的纯化不是产生抗体所必需的。可以用携带感兴趣抗原的细胞免疫动物。然后可以从免疫动物中分离脾细胞,脾细胞可以与骨髓瘤细胞系融合以产生杂交瘤(参见,例如,Meyaard等(1997)Immunity 7:283-290;Wright等(2000)Immunity 13:233-242;Preston等,supra;Kaithamana等(1999)J.Immunol.163:5157-5164)。

[0362] 抗体可以缀合至例如小药物分子、酶、脂质体、聚乙二醇(PEG)。抗体可用于治疗剂、诊断剂、试剂盒或其他目的,并且包括偶联至例如染料、放射性同位素、酶或金属(例如胶体金)的抗体(参见,例如,Le Doussal等(1991)J.Immunol.146:169-175;Gibellini等(1998)J.Immunol.160:3891-3898;Hsing和Bishop(1999)J.Immunol.162:2804-2811;Everts等(2002)J.Immunol.168:883-889)。

[0363] 流式细胞术的方法,包括荧光激活细胞分选术(FACS)是可用的(参见,例如,Owens,等(1994)Flow Cytometry Principles for Clinical Laboratory Practice,John Wiley and Sons,Hoboken,NJ;Givan(2001)Flow Cytometry,2nd ed.;Wiley-Liss,Hoboken,NJ;Shapiro(2003)Practical Flow Cytometry,John Wiley and Sons,Hoboken,NJ;Givan(2001)Flow Cytometry,2nd ed.;Wiley-Liss,Hoboken,NJ;Shapiro(2003)Practical Flow Cytometry,John Wiley and Sons,Hoboken,NJ)。适用于修饰核酸(包括



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核酸引物和探针、多肽和抗体)以用作例如诊断试剂的荧光试剂是可获得的(Molecular Probesy(2003)Catalogue,Molecular Probes,Inc.,Eugene,OR;Sigma-Aldrich(2003)Catalogue,St.Louis,MO)。

[0364] 描述了免疫系统的标准组织学方法(参见,例如,Muller-Harmelink(ed.)(1986)Human Thymus:Histopathology and Pathology,Springer Verlag,New York,NY;Hiatt,等(2000)Color Atlas of Histology,Lippincott,Williams,and Wilkins,Phila,PA;Louis,等(2002)Basic Histology:Text and Atlas,McGraw-Hill,New York,NY)。用于确定例如抗原片段、前导序列、蛋白质折叠、功能结构域、糖基化位点和序列比对的软件包和数据库是可获得的(参见,例如,GenBank,Vector NTI® Suite(Informax,Inc,Bethesda,MD);GCG Wisconsin Package(Accelrys,Inc.,San Diego,CA);DeCypher®(TimeLogic Corp.,Crystal Bay,Nevada);Menne,等(2000)Bioinformatics 16:741-742;Menne,等(2000)Bioinformatics Applications Note 16:741-742;Wren,等(2002)Comput.Methods Programs Biomed.68:177-181;von Heijne(1983)Eur.J.Biochem.133:17-21;von Heijne(1986)Nucleic Acids Res.14:4683-4690)。

[0365] 出于描述和公开可能与本发明结合使用的方法和材料的目的,本文中提及的所有出版物均通过引用并入本文。

[0366] 已经参考附图描述了本发明的不同实施方式,应该理解,本发明不限于那些严格的实施方式,并且本领域技术人员可以在其中实现各种改变和修饰而不偏离如所附权利要求中限定的本发明的范围或精神。

[0367] 实施例1

[0368] 一项旨在表征黑色素瘤受试者中派姆单抗+伊匹单抗的组合的安全性、耐受性和初步功效的I/II期研究。

[0369] 临床前研究已显示,伴随CTLA-4和PD-1阻断引起的响应率在MC38小鼠模型中高于任一单个药剂(Selby等Antitumor activity of concurrent blockade of immune checkpoint molecules CTLA-4 and PD-1 in preclinical models.[Abstract 3061].2013 ASCO Annual Meeting,General Poster Session,Developmental Therapeutics-Immunotherapy;2013May 31-Jun 3.Chicago,IL,2013)。值得注意的是,虽然在实验结束时用抗CTLA-4或抗PD-1处理的小鼠没有不患病的,但是用不同剂量水平的组合处理的小鼠中有7-60%是无肿瘤的。

[0370] 在患有MEL的受试者的1期试验中,IPI(抗CTLA-4)与纳武单抗(抗PD-1)组合导致响应率范围为21-53%。这些可能代表比纳武单抗单一疗法(Sznol等[Abstract 3734].European Society for Medical Oncology 2013 Annual Meeting;Sep 27-Oct 1,2013;Amsterdam,Netherlands;Topalian等[Abstract 3002].2013 ASCO Annual Meeting;2013 May 31-Jun 4.Chicago,IL)和在单一疗法中对IPI的历史响应(分别约为20-31%和10%)更大的响应(Hodi等,N Engl J Med 363(8):711-23(2010))。抗PD-1抗体纳武单抗和派姆单抗两者在先前未处理的受试者中证实优于单一药剂IPI(派姆单抗),并且在未治疗的受试者和IPI失败后两者中优于化学疗法(纳武单抗、派姆单抗)。向纳武单抗添加IPI与更高的响应率和更好的PFS相关,然而,它与高比率的3-4级DRAE相关。

[0371] 在该研究中,派姆单抗与1mg/kg的IPI组合进行评估。IPI的计划(schedule)遵循



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批准的q3w药物标签(label)。在先前的I期研究中,3mg/kg纳武单抗+1mg/kg IPI(ORR 40%(95%CI 16-68%)的功效和总临床活动率(Aggregate Clinical Activity Rate)(ACAR:定义为CR+PR+未确诊的CR+未确诊的PR+免疫相关的PR+SD>24周的+irSD>24周)73%]似乎具有与1mg/kg纳武单抗+3mg/kg IPI(ORR 53%相似的效果(ORR 53%(95%CI 28-77%)和ACAR 65%)(Sznol等,Combined nivolumab(anti-PD-1,BMS-936558,ONO-4538)and ipilimumab in the treatment of advanced melanoma patients:Safety and clinical activity.[Abstract 3734].European Society for Medical Onocology 2013Annual Meeting;Sep 27-Oct 1,2013;Amsterdam,Netherlands)。此外,3mg/kg纳武单抗+1mg/kg IPI的组合显示相比于1mg/kg纳武单抗+3mg/kg IPI更低的3-4级DRAE的比率(分别为44%相对于65%)。共有6/28(21%)位患者经历发现为剂量限制性的3-4级毒性。

[0372] 本研究的总体剂量(dosing)策略旨在强调派姆单抗而非IPI的剂量强度;此外,抗PD-1+抗CTLA4组合的剂量范例探索了抗PD-1的最低治疗性剂量(2mg/kg q3w派姆单抗)的和低于抗CTLA4的推荐单疗法剂量的剂量(1mg/kg q3w x 4剂量的IPI)。

[0373] 153名MEL患者结果表明,较低剂量的IPI(1mg/kg Q3W)与2mg/kg q3w的派姆单抗的组合具有通过独立中心审查确认的57%ORR和与3mg/kg纳武单抗+1mg/kg IPI可比较的3-4级DRAE比率(42%vs.44%)(Larkin等,Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma.N Engl J Med.373(1):23-34(2015))或与3mg/kg纳武单抗+3mg/kg IPI可比较的3-4级DRAE比率(对CheckMate 067和CheckMate 069而言分别为55%和54%)(同上,Hodi等Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma:2-year overall survival outcomes in a multicenter,randomized,controlled,phase 2 trial.Lancet Oncol.17(11):1558-1568(2016))。72%的受试者能够完成所有4个计划的IPI剂量,然而,42%的受试者中存在DRAE(Long等Presented at 2016ASCO Annual Meeting June 3-7,2016;Chicago,IL;abstract 9506)。

[0374] 实施例2

[0375] 派姆单抗加上伊匹单抗的2个固定剂量方案用于晚期黑色素瘤的I/II期随机试验

[0376] 目标:

[0377] 本研究的主要目标是建立200mg固定剂量的派姆单抗与2个计划(schedule)的降低固定剂量的伊匹单抗的组合的安全性和耐受性,并且依据RECIST v1.1通过对200mg固定剂量的派姆单抗与2个计划的降低固定剂量的伊匹单抗的组合的独立中心审查来评估总体响应比率(ORR)。

[0378] 本研究的次要目标是:(1)评估与顺序响应评分(定义为以完全响应(CR)计算的最佳ORR,非常好的部分响应(VGPR);定义为依据RECIST v1.1肿瘤大小从基线的变化>60%,中等部分响应(PR)(定义为肿瘤大小从基线的变化>30%且≤60%),稳定疾病(SD)、或基于肿瘤萎缩程度的进行性疾病(PD))相关的组合方案的功效;(2)依据RECIST v1.1通过独立中心审查评估组合方案的响应持续时间(DOR)和无进展存活期(PFS);(3)评估组合方案的总体存活期(OS);(4)评估用组合方案治疗的患者中PD-L1表达与ORR、PFS和OS之间的关系。

[0379] 本研究的另外的探索性目标是:(1)依据RECIST v1.1通过中心审查使用肿瘤长度和肿瘤体积变化来评估组合方案的ORR;(2)依据RECIST v1.1通过独立中心审查,对停用派



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姆单抗具有SD或更好的患者以及在随后的疾病进展后用组合疗法或派姆单抗单疗法进行再治疗的患者进行ORR评估；(3)研究潜在生物标志物与派姆单抗的抗肿瘤活性之间的关系；(4)研究派姆单抗抗药物抗体的比率及对药代动力学的影响；(5)将进行生长建模和模拟所用时间与以组合方案治疗的患者的临床结果关联起来。

[0380] 研究设计：

[0381] 将约100名患者1:1随机分配以接受：(1)200mg Q3W派姆单抗加50mg Q6W伊匹单抗，≤4剂量(组1)、或(2)200mg Q3W派姆单抗加100mg Q12W伊匹单抗，≤4剂量(组2)。组合治疗将在第1组持续≤24周或在第2组持续≤48周，然后是派姆单抗单疗法≤24个月或直至记录有PD、不可接受的毒性、患者撤回同意书或研究者的决定。

[0382] 用派姆单抗治疗≥24周且在初始确定CR后接受≥2剂量的派姆单抗、依据改良的RECIST v1.1具有研究者确定的确认CR的患者可停用派姆单抗；接受过≥1次伊匹单抗剂量、具有由研究者确定的确认CR或VGPR的患者可停用伊匹单抗。

[0383] 依据改良的RECIST v1.1经历SD或更好、随后经历PD的患者可以适格用派姆单抗加伊匹单抗或派姆单抗单疗法进行再治疗，最多17剂量的派姆单抗和4剂量的伊匹单抗。

[0384] 依据改良的RECIST v1.1经历放射性PD的适格患者可以继续接受治疗，直到≥4周后的确认性扫描。依据改良的RECIST v1.1具有确认的PD的临床稳定、正获得临床益处的患者可以由研究者自行决定继续治疗。

[0385] 评估和随访

[0386] 响应将通过每6周一次的肿瘤成像评估，直至第24周，然后依据RECIST v1.1通过独立中心审查(对功效)和依据改良的RECIST v1.1通过研究者审查(对适格和治疗决定)每12周评估一次。依据RECIST v1.1通过独立中心审查的的5类顺序响应评分将被用于评估以CR、VGPR、中等PR、SD、或PD计算的最佳总体响应。不良事件(AE)将在整个治疗期间和此后30天(对于严重AE 90天)评估，并且将依据美国国家癌症研究所不良事件通用术语标准4.0版(National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0)进行分级。确认PD或开始新的抗癌疗法后，将每12周通过电话联系患者以监测存活。

[0387] 分析

[0388] 为了分析功效，具有基线处的可测量疾病的全部患者将作为主要功效人群。对于真实ORR，将计算ORR的精确的90％置信区间。描述性统计将用于评估顺序响应评分。Kaplan-Meier方法将用于估计PFS、OS和DOR。只有达到CR或PR的患者才会被纳入DOR分析。

[0389] 对于安全性的分析，接受≥1剂量的研究治疗的所有随机分配的患者将作为主要安全性人群。目前正在进行招募。

[0390] 所有参考文献通过引用并入本文，其程度如同每个个体的出版物、数据库条目(例如Genbank序列或GeneID条目)、专利申请或专利被特定和个体地指出通过引用并入。根据37CFR§1.57(b)(1)，申请人打算通过进行通过引用并入的声明，以关联每一个个体的出版物、数据库条目(例如Genbank序列或GeneID条目)、专利申请或专利，根据37CFR§1.57(b)(2)明确标识每一项，即使这样的引用不是与通过引用并入的声明直接相邻。在说明书中包含通过引用并入的专用声明(如果有的话)并不以任何方式削弱通过引用并入的一般性声明。本文引用的参考文献并非旨在承认该参考文献是相关的现有技术，也不构成对这些出



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版物或文献的内容或日期的任何认可。

[0391] 表5提供了序列表中PD-1相关序列的简要描述。

[0392]

| SEQ ID NO: | 描述 | SEQ ID NO: | 描述 |
|---|---|---|---|
| 1 | hPD-1.08A 轻链 CDR1 | 18 | K09A-L-11 轻链全长 |
| 2 | hPD-1.08A 轻链 CDR2 | 19 | K09A-L-16 轻链全长 |
| 3 | hPD-1-08A 轻链 CDR3 | 20 | K09A-L-17 轻链全长 |
| 4 | hPD-1.08A 重链 CDR1 | 21 | 派姆单抗重链 |
| 5 | hPD-1.08A 重链 CDR2 | 22 | 派姆单抗轻链 |
| 6 | hPD-1.08A 重链 CDR3 | 23 | 纳武单抗重链 |
| 7 | hPD-1.09A 轻链 CDR1 | 24 | 纳武单抗轻链 |
| 8 | hPD-1.09A 轻链 CDR2 | 25 | 人 PD-L1 前体 |
| 9 | hPD-1.09A 轻链 CDR3 | 26 | 纳武单抗重链可变区 |
| 10 | hPD-1.09A 重链 CDR1 | 27 | 纳武单抗轻链可变区 |
| 11 | hPD-1.09A 重链 CDR2 | 28 | 纳武单抗重链 CDR1 |
| 12 | hPD-1.09A 重链 CDR3 | 29 | 纳武单抗重链 CDR2 |
| 13 | 109A-H 重链可变区 | 30 | 纳武单抗重链 CDR3 |
| 14 | 409A-H 重链全长 | 31 | 纳武单抗轻链 CDR1 |
| 15 | K09A-L-11 轻链可变区 | 32 | 纳武单抗轻链 CDR2 |
| 16 | K09A-L-16 轻链可变区 | 33 | 纳武单抗轻链 CDR3 |
| 17 | K09A-L-17 轻链可变区 | | |

[0393] 表6提供了序列表中CTLA4相关序列的简要描述。

[0394]

| SEQ ID NO: | 描述 | SEQ ID NO: | 描述 |
|---|---|---|---|
| 34 | CTLA4 Ab 重链 CDR1 | 67 | 4G10H1 人源化，重链可变区 |
| 35 | CTLA4 Ab 重链 CDR2 | 68 | 4G10H3 人源化，重链可变区 |
| 36 | CTLA4 Ab 重链 CDR3 | 69 | 4G10H4 人源化，重链可变区 |
| 37 | CTLA4 Ab 轻链 CDR1 | 70 | 4G10H5 人源化，重链可变区 |
| 38 | CTLA4 Ab 轻链 CDR2 | 71 | 4G10H 共有序列人源化，重链可变区 |
| 39 | CTLA4 Ab 轻链 CDR3 | 72 | 4G10L1 人源化，轻链可变区 |
| 40 | CTLA4 Ab 轻链 CDR3 | 73 | 4G10L3 人源化，轻链可变区 |
| 41 | CTLA4 Ab 轻链 CDR3 | 74 | 4G10L 共有序列人源化、轻链可变区 |



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[0395]

| | | | |
|---|---|---|---|
| 42 | 8D2/8D2 (RE)重链可变区 | 75 | 4G10H1L1 重链可变区 |
| 43 | 8D2/8D2 (RE)轻链可变区 | 76 | 4G10H1L1 轻链可变区 |
| 44 | 8D2H1L1 重链可变区 | 77 | 4G10H3L3 重链可变区 |
| 45 | 8D2H1L1 轻链可变区 | 78 | 4G10H3L3 轻链可变区 |
| 46 | 8D2H2L2 重链可变区 | 79 | 4G10H4L3 重链可变区 |
| 47 | 8D2H2L2 轻链可变区 | 80 | 4G10H4L3 轻链可变区 |
| 48 | 8D3H3L3 重链可变区 | 81 | 4G10H5L3 重链可变区 |
| 49 | 8D3H3L3 轻链可变区 | 82 | 4G10H5L3 轻链可变区 |
| 50 | 8D2H2L15 重链可变区 | 83 | 伊匹单抗重链 |
| 51 | 8D2H2L15 轻链可变区 | 84 | 伊匹单抗轻链 |
| 52 | 8D2H2l17 重链可变区 | 85 | 伊匹单抗 HCDR1 |
| 53 | 8D2H2l17 轻链可变区 | 86 | 伊匹单抗 HCDR2 |
| 54 | 鼠 4G10 重链可变区 | 87 | 伊匹单抗 HCDR3 |
| 55 | 鼠 4G10 轻链可变区 | 88 | 伊匹单抗 LCDR1 |
| 56 | CTLA4 Ab 重链 CDR1 | 89 | 伊匹单抗 LCDR2 |
| 57 | CTLA4 Ab 重链 CDR1 | 90 | 伊匹单抗 LCDR3 |
| 58 | CTLA4 Ab 重链 CDR2 | 91 | 伊匹单抗重链可变区 |
| 59 | CTLA4 Ab 重链 CDR2 | 92 | 伊匹单抗轻链可变区 |
| 60 | CTLA4 Ab 重链 CDR3 | 93 | 8D2/8D2 (RE) VH 共有序列（consensus） |
| 61 | CTLA4 Ab 重链 CDR3 | 94 | 8D2/8D2 (RE) VH 变体 1 |
| 62 | CTLA4 Ab 轻链 CDR1 | 95 | 8D2H1L1 VH 共有序列 |
| 63 | CTLA4 Ab 轻链 CDR1 | 96 | 8D2H1L1 VH 变体 1 |
| 64 | CTLA4 Ab 轻链 CDR2 | 97 | 8D2H2L2 VH 共有序列 |
| 65 | CTLA4 Ab 轻链 CDR3 | 98 | 8D2H2L2 VH 变体 1 |
| 66 | CTLA4 Ab 轻链 CDR3 | 99 | 8D2H1L15 VH 共有序列 |
| | | 100 | 8D2H1L15 VH 变体 1 |
| | | 101 | 8D2H1L17 VH 共有序列 |
| | | 102 | 8D2H1L17 VH 变体 1 |



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序列表

<110> Merck Sharp & Dohme C或p.
Nomet M或eno, Blanca
Ibrahim, Nageatte
Ebbinghaus, Scot W.
Jain, Lokesh
Diede, Scott

<120> 用PD-1的拮抗剂和抗CTLA4抗体的组合治疗癌症的组合物和方法

<130> 24437

<150> US 62/479,784
<151> 2017-03-31

<160> 106

<170> PatentIn version 3.5

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1 5 10 15

<210> 2
<211> 7
<212> PRT
<213> 人工序列

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1 5

<210> 3
<211> 9
<212> PRT
<213> 人工序列

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1 5

<210> 4
<211> 5
[0002] <212> PRT
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<400> 4

Ser Tyr Tyr Leu Tyr
1 5

<210> 5
<211> 17
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Gly Val Asn Pro Ser Asn Gly Gly Thr Asn Phe Ser Glu Lys Phe Lys
1 5 10 15

Ser

<210> 6
<211> 11
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1 5 10

[0003]

<210> 7
<211> 15
<212> PRT
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1 5 10 15

<210> 8
<211> 7
<212> PRT
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<220>
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<400> 8

Leu Ala Ser Tyr Leu Glu Ser
1 5

<210> 9
<211> 9
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<213> 人工序列

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<400> 9

Gln His Ser Arg Asp Leu Pro Leu Thr
1 5

[0004] <210> 10
<211> 5
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<400> 10

Asn Tyr Tyr Met Tyr
1 5

<210> 11
<211> 17
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<220>
<223> hPD-1.09 A HC CDR2

<400> 11

Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn Glu Lys Phe Lys
1 5 10 15

Asn

<210> 12
<211> 11
<212> PRT
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<220>
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<400> 12

Arg Asp Tyr Arg Phe Asp Met Gly Phe Asp Tyr
[0005] 1 5 10

<210> 13
<211> 120
<212> PRT
<213> 人工序列

<220>
<223> 109A-H HC 可变

<400> 13

Gln Val Gln Leu Val Gln Ser Gly Val Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Tyr Met Tyr Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met



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35 40 45

Gly Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn Glu Lys Phe
50 55 60

Lys Asn Arg Val Thr Leu Thr Thr Asp Ser Ser Thr Thr Thr Ala Tyr
65 70 75 80

Met Glu Leu Lys Ser Leu Gln Phe Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Asp Tyr Arg Phe Asp Met Gly Phe Asp Tyr Trp Gly Gln
100 105 110

Gly Thr Thr Val Thr Val Ser Ser
115 120

[0006]

<210> 14
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<212> PRT
<213> 人工序列

<220>
<223> 409A-HC 全长

<400> 14

Gln Val Gln Leu Val Gln Ser Gly Val Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Tyr Met Tyr Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn Glu Lys Phe
50 55 60

Lys Asn Arg Val Thr Leu Thr Thr Asp Ser Ser Thr Thr Thr Ala Tyr
65 70 75 80

Met Glu Leu Lys Ser Leu Gln Phe Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Arg Asp Tyr Arg Phe Asp Met Gly Phe Asp Tyr Trp Gly Gln
100 105 110

Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115 120 125

[0007] Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala
130 135 140

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145 150 155 160

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
165 170 175

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
180 185 190

Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp His Lys
195 200 205

Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro

210 215 220

Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val
225 230 235 240

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
245 250 255

Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu
260 265 270

Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
275 280 285

Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser
[0008] 290 295 300

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
305 310 315 320

Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile
325 330 335

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
340 345 350

Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
355 360 365

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
370 375 380

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
385 390 395 400

Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg
405 410 415

Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
420 425 430

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
435 440 445

<210> 15
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<213> 人工序列

[0009] <220>
<223> K09A-L-11 LC 可变

<400> 15

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Lys Gly Val Ser Thr Ser
20 25 30

Gly Tyr Ser Tyr Leu His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
35 40 45

Arg Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly Val Pro Ala
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser



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65 70 75 80

Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln His Ser Arg
85 90 95

Asp Leu Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105 110

<210> 16
<211> 111
<212> PRT
<213> 人工序列

<220>
<223> k09A-L-16 LC 可变

<400> 16

[0010] Glu Ile Val Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1 5 10 15

Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val Ser Thr Ser
20 25 30

Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro
35 40 45

Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly Val Pro Asp
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser
65 70 75 80

Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Gln His Ser Arg
85 90 95



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Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
100 105 110

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Asp Ile Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Thr Pro Gly
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Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val Ser Thr Ser
20 25 30

[0011]

Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro
35 40 45

Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly Val Pro Asp
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Lys Ile Ser
65 70 75 80

Arg Val Glu Ala Glu Asp Val Gly Leu Tyr Tyr Cys Gln His Ser Arg
85 90 95

Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
100 105 110

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Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
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Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Lys Gly Val Ser Thr Ser
20 25 30

Gly Tyr Ser Tyr Leu His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro
35 40 45

[0012]

Arg Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly Val Pro Ala
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
65 70 75 80

Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln His Ser Arg
85 90 95

Asp Leu Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg
100 105 110

Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
115 120 125

Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
130 135 140

Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
145 150 155 160

Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
165 170 175

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
180 185 190

His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
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Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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Glu Ile Val Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1 5 10 15

Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val Ser Thr Ser
20 25 30

Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro

35 40 45

Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly Val Pro Asp
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser
65 70 75 80

Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Gln His Ser Arg
85 90 95

Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys Arg
100 105 110

[0014]

Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
115 120 125

Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
130 135 140

Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
145 150 155 160

Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
165 170 175

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
180 185 190

His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
195 200 205