

REVIEW

Differences and evolution of the methods for the assessment of microsatellite instability

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Microsatellite instability (MSI) originates from the systematic accumulation of uncorrected deletion/insertion in repetitive DNA tracts in cancer cells with a deficient mismatch repair system. Among colorectal cancers, the MSI signature identifies hereditary cases arising in patients with germline mutations in *hMLH1*, *hMSH2*, *PMS2* and a fraction of those with *hMSH6* mutations, as well as sporadic cancers with epigenetic *hMLH1* promoter hypermethylation. Considering the specific pathogenesis, pathological features, natural history and response to 5-fluoro-uracil-based chemotherapy of the MSI cancers, confusion about the genetic markers for MSI recognition seems surprising. In this clinically relevant field, an agreement has not been reached concerning the use of di- or mononucleotide markers for MSI assessment. The Revised Bethesda Guidelines still recommend a panel of markers consisting of mono- and dinucleotides, despite being questioned whether it is congruous to continue to use dinucleotide markers for MSI identification. In any event, no single marker is accurate enough for MSI testing, and an awareness of their pros and cons is required for proper interpretation of results. In recent years, several papers have reported different prevalence of MSI in unrelated series, largely depending on the detection and classification method, suggesting that MSI test interpretation also requires the understanding of the phenomenon rather than simply the crude satisfaction of panel recommendations. Inaccuracies can otherwise lead to under- or overdiagnosis and inaccurate disease classification, which always have a negative impact on the clinical practice of medicine.

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Introduction

Short repetitive DNA tracts, also named as microsatellites, mostly interspread in the non-coding portions

of the genome composed of variable numbers of tandem repeats, such as (CA)_n (Strachan, 1999; de la Chapelle, 2003). Owing to the fact that microsatellites can be highly polymorphic loci, their analysis has been and is exploited to genotype individuals, populations and tumors. The polymerase chain reaction (PCR)-based analysis of microsatellite regions is based on the comparison of their presence and length among subjects or between normal and tumorous cells (Weinberg, 2006) (Figure 1). Microsatellite instability (MSI) (Thibodeau *et al.*, 1993) is a molecular phenotype peculiar to human cancers with defects in the post-replicative DNA mismatch repair (MMR) system (Jiricny, 2006). In cancer cells, the presence of a defective MMR system leads to the accumulation of unrepaired mitotic errors, which occur more frequently in repetitive DNA tracts, mainly owing to the slippage of error-prone DNA polymerases (such as the β one), and owing to the occurrence of DNA helix hairpins at the replication fork (Weinberg, 2006). The accumulation of such uncorrected errors over time in the progeny of cancer cells during their clonal expansion (Boland and Goel, 2005) leads to the progressive shortening of these repetitive sequences, which results in the appearance of new shorter alleles compared with those represented in the normal cells. Owing to the fact that microsatellite sequences are intrinsically unstable, MSI could be a misnomer (Perucho *et al.*, 1999), and what is relevant is not the presence of microsatellite alterations, but their number, which has been estimated to be in the magnitude of hundreds of thousands (Ionov *et al.*, 1993) in the presence of a deficient MMR system. However, over time, MSI became the standard term employed to refer to the DNA signature of such a deficiency, although its adoption has not been immediate, largely owing to the different definitions proposed and variably received from the scientific community (see below).

The discovery of MSI

Since 1993, when independent research groups discovered MSI in colorectal cancer (CRC), reports of this cancer phenotype mirrored the different scientific questions and methodological approaches that had led to its identification, readily reflected in the various

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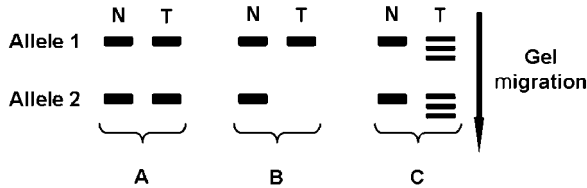


Figure 1 The illustration exemplifies the possibility of discriminating between the two alleles (paternal and maternal) of an informative (heterozygous) microsatellite locus, taking advantage of their different electrophoretic mobilities in a polyacrylamide gel. Following amplification by polymerase chain reaction of a hypothetical microsatellite composed of a CT tandem, electrophoresis shows that allele 1 is composed of the (CT) unit repeated 16 times, that is, (CT)₁₆, whereas the shorter allele 2 contains (CT)₁₂, thereby resulting in a faster migrating band (from top to bottom). (A) Both alleles retain the same migration pattern of normal (N) tissue in the tumor (T), indicating no difference at this locus between the germline and the cancer lineage. (B) The loss of allele 2 in cancer cells, a situation in which the microsatellite locus itself no longer appears heterozygous (that is, loss of heterozygosity). (C) The instance where both alleles in cancer cells appear in multiple different forms, differing in their length by one or multiple units of the tandem repeat, and allele 1 is present in its original (CT)₁₆ length but also in other forms, such as (CT)₁₅, (CT)₁₄, and so on. Similarly, allele 2 can be detected as (CT)₁₂, (CT)₁₁, (CT)₁₀, and so on. The presence of these additional shifted microsatellite bands in cancer cell clones has been designated as microsatellite instability.

terminologies employed to designate it. Back then, almost contemporarily, three groups reported that a fraction of CRCs displayed a distinct molecular phenotype (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993). The times of their appearance in the press might not have resembled the true paces leading to the discovery of MSI (Maddox, 1993; Perucho *et al.*, 1999); however, they shaped the scenario for more than a decade, with researchers debating on methodological issues concerning the definition and identification of MSI, as well as its interpretation and epidemiology (Maddox, 1993; Rodriguez-Bigas *et al.*, 1997; Perucho *et al.*, 1999). Two reports appeared as companion papers in *Science* (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993), whereas the third paper was published in *Nature* (Ionov *et al.*, 1993). The report by Thibodeau *et al.* (1993), which introduced the term MSI, originated from the analysis of the chromosomal arms 5q, 15q, 17p and 18q in CRC by using microsatellite markers, while looking for loss of heterozygosity (LOH) at these chromosomal arms. Thibodeau *et al.* (1993) reported that 25 out of 90 investigated CRCs (28%) displayed somatic changes in the dinucleotide (CA)_n repeat length of the tested microsatellites. Along similar lines, having genotyped individuals with familial CRC (whose germline defect was linked to chromosome 2 (Peltomaki *et al.*, 1993)) by microsatellites, Aaltonen *et al.* (1993) reported that familial cancers displayed widespread alterations in short repetitive DNA sequences. The authors concluded that numerous replication errors had occurred during tumor development, a phenomenon observed in 13% of sporadic CRCs as well (Aaltonen *et al.*, 1993). The group led by Perucho had a radically different approach. By using an unbiased

fingerprinting technique, rather than by employing specific microsatellites, this group showed that 12% CRCs harbor deletions in poly(dA.dT) tracts, which they named as ubiquitous somatic mutations (Ionov *et al.*, 1993). The authors suggested that a reduced fidelity of replication or repair, predisposition to which may be inherited, accounted for this phenotype (Ionov *et al.*, 1993). Although the paternity of the discovery of MSI was a disputed issue (Maddox, 1993), the competing researchers realized that MSI CRCs had a peculiar molecular pathogenesis, with a low prevalence of mutations in *KRAS* and *P53*, and not going through LOH at tumor suppressor genes (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993), but rather involving the disruption of a DNA repair system deputed to maintain the genome integrity (Ionov *et al.*, 1993). In addition, these seminal papers have already pointed to the fact that the molecular phenotype of MSI identifies a set of CRCs sharing unique clinical and pathological features. Namely, these cancers preferentially arise in the proximal colon, account for a high proportion of hereditary nonpolyposis colorectal cancers (HNPCCs), have a low metastatic potential and exhibit better survival. Owing to these intrinsic differences between CRC with and without MSI (that is, microsatellite stable (MSS)), it became common to classify CRC on the molecular ground taking advantage of the presence or absence of this feature. It was also shown that MSI CRCs are mostly near-diploid, whereas MSS ones are usually aneuploid (Lothe *et al.*, 1993), and following a concept similarity, MSS CRCs have also been referred to as having chromosomal instability (Grady, 2004), although the molecular mechanisms underlying chromosomal instability in cancers with gross chromosomal alterations (like LOH) remain elusive (Boland and Goel, 2005).

Linking MSI to inherited and sporadic defects of the DNA MMR system

Perucho's reasoning that MSI should be ascribed to mutation of a DNA repair gene turned out to be correct shortly afterward in the light of experimental data. At the end of 1993, two more companion papers published in *Cell* provided evidence that MSI is a mutator phenotype, by identifying the biochemical basis for this phenotype in strand-specific MMR defects (Fishel *et al.*, 1993), and by detecting the first germline mutation of the MMR gene *hMSH2* in CRC-affected members of HNPCC kindred (Parsons *et al.*, 1993). This was followed by the identification of mutations in the other MMR gene *hMLH1* (Papadopoulos *et al.*, 1994), together with the evidence that the transfer of a normal copy of *hMLH1* into a human cancer cell line with a mutant *hMLH1* gene completely restored MMR activity and reversed the MSI phenotype (Koi *et al.*, 1994). As the genetics, biochemistry and functions of the MMR components had been first characterized in unicellular organisms, the highly conserved human homologue (h) genes were named after those of *Escherichia coli* (Fishel

et al., 1993; see also individual OMIM entries online (NCBI, 2007)). Within a short time, mutations in the other MMR genes, *PMS2* (Nicolaidis *et al.*, 1994) and *hMSH6* (previously named GTBP) (Papadopoulos *et al.*, 1995), were documented in HNPCCs, completing the picture of the molecular defects that can be responsible for the MSI (Liu *et al.*, 1996). It should be noted that the *hMSH6* germline defects, unlike those in other MMR genes, are not necessarily associated with cancers showing the typical MSI phenotype (Miyaki *et al.*, 1997), and that *PMS2* mutations can also be associated with Turcot syndrome, similar to those in *APC* and *hMLH1* (Hamilton *et al.*, 1995). A MMR gene germline mutation plus a second hit inactivating the wild-type allele in somatic cells (Hemminki *et al.*, 1994) were thus established as the molecular basis fueling the mutator phenotype in HNPCCs; yet, the mechanism leading to sporadic MSI CRCs remained to be understood (Kinzler and Vogelstein, 1996). It turned out that in most sporadic MSI CRCs, mutations could not be identified (Moslein *et al.*, 1996; Wu *et al.*, 1997), leading to the speculation that non-mutational mechanisms were responsible for the defect. Thus, epigenetic changes in DNA were explored as the mechanism leading to gene silencing in cancer cells, alternate to mutational events. It emerged that *hMLH1* promoter hypermethylation was associated with the lack of *hMLH1* expression in primary CRC and colon cancer cell lines (Kane *et al.*, 1997), a phenomenon that could be reversed *in vitro* by demethylating agents (Herman *et al.*, 1998). Other studies showed that MSI CRCs can display the epigenetic signature of a widespread hypermethylation of the CpG islands in gene promoters (Ahuja *et al.*, 1997). It was later shown that some overlap exists between the sporadic CRC with the microsatellite mutator phenotype and a hypermethylator phenotype (Toyota *et al.*, 1999; Samowitz *et al.*, 2005), which would be inversely correlated with chromosomal instability and would precede MSI (Goel *et al.*, 2007), although other data challenge the concept of a methylator phenotype as the molecular ground for MSI development in sporadic CRCs (Yamashita *et al.*, 2003).

The operative definition of MSI

Owing to its multiple paternity, the discovery of MSI was not marked by a homogeneous methodological approach. Thus, lacking an original standard, investigators continued to look for the MSI signature using disparate dinucleotide markers, whose sensitivity and specificity to MSI recognition had not been evaluated. Only a few authors systematically assessed the prevalence of shifted shorter alleles in CRCs by testing panels of microsatellites, to pursue a stringent operative MSI definition based on the ratio of microsatellite markers with shifted bands over the total number of tested markers (Cawkwell *et al.*, 1995; Bocker *et al.*, 1997; Dietmaier *et al.*, 1997; Thibodeau *et al.*, 1998). The main concept emerging from these studies is that an

isolated length alteration of a microsatellite represents only a clonal alteration, whereas the coexistence of multiple shifts at more than one marker identifies MSI. In the first instance, termed low-level MSI (MSI-L), no MMR defects are found, whereas in the second instance, named high-level MSI (MSI-H), a defect can be identified in the MMR proteins (that is, lack of expression upon immunohistochemistry) and genes (Thibodeau *et al.*, 1996). In the late 1990s, most studies were still performed manually, using denaturing polyacrylamide gel and radiolabeled primers, although the feasibility of MS-status assessment with fluoresceinated primers and automatic sequencer had been already reported (Cawkwell *et al.*, 1995) and then used in population studies (Aaltonen *et al.*, 1998) (Figure 2). In the meantime, the role of immunohistochemistry had been delineated particularly in identifying the defective protein (particularly *hMLH1* and *hMSH2*) (Thibodeau *et al.*, 1996, 1998). In 1996, the first workshop held at the NIH focused on clarifying the role of genetics in the pathology of HNPCCs, and new clinical criteria (the Bethesda guidelines) were proposed to select potential HNPCC patients not identified by the Amsterdam criteria (Vasen *et al.*, 1991), and who should be candidates for molecular MSI testing and, when tested positive, for further germline sequencing of the MMR genes (Rodriguez-Bigas *et al.*, 1997). The workshop participants also discussed how to differentiate 'true' MSI from clonal alterations observed at microsatellite markers, but no operative statements were proposed at this point (Rodriguez-Bigas *et al.*, 1997). However, at the crossroads between high-tech medicine, focused on setting the rules for the proper way to diagnose HNPCC, and translational research exploring the molecular basis of MMR-deficient cancers, the reliability and reproducibility of the results obtained with different markers were a central issue, together with their relationships with the immunohistochemical analysis of the underlying MMR defects (Thibodeau *et al.*, 1996; Dietmaier *et al.*, 1997). On the same lines, a further workshop was held in 1997, with the aim to define uniform criteria for MSI recognition and to propose technical guidelines for its detection, introducing a shared nomenclature and encouraging the use of a working reference panel of microsatellite markers best suited for the molecular recognition of the MSI phenotype (that is, International Guidelines for Evaluation of MSI in CRC) (Boland *et al.*, 1998). The meeting had the unquestionable merit to address the call for a standard approach to the recognition of MSI; yet, although its suggestions ended up being broadly accepted, they were also a matter of criticism (see below). The reference panel included two mononucleotide markers (*BAT25* and *BAT26*) and three dinucleotide microsatellites (*D5S346*, *D2S123* and *D17S250*), previously tested by Fishel and co-workers (Dietmaier *et al.*, 1997), plus a list of several alternative loci. Comparing the interpretation of the microsatellite patterns among different laboratories, only some were easily and unambiguously classified (*BAT25*, *BAT26* and *D2S123*), whereas others appeared difficult to

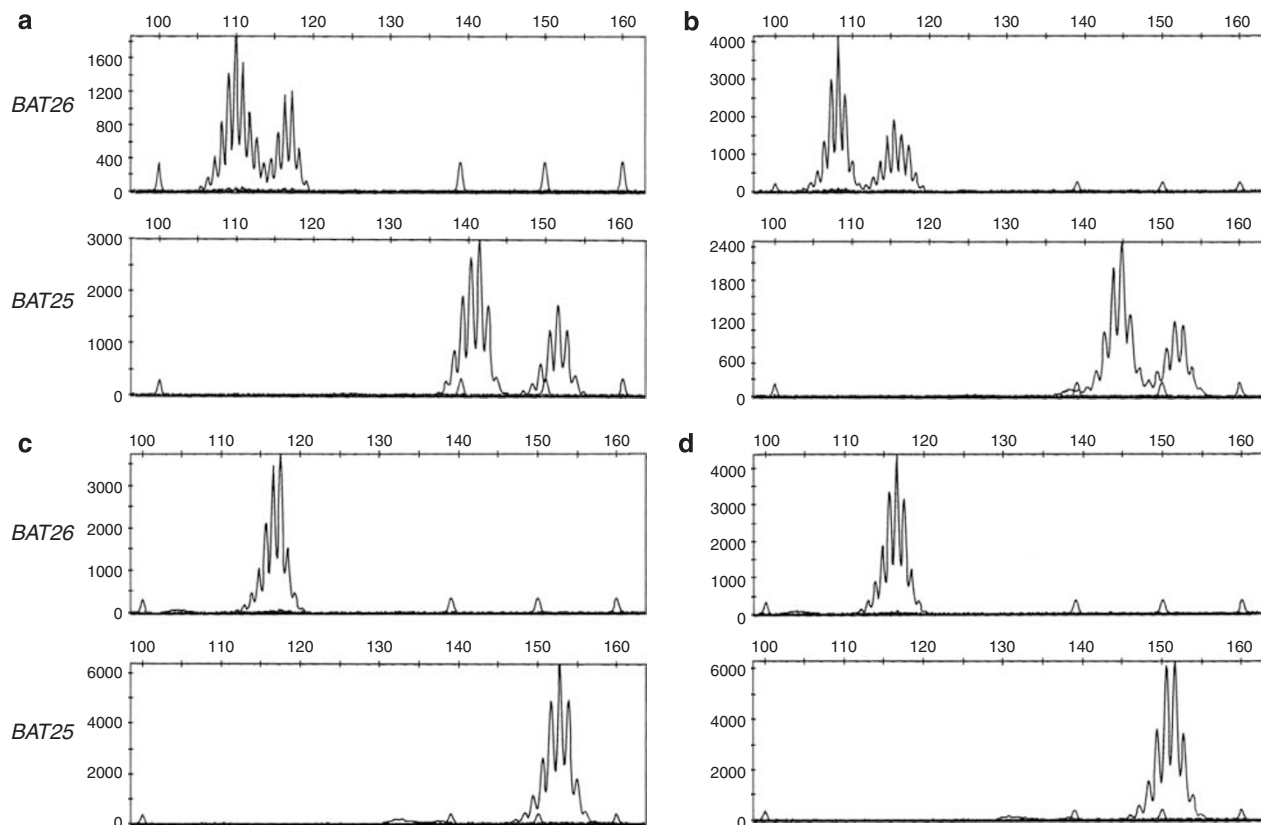


Figure 2 Capillary electrophoresis of microsatellite instability (MSI) and microsatellite stable (MSS) cancers. (a–d) Electropherograms of the fluoresceinated amplification products for the loci *BAT26* (upper rectangle of each panel) and *BAT25* (lower rectangle of each panel) from colorectal cancer (CRC) tissue. These two loci contain (A)₂₆ and (A)₂₅ repeats, respectively. Being nearly monomorphic, they are not suitable for heterozygosity analysis, and their amplification normally gives rise to a central peak plus several stuttering peaks, which originating from slippage through the mononucleotide repeat of the polymerase during the amplification reaction. (a and b) Electropherograms identifying two MSS CRCs, as only a main peak is seen at 116 bp length for *BAT26* and at 153 bp for *BAT25*, with an nearly Gaussian distribution of the stuttering peaks. (c and d) Electropherograms show two MSI CRCs, recognizable by the appearance of new shorter peaks, owing to the shortening of the adenine repeats in cancer cells. It should be appreciated that the residual normal signal is underrepresented with respect to the new unstable one, as most of the analyzed tissue was composed of neoplastic cells. This methodological approach allows to overcome the need for a comparative analysis of matched normal tissue.

interpret (Bocker *et al.*, 1997). Just as in the above-mentioned studies, the operative definition of MSI held on the distinction between MSI-H and MSI-L, depending on the number of markers exhibiting instability: MSI-H if more than two out of five analysed loci show instability vs MSI-L when instability is detected at only one locus; the definition of MSS was reserved only for those cancers with no instability at any tested marker. In the event that more than five markers were to be tested, instability at ≥ 30 –40% of analyzed markers would define MSI-H (Boland *et al.*, 1998). Even though the workshop participants acknowledged that the MSI-H cancers are distinct from the MSI-L ones, and that the latter do not show any MMR defect, it remained a matter of debate whether to separate or combine MSI-L and MSS tumors. In addition, although immunohistochemistry was considered a useful alternative strategy to MSI testing, its main role was addressed mainly as a step directing the further sequence analysis of the deficient MMR protein, rather than as a true alternative to MS testing (Thibodeau *et al.*, 1996; Boland *et al.*, 1998).

The potential pitfalls of using dinucleotides to define the MSI phenotype were highlighted by Perucho in a correspondence to *Cancer Research*, underlining that the contraction of mononucleotide repeats is sufficient in most cases to detect true MSI (that is, MSI-H), distinguishing this phenotype from the MSI-L one, which does not show a similar mononucleotide shortening (Perucho *et al.*, 1999). The use of mononucleotide repeats for determining the MS status had already been proposed by others (Hoang *et al.*, 1997), taking advantage of *BAT26*. Nearly monomorphic mononucleotides (that is, *BAT25* and *BAT26*) offer the vantage of being testable even in the absence of normal tissue, as the repeat contractions are readily picked up by the examiner (Zhou *et al.*, 1997). This relevant technical aspect allows the characterization of large CRC collections, and was tested in the first molecular screening aimed at estimating the HNPCC incidence (Aaltonen *et al.*, 1998). However, one has to be aware that *BAT26* polymorphisms exist along the length of the repeat (particularly in Afro-Americans) (Perucho *et al.*, 1999;

Pyatt *et al.*, 1999), which can be mistaken as a repeat contraction if normal tissue is not available for the analysis, making it advisable to test two mononucleotide markers. It was not stressed at that time that *BAT26*, which lies intragenically in *hMSH2*, might not show any instability in CRC with *hMSH2* biallelic deletion, a relevant and ethnicity-independent reason to employ two different mononucleotides for testing the MS status (see next paragraph). The other important point is that instability restricted at dinucleotide markers might lead to misclassifying MSS/MSI-L CRC as MSI-H (that is, dinucleotide-dependent MSI), generating false-positive cases (Perucho *et al.*, 1999). Consequently, the detection of MSI-L has a limited value, as sporadic microsatellite alterations in these cases are simply markers of clonality (Perucho *et al.*, 1999). Nevertheless, the MSI-L characterization of CRCs continued to be pursued in the literature, as were the efforts to link this clonality marker to other molecular defects or to specific phenotypic changes of CRCs. Indeed, in 2002, it was shown that MSI-L could be detected in the vast majority of CRCs simply by expanding the number of tested microsatellites (Laiho *et al.*, 2002), strengthening the idea that increasing the number of tested markers is not of any help in the MS-status assessment. The possibility that the distribution of MSI can be bimodal (MSI vs MSS) or trimodal (MSI-H vs MSI-L vs MSS) found no evidence for a separate subset comprising MSI-L CRCs (Gonzalez-Garcia *et al.*, 2000). Accordingly, the MSI-L phenotype was not associated with any clinico-pathological or molecular feature (such as, *K-RAS* mutations or *APC* LOH) (Laiho *et al.*, 2002). Although other groups had linked MSI-L to the so-called 'serrated adenoma' pathway of CRCs (Iino *et al.*, 1999), it seems unlikely that MSI-L represents the initial accumulation of clonal alterations that might later culminate in MSI-H (Tomlinson *et al.*, 2002). Thus, the existence of MSI-L CRCs remains to a large extent dubitative, originating from a temporary uncertainty in defining the nomenclature of true MSI. It should be added that true MSI cancers show frameshift mutations in coding mononucleotide runs of several cancer-related genes (for example, *TGF β R-II*; the MMR genes themselves, *hMSH3* and *hMSH6*; *BAX* and others referred to as 'target genes') (Markowitz *et al.*, 1995; Malkhosyan *et al.*, 1996; Rampino *et al.*, 1997; Yamamoto *et al.*, 1997), leading to truncated proteins and gene haploinsufficiencies (Ohmiya *et al.*, 2001) not encountered in the MSS or MSI-L cancers. Thus, it appears that MSI-H tumors follow a distinct microsatellite mutator pathway to cancer, which, in addition to the low prevalence of LOH at tumor suppressor genes, is marked by these specific mutations at target genes (Figure 3). Currently most important, the direct implication of the molecular test discrepancies in the different methodological approaches employed to look for MSI led to different prevalence of the phenotype in different CRC series. In a review article presenting the MSI frequency from selected papers, true MSI (MSI-H) CRC ranged from 3 to 23% (Tomlinson *et al.*, 2002), whereas previously it had been estimated that out of 50 papers on CRCs,

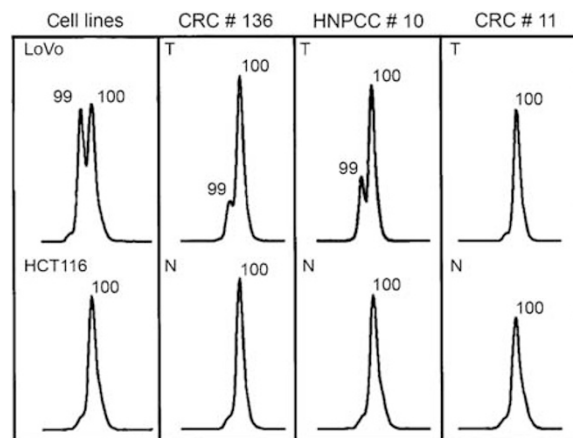


Figure 3 Mutations of target genes in microsatellite instability (MSI) cancers. Identification of frameshift mutations in the *gastrin receptor gene* (*hGARE*). Electropherograms of fluoresceinated PCR products from MSI CRC cell lines (*LoVo* and *HCT116*) and from MSI sporadic (*CRC#136* and *CRC#11*) and hereditary (*HNPCC#10*) CRCs. Numbers indicate the length of the amplicons in base pairs. Frameshift mutations are present in *LoVo* cells and in tumor DNA from MSI *CRC#136* and *HNPCC#10*. CRC, colorectal cancer; N, DNA from normal tissue; T, tumor DNA. Reprinted with permission from Laghi *et al.* (2002) *Lab Invest* 82: 265.

more than 50% overestimated the prevalence of MSI by more than two times (Perucho *et al.*, 1999). This confusion has practical implications in classifying MSI CRCs with respect to their clinico-pathological features, natural history and response to chemotherapy (Watanabe *et al.*, 2001; Ribic *et al.*, 2003; Carethers *et al.*, 2004); yet, nowadays it is not infrequent to encounter prevalence of the MSI CRCs close to or above 20%, which is likely an off-the-mark picture (Boland, 2007; Kim *et al.*, 2007). It should be emphasized that immunohistochemical analysis performed alongside MS-status determination can also help to elucidate whether a CRC is really MMR-deficient and, thereby, a true MSI CRC rather than an MSS one (Thibodeau *et al.*, 1996, 1998; Dietmaier *et al.*, 1997). Using immunohistochemistry for MMR defects rather than MS-status determination of CRCs depends on several factors, including local technical expertise (Boland *et al.*, 1998). However, most work has been based on DNA MS-status determination, particularly in population-based studies (Aaltonen *et al.*, 1998; Gryfe *et al.*, 2000; Samowitz *et al.*, 2001; Hampel *et al.*, 2005), although immunohistochemistry has also been employed in this setting (Hampel *et al.*, 2005; Truninger *et al.*, 2005). Conversely, in a diagnostic setting, MMR protein immunohistochemical analysis of CRC is the step preceding sequencing analysis and is aimed at identifying which of the MMR gene should be searched for germline alterations (Gruber, 2006).

Improving the standards for MSI identification by evaluating marker sensibility and specificity

The persistence of confusion in the field seems somehow surprising, considering that the basics for pitfalls in MSI

recognition had been pinpointed early (Perucho *et al.*, 1999). The use of mono- rather than dinucleotide repeats had been advocated, advising against potential false-positives when using *BAT26* or *BAT25*, owing to potential low-frequency polymorphisms (Pyatt *et al.*, 1999). Beyond this limitation, *BAT26* also has the opposite potential to originate false-negative cases, being located intragenically in *hMSH2* (downstream exon 5), and thus being no longer amplifiable from MSI CRCs that have a biallelic deletion of *hMSH2*. This, being a long-known fact (Hoang *et al.*, 1997), has been elegantly re-addressed in a well-selected group of CRC by Viel and co-workers (Pastrello *et al.*, 2006), although we do not know the prevalence of the subset CRCs with biallelic *hMSH2* deletion within the MSI group. Loukola *et al.* (2001) compared the MSI rate in 494 CRCs obtained by using *BAT26* or by the Bethesda panel and found that *BAT26* identified all the 27 *hMLH1* and *hMSH2* mutation positive cases, but two CRCs, otherwise classified as MSI-H, were expressing MMR proteins. Hamelin and co-workers compared the sensitivity and specificity of *BAT26* and *BAT25* with other mononucleotide repeats (Suraweera *et al.*, 2002), showing that *BAT26* and *BAT25* were unsurpassed in their performance by any other single microsatellite. Investigating 104 CRCs, they reported 100% specificity for both the absence of *BAT26* alterations in determining the MSS phenotype of CRC and for *BAT26* size variations in determining the MSI-H status of CRCs, with the only missed MSI case being the LoVo colon cancer cell line, which has a homozygous deletion of *hMSH2*. *BAT25* had similar performances but identified MSI even in the LoVo cells. To overcome limitations linked to the availability of cancer tissue alone, or to circumvent the need for matched normal tissue, they showed that, including three other mononucleotide markers (*NR21*, *NR22* and *NR24*), a unique amplification reaction (named pentaplex PCR) was sufficient to correctly identify MSI cases employing tumor tissue only (Suraweera *et al.*, 2002). This option was included in the 2004 Revised Bethesda Guidelines for HNPCC and MSI, although the original panel of mono- and dinucleotide markers remained unchanged, as did the definition of MSI-L (Umar *et al.*, 2004). This position appears partially conservative, and others have questioned whether it was appropriate to continue using dinucleotide markers for the purpose of MSI identification (Laghi *et al.*, 2004). Furthermore, the appropriate use of mononucleotide markers was instrumental in ruling out MSI as a frequent cancer phenotype in organs other than the colon, in which it was improperly assessed merely by taking advantage of dinucleotide microsatellites used for LOH assessment (Roncalli *et al.*, 2000). Taking for granted that using *BAT26* alone might lead to some (unknown) underestimation of the true MSI, owing to the non-recognition of the cases with biallelic *hMSH2* deletion, we screened by this marker probably the largest mono-institutional series of consecutive surgically resected CRCs and found a 10% MSI prevalence (Malesci *et al.*, 2007). In the study, we did not find any CRCs with an unstable *BAT25* out of the

120 cases comprising juvenile CRC patients (Bethesda criterion 1, $n=113$) and patients fulfilling the Amsterdam Criteria for HNPCC ($n=7$) who were scored as having MSS CRCs by *BAT26* analysis. Having expanded our published series, we have now encountered 1 *hMSH2*-deficient case with stable *BAT26* and unstable *BAT25* out of a total of 155 *BAT26*-stable CRCs (148 of which are juvenile cases) retested for MSI with *BAT25* (Figure 4). Very recently, a collaborative effort of the Gastrointestinal Oncology Group of the Spanish Gastroenterological Association readdressed the issue of the performance of different microsatellite panels for the detection of MMR-deficient CRCs (Xicola *et al.*, 2007). Out of 1058 patients, 531 were tested by the Bethesda panel (fresh frozen tissue available) and 527 by the pentaplex PCR (on paraffin-embedded tumor tissue). The authors reported that 7.5 and 9.9% of CRCs were scored as MSI-H by using the Bethesda panel and the pentaplex PCR, respectively. The sensitivity of the Bethesda panel was 76.5%, with a specificity of 97.2%, whereas the values scored by the pentaplex PCR were 95.8 and 98.7%, respectively. Furthermore, the authors found 7 cases with loss of *hMLH1* or *hMSH2* out of 462 (1.5%) CRCs classified as MSS according to the Bethesda panel, and 2 *hMSH2*-deficient cases out of the 475 CRCs classified as MSS by the pentaplex PCR ($P=0.09$ by χ^2 -test). Unfortunately, despite the large number of patients, this study did not compare the two panels in the same patient population, but restricted the crossover analysis to the MSI-H (and MSI-L) and to 100 more randomly selected MSS cases.

A different problem is whether MSI can be missed because of inadequate tissue sampling. The recommendations for molecular genetic analyses of cancer apply to MS-status determination as well, with result reliability requiring that the tissue specimen contains a major proportion of neoplastic cells (at least >50%) (Aaltonen *et al.*, 1998), eventually microdissecting cancer from stromal cells prior to DNA extraction (Boland *et al.*, 1998). In this respect, an intriguing study by the German HNPCC Consortium pointed out the fact that laser microdissection of adenomas in HNPCC patients with MSI CRCs, either synchronous or metachronous, enhanced the rate of MSI lesions from 50% (9 of 18) to 83% (15 of 18) (Giuffrè *et al.*, 2005). At any event, almost 20% of the adenomas arising in HNPCC patients might not be classically MSI and/or MMR-deficient. Specifically, Muller *et al.* investigating 71 colorectal adenomas in a cohort of 36 HNPCC patients (12 with identified germline mutation) reported that 3 adenomas (4%) were true MSI with retained MMR protein expression, whereas 3 others were MSS with MMR protein loss, and as many as 7 (10%) were MSS and retained MMR protein expression. Explanations advocated for these discrepancies include the delayed development of MSI as compared with MMR deficiency during the adenoma progression, and the occurrence of MSS adenomas up to the grade of severe dysplasia in these patients (Muller *et al.*, 2006). We also found one MSS Tis cecal cancer with *hMSH2*-negative immunostaining in a young male who turned out to have a

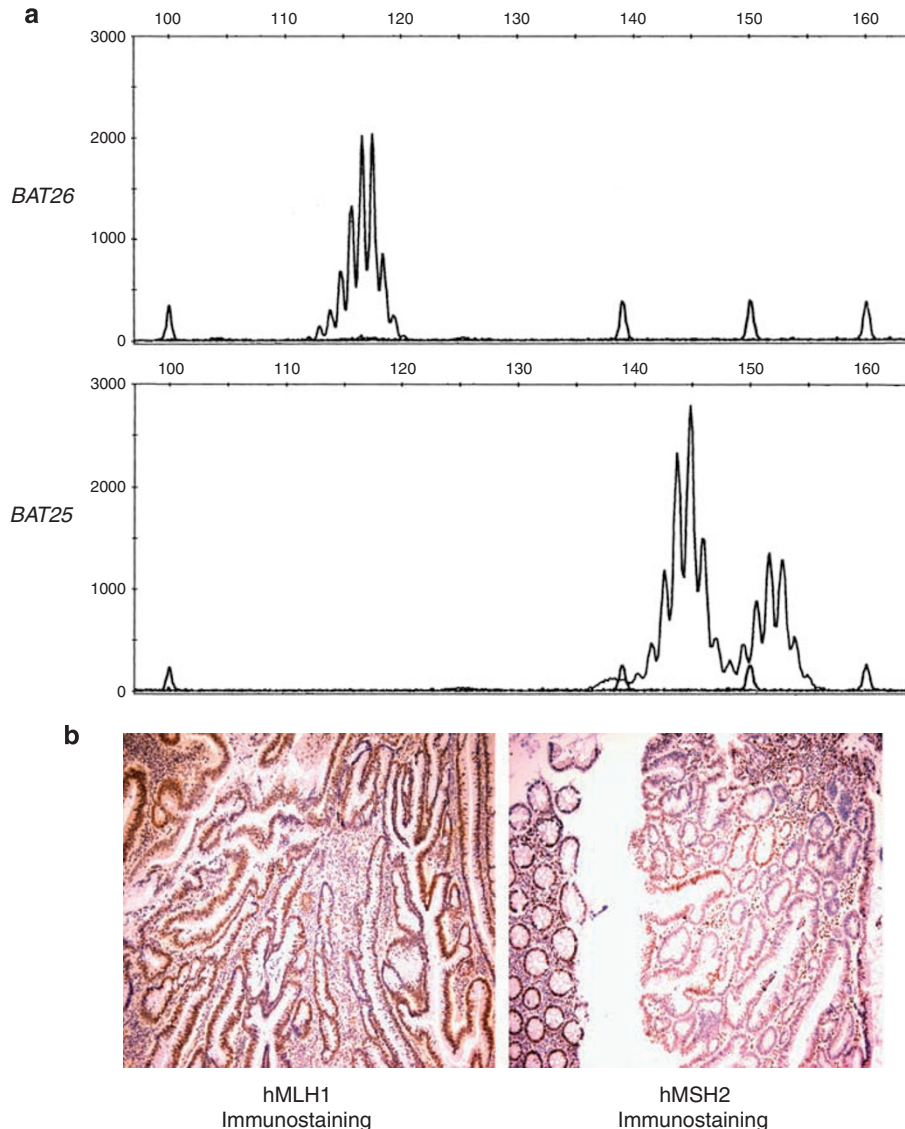


Figure 4 Identification of a microsatellite instability colorectal cancer (MSI CRC) with biallelic deletion of *hMSH2*, leading to the lack of its intragenic *BAT26* locus. **(a)** False-negative stability at *BAT26* (upper rectangle), as opposed to unstable *BAT25* (lower rectangle), in a CRC lacking hMSH2 immunostaining **(b)**. Owing to the contemporary analysis of both loci, the identification of this case as MSI was possible, even though only *BAT26* normal signal was seen, as the amplification of the *locus* occurs exclusively from contaminant normal cells, with no amplification of *BAT26*, located 3' from the *hMSH2* exon 5, taking place from cancer cells lacking both copies of the gene.

germline deletion of the exons 4–7 of *hMSH2* (Laghi L and Bianchi P, personal observation). Thus, contrary to what is known about CRCs, caution is warranted when looking for MSI and/or MMR protein defects in colorectal adenomas with the aim of identifying HNPCC subjects.

In conclusion, it should be first addressed that for a long time, and to a lesser extent even today, confusion in the field of the tools for MS-status determination led to MSI overestimation owing to dinucleotide-dependent MSI in certain settings. In any event, methods and interpretations most likely differ among research labs even in 2007, as two papers reported MSI-H prevalence of 9.9% (52 out of 527 CRCs (Xicola *et al.*, 2007)) and 18.1% (98 out of 542 CRCs (Kim *et al.*, 2007); $P=0.0002$ by χ^2 -test), using the pentaplex and the

Bethesda panel, respectively. The alternative explanation would be that the MSI phenotype has largely different prevalence in North American and European CRC patients. The second contrary remark implies the awareness that the most cited mononucleotide *BAT26* is inadequate to detect MSI CRCs with biallelic deletion of *hMSH2*, thereby requiring the side-by-side use of at least one other mononucleotide marker (such as, *BAT25* or *NR24*) to specifically detect such cases, which would also be readily classified as to the underlying MMR defect by doing so. Clearly, dealing with MSI CRCs for more than a decade led to improved care in the field, and a recent study found that pathology criteria had a sensitivity of 93% and a specificity of 55% for the MSI phenotype and that it can identify almost all MSI CRCs diagnosed before the age of 60 years (Jenkins *et al.*,

2007). Yet, testing MSI, and DNA MMR loss in tumors, followed by germline MMR mutation analysis should be undertaken not simply as a proof of principle in such cases, but also for the proper genetic counseling of those patients most likely having HNPCC, and of

their relatives. Thus, in our opinion, the detection of MSI still relies on the understanding of the basics of the phenomenon rather than on the plain mathematical satisfaction of panel recommendations, which can otherwise lead to inaccurate estimations.

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