Microsatellite Marker Analysis in Screening for Hereditary Nonpolyposis Colorectal Cancer (HNPCC)

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ABSTRACT

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant cancer predisposition syndrome caused by genetic mutations in DNA mismatch repair genes. It is relevant to identify HNPCC patients because colonoscopic screening of individuals with HNPCC reduces cancer morbidity and mortality. Microsatellite instability (MSI) is characteristic of HNPCC tumors. A panel of five markers (BAT25, BAT26, D2S123, D5S346, and D17S250, the so-called Bethesda markers) has been proposed for screening for MSI. To test a hypothesis that the use of BAT26 alone is feasible in screening for MLH1/MSH2 mutation-positive HNPCC patients, we compared the MSI results of 494 colorectal cancer patients obtained using BAT26 with results obtained using the Bethesda markers. BAT26 was able to identify all 27 mutation-positive individuals in this series. The marker failed to identify 2 high MSI tumors and 20 low MSI tumors, all of which expressed MLH1, MSH2, and MSH6 when scrutinized by immunohistochemistry.

INTRODUCTION

HNPCC is an autosomal dominant inherited cancer susceptibility syndrome. The condition is characterized by the development of CRC at an early age and by frequently occurring extracolonic tumors, e.g., cancers of the endometrium, stomach, ovaries, small bowel, ureter, biliary tract, and renal pelvis (1, 2). Early removal of benign and malignant tumors reduces cancer morbidity and mortality (3, 4). HNPCC is caused by an inherited mutation in one of five mismatch repair genes: (a) MLH1; (b) MSH2; (c) PMS1; (d) PMS2; and (e) MSH6 (5). Defective DNA mismatch repair results in RERs and genetic instability, which can easily be observed in short repetitive sequences such as microsatellites (7) and is thus referred to as MSI. In the majority of families with HNPCC, the mutations affect MLH1 or MSH2 (10), and few mutations have been reported in PMS1, PMS2, and MSH6.

A total of 85–90% of HNPCC patients show MSI, and this proportion is even higher in mutation-positive families (7, 11, 12), whereas only 10–15% of sporadic colorectal tumors do so (7–9). Thus, MSI is a relatively sensitive but unspecific marker for HNPCC. In 1997, the International Workshop on MSI and RER Phenotypes in Cancer Detection and Familial Predisposition proposed a panel of five microsatellite markers to be used in MSI analysis (13). For the purpose of providing some uniformity, two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250) were recommended. Using this reference panel (known as the Bethesda markers), tumors with instability in two or more markers are defined as MSI-H, tumors with instability in one marker are defined as MSI-L, and tumors in which none of the markers exhibit MSI are defined as MSS. Arguments in favor of combining the MSI-L and MSS groups include the facts that the baseline mutation rate for microsatellites in apparently stable CRCs is not precisely known and that the clinical features in the two groups are similar (14, 15). The distinction between these two groups is dependent on both the type and the number of microsatellites analyzed. If a large number of markers are used, all CRCs may exhibit some level of MSI (13).

Nevertheless, MSI-L CRCs seem to be distinct from both MSI-H and MSS CRC. They appear to have more in common with MSS cancers (16), although they can be distinguished on the basis of a higher frequency of K-ras mutations (16, 17) and reduced expression of BCL-2 (16, 18). MSI-L may represent a subtype of CRC combining features of the common chromosomal instability and mild mutator pathways (16, 19). However, MLH1 and MSH2 do not appear to be implicated in the MSI-L subset (20–22); thus, for the purpose of identifying patients with defects in these two genes, MSI-L and MSS cases may not need to be distinguished.

BAT26 has some advantages over many other markers in MSI analysis. It is extremely sensitive in detecting tumors with instability (20, 23–26) and shows negligible size variation either between both alleles of one individual or among individuals (23). Several studies support the use of BAT26 on tumor DNA alone (23, 24, 27). However, a germ-line polymorphism in the BAT26 locus has been detected in 7.7–12.6% of African Americans (26, 28) and in 0.8% of Caucasians (26). The presence of allelic variations, although rare in some populations, emphasizes the need for matching normal DNA in MSI-positive cases to avoid misclassifications.

This work was performed to test MSI analysis using the Bethesda panel of five markers in a series of 494 CRCs including 27 patients with identified MLH1 or MSH2 mutations. This series is part of a larger population-based study (24, 29). The MSI status of these samples had already been determined using BAT26 alone. The aim of this study was to evaluate the possible benefit of using the Bethesda set, as compared with using BAT26 alone, to identify MLH1 or MSH2 mutation-positive HNPCC patients.

MATERIALS AND METHODS

Patients. The study was approved by the appropriate ethics review committees. Colorectal tumor and normal tissue specimens were derived from 494 CRC patients treated at nine large regional hospitals in southeastern Finland. Altogether, 484 patients were derived from a consecutive series, and 10 patients with a germ-line MLH1 or MSH2 mutation were added to enrich the proportion of mutation-positive patients (24, 29). The individuals ranged in age from 34–92 years, with a mean age of 67 years. The freshly frozen samples...
were evaluated histologically by a pathologist before DNA extraction to document the proportion of tumor tissue. A total of 480 of 494 (97%) samples displayed 50% or more carcinoma tissue. The specimens representing normal mucosa were always derived from a separate site rather than from the tumor margins.

**MSI Analysis.** All 494 samples had already been analyzed for MSI using BAT26 and tumor DNA (24, 29). In this study, these tumor DNAs and respective normal tissue DNAs were used to independently study MSI status using five fluorescence-labeled microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250, the Bethesda panel). Primer sequences are presented in Table 1. PCR reactions were carried out in a 10-μl reaction volume containing 50–100 ng of genomic DNA, 1× PCR buffer (Perkin-Elmer Applied Biosystems Division, Foster City, CA), 250 μM each dNTP, and 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer). The MgCl₂ concentration was 2.5 mM for BAT25 and 2.75 mM for BAT26, D2S123, D5S346, and D17S250. The PCR cycles for each marker are indicated in Table 1. Predenaturation was performed at 95°C for 10 min, and final extension was performed at 72°C for 10 min in all reactions. PCR products were loaded on a 5% Long Ranger 6M urea gel (FMC BioProducts, Rockland, ME) and run in an ABI PRISM 377 DNA Sequencer (Perkin-Elmer) according to the manufacturer’s instructions. The data were collected automatically and analyzed by GeneScan 3.1 software (Perkin-Elmer).

Patients whose tumor DNA showed alleles that were not present in the corresponding normal DNA were classified as MSI positive. With BAT25 and BAT26, shifts of 3 or more bp were considered MSI positive. For the dinucleotide markers (D2S123, D5S346, and D17S250), all deviations starting from shifts of one CA repeat were acknowledged. If only one of the five markers showed MSI, the tumor was classified as MSI-L, and if two or more markers showed MSI, the tumor was classified as MSI-H. The results were evaluated visually by three independent reviewers (A. L., V. L., L. A. A.). All samples showing MSI-L were analyzed using five additional markers (TGF-βRII, D18S363, D18S1156, D5S318, and TP53). Primer sequences and PCR conditions are available on request.

In 16 cases, the distinction between MSI-positive and MSS in individual markers was difficult to perform visually, and in these cases, a previously described (30) mathematical model for the calculation of a RER score was used. The RER or MSI score was calculated individually for each dinucleotide marker and used to determine the MSI status of each marker separately. A MSI score of 25% or higher was used as a cutoff level for positivity (30).

**Immunohistochemistry.** Immunohistochemistry for MLH1, MSH2, and MSH6 was performed for MSI-L cases and novel MSI-H cases. Paraffin-embedded surgical resection specimens were collected from the files of pathology departments in different hospitals.

The tissue sections were mounted on ChemMate Capillary Gap microscopy slides (DAKO A/S, Glostrup, Denmark; BioTek Solutions) and dried at 37°C. The sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. The samples were then microwaved at high power four times (5 min each) in citrate buffer and then cooled and washed in PBS.

The following monoclonal antibodies were used: (a) MLH1 (clone G168-15; catalogue number 13271; Pharmingen); (b) MSH2 (clone FE 11; catalogue number NA27; Oncogene Sciences); and (c) MSH6 (clone 44; G70220; Transduction Laboratories). For immunohistochemical analysis, avidin-biotin complex immunoperoxidase technique was performed using a commercial ChemMate detection kit (DAKO A/S) in a Techmate automate machine. Endogenous peroxidase was blocked by incubation in hydrogen peroxide with methanol. Incubation with nonimmune horse serum was followed by incubation with primary antibody. The sections were then incubated in biotinylated second antibody and peroxidase-labeled avidin-biotin complex. All dilutions were made in PBS (pH 7.2). The stainings were visualized with diaminobenzidine tetrahydrochloride solution. The sections were counterstained in Mayer’s hematoxylin, rinsed with water, and mounted in an aqueous mounting media (Aquamount, BDH, Poole, United Kingdom). The percentage of positive nuclei was evaluated and scored as follows: (a) −, 0%; (b) +, 1–10%; (c) ++, 11–50%; (d) +++, 51–80%; and (e) ++++, 81% or more. The slides were analyzed by one pathologist (R. S.).

**Detection of Germline Mutations.** All 494 patients had been scrutinized previously for the two most common mismatch repair gene mutations in Finland (24, 29). Founder mutation 1 is a 3.5-kb genomic deletion of MLH1 comprising exon 16, and founder mutation 2 is MLH1 exon 6 splice site mutation G→A at 454-1 (last intronic base before exon 6). If neither founder mutation was detected, but the patient’s tumor displayed MSI (analyzed using BAT26 alone), mutation analysis of MLH1 and MSH2 was performed by direct genomic sequencing of the coding exons including the flanking intronic regions and promoter region, as described previously (24, 29).

The new MSI-H cases appearing after MSI analysis using the Bethesda markers were similarly analyzed for mutations in MLH1 and MSH2. To exclude the possibility of large deletions of MLH1 and MSH2, the new MSI-H cases were analyzed by Southern blotting according to standard procedures. Genomic DNA was digested with EcoRI and analyzed with two different cDNA probes encompassing MLH1 exons 11–19 and MSH2 exons 1–8. The new MSI-H cases were also screened for MSH6 mutations by direct sequencing covering 98% of the coding region. Primer sequences and PCR conditions are available on request. Direct sequencing of the PCR products was performed using cycle sequencing with Big Dye Terminator kit (Perkin-Elmer), and reactions were run on an ABI 3100 capillary sequencer (Perkin-Elmer) according to the manufacturer’s instructions.

A total of 182 cancer-free control individuals and 83 CRC patients were analyzed for a MSH6 variant in exon 2 by single-strand conformational polymorphism analysis using mutation detection enhancement gel solution (BioWhittaker Molecular Applications, Rockland, ME). PCR products were run on 0.6× mutation detection enhancement gels at 4 W for 22 h. The running buffer was 0.6× Tris-borate EDTA. Single-strand conformational polymorphism gels were silver-stained according to standard procedures.

**RESULTS AND DISCUSSION.**

HNPCC is the most common hereditary CRC syndrome. The patients benefit greatly from early diagnosis because CRC deaths can be efficiently reduced by removing adenomas and early carcinomas (3, 4), emphasizing the need for development of molecular identification procedures for HNPCC. MSI analysis is a practical tool for prescreening HNPCC (24, 29, 31–33). A panel containing two mononucleotide markers (BAT25 and BAT26) and three dinucleotide markers (D2S123, D5S346, and D17S250), the so-called Bethesda markers, has been proposed for MSI analysis (13). BAT26 has some advantages over dinucleotide markers. It is quasimonomorphic (23), and germ-line polymorphisms are rare in the Caucasian population (26). If analysis of tumor tissue DNA reveals no putative shifts, use of normal

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**Table 1** Primer sequences (http://www.gdb.org) and PCR cycles for the five Bethesda markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequences</th>
<th>PCR cycles</th>
</tr>
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<tbody>
<tr>
<td>BAT25</td>
<td>TCG-TCT-CCA-AGA-ATG-TGA-ATT</td>
<td>28 cycles of 95°C for 1 min, 56°C for 45 s, 72°C for 45 s</td>
</tr>
<tr>
<td>BAT26</td>
<td>TCT-GGA-TTT-TAA-CTA-TGA-GTC</td>
<td>32 cycles of 95°C for 45 s, 55°C for 1 min, 72°C for 30 s</td>
</tr>
<tr>
<td>D2S123</td>
<td>GCA-TCA-ATC-AGC-TCA-GCC</td>
<td>35 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 45 s</td>
</tr>
<tr>
<td>D5S346</td>
<td>ACT-CAC-TCT-GAT-GAG-TAA-TCG-G</td>
<td>30 cycles of 95°C for 1 min, 57°C for 45 s, 72°C for 45 s</td>
</tr>
<tr>
<td>D17S250</td>
<td>AGC-AGA-TAA-GAC-AAG-TAT-TAC-TAG</td>
<td>35 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 45 s</td>
</tr>
</tbody>
</table>
control DNA is not necessary. The use of Bethesda markers, on the other hand, harbors some technical difficulties. Reamplifications are frequently needed to obtain successful amplifications and unambiguous results for all markers and for both normal and tumor DNA; in this study, there were 31 reamplifications for BAT25, 68 reamplifications for BAT26, 119 reamplifications for D2S123, 57 reamplifications for D5S346, and 182 reamplifications for D17S250. In addition, the interpretation of D2S123, D5S346, and D17S250 is not trivial and cannot be accomplished without the matching normal DNA, even if the sample is MSS (see Fig. 1). The cutoff level for MSI positivity for the mononucleotide markers was set at shifts of 3 bp or greater. The cutoff level of 3 bp was chosen because all 10 cases displaying such deviation also had instability at other loci. Eight and seven patients showed 1–2-bp deviations at BAT25 and BAT26, respectively, and none of these cases displayed additional evidence of MSI at other loci. In 31 individual cases, the three reviewers had discrepancies when scoring dinucleotide markers, whereas not a single scoring discrepancy occurred with BAT25 and BAT26.

A total of 494 CRC patients were successfully analyzed for MSI using the Bethesda panel of five microsatellite markers (13). A total of 73 of 494 patients (14.8%) had previously shown MSI when analyzed using BAT26 alone (24, 29). When the five Bethesda markers were used, all 73 appeared as MSI-H (Table 2). A total of 95 of 494 patients (19.2%) were classified as MSI with the Bethesda panel; 75 patients (15.2%) were classified as MSI-H, and 20 patients (4.0%) were classified as MSI-L (Table 2).

Twenty-two new MSI-positive cases appeared when the panel of five markers was used (2 MSI-H cases and 20 MSI-L cases; Table 2). Germ-line mutation analysis of MLH1 and MSH2 was performed for the two new MSI-H cases. No mutations were found in the coding regions, exon-intron boundaries, or the promoter region of these genes. Neither case had a family history of cancer, and the ages at

![Image](https://cancerres.aacrjournals.org/)

Fig. 1. MSI analysis using BAT25, BAT26, D2S123, DSS346, and D17S250. For each marker, the top graph represents tumor DNA, and the bottom graph represents matching normal DNA. A, case c145 (Finnish founder mutation 1, 70% tumor tissue) shows instability at all markers except DSS346. B, case c1034 (no MLH1/MSH2 germ-line mutation identified, 70% tumor tissue) shows instability only at BAT25 and BAT26. The novel allele in BAT25 represents the cutoff level for positivity (shift of 3 bp in tumor DNA). Marker DSS346 shows a weak extra peak on this patient’s tumor (indicated by an arrow). The RER score (30) was calculated to confirm the MSI status and resulted in a score of 3.2%, consistent with visual MSI scoring, excluding MSI positivity. However, considering the patterns in other loci, the peak indicated by the arrow may reflect true MSI. Scoring is much more trivial with the mononucleotide markers because the cutoff level can be determined as the number of bp deleted. The BAT25 and BAT26 patterns in this study were highly reproducible. C, examples of shifts in BAT26 that are below the cutoff level: sample c859, top graph pair; and sample c1079, bottom graph pair.

Table 2: Comparison of MSI analysis based on BAT26 alone and MSI analysis based on the Bethesda markers (BAT25, BAT26, D2S123, DSS346, and D17S250)

<table>
<thead>
<tr>
<th>MSI with BAT26 alone</th>
<th>0 positive markers</th>
<th>1 positive marker</th>
<th>2 positive markers</th>
<th>3 positive markers</th>
<th>4 positive markers</th>
<th>5 positive markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT26 positive (n = 73)</td>
<td>n = 399</td>
<td>n = 20</td>
<td>n = 4</td>
<td>n = 8</td>
<td>n = 25</td>
<td>n = 36</td>
</tr>
<tr>
<td>BAT26 negative (n = 421)</td>
<td></td>
<td></td>
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The total number of samples studied was 494.
diagnosis were 64 and 65 years. To examine the molecular back-
ground of the observed MSI-H phenotype in more detail, additional
experiments were performed on the two cases. Because direct
genomic sequencing cannot detect defects such as genomic deletions
affecting whole exons, we examined both MLH1 and MSH2 by
Southern hybridization. No aberrations were detected. Direct genomic
sequencing of MSH6 revealed a missense change in one case; MSH6
exon 2 S144I (AGC → ATC). The tumor DNA showed no loss of
heterozygosity at this change. This variant has been reported previ-
ously as a pathogenic mutation (34). We analyzed 182 cancer-free
controls and 83 individuals with CRC for the change and found it in
1 control sample. It thus appears to be a rare polymorphism, although
additional studies are needed to confirm its nature.

Five additional microsatellite markers (TGF-βRII, D18S363,
D18S1156, D5S318, and TP53) were used to confirm the status of the
20 MSI-L cases. Three samples showed further instability with one of
the markers. This degree of instability (2 of 10 or 20%) should be
considered as MSI-L (13).

In addition, immunohistochemistry of MLH1, MSH2, and MSH6
was performed for the 20 MSI-L and the 2 new MSI-H cases. Because
no lack of expression was revealed in the MSI-L cases (all scored
++, +++, or ++++), no further procedures were performed. Also,
previous studies have confirmed that the expression of MLH1 and
MSH2 is not altered in MSI-L cases, whereas loss of expression in one
of them, typically MLH1, can be seen in most MSI-H cases (20, 21).
However, because MLH1 antibody has a tendency to show a weak
positive staining even in cases harboring deleterious mutations (35),
we must acknowledge the possible involvement of a gene defect even
in cases showing MLH1 immunostaining. Importantly, the two new
MSI-H cases expressed MLH1, MSH2, and MSH6 (all scored ++++
or ++++). This result, together with their BAT26 negativity, im-
plies that they are possibly MSI-L despite the two unstable dinucle-
otide markers.

All 27 mutation-positive CRCs in the series of 494 tumors showed
instability at both the BAT26 and BAT25 loci. However, in other
studies, we have detected one sample derived from a mutation-
positive patient with no instability at BAT26. The patient was diag-
nosed at 37 years of age with a proximal tumor, has Finnish founder
mutation 1, and has a strong family history of CRC. Thus, the lesion
is most likely a HNPCC tumor. The degree of MSI in tumors is related
to past patterns of tumorigenesis, primarily to mutation rate and the
number of divisions since loss of mismatch repair. Relatively young
lesions are likely to show less MSI, and rare cases of HNPCC may
display MSI at relatively few loci (36). In this study, D21S23,
D5S346, and D17S250 identified 24 (89%), 16 (59%), and 22 (81%)
of 27 mutation-positive individuals, respectively. Despite negative
results in genomic sequencing, some of the 46 cases that showed
BAT26 instability may have had a MSH2 or MLH1 mutation that was
not detected by the method. Of these 46 individuals, 35 had no family
history of CRC or endometrial cancer, but undetected mutations may
underlie a subset of the 11 cases with some HNPCC features (1–3
relatives with CRC or endometrial cancer). None of the 11 pedigrees
fulfilled the Amsterdam criteria for HNPCC (2).

It is possible that the two common founder mutations in Finland
cause a bias toward MSI-H phenotypes in our HNPCC series. To-
gether, these two mutations accounted for 18 of 27 (67%) mutations in
this series. However, all of the nine other patients with five
different mutations exhibited MSI-H phenotypes, supporting the no-
tion that deleterious MLH1 and MSH2 mutations tend to cause MSI-H
phenotypes. A minority of HNPCC tumors are MSS. The genes
responsible for HNPCC are not yet fully known, and some of these
appear to be associated with more attenuated phenotypes (13). It is
possible that MSH6, TGF-βRII, and perhaps other currently uniden-
tified predisposing genes cause a MSI-L or MSS phenotype (34).

Relying on BAT26 alone in MSI analysis has prompted the ques-
tion of possible loss of sensitivity and specificity. The proposed panel of
five microsatellite markers (13) aims at dividing tumors into
categories of MSI-H, MSI-L, and MSS, of which typically only
MSI-H tumors are considered as candidates for MLH1/MSH2
mutation analysis (20–22). When relying on one marker, distinguishing
between MSI-H and MSI-L is impossible. According to this study,
BAT26 detects MSI-H cases with high sensitivity because 73 of 75
(97%) MSI-H cases were BAT26 positive. The sensitivity in detecting
MLH1/MSH2 mutation-positive individuals was 100% (27 of 27).

Only 2 of 421 (0.5%) BAT26-negative tumors were found to be
MSI-H, and no MLH1/MSH2 mutations could be found in these
patients. Twenty of 421 (4.8%) BAT26-negative tumors were found
to be MSI-L. These 22 BAT26-negative tumors expressed MLH1,
MSH2, and MSH6 according to immunohistochemistry and are un-
likely to represent HNPCC. The specificity of BAT26 in detecting
mutation-positive individuals (27 of 73 cases, 37%) was very similar
to the specificity of the Bethesda panel (27 of 75 cases, 36%). The
data suggest that there is little loss of specificity when relying on
BAT26 alone.

On the basis of analyzing 494 CRC patients for MSI using the
Bethesda markers, we conclude that BAT26 alone was sufficient to
identify 97% of MSI-H cases but seemingly fails to detect MSI-L
cases. Thus, additional markers are needed when aiming at distin-
guishing MSI-L and MSS subgroups. However, this may not be
necessary when prescreening patients for MLH1/MSH2 germ-line
mutations. It seems that BAT26 identifies MLH1 or MSH2 mutation-
positive CRC patients with high sensitivity. Whereas utilization of
more markers may be useful if extensive resources are available, the
benefit appears marginal and may not be cost effective. Utilization of
numerous markers resulted in increased workload and expense, as
well as difficulties in scoring even in experienced hands. Our data
indicate that BAT26 alone can be used as a tool in detection of
MLH1/MSH2-associated HNPCC in circumstances where resources
and experience in interpretation are limited.

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