Detection of Microsatellite Instability in Colorectal Cancer Using an Alternative Multiplex Assay of Quasi-Monomorphic Mononucleotide Markers

Vanessa Deschoolmeester,* Marc Baay,*
Wim Wuyts,[†] Eric Van Marck,[‡]
Nancy Van Damme,[§] Peter Vermeulen,[¶]
Krzysztof Lukaszuk,^{||} Filip Lardon,*
and Jan B. Vermorken*

From the Laboratory of Cancer Research and Clinical Oncology, Department of Medical Oncology, and the Department of Medical Genetics,[†] University of Antwerp (UA/UZA), Wilrijk, Belgium; the Department of Pathology,[‡] University Hospital of Antwerp (UZA), Edegem, Belgium; the Department of Gastroenterology,[§] Ghent University Hospital, Ghent, Belgium; the Translational Cancer Research Group[¶] (Laboratory Pathology, University of Antwerp/University Hospital Antwerp, Oncology Center, St. Augustinus General Hospital), Wilrijk, Belgium; and the Department of Gynaecological Endocrinology,^{||} University of Gdansk and INVICTA, Prophylactic Centre, Gdansk, Poland*

Colorectal malignancies demonstrating microsatellite instability (MSI) have a very heterogeneous histological appearance, better prognosis, and altered response to therapy. Consequently, identification of the MSI phenotype is both relevant and interesting as a screening and prognostic tool and as a potential predictive factor of chemotherapeutic response. Several groups have argued for the exclusive use of mononucleotide markers for MSI analysis. In this study, an alternative MSI typing multiplex system of mononucleotide microsatellite repeats was developed. This system obviates the need to compare allelic profiles between tumor and matching normal DNA, rendering MSI analysis amenable to high throughput. The quasi-monomorphic allelic distribution of five alternative mononucleotide markers was evaluated in genomic DNA. Only SEC63 and CAT25 were found to be quasi-monomorphic and were thus combined with BAT25 and BAT26 from the Bethesda panel. Consequently, 177 colorectal cancer samples previously analyzed by the Bethesda panel were tested for MSI using this alternative mononucleotide panel. In an attempt to resolve discordant cases, immunohistochemistry of MLH1, MSH2, and MSH6 was performed. The concordance between both panels reached 99.4% when microsatellite stability and MSI-L were grouped together. These new markers were subsequently multiplexed in a single polymerase chain reaction assay. The resulting mononucleotide fluorescent multiplex MSI assay has high accuracy, reliability, and throughput, thus reduc-

ing the time and cost involved in MSI testing. (*J Mol Diagn* 2008, 10:154–159; DOI: 10.2353/jmoldx.2008.070087)

High-frequency microsatellite instability (MSI-H) is a genetic instability observed in virtually all tumors from patients with hereditary nonpolyposis colorectal cancer and in a subset of sporadic colorectal cancers (CRCs). Its hallmark is extensive instability in simple tandem repeat nucleotide sequences (microsatellites) caused by a defective DNA mismatch repair function.

Colorectal malignancies demonstrating MSI have a very heterogeneous histological appearance, improved prognosis, and altered response to chemotherapy and radiotherapy.^{1,2} Consequently, identification of the MSI phenotype can both be relevant and interesting as a screening tool for hereditary nonpolyposis colorectal cancer,³ as a prognostic marker, and as a potential predictive factor of chemotherapy response.^{4,5}

MSI is defined as alterations in lengths of microsatellites due to deletions or insertions of repeating units to produce novel length alleles in tumor DNA when compared with normal DNA from the same individual.⁶ The absence of consensus markers for MSI analysis for several years resulted in conflicting data. Presently, the diagnosis of MSI in CRC is based on a set of five microsatellite markers (two mononucleotide and three dinucleotide repeats) proposed by the National Cancer Institute Research Workshop in Bethesda.⁷ However, it has been argued during a second consensus workshop held at the end of 2002 that the original microsatellite panel has limitations resulting from the inclusion of dinucleotide markers, which are less sensitive and specific for detection of tumors with mismatch repair deficiencies. Among the suggested changes was the exclusive use of mononucleotide repeats improving the sensitivity of MSI detection in CRC.⁸ An additional argument to use mononucleotide markers is that they are more commonly quasi-monomorphic, potentially obviating the need to test the corresponding normal DNA.⁶

Accepted for publication October 19, 2007.

Address reprint requests to Vanessa Deschoolmeester, Laboratory of Cancer Research and Clinical Oncology, Department of Medical Oncology, University of Antwerp (UA/UZA), Universiteitsplein 1, 2610 Wilrijk, Belgium. E-mail: vanessa.deschoolmeester@ua.ac.be.

Several groups have studied the use of mononucleotide loci to identify MSI in CRC tumors^{9–12} and very recently a mononucleotide pentaplex MSI analysis system kit is commercially available from Promega Corp. (Madison, WI).⁶ In this study we developed an alternative easy, in particular a cost-effective and simplified MSI typing system of mononucleotide microsatellite repeats requiring only a single polymerase chain reaction (PCR) and obviating the need to compare allelic profiles between tumor and matching germline DNA rendering MSI analysis amenable to high throughput.

BAT26 and BAT25 are the best known quasi-monomorphic mononucleotide repeats and have been proven very useful for the identification of MSI even without the use of corresponding germline DNA. Second, these markers appear to undergo significant deletions in the large majority of tumors with MSI.^{13,14} Nevertheless, to establish an MSI analysis system with a similar or even higher sensitivity than the Bethesda panel, the inclusion of additional mononucleotide markers is recommended.⁸

Woerner et al¹⁵ identified a new set of genes frequently affected by mutations in MSI-positive tumor cells. Four of these mononucleotide repeats (PTHL3, SEC63, HPDMPK, and U79260) showed mutation rates in 80% or more of MSI-H CRCs and were suggested as new candidate genes for diagnostic purposes. Similarly, Findeisen et al¹⁶ described a novel T₂₅ mononucleotide marker in the 3' untranslated region of the *CASP2* gene (CAT25) that displayed a quasi-monomorphic repeat pattern in normal tissue and represented a highly promising candidate marker for future high-throughput MSI testing.

These five alternative mononucleotide markers were tested for monomorphic or quasi-monomorphic allele distribution, and their sensitivity in detecting MSI tumors was studied. The most suitable markers were multiplexed with BAT26 and BAT25, according to the step-by-step protocol of Henegariu et al,¹⁷ to establish an alternative, simple and sensitive MSI analysis system of mononucleotide microsatellite repeats.

Materials and Methods

Sample Preparation and DNA Extraction

Genomic DNA was obtained from peripheral blood lymphocytes of 66 healthy Caucasian individuals at the University of Antwerp. Normal and tumor DNA were obtained separately from formalin-fixed, paraffin-embedded tissue blocks of 95 cervical cancers at the University of Gdansk, Poland. Tumor DNA was obtained from 177 colorectal cancer specimens at the University Hospital Antwerp, the St. Augustinus Hospital, and the Ghent University Hospital. After manual microdissection, DNA was isolated as described previously.¹⁸

Evaluation Monomorphic State

The quasi-monomorphic allele distribution of PTHL3, SEC63, HPDMPK, U79260, and CAT25 was evaluated in the genomic DNA of the peripheral white blood cells of

the 66 healthy control individuals mentioned above. Additionally, normal DNA of 95 cervical carcinoma samples was analyzed to verify the quasi-monomorphic allele distribution of these markers. Polymorphism was scored if smaller or larger size amplicons were detected after calculating the ratios of the peak areas of wild-type and novel alleles in normal tissues. A twofold difference was defined as threshold for allelic shifts.¹⁵

Bethesda Panel Assay

From an ongoing retrospective study on MSI in sporadic CRC, 177 formalin-fixed, paraffin-embedded colorectal cancer specimens were selected. These specimens were typed for MSI using the standard Bethesda marker panel as described previously.¹⁸ Fluorescent PCR products were analyzed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium) and Genemapper software 3.7. For interpretation purposes, MSI at two or more loci was defined as MSI-H, instability at a single locus was defined as MSI-L, and no instability at any of the loci was defined as microsatellite stability (MSS).

Mononucleotide Marker Assay

The MSI analysis system consists of nearly monomorphic mononucleotide markers. PCR primers for the amplification of these markers were described elsewhere.^{15,16} The sense primers were chemically labeled at the 5' end with 5-carboxyfluorescein fluorescent dyes. PCR was performed in a total volume of 25 μ L using a final concentration of 200 μ mol/L deoxyribonucleotide triphosphates (MBI Fermentas, St. Leon-Rot, Germany), 500 nmol/L each sense and antisense primer (Eurogentec, Seraing, Belgium), 1X PCR buffer (60 mmol/L Tris-SO₄, pH 8.9; 18 mmol/L (NH₄)SO₄; 2 mmol/L MgSO₄) and 1 unit of Discoverase denaturing high performance liquid chromatography DNA polymerase (Invitrogen, Merelbeke, Belgium). Again, as described for the Bethesda panel assay, fluorescent PCR products were analyzed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems) and Genemapper software 3.7.

Immunohistochemistry

Discordant cases were examined by immunohistochemistry for MLH1, MSH2, and MSH6. Sections, 4 μ m thick, were prepared from formalin-fixed paraffin-embedded tissue for immunohistochemistry. Sections were deparaffinized, dehydrated, and subjected to heat antigen retrieval by microwave in EDTA buffer for 10 minutes at 600 W and for 10 minutes at 300 W for MLH1 detection. Antigen retrieval for MSH2 and MSH6 was performed by EDTA buffer in a heating bath for 15 minutes at 100°C. Endogenous peroxidase activity was quenched by incubating the slides in peroxidase block EnVision (DAKO EnVision kit, DakoCytomation, Copenhagen, Denmark). Incubations with primary monoclonal antibodies were performed as follows: anti-MLH1 (clone G168–15, diluted

1:100, BD Biosciences PharMingen, San Diego, CA); anti-MSH2 (clone FE11, diluted 1:250, Oncogene Research Products, San Diego, CA); and anti-MSH6 (clone 44, diluted 1:250, BD Biosciences PharMingen), all for 1 hour at room temperature. After incubation, the slides were washed, and anti-mouse secondary antibodies conjugated to peroxidase-labeled polymer (DAKO) were applied for 30 minutes. The peroxidase activity was developed by incubation with 3,3'-diaminobenzidine chromogen solution (DAKO) for 10 minutes. The sections were then counterstained with hematoxylin. Loss of protein expression was scored as absence of nuclear staining in tumor cells despite nuclear staining in proliferating cells in normal crypts and stroma.

Multiplex PCR Assay

The two most sensitive quasi-monomorphic mononucleotide markers were combined with Bat26 and Bat25 in a multiplex assay. Again, all sense primers were chemically labeled at the 5' end with 5-carboxyfluorescein, with the exception of Bat26 primer, which was labeled with hexachlorofluorescein. To perform four PCR reactions in one tube, the step-by-step protocol of Henegariu et al¹⁷ was used. First, a PCR program was designed to amplify all loci individually under the same conditions. Then, these primers were combined in various mixtures to amplify all loci simultaneously. This required alterations and optimization of several parameters of the reaction. Primer, buffer and MgSO₄ concentrations were all optimized to perform a multiplex reaction of the four markers. PCR was performed in a total volume of 25 µl using a final concentration of 200 µmol/L deoxyribonucleotide triphosphates (MBI Fermentas), 0.8X PCR buffer (48 mmol/L Tris-SO₄ pH 8.9, 14.4 mmol/L (NH₄)SO₄, and 1.6 mmol/L MgSO₄), 3.5 mmol/L MgSO₄, 1 unit of Discoverase denaturing high performance liquid chromatography DNA polymerase (Invitrogen) and 500 nmol/L BAT25 and CAT25 sense and antisense primer, 600 nmol/L BAT26 sense and antisense primer, and 80 nmol/L SEC63 sense and antisense primer (Eurogentec). The multiplex assay was verified in all 177 CRC and in 10 cervical carcinoma samples previously analyzed by the Bethesda panel and the mononucleotide markers. Although only four markers were analyzed, MSI at two or more loci was defined as MSI-H, instability at a single locus was defined as MSI-L, and no instability at any of the loci was defined as MSS.

Statistical Analysis

To quantify the degree of agreement between the Bethesda panel and the new mononucleotide panel, the kappa statistical test was used.

Results

Monomorphic State

The monomorphic or quasi-monomorphic allele distribution of PTHL3, SEC63, HPDMKP, U79260, and CAT25 was evaluated in genomic DNA of peripheral white blood

cells of the 66 control individuals and in normal DNA of the 95 cervical carcinoma samples. U79260 did not provide reproducible results in the peripheral blood samples. HPDMKP and PTHL3 were polymorphic in 42.9% and 29.4%, respectively, whereas SEC63 and CAT25 showed monomorphism in 100% and 98.8% of all samples examined, respectively. Therefore, CAT25 and SEC63 were selected to be combined with BAT25 and BAT26 from the Bethesda panel to form a new quasi-monomorphic mononucleotide marker panel.

MSI Analysis Using Bethesda Panel

All samples could be typed for MSI using the Bethesda panel. Thirty of the 177 colorectal tumors, selected from a larger ongoing retrospective study, showed MSI-H, while four of 177 showed MSI-L and 143 of 177 MSS (data not shown). For all but one of the MSI-H samples, the Bethesda panel showed instability at both mononucleotide markers (BAT26 and BAT25).

MSI Analysis Using Mononucleotide Markers

All samples could be typed for MSI using the quasi-monomorphic mononucleotide markers selected. In the 177 cases of CRC there was a good overall concordance between both assays, since 29 of 30 MSI-H tumors identified by the Bethesda panel were also recognized as MSI-H by this new panel (Table 1). The one MSI-H sample missed by the quasi-monomorphic mononucleotide panel showed instability in only two dinucleotide markers of the Bethesda panel. Of the four cases identified as MSI-L by the Bethesda assay, all were classified as MSS using the new mononucleotide panel (Table 1). These samples only showed instability in one dinucleotide marker of the Bethesda panel. To resolve the discordant cases, immunohistochemistry was performed for MLH1, MSH2, and MSH6 on the one discordant MSI-H case and two of four cases identified as MSI-L by the Bethesda assay. The discordant MSI-H case seemed to have lost expression of the MSH2 protein, while one MSI-L showed loss of expression of MSH6 (data not shown). The second MSI-L case showed no loss of expression of mismatch repair genes (data not shown). Regrettably, from the remaining MSI-L cases insufficient material was available to perform immunohistochemistry.

Additionally, the sensitivity and specificity of the mononucleotide markers and the Bethesda panel for MSI analysis was calculated. MSI analysis using all seven markers was used as a gold standard, and MSI-H was scored

Table 1. Comparison of MSI Analysis by Bethesda Panel and Quasi-Monomorphic Mononucleotide Panel

Mononucleotide panel	Bethesda panel		
	MSI-H	MSI-L	MSS
MSI-H	29	0	0
MSI-L	0	0	0
MSS	1	4	143

Kappa: 0.905 (95CI: 0.823–0.987).

Table 2. Comparison of MSI Analysis by Bethesda Panel and Quasi-Monomorphic Mononucleotide Panel if MSS and MSI-L Are Combined

Mononucleotide panel	Bethesda panel	
	MSI-H	MSI-L/MSS
MSI-H	29	0
MSI-L/MSS	1	143

Kappa: 0.980 (95CI: 0.940–1.019).

when at least three of seven (>40%) samples were unstable and MSI-L/MSS were grouped together. All mononucleotide markers showed a specificity of 100%. BAT25, BAT26, and CAT25 showed a sensitivity of 100%, while SEC63 showed a sensitivity of 56.7%. For the Bethesda panel, D2S123 had a sensitivity of 53.6% and a specificity of 97.3%, while D5S346 and D17S250 both showed a specificity of 99.3% but a sensitivity of only 25% and 34.5%, respectively, indicating a superiority of mononucleotide over dinucleotide repeats in detecting MSI.

The overall concordance between the quasi-monomorphic mononucleotide MSI analysis assay and the Bethesda assay was 97.1%: kappa, 0.905 (95CI, 0.823–0.987) (Table 1). If MSS and MSI-L tumors were grouped together the agreement reached 99.4%: kappa, 0.980 (95CI, 0.940–1.019) (Table 2).

The four quasi-monomorphic mononucleotide markers were combined in a single multiplex assay as described by the step-by-step protocol of Henegariu et al.¹⁰ All CRC and 10 cervical carcinomas were used for evaluation of the multiplex PCR. All samples could be typed for MSI using the quasi-monomorphic mononucleotide multiplex PCR assay. All (100%) of the MSI-H CRC tumors identified by the Bethesda and the mononucleotide panel were recognized by the multiplex assay. None of the MSS colorectal tumors showed instability in any mononucleotide marker of the multiplex assay and were therefore scored correctly. Additionally, the cervical carcinoma samples were also scored correctly using the multiplex assay. Examples of MSI-H and MSS profiles generated with the quasi-monomorphic mononucleotide multiplex PCR assay are shown in Figure 1.

Discussion

The current standard method of MSI analysis is relatively time-consuming, laborious, and expensive, due to the need to compare allelic profiles between tumor and matching germline DNA. Fluorescent multiplex PCR of mononucleotide repeats and the computerized fragment analysis method, as described here and by others^{6,9–11} was designed to render MSI typing feasible for high-throughput application. This type of assay allows screening of large sample numbers with high specificity and sensitivity and clear interpretation of the data.

The ideal markers for MSI typing should match the following criteria: 1) a quasi-monomorphic allele pattern in all human populations, 2) 100% mutation frequency in MSI-H tumors, and 3) no mutations in MSS tissue specimens.¹⁶ BAT26 and BAT25 mononucleotide markers

Figure 1. Examples of MSS (A) and MSI-H (B and C) profiles generated with the quasi-monomorphic mononucleotide multiplex PCR assay. B: Example of MSI-H where all markers are unstable. C: Example of MSI-H where three of four markers are unstable.

have already proved to be very useful for the identification of MSI due to the quasi-monomorphic nature in Caucasian populations of both loci and the sensitivity of both markers to MSI.^{13,14,19,20} Although adequate in the great majority of circumstances, analysis of additional repeats may be needed in some cases, especially in individuals of African origin.^{21,22}

Five additional mononucleotide markers (PTHL3, SEC63, HPDMPK, U79260, and CAT25) were selected after in-depth review of the literature.^{15,16} Two of these selected markers (SEC63 and CAT25) were identified as quasi-monomorphic and, similar to BAT26 and BAT25, highly sensitive to somatic deletions in MSI-H tumors. In contrast to Woerner et al,¹⁵ PTHL3 and HPDMPK were found to be polymorphic and U79260 could not be analyzed. Even so, PTHL3 and HPDMPK can be used for MSI analysis if corresponding normal tissue is available. Finally, it should be noted that the monomorphic state of SEC63 and CAT25 was investigated in a limited number of European (ie, Belgian and Polish) individuals. Use of this panel in populations of other ethnic origin would require confirmation of the monomorphic nature in that population under study.

Distinction between MSI-H and MSS tumors is unambiguous when SEC63 and CAT25 are used in conjunction with BAT26 and BAT25. This MSI analysis is based on the classification of allelic size variation in more than or equal to two of four markers as MSI-H and no variation in any marker as MSS. Although most panels consist of five markers, this tetraplex assay scored instability in at least three of four markers in MSI-H samples, suggesting that the use of a tetraplex mononucleotide panel is sufficient to detect MSI in CRC correctly. It seems to be unlikely that solely one mononucleotide marker of this panel would be unstable. Multiplex PCR of these mononucleotide markers has the additional advantage of

avoiding the need for simultaneous analysis of corresponding normal DNA. Since the monomorphic nature of these new mononucleotides is not known for all ethnicities, we advise testing the corresponding normal sample in retrospect when the tumor demonstrates MSI to confirm that the change was somatically acquired. Moreover, because the reaction is performed in a single PCR, the method is simple to use and free of errors arising from the mixing of samples. For all but one MSI-H case, the Bethesda panel assay demonstrated instability at both mononucleotide loci (BAT26 and BAT25), the one remaining MSI-H case was unstable in two of three dinucleotide markers (D2S123 and D17S250).

Using the mononucleotide panel, 29 of 30 tumors previously classified as MSI-H showed deletions in at least three of four mononucleotide markers and all cases were indicative without the analysis of corresponding normal tissue. The shifts for all mononucleotide loci in the MSI-H cases resulted in products that were smaller in size than the germline allele, which is consistent with data of others demonstrating that deletions in poly(A) sequences are much more common than insertions.^{6,23,24} The one MSI-H case solely instable in two of the dinucleotide markers of the Bethesda panel showed stability in all four mononucleotide markers. This strongly suggests the sample may represent a MSS tumor misclassified by the dinucleotide markers of the Bethesda panel, a phenomenon that has been described previously.^{8,9,25} However, immunohistochemistry for MHL1, MSH2, and MSH6 showed loss of expression of MSH2 for this discordant case. To be absolutely certain, gene sequencing should be performed for this mismatch repair gene. Unfortunately, due to the formalin-fixed, paraffin-embedded origin of the sample, which resulted in DNA fragmentation, it was impossible to perform sequencing analysis.

All of the cases interpreted as MSI-L by the Bethesda panel assay were scored as MSS by the new mononucleotide panel. Interestingly, all of these shifts were to a larger allele size, which is less common in MSI-H cases (Figure 2), and again two of three MSI-L cases showed evidence of instability in D2S123 locus. These findings are consistent with those of Murphy et al.⁶ Furthermore, D2S123 and D17S250 were found to have the lowest specificity for MSI analysis when compared to 24 other microsatellite loci by Bacher et al.¹⁰ The findings that all MSI-L cases resulted from shifts of increasing size in a single dinucleotide marker without instability in either of the mononucleotide markers is consistent with the theory that MSI-H and MSI-L cancers result from distinct processes.⁶

Additionally, immunohistochemistry for MHL1, MSH2, and MSH6 could be performed in two of four MSI-L cases. One case showed expression of all mismatch repair proteins indicating microsatellite stability, while the second sample showed loss of MSH6 protein, which is described to play a possible role in MSI-L tumors.²⁶ However, the MSI-L phenomenon is poorly defined and its significance is not well understood, so controversy remains.²⁷ There is also evidence to suggest that all CRC have some inherent instability and, if enough markers are

Figure 2. Examples of allelic shift patterns in dinucleotide markers D2S123 (A) and D17S250 (B) in an MSI-H sample (A), and an MSI-L (B) sample. N, normal tissue; T, tumor tissue.

tested, all tumors will have some degree of instability.²⁸ It has therefore been suggested that MSI-L cancer should be included with MSS cancers for clinical purposes.⁸ Thus grouping MSS and MSI-L tumors together, the agreement between the Bethesda assay and the mononucleotide multiplex assay reached 99.4%. Keeping in mind that the one MSI-H sample missed by the mononucleotide multiplex assay might have been misclassified by the dinucleotide markers of the Bethesda panel, this alternative simple and straightforward MSI analysis system of mononucleotide microsatellite repeats can identify MSI-H tumors with a high sensitivity and specificity. It also offers several distinct advantages in that it has high accuracy, reliability, and throughput and no need to compare tumor with matching germline DNA. Therefore, it reduces time and costs involved in MSI testing. Currently, we are using this multiplex PCR to identify MSI in a large cohort of colorectal cancer patients to elucidate the clinical and molecular correlation with MSI in CRC.

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