Comparison of the Microsatellite Instability Analysis System and the Bethesda Panel for the Determination of Microsatellite Instability in Colorectal Cancers

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Microsatellite instability (MSI) analysis of colorectal cancers is clinically useful to identify patients with hereditary nonpolyposis colorectal cancer (HNPCC) caused by germline mutations of mismatch repair genes. MSI status may also predict cancer response/resistance to certain chemotherapies. We evaluated the MSI Analysis System (Promega Corp.; five mononucleotide and two pentanucleotide repeats) and compared the results to the Bethesda panel, which interrogates five microsatellite loci recommended by the 1997 National Cancer Institute-sponsored MSI workshop (three dinucleotide and two mononucleotide repeats). Thirty-four colorectal cancers were analyzed by both assays. The overall concordance between the two assays was 85% (29 of 34). There was complete concordance between the two assays for all of the MSI-high (11 of 11) and microsatellite stable (MSS; 18 of 18) cases. In the 11 MSI-high cases, all 5 of the mononucleotide loci in the MSI Analysis System demonstrated shifted alleles (100% sensitivity), and each shift resulted in products that were smaller in size than the germline alleles. All (5 of 5) of the cases interpreted as MSI-low by the Bethesda assay were interpreted as MSS by the MSI Analysis System. Our results suggest that the MSI Analysis System is generally superior and may help resolve cases of MSI-low into either MSI-high or MSS. (*J Mol Diagn* 2006, 8:305–311; DOI: 10.2353/jmoldx.2006.050092)

Microsatellite instability (MSI) is caused by DNA mismatch repair (MMR) deficiency, resulting in failure to repair the errors that normally occur during replication of repetitive DNA sequences.^{1–3} Inactivation of any of several MMR genes, including *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2*, can result in MSI.⁴ Originally, MSI was shown to correlate with germline/inherited defects in MMR genes in

families with HNPCC, where greater than 90% of colon cancers from patients exhibit MSI. However, it is now recognized that MSI also occurs in the absence of germline MMR mutations as the result of epigenetic inactivation of *hMLH1* expression by promoter methylation.⁵ Certain histological features are also associated with MSI cancers.⁶ Overall, MSI is detected in approximately 15% of all colorectal cancers and in unselected cases of colorectal cancer; in fact, the majority of MSI cases are due to *hMLH1* promoter methylation rather than to MMR gene mutation.⁷

Determining the MSI status of a colonic adenocarcinoma has clinical use for identifying patients with HNPCC/Lynch Syndrome.⁸ In addition, MSI status, regardless of whether the causative defect is inherited or sporadic, may have use for prognostic and therapeutic decision-making purposes. Many studies have demonstrated an improved prognosis for MSI-high cancers relative to microsatellite stable (MSS) cancers.^{2,9} A recent report by Ribic et al¹⁰ confirmed the superior survival for patients with MSI-positive colorectal cancers but also demonstrated that MSI cancers are less responsive to 5FU-based adjuvant chemotherapy. In fact, in this study, patients with MSI-positive cancers actually had a worse clinical outcome when treated with adjuvant chemotherapy, although these findings did not achieve statistical significance. If this finding is independently confirmed, MSI testing may be required before initiating 5FU-based treatment.

MSI is defined as alterations in the lengths of microsatellites due to deletion or insertion of repeating units to produce novel length alleles in tumor DNA when compared with the normal/germline DNA from the same individual. There are hundreds of thousands of microsatellite loci throughout the genome that could potentially be used for MSI analysis.¹¹ In the mid-1990s, many investigators were using different markers and obtaining very different frequencies of MSI in the same type of cancer, most likely due to variable sensitivity of individual markers to detect

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MSI.^{12,13} In an attempt to standardize MSI analysis, a 1997 National Cancer Institute (NCI) workshop recommended a "reference panel" of five microsatellite markers for the detection of MSI and established MSI classification guidelines based on the results.¹⁴ The reference panel, referred to as the Bethesda panel, consists of two mononucleotide loci (Big Adenine Tract [*BAT*]-25 and *BAT*-26) and three dinucleotide loci (*D2S123*, *D5S346*, and *D17S250*). Many alternate microsatellite markers were also recommended, and it was acknowledged that the reference panel would likely change over time with additional data. Using the Bethesda panel, cancers with instability at two or more of these loci were interpreted as MSI-high, and cancers with no instability at any of the five loci were considered MSS. Cancers showing instability at only one of the five recommended loci were interpreted as MSI-low, although it was unclear at the time whether MSI-low represented a biologically distinct category or whether this simply reflected the inherent limitations of faithfully replicating these repetitive sequences.

A recent follow-up NCI workshop was convened to further address MSI testing and identification of patients with HNPCC.¹⁵ The panel recognized the limitations of the original Bethesda panel due to the inclusion of dinucleotide repeats, which are less sensitive and specific than mononucleotide repeats for the identification of cancers with MMR deficiencies. It has been suggested that a panel of five mononucleotide repeats may be more sensitive for the detection of MSI-high cancers.¹⁶ An additional argument for the use of mononucleotide loci is that they are more commonly monomorphic or quasi-monomorphic, potentially obviating the need to test the corresponding normal sample.¹⁶⁻¹⁹ Immunohistochemistry for the four MMR proteins can also be performed in the workup of suspected HNPCC cancers but should probably not be considered a substitute for MSI testing because it is well-documented to miss approximately 5% of MSI cancers that test IHC-positive for all of the four gene products, presumably because they are functionally defective while retaining antigenicity.^{13,15,20}

Bacher et al¹³ have recently evaluated a set of 266 mono-, di-, tetra-, and penta-nucleotide microsatellite loci for potential use for MSI screening. This group determined that mononucleotide markers were more sensitive and specific than dinucleotide markers for the detection of MSI and therefore developed a fluorescent multiplex assay that analyzes five nearly monomorphic mononucleotide microsatellite loci (*BAT*-25, *BAT*-26, *NR*-21, *NR*-24, and *MONO*-27). This assay, the MSI Analysis System, is commercially available from Promega Corp. (Madison, WI).

In this study, we have independently evaluated 34 matched normal and colorectal tumor samples for MSI using both a multiplex PCR assay of the Bethesda panel markers²¹ and the MSI Analysis System.¹³ Our results are reported here with special attention paid to the MSI-low categorization.

Materials and Methods

Samples and DNA Extraction

Approval from the institutional review board of the Johns Hopkins Hospital was granted for this study. The tumors used in this study were all submitted to the Johns Hopkins University Clinical Molecular Diagnostics Laboratory for evaluation of MSI. The cases were sent with a clinical suspicion of HNPCC. The ratio of males to females in this study is approximately 1:1. Cases were coded as required by the institutional review board, and this precluded more detailed analysis of the clinical background. Hematoxylin and eosin-stained slides cut from formalin-fixed, paraffin-embedded tissues were evaluated by a pathologist, and the areas of the slide representing "tumor" (highest numbers of cancer cells present) and "normal" (no malignant tissue identified) were identified. The normal samples were most commonly an uninvolved proximal or distal margin of resection. Unstained slides with tissue immediately adjacent to the hematoxylin and eosin-stained tissue were then superimposed on top of the marked slides, and cancer tissue was microdissected. DNA was extracted using Pinpoint DNA Extraction kit (ZymoResearch, Orange, CA) according to the manufacturer's directions.

Bethesda Panel Assay

MSI analysis was performed as previously described.²¹ Briefly, the five microsatellite loci (*BAT*-25, *BAT*-26, *D2S123*, *D5S346*, and *D17S250*) recommended by the 1997 NCI-sponsored MSI workshop were amplified in a single multiplex PCR reaction. PCR products were analyzed by capillary electrophoresis (CE) using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). For interpretation purposes, microsatellite instability at ≥ 2 loci was defined as MSI-high, instability at a single locus was defined as MSI-low, and no instability at any of the loci tested was defined as MSS.

Promega MSI Multiplex System Version 1.1

The MSI Analysis System consists of five nearly monomorphic mononucleotide markers (*BAT*-25, *BAT*-26, *NR*-21, *NR*-24, and *MONO*-27) for MSI determination and two polymorphic pentanucleotide markers (*Penta C* and *Penta D*) for sample identification. MSI analysis was performed according to the manufacturer's directions (Promega Corp.). Products were analyzed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems). We interpreted microsatellite instability at ≥ 2 mononucleotide loci as MSI-high, instability at a single mononucleotide locus as MSI-low, and no instability at any of the loci tested as microsatellite stable (MSS). To approximate the percentage of alleles that were shifted to a novel position at each of the MSI Analysis System mononucleotide loci, the peak height for each of the allele positions that were considered to be unique to the cancer sample were added together, and the total was divided

Table 1. Comparison of the MSI Analysis System with the Bethesda Panel Assay

		Bethesda Panel Assay		
		Stable	Low	High
MSI analysis system	Stable	18	5	–
	Low	–	–	–
	High	–	–	11

by the total peak heights for all alleles (unique and germline).

Results and Discussion

We evaluated tissue from 34 paraffin-embedded colorectal cancers and microdissected areas of high cancer density. DNA was extracted from the microdissected tissue and analyzed for MSI using the Bethesda panel assay and the MSI Analysis System. Both assays were performed on DNA obtained from the same extraction. MSI results generated by the two assays were interpreted and compared. Table 1 summarizes our findings. There was good overall concordance between the two assays for 29 of 34 samples (85%). There was complete concordance between the two assays for all of the MSI-high (11 of 11) and MSS (18 of 18) cases. Of the five cases identified as MSI-low by the Bethesda assay, all (5 of 5) were shown to be MSS by the MSI Analysis System. Figure 1 demonstrates an MSI-high result using both the MSI Analysis System (Figure 1A) and the Bethesda panel assay (Figure 1B).

For all of the MSI-high cases ($n = 11$), the Bethesda panel assay demonstrated instability at both mononucleotide loci (*BAT-25* and *BAT-26*). Additionally, in all 11 cases, at least one dinucleotide locus was shifted as well. Interestingly, because the Bethesda panel criteria for MSI-high is ≥ 2 of 5 loci exhibiting shifted alleles, all of the MSI-high cases would have been interpreted as MSI-high even without testing the additional dinucleotide loci in the panel. Thus, the dinucleotide loci provided very little additional information for the interpretation of MSI-high cases.

Using the MSI Analysis System, 11 of 11 MSI-high cases (100%) demonstrated instability at all five (5 of 5, 100%) of the mononucleotide loci tested. Thus, in this limited series of cases, the sensitivity of each of the five mononucleotide loci in the MSI Analysis System for the detection of MSI was 100%. Based on these findings, we suggest that a shift in only one of the five mononucleotide markers would be highly suspicious and warrant additional investigation and follow-up testing. The shifts for all five mononucleotide loci in the 11 MSI-high cases resulted in products that were smaller in size than the germline allele, which is consistent with the data of others demonstrating that deletions in polyA sequences are much more common than insertions.^{22,23} Although the dominant novel product lengths varied from 2 to 12 bases smaller than the germline allele length, shifts to dominant allele sizes that were 6 to 8 bases smaller than the germ-

line allele were most frequent when all markers are considered. For *BAT-25* and *BAT-26*, the average shift was -5.4 and -8.8 bases, respectively, which is remarkably consistent with previous data demonstrating average deletions of these markers in MSI+ cancers of -5.3 and -9 bases, respectively.²³ The average shift for NR21, Mono27, and NR24 was -6.9 , -5.6 , and -7.3 bases, respectively. Thus, it appears that shifts to larger size products for each of these five mononucleotide loci are uncommon. Interestingly, there seems to be a lower boundary on the maximum size of the loss that is significantly smaller than the 21 to 27 bases in the repeat. None of the mononucleotide loci in the MSI Analysis System loci were shifted in the MSS or MSI-low cases (as classified using the Bethesda panel).

To evaluate whether any of the mononucleotide loci tested typically shifted to a greater extent than the others, we calculated the percentage of alleles that were shifted at each of the MSI Analysis System mononucleotide loci. This analysis can potentially provide insight into how difficult it is to detect MSI, how early in tumorigenesis MSI occurs, and whether any particular marker is more informative than the others. To do this, we added the peak height for all of the allele positions that were shifted and divided that value by the total height for all alleles (including both germline and shifted). The data are summarized in Figure 2. In the majority of cases, the percentage of shifted alleles relative to germline alleles was similar for all five loci evaluated. In some cases, however, the percentage of shifted alleles relative to germline alleles was more variable between the loci. For example, specimen 1 displayed much more variability in the percentage of shifted alleles at the five loci (76, 45, 20, 92, and 40%) compared with specimen 2, which was much more consistent (81, 63, 75, 86, and 86%; Figure 2). It is unclear whether the observed variation between markers, such as that seen in specimen 1, is simply due to chance or whether patterns might emerge when larger numbers of samples are examined. Several samples, particularly 2, 4, and 5, demonstrated very high percentages of shifted alleles (generally $>70\%$), which is most consistent with the shifts having occurred early in tumorigenesis.^{23,24} Almost all of the mononucleotide loci had $>20\%$ of alleles shifted to a novel position, supporting the notion that true MSI is generally not a subtle finding, at least for these cancers. Importantly, a value of 20% is well above the limit of detection of these assays as determined in serial titration experiments, typically cited as about 2 to 10%.²¹ There was no evidence that any specific locus was typically shifted to a greater or lesser extent than the other loci, consistent with the fact that these five loci were selected for high diagnostic sensitivity and supporting the concept that there are not intrinsic stability differences between the five loci.

The percentage of alleles shifted should be affected by the proportion of cancer cells in the specimen, but this proportion should in theory influence all loci equally. We compared the estimated percentage of shifted alleles with the estimate of the relative amount of cancer and normal cells in the specimens analyzed and found a general correlation between the percentage of cancer

Figure 1. Capillary electrophoresis results of the MSI Analysis System (A) and the Bethesda panel assay (B). x axis is size in bases; y axis is fluorescence intensity. Red peaks are internal size standards. Green, blue, and black peaks are amplification products from microsatellite loci. Note the shift in the size (bases) of the amplification products in the tumor specimen when compared with normal. An example of one shifted locus is demonstrated (arrow) in the tumor sample for each assay.

cells identified histologically in the microdissected specimen and the average percentage of shifted alleles identified in MSI analysis. This underscores the importance of having a pathologist review the slides to identify the optimal area for testing, thereby assuring that the minimal percentage of tumor cells is present in the sample to be tested. Importantly, even for specimens that were estimated to be composed of only 25 to 50% cancer cells (50 to 75% normal cells), MSI was easily identified.

All (five of five) of the cases interpreted as MSI-low by the Bethesda panel assay were interpreted as MSS by the MSI Analysis System. Interestingly, all of these cases

had shifted a single dinucleotide marker in the Bethesda panel assay, and all of the shifts were to a larger allele size, which is less common in MSI-high cases. Of the MSI-low cases, three of five showed evidence of instability at locus *D2S123*. In each case, the shift was characterized by an increase of 2 bases compared with the germline allele. Two of the five MSI-low cases showed evidence of instability at locus *D17S250*. Our findings are consistent with those of Whitehall et al,²⁵ who found that *D2S123* was frequently mutated in MSI-low cancers, showing instability in 24% (10 of 42) of MSI-low cancers. Also consistent with our data, *D2S123* and *D17S250* were

Figure 2. Percentage of alleles shifted at each of the five mononucleotide loci in the MSI Analysis System. The percentage of alleles shifted at each of the five mononucleotide loci was estimated as described in Materials and Methods. Results are demonstrated for 10 of the 11 MSI-high cases. For one MSI-high case, the percentage of shifted alleles could not be estimated.

found to have the lowest specificity (86 and 93%, respectively) for MSI analysis when compared with 24 other microsatellite loci.¹³ The percentage of shifted alleles in these cases (20 to 35%, average 28%) was generally lower compared with the MSI-high cases.

The finding that all (five of five) of the MSI-low cases (Bethesda panel) resulted from shifts of increasing size in a single dinucleotide marker without shifting either of the mononucleotide marker is consistent with the theory that MSI-high and MSI-low cancers result from biologically distinct processes. The MSI-low phenomenon is poorly defined, and its significance is still not well understood. Although it is generally accepted that MSI-high and MSI-low are distinct entities, controversy remains regarding the separation of MSI-low and MSS. There is increasing evidence that MSI-low cancers may be biologically distinct from MSS cancers.^{26,27} However, there is also evidence to suggest that all colorectal cancers have some inherent instability, such that if enough markers are tested, all cancers will have at least some degree of MSI.^{28,29} Even if it is ultimately demonstrated that MSI-low cancers portend a worse prognosis,^{30,31} it will remain to be demonstrated whether the difference is great enough to justify its detection. It has therefore been suggested that for current clinical purposes, MSI-low cancers should be included with MSS cancers.¹⁵ Research applications investigating the origin and significance of MSI-low should still include dinucleotide markers in their analyses.

MSI-low cancers can be due to mutations in *hMSH6* (*GTBP*), especially those demonstrating instability in mononucleotides.³² With the increased number of mononucleotides in the MSI Analysis System, one would anticipate that *hMSH6*-defective tumors may display enough shifted loci to be characterized as MSI-high, although this will need to be systematically investigated.

The pentanucleotide markers in the MSI Analysis System were included in the assay not for evaluation of MSI but rather for specimen identity purposes, confirming that the normal and tumor specimens originated from the same individual. In all 34 samples, the germline alleles were present in corresponding cancer samples, indicating a match between normal/tumor pairs and supporting

the concept that these markers can be used to confirm identity. However, the presence of loss of heterozygosity (LOH) and shifted pentanucleotide alleles can somewhat complicate sample identification.

Three of the MSS cases (3 of 18, 17%) demonstrated allelic imbalance/LOH at one pentanucleotide locus (two *Penta C*, one *Penta D*), as indicated by an altered ratio of the heights of the two alleles in the tumor specimen when compared with the ratio of the alleles in the normal sample. One MSS case (1 of 18, 5.6%) demonstrated a shift at the *Penta D* locus. Data from Bacher et al¹³ suggest that *Penta D* has 100% specificity for the detection of MSI (although the specificity of *Penta C* was reported to be 98%), and therefore the significance of this finding is not immediately clear.

Of the five cases classified as MSI-low by the Bethesda panel and MSS by the MSI Analysis System, none exhibited instability at either of the pentanucleotide loci, whereas one of five (20%) demonstrated LOH at *Penta C*. Thus of the 23 total cases classified as MSS by the MSI Analysis System, only 4 of 23 (17%) demonstrated LOH. The LOH occurred in only one of the two markers, and the residual allele was sufficiently present to correlate with the normal sample. The dinucleotide loci in the Bethesda panel also have use for specimen identity evaluation purposes. Importantly, the dinucleotide loci in the Bethesda panel are all situated in or near tumor suppressor genes with significant importance in colorectal carcinogenesis (*APC*, *p53*, and *hMSH2*). Therefore, these dinucleotide loci frequently demonstrate LOH, which can somewhat complicate the interpretation of these loci both for MSI determination and for identity determination purposes.

Of the MSI-high cases, 4 of 11 (36%) shifted *Penta C*, 5 of 11 (45%) shifted *Penta D*, and 2 of 11 (18%) did not shift either pentanucleotide locus. The frequency of instability at the *Penta C* and *Penta D* loci in this study was higher than that determined by Bacher et al¹³ (14 and 35%, respectively). Although the pentanucleotide loci are not included for assessing MSI status, the combined sensitivity of the pentanucleotide loci *Penta C* and *Penta D* for MSI determination was surprisingly 82% (9 of 11). For both *Penta C* and *Penta D*, the most common shift identified was to a position one repeating unit smaller (−5 bases) than the germline allele.

Overall, the MSI Analysis System offers several advantages over the Bethesda panel assay. First, we found the MSI Analysis System to be a well-balanced multiplex reaction, resulting in relatively equivalent CE peak heights for each of the microsatellite loci tested. This feature of the assay is valuable because it allows for interpretation of all of the loci from a single capillary injection of the PCR products. In contrast, we have found with the “homebrew” Bethesda panel assay that it is often necessary to inject at different times to obtain optimal peak heights for all of the loci and that it may require re-optimization of the molar ratios when a new primer is synthesized to balance the CE peak heights. We also found the MSI Analysis System data easier to visually interpret because the assay has less overlap of PCR product sizes for each of the five informative loci. Finally,

the PCR amplicon sizes in the MSI Analysis System are shorter (longest = 154) than in the Bethesda panel assay (longest = 227), which is advantageous for the analysis of formalin-fixed, paraffin-embedded tissue samples.

A note of caution should be made for the interpretation of both assays. Because both assays are fluorescent multiplex assays, fluorescent pull-up (bleed) can be problematic. In both assays, we have identified instances where fluorescent pull-up can complicate interpretation of the results. In the worst case, fluorescent pull-up could produce a false positive by erroneously being interpreted as instability. The instructions for the MSI Analysis System assay suggest that amplified peak heights of less than 2000 relative fluorescence units are ideal; adherence to this recommendation and the use of an appropriate CE matrix should minimize problems with pull-up. An important design feature of the MSI Analysis System is the spacing between the different loci, which generally exceeds the typical number of bases shifted (average 5 to 9 bases) when MSI is present. When inspecting the electropherogram, potentially shifted alleles that perfectly underlie germline alleles may represent pull-up and should be viewed with significant skepticism.

In summary, the MSI Analysis System and the Bethesda panel assay had excellent correlation of the MSI-high and MSS cases. All Bethesda panel MSI-low cases were classified as MSS by the MSI Analysis System. Based on current data, this reclassification is probably clinically justified.

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