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Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies

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Abstract

The development of noninvasive methods to detect and monitor tumors continues to be a major challenge in oncology. We used digital polymerase chain reaction–based technologies to evaluate the ability of circulating tumor DNA (ctDNA) to detect tumors in 640 patients with various cancer types. We found that ctDNA was detectable in >75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers, but in less than 50% of primary brain, renal, prostate, or thyroid cancers. In patients

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www.sciencetranslationalmedicine.org/cgi/content/full/6/224/224ra24/DC1
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Competing interests: K.W.K. and B.V. are consultants for Inostics. L.A.D. is a consultant for Amgen and Anaeropharma. L.A.D. and V.E.V. are co-founders and on the board of directors of Personal Genome Diagnostics. K.W.K., B.V., L.A.D., N.P., and V.E.V. all own Personal Genome Diagnostics stock, which is subject to certain restrictions under University policy. L.D.W. works as a paid consultant for Personal Genome Diagnostics. Johns Hopkins University has several patents related to the work presented in this paper. The terms of all these arrangements are managed by the Johns Hopkins University in accordance with its conflict-of-interest policies. A.B. is a shareholder and advisory board member of Horizon Discovery, and an advisory board member or Biocartis. L.A.F. is an advisory board member of Genentech-Roche. C.M.S. is a scientific advisor to Asuragen Inc. and a consultant to Redpath Inc.

Data and materials availability: A complete self-contained software script that can be used to reproduce all data manipulation, transformation, and analysis steps reported in this paper is available as part of the Supplementary Materials.

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with localized tumors, ctDNA was detected in 73, 57, 48, and 50% of patients with colorectal cancer, gastroesophageal cancer, pancreatic cancer, and breast adenocarcinoma, respectively. ctDNA was often present in patients without detectable circulating tumor cells, suggesting that these two biomarkers are distinct entities. In a separate panel of 206 patients with metastatic colorectal cancers, we showed that the sensitivity of ctDNA for detection of clinically relevant *KRAS* gene mutations was 87.2% and its specificity was 99.2%. Finally, we assessed whether ctDNA could provide clues into the mechanisms underlying resistance to epidermal growth factor receptor blockade in 24 patients who objectively responded to therapy but subsequently relapsed. Twenty-three (96%) of these patients developed one or more mutations in genes involved in the mitogen-activated protein kinase pathway. Together, these data suggest that ctDNA is a broadly applicable, sensitive, and specific biomarker that can be used for a variety of clinical and research purposes in patients with multiple different types of cancer.

INTRODUCTION

Cancer will occur in more than 1.6 million individuals this year in the United States alone, but a clinically proven circulating biomarker that can be used to help guide patient management will be available for only a minority of them, even in the setting of widespread metastasis (1–6). Although serum-based protein biomarkers such as carcinoma antigen-125 (CA-125), carcinoembryonic antigen (CEA), and prostate-specific antigen (PSA) are commonly used for this purpose, these proteins are also found in the serum of individuals without cancer, albeit in lower concentrations (2–4). Additionally, these markers are not found to be elevated in a substantial portion of patients with advanced cancers (5, 6).

A new generation of biomarkers has become available with the discovery of the genetic alterations that are responsible for the initiation and progression of human cancers (7–11). With the influx of genomic information from recent cancer genome sequencing studies, it is now known that virtually all cancers of every type harbor somatic genetic alterations. These alterations include single-base substitutions, insertions, deletions, and translocations (the latter including those associated with the creation of gene fusions, gene amplifications, or losses of heterozygosity). These somatic mutations occur at negligible frequencies in normal cell populations and therefore provide exquisitely specific biomarkers from a biological perspective (9).

There are two sources of tumor DNA that can be noninvasively assessed in the circulation: cell-free circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) (12, 13). ctDNA is composed of small fragments of nucleic acid that are not associated with cells or cell fragments (14). In contrast, CTCs represent intact, often viable, cells that can be purified from blood by virtue of physicochemical characteristics or cell surface molecules that distinguish them from normal blood cells (15). Many studies have shown that both ctDNA and CTCs are present in advanced neoplasia, although only a few studies have compared the amounts of CTCs and ctDNA templates in the same patients (16–19). The studies comparing the two approaches have reached opposing conclusions, likely because of technical issues that limited interpretation of either the ctDNA or CTC content. Furthermore, the mechanism by which CTCs or ctDNA is released into the circulation is unclear, although it is possible

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that ctDNA actually comes from CTCs. One of the purposes of the current study was to compare the quantities of ctDNA and CTCs in the circulation of the same patients using an unbiased approach.

Most studies of ctDNA published to date have each evaluated patients with a single tumor type. In light of considerable differences in DNA preparation and analytic techniques in these studies, it has been difficult to directly compare the amounts of ctDNA among tumor types (16, 20–26). Comparisons of studies are also challenging because of differences in the types of data that are reported. For example, it is often impossible to compare real-time polymerase chain reaction (PCR) results with those reporting the fraction of mutant template molecules assessed, or to compare results based on the analysis of serum with those based on plasma. To directly compare different tumor types and to determine the spectrum of cancers in which ctDNA measurements could prove clinically useful, we evaluated a large number of tumor types in the current study. We purified plasma and tumor DNA using regimented protocols for all samples and used digital technologies to evaluate ctDNA levels from each tumor so that we could report the number of mutant templates per milliliter of plasma in each case (Fig. 1). This approach also allowed us to directly compare the two most commonly used types of tumor-specific genetic alterations found in the circulation: single-base substitutions and rearrangements.

One of the most immediate applications of ctDNA has been termed the “liquid biopsy” (20). In research studies as well as in clinical practice, it is often difficult to obtain tumor samples for genetic analyses. Some tumors are only accessible through fine-needle aspirates (lung cancer, for example) with insufficient material available for genotyping, whereas in other cases it can be challenging or time-consuming to acquire samples from different medical centers (27). Additionally, once a targeted therapy is initiated in a patient with multiple metastases, clinicians frequently search for early evidence of recurrence or mechanisms underlying resistance, a scenario in which liquid biopsies are particularly valuable. For example, they can provide temporal measurements of the total tumor burden as well as identify specific mutations that arise during therapy (16, 20, 21, 23, 28, 52). Although the liquid biopsy approach has been shown to be promising, its sensitivity and specificity with respect to conventional tumor biopsies have not been evaluated in a large, clinically relevant cohort. Here, we evaluated the sensitivity and specificity of this approach in patients with colorectal cancers (CRCs) who were candidates for epidermal growth factor receptor (EGFR) blockade. We also used liquid biopsies to identify mutations that were responsible for recurrence in patients who initially responded to EGFR blockade. In aggregate, these studies provide a wealth of information on the potential utility, as well as the limitations, of ctDNA measurements for the assessment of patients with various cancers.

RESULTS

Patients with metastatic cancers

We began this study with an evaluation of 136 metastatic tumors originating from 14 different tissue types, as well as of 41 patients with primary brain tumors (glioma and medulloblastoma). Primary brain tumors were included in this evaluation because they are generally lethal even though they rarely metastasize. We also included 10 additional cases,

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composed of stage III ovarian (n = 7) and hepatocellular carcinomas (n = 3), in this particular evaluation because stage IV cases are rare and stage III disease is more representative of advanced disease in these two tumor types. The clinical characteristics of these patients are summarized in Table 1. Targeted sequencing, exomic sequencing, or whole-genome sequencing was used to identify mutations in the tumors, as described in Supplementary Materials and Methods. In these advanced cases, at least one genetic alteration—a point mutation (151 cases) or genetic rearrangement (36 cases)—was found in each of the tumors studied (table S1). Except for a subset of mutations at the known hotspots of the *KRAS*, *NRAS*, *PIK3CA*, and *BRAF* genes (which are well known to be somatic), all other genetic alterations were demonstrated to be somatic through evaluation of DNA from non-neoplastic cells of the same patients. ctDNA was assessed by one of three digital methods (see Supplementary Materials and Methods). These methods yielded comparable results when applied to the same plasma samples (fig. S1) and all were able to detect one mutant template in the DNA purified from up to 5 ml of plasma. The amounts of plasma available from each patient are listed in table S1.

ctDNA was detected in most of the studied patients with solid tumors outside the brain (112 of 136; 82%). However, the fraction of patients with detectable ctDNA varied with tumor type (likelihood ratio test, $P < 0.001$). As shown in Fig. 2A and fig. S2, most patients with stage III ovarian and liver cancers and metastatic cancers of the pancreas, bladder, colon, stomach, breast, liver, esophagus, and head and neck, as well as patients with neuroblastoma and melanoma, harbored detectable levels of ctDNA. In contrast, less than 50% of patients with medulloblastomas or metastatic cancers of the kidney, prostate, or thyroid, and less than 10% of patients with gliomas, harbored detectable ctDNA. The number of patients with some of the tumor types depicted in Fig. 2A was small, limiting the statistical significance of comparisons among tumor types, but patients with gliomas (low or high grade; table S1) were less likely to harbor ctDNA than those with metastatic cancers of the pancreas, colon, breast, esophagus/stomach, or ovary (Fig. 2A and fig. S2).

Although ctDNA was detectable in most patients with metastatic cancers, the concentration of ctDNA varied among patients, even those with the same tumor type (Fig. 2B and table S1). Some of this variability was due to differences in copy number of the genes assayed in different tumors. For example, if the queried gene was amplified 50-fold in the tumor of patient A, whereas the queried gene in the tumor of patient B was present at normal copy number, the amount of ctDNA would be expected to be 50-fold higher in patient A than in patient B (see “Comparison of rearrangements with single-base substitutions in ctDNA”). However, great variability was also observed among cancers in which only nonamplified genes (such as *TP53*) were assessed.

Patients with localized disease

We next evaluated ctDNA in patients with localized disease, that is, no clinical or radiographic evidence of distant metastasis at the time of sample collection. Among 223 patients with localized cancers of all types evaluated, detectable levels of ctDNA were found in 55% (122 of 223 patients; table S1). This fraction was lower than observed in patients with metastatic disease from all tumor types in which a sufficient number of samples were

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available (breast, colon, pancreas, and gastroesophageal; Fig. 3A; Cochran-Mantel-Haenszel χ^2 test, $P < 0.001$). Detectable levels of ctDNA were present in 49 to 78% of patients with localized tumors and in 86 to 100% of patients with meta-static tumors of these four types (Fig. 3A).

Differences in the fraction of patients with detectable levels of ctDNA also correlated with stage: 47% of patients with stage I cancers of any type had detectable ctDNA, whereas the fraction of patients with detectable ctDNA was 55, 69, and 82% for patients with stage II, III, and IV cancers, respectively (Fig. 3B; Somers' Dxy rank correlation = 0.337). The concentration of ctDNA in the plasma similarly increased with stage (Fig. 3C).

Comparison of ctDNA with CTCs

For these experiments, DNA was isolated from the cellular compartment of blood obtained after centrifugation; these pellets contained CTCs as well as white blood cells (WBCs), platelets, and other cellular fragments. In each case, whole-genome sequencing of tumor DNA was used to identify somatic rearrangements. PCR-based assays were then used to identify these rearrangements in blood pellets (CTCs) or in the blood supernatants (plasma) of the same patients. This experiment could be performed with tumor-specific rearrangements, but not with tumor-specific point mutations, for the reasons given in the Discussion. We did not identify any cases in which CTCs were detected but in which ctDNA was absent. However, in many cases in which ctDNA was detected (13 of 16; 81%), no CTCs were detectable with the identical assay (Table 2). Moreover, in the three cases wherein both CTC and ctDNA levels were detectable, the average number of mutant fragments in the plasma was >50-fold higher than analogous levels in CTCs (Table 2).

Comparison of rearrangements with single-base substitutions in ctDNA

We were also interested in comparing the quantity of two different types of genetically altered DNA fragments in the circulation of the same patients. Although practical issues precluded us from identifying a rearrangement in all patients in this study (see Discussion), tumor-specific rearrangements as well as tumor-specific point mutations were identified in 19 patients (table S2). The rearrangements were identified by whole-genome sequencing of tumor DNA, and the point mutations identified by targeted sequencing. In each case, the alteration was shown to be somatic via evaluation of normal DNA from the same patients. In 18 of the 19 patients harboring a circulating point mutation, a circulating rearrangement was also detectable (table S2). The one exception was a patient (CRC 37) with a circulating point mutation in *TP53* in which the rearrangement identified in that patient's tumor could not be identified in her plasma (table S2). The absolute number of circulating DNA fragments with point mutations versus rearrangements was highly correlated (Fig. 4; correlation coefficient = 0.96). However, in four patients, the number of circulating fragments containing rearrangements was >10-fold that of the queried point mutation (table S2). The reason for this was that the rearrangements we chose for analysis often arose as a result of gene amplification in the tumor, whereas the point mutations were generally present only once per tumor genome.

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The sensitivity and specificity of liquid biopsy

The results described above were obtained by first identifying a mutation in a tumor and then determining whether that same mutation was detectable in the plasma. For certain liquid biopsy applications, the mutation in the tumor is not known a priori and all mutations of interest are queried at once. To determine the sensitivity of the liquid biopsy approach, we evaluated the plasma and tumors of 206 patients with metastatic CRC in a blinded fashion (table S3). This cohort of patients was completely distinct from the 410 patients described above and in tables S1 and S2. For each case, we determined whether mutations at codon 12 or 13 of *KRAS* were present in either the primary tumor or in 2 ml of plasma drawn before treatment. The *KRAS* gene was chosen for this study because of its clinical relevance; the absence of a *KRAS* gene mutation in the primary tumor is a prerequisite for treatment of metastatic CRC patients with antibodies that block EGFR (29). We identified 69 patients (33% of the 206) who harbored circulating mutant *KRAS* in their plasma. Circulating *KRAS* mutations were not detected in 127 of 128 patients with KRAS wild-type tumors, yielding an uncorrected specificity of 99.2%. The mutation identified in the 69 plasma samples was always identical to that identified in the tumors, further emphasizing the specificity of the liquid biopsy. In addition to these 69 tumors, we identified 10 cases (of 206) in which mutations were present in the primary tumors but not in the plasma, yielding a sensitivity of 87.2%. Percent concordance between *KRAS* mutation status in the plasma and tumor tissue was 95%, and the agreement was highly significant (κ statistic = 0.88, $P < 0.0001$).

We next evaluated 26 clinical and pathologic characteristics to better understand the observed false-negative results (tables S3 and S4). The factors associated with a false-negative ctDNA result (mutant KRAS in the tumor but no mutants detectable in the plasma) were low CEA level, mucinous histology, low alanine aminotransferase levels, low WBC count, and younger age (tables S4 and S5). CEA levels were also positively correlated with the concentration of mutant *KRAS* fragments in the plasma (tables S6 and S7). These observations are consistent with the idea that lower tumor burdens (reflected by normal CEA levels) are associated with lower ctDNA levels.

We next examined the relationship between the concentration of ctDNA and survival. Beginning with a model of known prognostic factors [age, Eastern Cooperative Oncology Group (ECOG) performance status (PS), and CEA], and assuming linearity for these adjustment variables, we found that ctDNA concentration provided added value in predicting survival (likelihood ratio test, $P = 0.00253$, df = 3). We then estimated the 2-year survival rate for differing concentration of ctDNA, holding the other predictors constant (Fig. 5). We observed a steady decrease in survival rate as ctDNA concentration increased.

Monitoring patients for resistance-conferring mutations

Liquid biopsies can also be used to monitor patients being treated with targeted agents, providing an early warning of recurrence and information about the genetic basis of resistance. For example, *KRAS* codon 12 and 13 mutations were shown to develop in 38% of 24 patients who first responded to EGFR blockade and then progressed (20). In each case, the *KRAS* gene mutation was not present in the primary tumor but had presumably arisen in a small population of cells within a metastatic lesion and expanded under the influence of

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the EGFR blockade. Here, we wished to determine whether other resistance mutations, besides those at *KRAS* codons 12 and 13, could be identified in liquid biopsies of patients treated with EGFR blockade. We therefore designed a multiplexed, sequencing-based assay to query known mutated hotspots of several genes in the EGFR pathway: the regions within and surrounding *KRAS* codons 12, 13, 59, 60, and 61; *NRAS* codons 12, 13, 59, 60, and 61; *BRAF* codons 599 and 600; *EGFR* codons 712 to 721, 738 to 748, 790 to 800, and 847 to 859; and *PIK3CA* codons 538 to 549 and 1039 to 1050. The 24 cases assessed included 17 of those previously assessed for *KRAS* mutations (20) plus 7 additional cases of patients who had first responded, then progressed, while being treated with blocking antibodies to EGFR (panitumumab or cetuximab). The primary tumors of nine of these cases were unavailable, so we used pretreatment DNA from plasma to assess whether any of the queried mutations were detected before administration of EGFR antibodies; none of the mutations listed in Fig. 6 were found before antibody treatment.

We identified emergent circulating mutations of at least one mitogen-activated protein kinase pathway gene in 23 of the 24 patients (96%). The number of different mutations identified in the circulation of individual patients averaged 2.9 (range, 0 to 12). The development of different mutations in the same patient is not surprising given that each of these patients had multiple lesions; each lesion that responds to EGFR blockade and then progresses is expected to harbor at least one resistance mutation (20, 30).

In total, we observed 70 somatic mutations that were not detected in the tumor or in the plasma before EGFR blockade and only appeared after therapy was initiated (table S8 and Fig. 6). Half of the mutations (34 of 70) occurred in *KRAS* codon 12. These mutations are known to cause resistance to EGFR blockade when present in the primary tumor, and have been observed to arise after EGFR blockade in vitro as well as in vivo (20, 30). One mutation in *BRAF* was observed. Several previous studies have shown that BRAF V600E mutations, when present in primary tumors, are associated with failure to achieve a response to EGFR blockade (31–33). Two other patients developed mutations in the kinase domain of EGFR (codons 714 and 794; table S8 and Fig. 6). Mutations at these residues have been previously observed in primary CRC, albeit infrequently, and resistance to EGFR blockade has been shown to result from genetic alterations in the *EGFR* gene (34, 35). We did not identify treatment-related mutations in the known PIK3CA gene hotspots (exons 9 and 20) (36).

The most surprising observation in the EGFR blockade component of our study was the large number of mutations in codon 61 of either the *KRAS* or *NRAS* gene (table S6 and Fig. 6). Fifteen of the 24 patients (62.5%) harbored at least one codon 61 mutation, and the 31 mutations in these 15 patients comprised 45% of the total (69) mutations observed. Forty-eight percent of the codon 61 mutations were in *NRAS* and the remainder were in *KRAS* (table S6 and Fig. 6).

DISCUSSION

Through the study of 640 patients, we have learned that mutant DNA fragments are found at relatively high concentrations in the circulation of most patients with metastatic cancer and

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at lower but detectable concentrations in a substantial fraction of patients with localized cancers. These results have several translational implications and suggest important avenues of future research.

Monitoring disease in advanced cancer patients

A genetic alteration could be identified in the tumor of all 410 patients evaluated in this part of study, making ctDNA a widely applicable bio-marker for cancer patients. Moreover, >80% of patients with metastatic disease had detectable levels of ctDNA, higher than that reported for most conventional biomarkers (37). Unlike proteins such as CEA or CA19-9, which are expressed in normal cells as well as in neoplastic cells, genetic alterations of a clonal nature are only found in neoplasms. Our data indicate that measurements of ctDNA can also provide therapeutic, predictive, and prognostic information in patients with metastatic disease. As shown in Fig. 5, metastatic CRC patients with relatively low levels of ctDNA lived significantly longer than patients with higher levels, and there was a marked correlation between ctDNA concentration and survival. A similar association between survival and ctDNA concentration has recently been reported in patients with advanced breast cancers (16).

Although these advantages of ctDNA render it promising for monitoring patients, there are potential limitations. The specific mutations are defined by evaluation of the primary tumor, adding both time and expense to patient management. This may be less of an obstacle in the future because more cancer patients will have their tumors genetically analyzed to guide therapeutic decisions. The genetic alterations used to guide therapies can also be used for ctDNA analysis. A more serious issue relates to the utility of monitoring patients with advanced cancers, either with ctDNA or with other biomarkers (38, 39). On one hand, patients and their physicians are anxious to know, as soon as possible, whether disease has progressed. Imaging studies are often noninformative or slow to reflect progression. Repeated imaging also subjects patients to radiation, whereas monitoring ctDNA is noninvasive. On the other hand, it has not yet been shown that monitoring patients with advanced disease with any biomarker provides clinical as opposed to psychological benefits. Knowing that progression (or response) has occurred prior to changes in clinical symptoms may not prolong survival or improve quality of life.

Methodological comparisons

There are two sources of tumor DNA accessible in the blood (CTCs and ctDNA), and two types of genetic alterations that can be most easily assessed in either source (point mutations and translocations). Previous studies that compared ctDNA with CTCs reached mixed conclusions. For example, one group concluded that ctDNA was present less often than CTCs (17); this group used state-of-the-art methods to detect CTCs but did not use a highly sensitive method to detect ctDNA. The second group concluded that ctDNA was present more often than CTCs (16); this group used a sensitive method for analyzing ctDNA but used a relatively insensitive method for analyzing CTCs. More recently, much higher levels of ctDNA than CTCs were found in two of three pediatric patients with neuroblastomas (19).

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To investigate this issue further, we assessed both ctDNA and CTCs in the same blood sample from patients with typical solid tumors. We simply separated the cellular component from plasma and determined the fraction of cells or cell equivalents, respectively, in which tumor-specific rearrangements could be identified. Because we did not attempt to physically separate tumor cells from normal WBCs, technical issues related to the efficiency of CTC purification were eliminated. The comparison between DNA from CTCs and ctDNA cannot easily be performed with point mutations because the background level of point mutations in PCR-based assays is too high, even with the sensitive methods used in our study. This background precludes the detection of point mutations at levels less than 1 in 100,000 cells (40, 41). Because several million normal cells but only a few CTCs are present per milliliter of blood, a technology that is more sensitive is required. The detection of rearrangements is well suited for this task because it has been shown that one mutation can be reliably detected among millions of wild-type template molecules; PCR errors do not generate specific rearrangements (42).

Using patient-specific rearrangements as a tool, we were able to show that the level of ctDNA was always higher than that of CTCs. In 13 of 16 patients, ctDNA levels were relatively high, whereas no CTCs at all could be detected. This does not mean that ctDNA is preferable to CTCs for the detection or monitoring of cancer. Rather, the optimal technology depends on many other factors, including cost and throughput, for which CTC detection has advantages. However, this comparison does suggest that the vast majority of ctDNA is not derived directly from CTCs. Because the half-life of ctDNA is short (<1.5 hours) (21), in fact shorter than that of CTCs (43), our work suggests that the mutant molecules in the plasma are generally not derived from the CTCs.

Another comparison of interest concerns translocations and point mutations. Our results (table S2) show that the number of ctDNA fragments per milliliter of plasma for translocations and point mutations was similar in most of the cases studied. However, in 1 of 19 cases, a point mutation was detected in a plasma sample in which the studied rearrangement was absent. The likely reason for this was that the point mutation was in a driver gene that occurred relatively early in tumorigenesis, whereas the rearrangement was subclonal, perhaps not contributing to the development of the tumor. In four other cases, rearrangements were detected at 10-fold higher levels than the point mutations (table S2). In these cases, the rearrangements were found to be components of somatically amplified genes.

From a practical perspective, these data suggest the following conclusions: Maximal sensitivity for detecting a genetic alteration can be achieved by using a rearrangement present within an amplicon. Many tumors, particularly advanced ones, contain such amplifications, making them relatively easy to detect with low-coverage (10×) genome sequencing. As with the comparison between CTCs and ctDNA, however, this greater sensitivity does not mean that rearrangements are preferred over point mutations for clinical use. The discovery of a rearrangement in a patient's tumor, and the work and time required to develop and test primer pairs that can efficiently detect the rearrangement(s) in the degraded DNA characteristic of plasma, is considerable. In contrast, a panel of assays

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detecting the most commonly mutated point mutations is currently simpler and less expensive to implement in the clinical setting.

Early detection of localized cancers

Until therapeutic agents with much greater potency and minimal side effects are developed, the current best hope for reducing cancer morbidity and mortality is early detection of neoplastic disease (9). Prior to metastasis, most solid tumors can be cured by extant surgical methods, and even when occult metastasis has occurred, adjuvant therapy or additional surgery can lead to cure in some patients. One of the encouraging results of our study is that ctDNA was found in most of the patients with localized disease, when their chances of a favorable outcome are highest (Fig. 3). Even in patients with stage I disease, who are nearly always curable by surgery alone, 47% of patients were shown to have detectable levels of ctDNA in their plasma. In stage III disease, which is curable in many patients with certain forms of cancer, more than two-thirds of patients had detectable ctDNA.

Although early detection strategies based on ctDNA are promising, numerous obstacles must be overcome before they can be applied clinically. The fraction of patients with detectable ctDNA represents the maximum obtainable with the amount of plasma collected in this study (table S1). In a screening setting, with the exception of pancreatic ductal adenocarcinomas [where one gene, *KRAS*, is mutated in almost all cases (44)], the mutation of interest would not be known a priori and a panel of genes would have to be assessed. Our study on the EGFR blockade cohort shows that it is indeed possible to assess several genes at once for the detection of relatively rare mutations in plasma (table S6).

In addition to these technical challenges, biomedical issues will have to be addressed by any ctDNA-based screening test. False-positive findings can be problematic for any screening assay (45). Experience thus far suggests that benign tumors and nonneoplastic conditions do not generally give rise to ctDNA (46), so the “overdiagnosis” of benign tumors is not likely to pose a major problem. However, other studies suggest that a tumor containing ~50 million malignant (rather than benign) cells releases sufficient DNA for detection in the circulation (20). A cancer of this size is far below that required for definitive imaging at present. How would a patient who had a positive ctDNA test be managed if follow-up imaging tests were negative? A related issue is the fact that the type of mutation does not provide many clues to the tumor type. For example, a patient with a circulating *TP53* mutation, in the absence of other mutations, could have a cancer in any of several organs. Another question concerns the value of detecting early cancers. In pancreatic ductal adenocarcinomas, for example, it might be argued that most patients with a positive ctDNA test will die from their disease anyway, given the aggressive nature of this form of cancer. Although these obstacles are formidable, we would argue that the presence of detectable amount of a mutant driver gene is a cause for serious concern given the known causal relationships between such mutations and cancer. Indeed, this point distinguishes mutation-based biomarkers from all other types of biomarkers yet described.

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Liquid biopsies

Our studies demonstrate two uses for liquid biopsies. The first—assessing plasma for the presence of specific mutations that can direct patient management—is clinically actionable. We show here that the sensitivity of the liquid biopsy for testing *KRAS* codon 12 is 88.2% in patients with metastatic CRC. Although conventional tumor biopsies are preferable, these often cannot be obtained for logistic or medical reasons. When tumor tissue specimens from metastatic cancer patients are unavailable, liquid biopsies offer an alternative that can be rapidly implemented without the pain, risk, and expense entailed by a biopsy of one of the metastatic lesions. Of note is the fact that ctDNA from neoplasms confined to the central nervous system (Fig. 2A) and those with mucinous features (table S4) was infrequently detectable. This suggests that physical obstacles such the blood-brain barrier and mucin could prevent ctDNA from entering the circulation.

Tracking resistance

A second use of liquid biopsies is for identifying resistance mutations that occur when patients first respond to therapy and then progress. The detection of ctDNA requires tumor cells to die, and even tumor cells that are resistant to therapy turn over rapidly; they die almost as frequently as they are born (20). Thus, it is expected, and in fact observed, that the DNA fragments from drug-resistant cancer cells are found in the plasma. Although this approach is mainly of interest for research purposes at present, the obtained information can be clinically informative. A good example of this principle is provided by our discovery of remarkably frequent mutations at codon 61 of *NRAS* and of *KRAS*, representing 46% of the detected mutations in patients resistant to EGFR blockade. Codon 61 mutations of *KRAS* and *NRAS* have previously been observed to occur in primary CRCs, but very infrequently compared to the prevalence at which we found them in patients progressing after EGFR blockade (33). *KRAS* codon 61 mutations have been observed to be associated with primary resistance to EGFR blockade when they occur in primary CRCs (32, 33, 47). There are no previous studies indicating that *NRAS* codon 61 mutations are associated with acquired resistance, but the results in Fig. 6 leave little doubt as to their role. This finding provides unequivocal evidence that these mutations confer resistance to therapy—the probability that recurrent mutations at these positions occurred by chance alone is essentially nil (20). It also supports studies showing that *KRAS*, *BRAF*, *NRAS*, and *EGFR* mutations compromise the efficacy of EGFR blockade in patients with CRC (47, 48).

Collectively, codon 600 mutations of *BRAF*, codon 61 mutations of *KRAS*, and codon 12 or 61 mutations of *NRAS* occur about half as often as mutations in *KRAS* codons 12 or 13 in primary CRCs (49). These data therefore strongly suggest that patients being considered for treatment with EGFR blockading agents should be tested for these additional mutations. This conclusion was independently supported by a clinical study reported during the review of our manuscript (50). Patients harboring mutations at these positions are unlikely to benefit from these agents and would be better served by other therapeutic approaches.

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SUMMARY

In summary, we demonstrate that ctDNA can be used as a feasible biomarker for a variety of different solid tumor types and clinical indications. The clinical utility of this biomarker, and the risks and benefits accruing from knowledge of ctDNA levels, can only be addressed through longitudinal studies of ctDNA in appropriate populations of patients, as is currently under way for CTCs (51). The studies reported here lay the groundwork for such future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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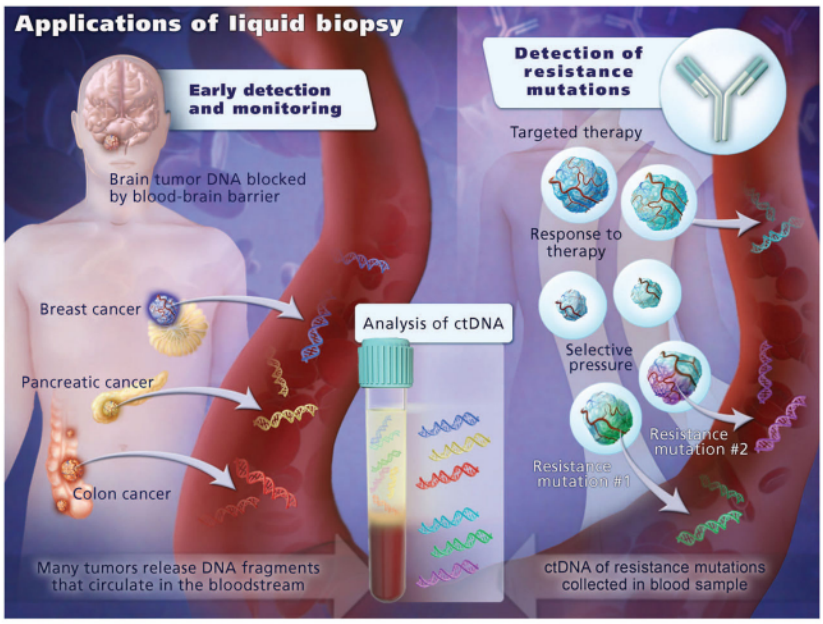

Fig. 1.
Potential applications of ctDNA.

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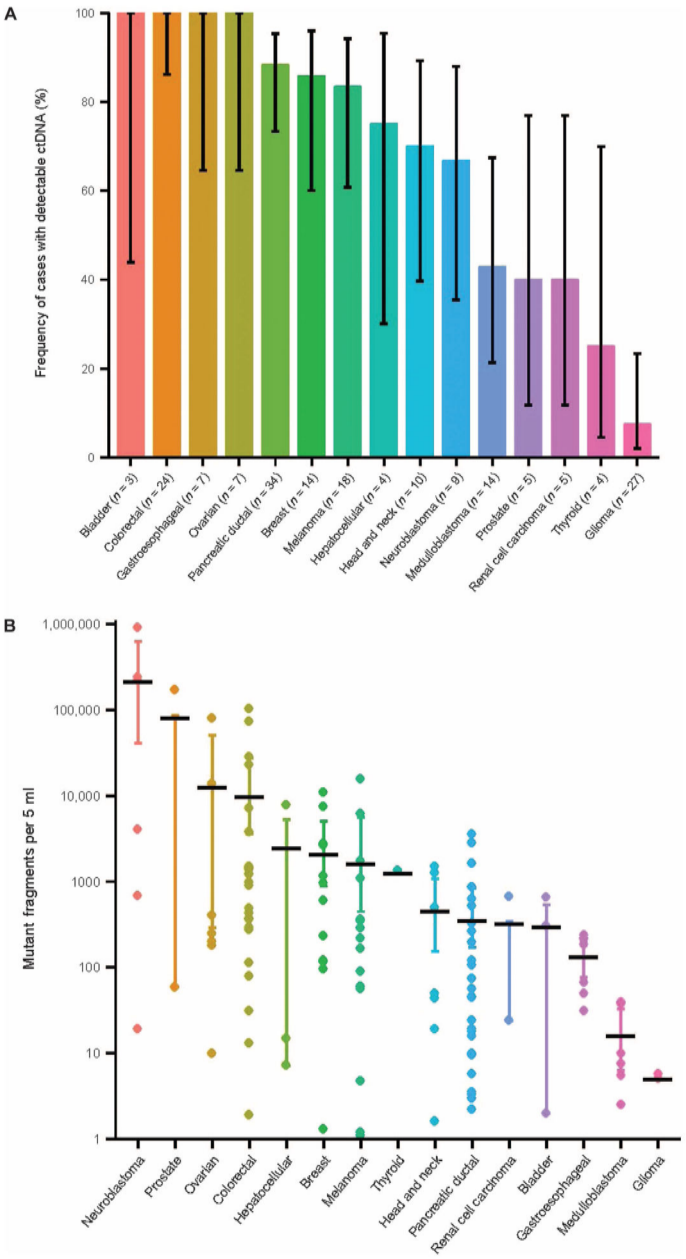

Fig. 2. ctDNA in advanced malignancies

(**A**) Fraction of patients with detectable ctDNA. (**B**) Quantification of mutant fragments. Error bars represent the 95% bootstrapped confidence interval of the mean (tumor types with <4 samples were excluded from this figure).

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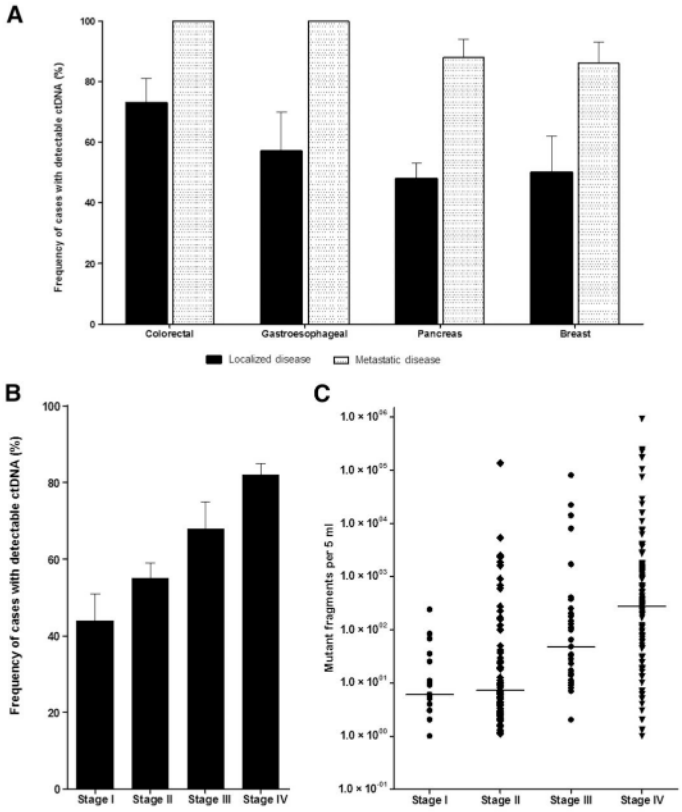

Fig. 3. ctDNA in localized and nonlocalized malignancies

(**A**) Fraction of patients with detectable ctDNA in localized (stages I to III) and metastatic (stage IV) colorectal, gastroesophageal, pancreatic, and breast cancers. (**B**) Fraction of patients with detectable ctDNA. (**C**) Quantification of mutant fragments in cancer cases categorized by stage. Error bars represent SEM.

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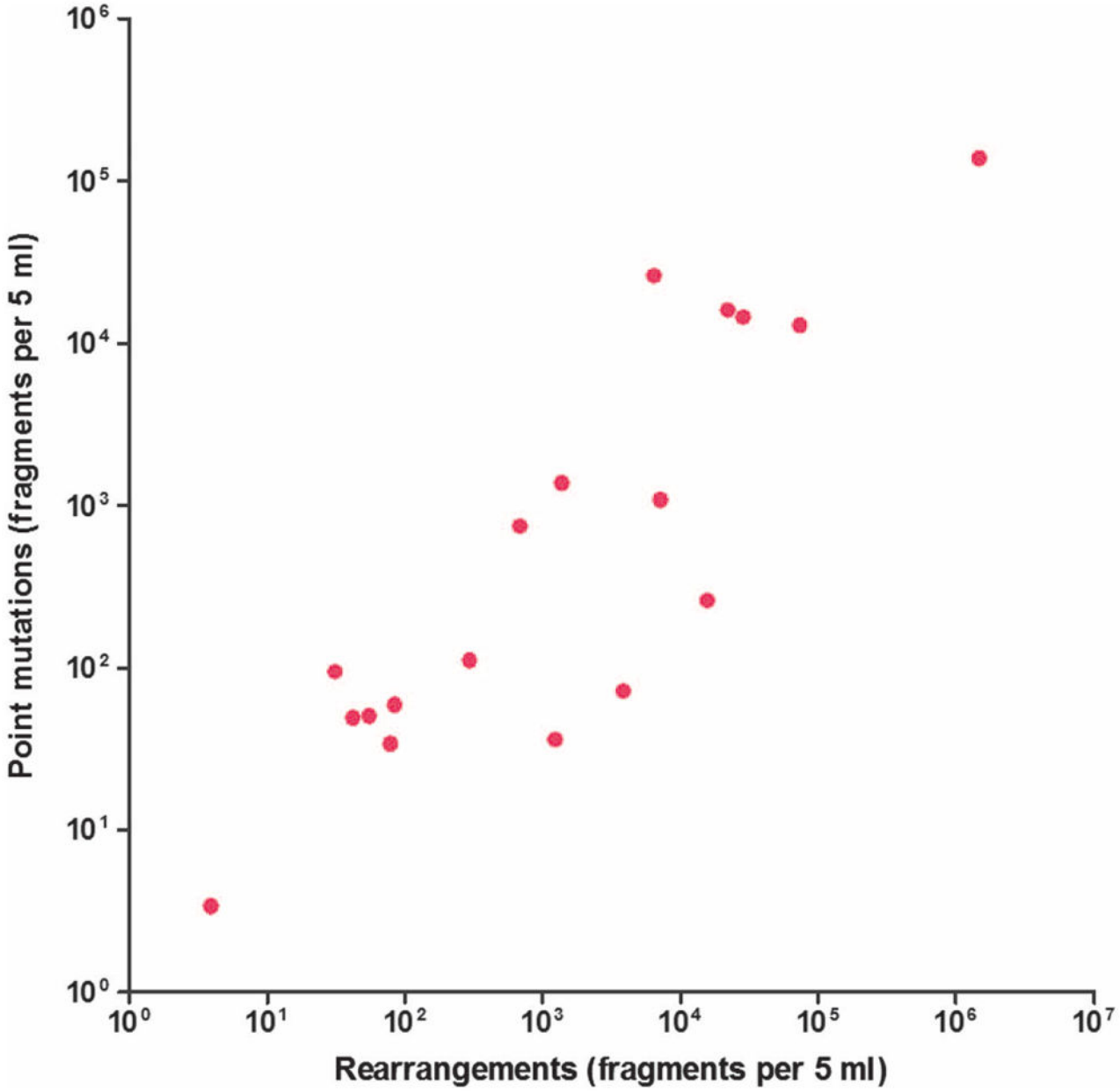

Fig. 4.
Scatter plot correlating point mutations with rearrangements in the same plasma specimens.

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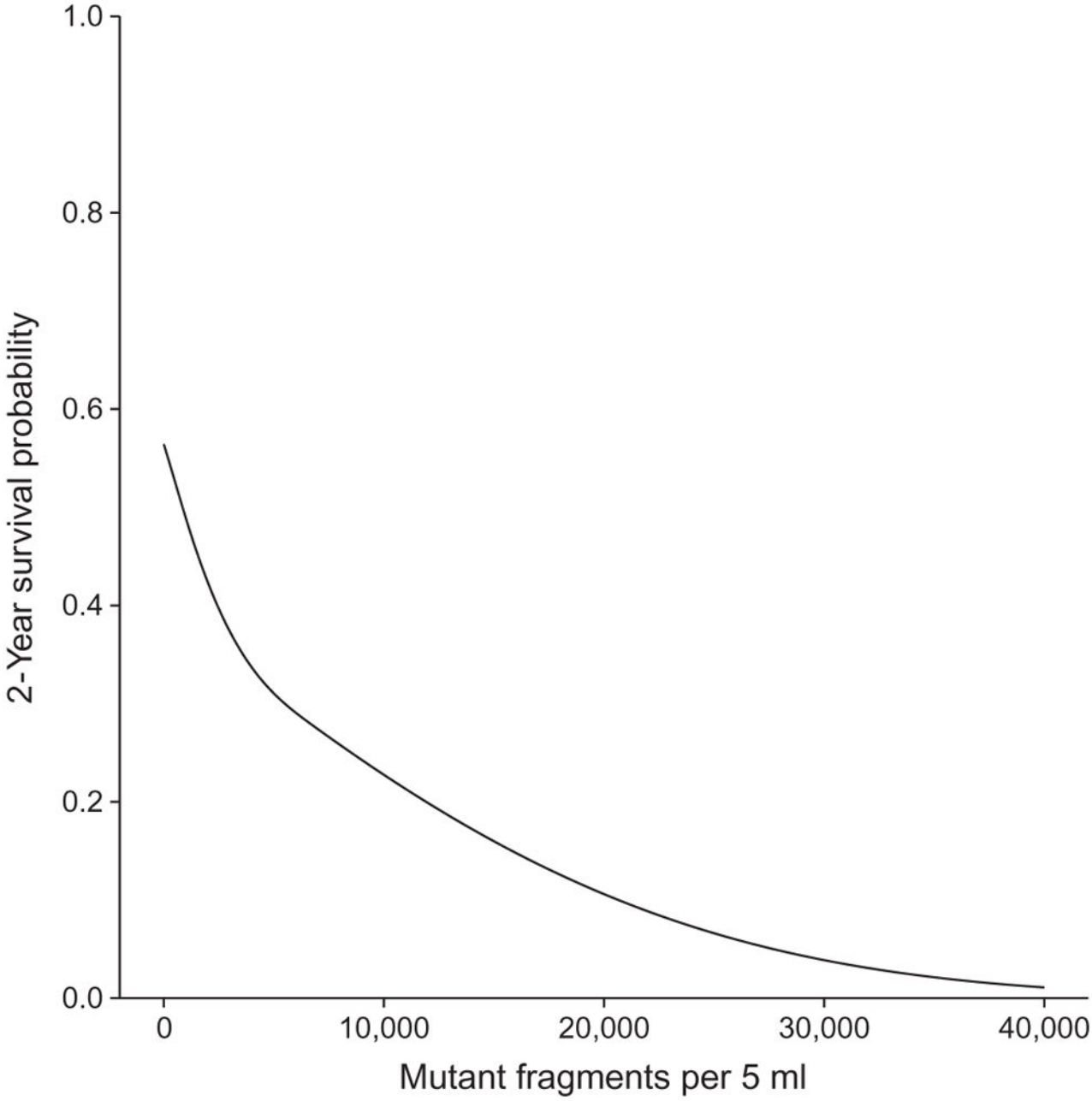

Fig. 5. The relationship between ctDNA concentration (mutant fragments per milliliter) and 2-year survival

The association between survival and ctDNA concentration was assessed, holding known prognostic factors (age, ECOG PS, and CEA) constant. The 2-year survival was estimated on the basis of a multivariable Cox regression model, in which ctDNA concentration level was transformed with a natural spline function.

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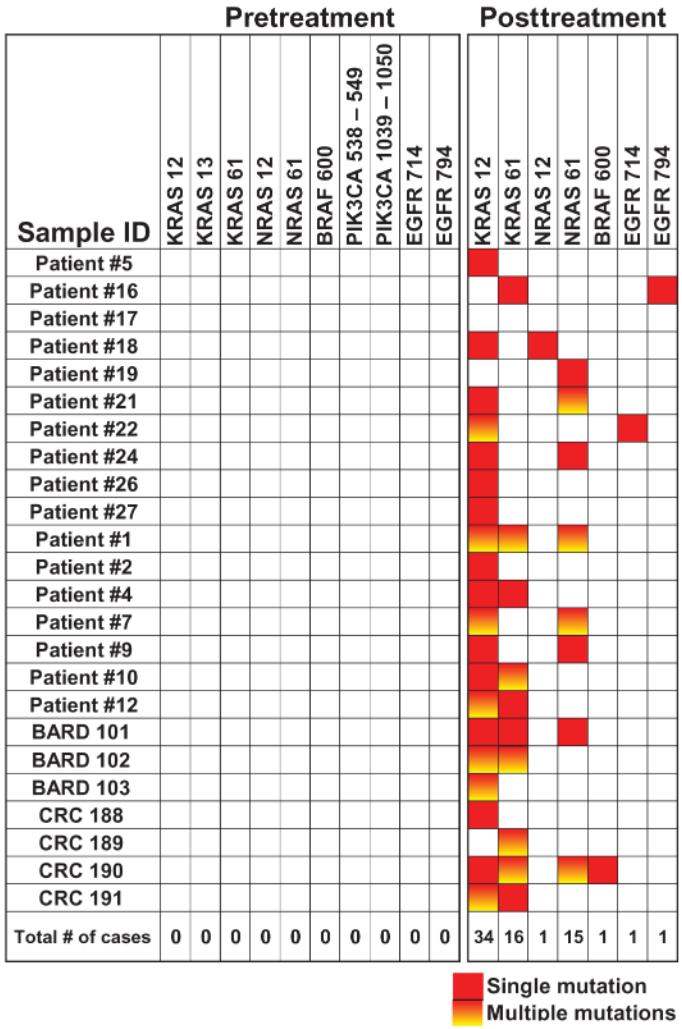

Fig. 6.

Heat map of acquired resistance mutations to EGFR blockade in ctDNA from patients with metastatic CRC.

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Table 1

Summary of clinical characteristics of 410 patients with various malignancies.

	Parameter value
Age, years	
Mean (SD)	63.0 (13.6)
Median (range)	64 (23–95)
No. unknown (%)	67 (16.3)
Gender, *n* (%)	
Female	163 (39.8)
Male	181 (44.1)
No. unknown (%)	66 (16.1)
Tumor type, *n* (%)	
Bladder	10 (2.4)
Breast	33 (8.0)
Colorectal	64 (15.6)
Endometrial	12 (2.9)
Gastroesophageal	21 (5.1)
Glioma	27 (6.6)
Head and neck	12 (2.9)
Hepatocellular	4 (1.0)
Medulloblastoma	14 (3.4)
Melanoma	20 (4.9)
Neuroblastoma	9 (2.2)
Non–small cell lung cancer	5 (1.2)
Ovary	9 (2.2)
Pancreas	155 (37.8)
Prostate	5 (1.2)
Renal cell carcinoma	5 (1.2)
Small cell lung cancer	1 (0.2)
Thyroid	4 (1.0)
Clinical stage*	
1	49 (13.3)
2	133 (36.0)
3	51 (13.8)
4	136 (36.9)

*Excludes 41 primary brain tumor patients.

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Table 2

Comparison of CTCs with ctDNA.

Sample ID	Tumor type	Clinical stage	Cellular DNA (mutant fragments per 5 ml)	Plasma DNA (mutant fragments per 5 ml)
BLD 21	Bladder cancer	2	0	226
BLD 24	Bladder cancer	2	0	4
CRC 12	Colorectal cancer	4	0	79
CRC 14	Colorectal cancer	4	0	31
CRC 31	Colorectal cancer	1	0	35
CRC 32	Colorectal cancer	2	0	37
CRC 35	Colorectal cancer	2	0	5
CRC 40	Colorectal cancer	1	0	25
CRC 60	Colorectal cancer	4	680	73,000
CRC BIO 23a*	Colorectal cancer	4	370	21,000
CRC BIO 23b*	Colorectal cancer	4	400	28,000
BR 833	Breast cancer	2	0	2,500
BR 834	Breast cancer	2	0	41
BR 837	Breast cancer	2	0	3
BR 841	Breast cancer	2	0	690
BR 848	Breast cancer	2	0	9,900

*Two independent blood samples from the same patient, drawn 2 months apart, were separately analyzed.

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