Comparison of Three Commonly Used PCR-Based Techniques to Analyze MSI Status in Sporadic Colorectal Cancer

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Several retrospective studies have shown that a high level of microsatellite instability (MSI-H) is an important prognostic factor of a more favorable outcome in stage II and III colorectal cancer (CRC) patients. In this study, three commonly used polymerase chain reaction (PCR)-based MSI analysis techniques were compared (polyacrylamide gel electrophoresis followed by silver-staining [SSPAGE], fluorescence capillary electrophoresis [FCE], and denaturing high-performance liquid chromatography [DHPLC]) on a limited group of CRC patients, to identify the most optimal detection technique. Pathology blocks of 26 CRC patients were subjected to microdissection and the Bethesda reference panel was used for MSI analysis. Considering the

samples analyzed by both SSPAGE and FCE, 8.7% were MSI-H, 8.7% were MSI-L, and 82.6% were MSS using SSPAGE. FCE resulted in 16% MSI-H, 4% MSI-L, and 80% MSS. Due to difficulties in analyzing the dinucleotide markers on DHPLC, we only analyzed the mononucleotide markers with this technique. The results were 100% concordant to those obtained by FCE. SSPAGE is time consuming, subjective, and less user-friendly and interpretable. DHPLC was not feasible due to interpretation difficulties for the dinucleotide markers. We recommend the use of FCE to analyze MSI status. This technique is sensitive, reproducible, user-friendly and leads to easy interpretation and high-throughput. *J. Clin. Lab. Anal.* 20:52–61, 2006. © 2006 Wiley-Liss, Inc.

Key words: colorectal cancer; MSI; PAGE; fluorescence capillary electrophoresis; DHPLC

INTRODUCTION

Colorectal cancer (CRC) poses a major public health problem in Europe as the fourth most common type of cancer (1). Carcinogenesis of CRC is a multistep process involving the accumulation of multiple mutations. Two major pathogenic mechanisms of genomic instability have been identified in colorectal cancer. The suppressor pathway is found in the majority of cancers (85%) and these tumors have a molecular profile characterized by specific chromosomal amplifications and transformations, aneuploidy and loss of heterozygosity (LOH) (2,3). Cancers originating from the mutator pathway have a defective DNA mismatch repair mechanism (MMR), which allows mutations to be accumulated at many times the normal rate. This inability to repair DNA mismatches can easily be demonstrated as it

results in cell-to-cell variability in the length of DNA microsatellites called microsatellite instability (MSI) (4).

Abbreviations: LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; SSPAGE, silver-staining polyacrylamide gel electrophoresis; DHPLC, denaturing high-performance liquid chromatography; TEAA, triethylammonium acetate; H&E, hematoxylin and eosin; MSI-H, microsatellite instability–high grade; MSI-L, microsatellite instability–low grade; MSS, microsatellite stable.

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The survival figures of patients with TNM stage II and III disease are unfavorable, but are not predictable on an individual basis. Consequently, there is a strong need for adjuvant therapies, which in turn enhances the need for prognostic factors able to discriminate between patients at low risk or high risk for relapse. Several retrospective studies have suggested that the presence of a high level of MSI may be an important prognostic factor of a more favorable outcome (5,6). In recent years several polymerase chain reaction (PCR)-based MSI analysis techniques have been developed for research purposes. For identifying the MSI status, the National Cancer Institute recommends the Bethesda reference panel composed of two mononucleotide and three dinucleotide markers validated by Rushoff and Fishel (7). The MSI analysis is based on the interpretation of the mobility shift of tumor DNA markers in comparison to normal DNA markers from the same patient.

In this study, three techniques for MSI detection were compared. With the first technique the amplified PCR products were separated by polyacrylamide gel electrophoresis and detected by silver-staining (SSPAGE) (8).

The second technique to identify the MSI status was the use of an automated capillary electrophoresis system (fluorescence capillary electrophoresis [FCE]) that can separate, detect, and analyze fluorescently labeled DNA fragments (ABI PRISM Genetic Analyzer). Fragment analysis was used to determine the length of each fragment (in base pairs) and to estimate their relative concentrations. The detection components collect the fluorescence of the labeled DNA fragments and convert the information in electronic form, which is then processed, stored, analyzed, and viewed with the GeneScan Analysis Software (ABI PRISM 3100 Genetic Analyzer, User's Manual, Applied Biosystems, Hitachi).

The last MSI analysis technique studied was a column-based method of separation (denaturing high-performance liquid chromatography [DHPLC]). In reversed-phase chromatography, the DNA fragments bind, with the aid of triethylammonium acetate (TEAA)—a hydrophobic and positively charged ion pairing reagent—to an immobilized electrostatically neutral and hydrophobic matrix (stationary phase) in a polar solvent (mobile phase). A buffer of increasing hydrophobicity (acetonitrile) is used to elute the DNA off the matrix. The concentration of acetonitrile needed for elution depends on the size and sequence of the DNA fragment. The absorbance is measured at 260 nm. The peak of maximum absorbance is the retention time of that DNA sample at a given acetonitrile concentration (Wave System, User's Manual, Transgenomic, 2002).

The aim of the study was to compare these three commonly used techniques (SSPAGE, FCE, and DHPLC) on a group of 26 colorectal cancer patients,

in order to identify the most objectively interpretable and most user-friendly high-throughput detection technique for MSI analysis.

MATERIALS AND METHODS

Patients and Specimen

Of all colorectal cancer patients seen between 1997 and 2001 at the University Hospital of Antwerp, 26 patients were selected by age from the cancer registries. Relative young patients were selected because MSI is related to age at diagnosis, as it is more common in slightly younger patients (6).

Patients were included in the study only if pathological material was available. Six 5- μ m sections were cut from formalin fixed, paraffin-embedded pathology blocks and placed onto slides. One slide was used for hematoxylin-eosin (H&E) staining.

DNA Extraction

DNA was extracted separately from tissue of the patient's colorectal tumor and the surrounding non-cancerous tissue. Separate areas of the tumor and the normal tissue were outlined with a black marker under the microscope on the reference H&E-stained slide. Unstained tissue sections adjacent to the reference slide were microdissected according to the outlined areas on the H&E-stained slide using a surgical scalpel blade. Separate blades were used for tumor and normal tissue (9,10).

The dissected specimen were collected in 1.5-mL centrifuge tubes and deparaffinized with xylene (Merck, Leuven, Belgium), the DNA was purified with ethanol (Merck, Leuven, Belgium) followed by a wash step with acetone (Fisher Chemicals, Leics, UK). The release of the DNA comprised an enzymatic degradation of the extracellular proteins by addition of 0.1 mg/mL proteinase K (Invitrogen, Merelbeke, Belgium) and incubation at 37°C overnight. Proteinase K was subsequently inactivated by boiling for 10 min followed by a centrifugation step. Samples were stored at -20°C.

Primers and PCR

For the determination of the MSI status, the National Cancer Institute recommended Bethesda panel of 5 microsatellite markers was used (Bat 25, Bat 26, D5S346, D2S123, and D17S250) (7). PCR primer sequences for amplifying the five markers were obtained from the genome database (www.gdb.org) (11). To perform the FCE analysis, the primers were chemically labeled with fluorescent dyes (6 FAMTM, HEXTM). All primers were obtained from Biosource (Nivelle, Belgium).

TABLE 1. Optimized Annealing Temperatures for PCR of the Bethesda panel of 5 Microsatellite Markers

Marker	T _m SSPAGE	T _m FCE	MgCl ₂ SSPAGE	MgCl ₂ ² FCE
	and DHPLC		and DHPLC	
Bat 25	54°C	56°C	2.6 mM	2 mM
Bat 26	56°C	53°C	5.1 mM	4.6 mM
D2S123	58°C	54°C	1.9 mM	2 mM
D5S346	56°C	60°C	2.2 mM	1.8 mM
D17S250	54°C	56°C	1.9 mM	1.9 mM

A high level of MSI (MSI-H) was defined when two or more markers showed new alleles compared to the normal tissue, a low level of MSI (MSI-L) was defined when only one marker showed a novel allele and microsatellite stability (MSS) was defined when no markers with a novel allele were found (7).

The amplification of each microsatellite marker was optimized for PCR conditions using AmpliTaq Gold DNA polymerase (Applied Biosystems, Lennik, Belgium) (3). The samples were amplified in 50-μL reaction volumes containing 5 μL of genomic DNA, 25 pmol of each sense and antisense primer, 200 μM dNTPs (MBI Fermentas, St. Leon-Rot, Germany), PCR buffer (Applied Biosystems, Lennik, Belgium), 1.25 U Taq polymerase (Applied Biosystems, Lennik, Belgium) and 1.8–4.6 mM MgCl₂, as appropriate (Applied Biosystems, Lennik, Belgium) (Table 1). For SSPAGE analysis, PCR amplification was performed for 35 cycles: 94°C for 1 min, the optimized annealing temperature (Table 1) for 1 min, and 72°C for 1 min. The final extension was performed at 72°C for 10 min. PCR amplification for FCE and DHPLC analysis was performed for 35 cycles: 95°C for 30 sec, the optimized annealing temperature (Table 1) for 30 sec, and 72°C for 30 sec. The final extension was performed at 72°C for 10 min.

Detection of MSI Status

Microsatellite instability analysis by SSPAGE

The PCR products were analyzed under denaturing conditions on a 6% polyacrylamide gel containing 7 M urea. The gel was preheated and the DNA samples were boiled in the presence of a formamide containing loading buffer before they were loaded on the gel. After electrophoresis, the polyacrylamide gel was fixed and the DNA amplicons were visualized using silver-staining (Promega, Leiden, The Netherlands). MSI analysis was based on the interpretation of the mobility shift of tumor DNA in comparison to normal DNA. MSI was defined as the presence of novel bands following PCR amplification of tumor DNA, which were not present in PCR products of corresponding normal DNA (12). Two

general types of patterns were defined. On the one hand a ladder-like expansion or contraction of the microsatellite repeat unit, and on the other hand a typical single repeat change above or below the exposed allele fragment size (13).

Microsatellite instability analysis by FCE

One microliter of each fluorescently labeled amplification sample was mixed with 0.15 μL ROX™ dye (standard) (Applied Biosystems, Lennik, Belgium) and 10 μL HI-DI™ formamide (injection solution) (Amresco, Franceville, France), 10 μL of this mix was pipetted into a 384-well plate. The thin, silica-fused capillaries filled with the replaceable sieving medium (POP-4) (Applied Biosystems, Lennik, Belgium) separate the DNA fragments by size during electrophoresis. As the DNA amplicons enter the detection cell, the dye fluoresces. The size of the PCR products was subsequently evaluated on the ABI PRISM 3100 Analyzer with GeneScan Software. MSI analysis was based on the fragment analysis pattern of tumor DNA in comparison to normal DNA of the same patient. MSI is recognized as a change in allele size(s) in tumor DNA from the constitutional allele pattern observed in corresponding non-tumoral DNA. The case was classified MSI positive if any novel allele was apparent (14).

Microsatellite instability analysis by DHPLC

Ten microliters of each amplification product was pipetted in a 96-well plate, which was placed in the autosampler of the Wave system. The samples were automatically introduced into the Wave system's liquid path. Due to the presence of TEAA (Transgenomic, Paris, France), the DNA fragments can bind the polystyrene divinylbenzene beads of the stationary phase. Subsequently, the mobile phase consisting of TEAA mixed with acetonitrile (Transgenomic, Paris, France) elutes the DNA off the cartridge. The system runs at 50°C and the number of base pairs determines the gradient.

The absorbance of the DNA fragment is measured at 260 nm and is plotted versus the elution time (Wave System, User's Manual, Transgenomic, 2002).

This technique can separate PCR products that differ as little as a few base pairs. MSI analysis also depends on the presence of a novel allele in tumoral DNA that is not represented in the corresponding normal DNA (11).

RESULTS

MSI Analysis by SSPAGE

The 26 cancer and matched normal samples were analyzed with SSPAGE. One sample (MSI 12) could not

be analyzed due to a lack of clarity to interpret the band patterns of Bat 25, Bat 26, and D2S123 markers. The mononucleotide markers (Bat 25 and Bat 26) showed MSI in 8% (2/25). MSI occurred in 4% (1/25) at D2S123 and D5S346 and in 12% (3/25) at D17S250. Out of 25 samples, 2 (8%) were found to be MSI-H, 2 (8%) were MSI-L, and 21 (84%) were MSS (Table 2).

MSI Analysis by FCE

Two samples (MSI 14 and MSI 19) were lost and one novel sample (MSI 27) was introduced for the MSI analysis of the 26 cancer and matched normal samples by FCE. Bat 25 and Bat 26 markers showed MSI in 12% (3/25). MSI was found in 16% (4/25) at D2S123, in 8% (2/25) at D5S346 and in 12% (3/25) at D17S250 (Fig. 2). Out of 25 analyzed samples, four (16%) were scored MSI-H, 1 (4%) was MSI-L, and 20 (80%) were MSS (Table 2).

Two markers (D5S346 and D17S250) also showed loss of heterozygosity (LOH) in some samples, these samples were scored as negative for MSI (Fig. 1; Table 2).

MSI Analysis by DHPLC

Due to difficulties interpreting the dinucleotide markers on DHPLC, we only analyzed the mononucleotide markers with this technique. The results were 100% concordant to the results obtained by FCE (Table 2; Fig. 2)

Comparison of the Techniques

Of the 26 cancer and matched normal samples selected, 23 samples could be analyzed by both SSPAGE and FCE. Using SSPAGE, two samples (8.7%) were MSI-H, 2 (8.7%) were MSI-L, and 19 (82.6%) were MSS. In contrast to this analysis, the MSI analysis with FCE resulted in three MSI-H samples (13.1%), one (4.3%) MSI-L, and 19 (82.6%) MSS.

The use of FCE in comparison to SSPAGE implied the reclassification of a few samples. One MSS sample was reclassified to the MSI-H group, while one sample in the MSS group was reclassified to the MSI-L group and two samples were reclassified from the MSI-L to the MSS group (Fig. 3, Table 3).

DISCUSSION

Microsatellite instability is a useful characteristic to assess the efficiency of the DNA MMR process and is potentially a useful prognostic marker in sporadic colorectal cancer. Therefore, it is of major importance to identify a sensitive, specific, user-friendly, and high-throughput analysis technique for the correct identification of the MSI status. PCR is the gold standard for

MSI analysis, but there is no consensus about the choice of post-PCR detection technique. This study compares three commonly used PCR-based techniques for MSI analysis on 26 patients selected by relative young age from the entire population of CRC patients treated in the University Hospital of Antwerp between 1997–2001. The combination of these three tests is used for the detection of the most optimal and reliable technique, i.e., a positive result in any test was taken as a proof for the existence of MSI-L or MSI-H status.

For this determination, the Bethesda markers were used. Loukola et al. (15) report difficulties using these markers, reamplifications were frequently needed and the interpretation of the results was not always trivial, although they used a fluorescence-based technique. We did not encounter tremendous problems using the Bethesda markers, although reamplifications were sometimes needed due to the presence of internal inhibitory factors derived from the formalin-fixed paraffin-embedded blocks.

SSPAGE, a gel-based electrophoresis and sequencing analysis technique, does not require a complicated protocol or expensive equipment and is therefore available in a large number of laboratories. However, this technique has been found to be very time-consuming and labor intensive. The protocol involves several experimental steps, which are not compatible with high-throughput analysis (11). Moreover, it is sometimes difficult to interpret the results due to the appearance of stutterbands that limit the accuracy of the MSI analysis. The resolution of this technique is in some cases too restricted to distinguish between the presence of stutterbands or actual instability within lane to lane variability of the band patterns (14). Furthermore, a relatively large amount of amplicon DNA needed to be loaded on the polyacrylamide gel to be detected with SSPAGE.

In this study, interpretation problems were also encountered; one sample (MSI 12) could not be analyzed because of the appearance of stutterbands. We found 8.7% of the samples to be MSI-H, 8.7% MSI-L, and 82.6% were stable using this technique, this contrasts with the results obtained using FCE, where 13.1% was MSI-H, 4.3% MSI-L, and 82.6% MSS. One sample was reclassified from the MSS group to the MSI-H group using FCE.

Several other studies, using techniques similar to SSPAGE, obtained variable results. Pedroni et al. (16), found 13.8% and 19.4% MSI-H in a synchronous group and a metachronous group of sporadic colorectal cancer samples, respectively. Another study reports a similar result as that found with SSPAGE in our study (17). The selection criteria, the size of the study population and the marker panel used for the MSI analysis are three very important factors that can be

TABLE 2. MSI status determination using SSPAGE, FCE, and DHPLC; comparison

Number	Bat25				Bat 26				D2S123				D5S346				D17S250				MSI status			
	SSPAGE	FCE	DHPLC	SSPAGE	FCE	DHPLC	SSPAGE	FCE	SSPAGE	FCE	SSPAGE	FCE	SSPAGE	FCE	SSPAGE	FCE	SSPAGE	FCE	SSPAGE	FCE	DHPLC ^a			
1		+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSI-H	MSI-H			
2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSI-L	MSS	MSS			
3		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MSI-H	MSI-H	MSI-H			
4		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	LOH	MSS	MSI-L	MSS			
6		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
7		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
8		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
9		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
10		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
11		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
12		NA	-	-	NA	-	NA	+	+	+	+	+	+	+	+	+	+	+	NA	MSI-H	MSS			
13		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
14		NA	NA	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	MSS	NA	NA			
15		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	LOH	MSS	MSS	MSS			
16		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
17		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
18		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
19		NA	NA	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	MSS	NA	NA			
20		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
21		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
22		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
23		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MSI-H	MSI-H	MSI-H			
24		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	LOH	MSS	MSS	MSS			
25		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSI-L	MSS	MSS			
26		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	MSS	MSS	MSS			
27		NA	-	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	MSS	MSS			

^aThe MSI status determined by DHPLC is only based on the results obtained by the mononucleotide markers Bat 25 and Bat 26 because the dinucleotide markers could not be analyzed using DHPLC.

NA: not analyzed.

Fig. 1. MSI analysis using FCE and Bat 25 (A), Bat 26 (B), D2S123 (C), D5S346 (D), and D17S250 (E). The upper curve of a pair represents normal DNA while the lower curve represents tumoral DNA. Each upper pair is an example of MSS and each lower pair of MSI except fig. D where the lower pair represents LOH.

responsible for the diversity of the results found in the literature.

Although the use of fluorescent techniques requires the use of rather expensive photolabile fluorescent PCR primers and additional hardware (GeneScan) and soft-

ware, it is a multifunctional system and the running costs associated with these analyses can be relatively low. The use of a cross-sectional visual representation of the allele products means that the interpretation of microsatellite instability is much easier and less sub-

Fig. 2. MSI analysis using DHPLC and Bat markers, Bat 25 (A) and Bat 26 (B). The top graph always represents normal DNA and the lower graph represents tumoral DNA. In (A) as in (B), the two upper pairs are an example of MSI and the lower pair is an example of MSS.

Fig. 3. Comparison of the results obtained by SSPAGE (A–E) and FCE (A'–E') for two dinucleotide markers, D17S250 (A, B, D and A', B', D') and D5S346 (E and E') and a mononucleotide marker Bat 25 (C and C'). N: normal DNA, T: tumoral DNA. A and A' represent MSS, and B and B' represent LOH, while C and C' and D and D' represent MSI. E and E' represent contradictory results. SSPAGE seems to have an additional allele in the T sample compared to the N sample, but this is not 100% indisputable because a very light shadow is seen in the N sample at the same location. FCE, on the other hand, clearly and undoubtedly shows two alleles in the N and T sample (MSS). It is obvious that the interpretation of the FCE pictures is more straightforward compared to the pictures obtained with SSPAGE.

jective. The accurate analysis of allele size ensures correct sample pairing and also allows to establish the numbers and sizes of the new alleles of each marker showing MSI (14). The appearance of stutterbands

limiting the accuracy of the MSI analysis in SSPAGE, especially when formalin-fixed paraffin-embedded material is used, was overcome in FCE because the appearance of shadow peaks in this technique are

TABLE 3. Comparison Results Obtained by SSPAGE and FCE

SSPAGE	FCE			TOTAL
	MSI-H	MSI-L	MSS	
MSI-H	2	0	0	2
MSI-L	0	0	2	3
MSS	1	1	17	18
TOTAL	3	1	19	23

relatively easy to distinguish from the peaks representing the true alleles.

FCE has a rapid throughput and the use of an internal standard in each lane ensures that lane-to-lane variation does not affect sizing of the PCR products (14).

This technique is very sensitive, so that only 1 μ L of the amplification product needs to be run. Even weak PCR amplicon products that could not be analyzed by SSPAGE could still be analyzed with FCE.

Two independent examiners scored the samples. When discrepancies occurred the samples were reanalyzed by a third independent experienced examiner. At the end complete agreement was achieved.

Like the groups of Cawkwell et al. (14), Ward et al. (17), Smyrk et al. (18), and many others, we believe this fluorescence-based PCR assay in combination with GeneScan analysis to be a sensitive, objectively interpretable, user-friendly, and reproducible high-throughput technique for the assessment of microsatellite instability in colorectal cancer. Using this technique, one additional sample (MSI 01) was identified as MSI-H compared to SSPAGE where it was classified as MSS. It is obvious from Fig. 3 that the interpretation of the results is much more unequivocal for FCE in comparison to SSPAGE. The appearance of stutterbands could more easily lead to misinterpretations using SSPAGE compared to FCE, presumably explaining the two MSS samples in FCE that were classified as MSI-L in SSPAGE. FCE analysis is widely used for different research purposes (19,20). Our results show that the use of the Bat markers alone is not sufficient for a correct MSI analysis. In consensus with the study of Halford et al. (21), we found a specificity of less than 100% (75%) for the analysis of high-level MSI using the mononucleotide markers alone. The addition of the dinucleotide markers led to an increase in the percentage of MSI-H samples. This was also established in studies by Loukola et al. (15) and Halford et al. (21). The reduced specificity of the mononucleotide markers found in this study, however, needs to be interpreted with caution because of the low percentage of MSI-H samples, one missed sample immediately leads to a decrease of 25% in specificity.

Over the years, DHPLC has been used frequently for genetic analysis. Kim et al. (11) developed a new method for MSI analysis using this technique. PCR products differing by a single base pair are distinguishable at 50°C by ion-pair reversed-phase chromatography. According to Kim et al. (11), DHPLC analysis is a simple and efficient process to detect the presence of a novel allele in cancer DNA that is not present in the corresponding normal tissue (11). The analysis of the quasimonomorphic Bat markers using DHPLC was sensitive, the results being in complete agreement with the results obtained by FCE with these markers. However, problems were encountered analyzing the dinucleotide markers. These results were not unambiguous and difficulties occurred during the interpretation. A possible explanation lies in the fact that the samples originated from formalin-fixed paraffin-embedded slides, which might hamper MSI analysis due to the presence of impurities, although this did not seem to have severe adverse effect on analysis by FCE. Using fresh frozen samples collected at the time of surgery for DHPLC analysis, these problems were not encountered by Kim et al. (11) and Pan et al. (22).

In conclusion, due to the absence of a consensus about the choice of post-PCR detection technique for MSI analysis, this study compares three commonly used PCR-based techniques for MSI analysis. SSPAGE is very time consuming. The interpretation of the SSPAGE results is difficult and very subjective. SSPAGE was found to be less user-friendly and interpretable than the other two techniques tested. One MSI-H sample was missed using this analysis method and a notable larger amount of amplicon DNA needed to be loaded on the polyacrylamide gel to be detected with SSPAGE in comparison to the other techniques. The DHPLC analysis of MSI-status was much easier to interpret using the mononucleotide markers, but difficulties arose for the detection of the dinucleotide markers. This technique is sensitive but still rather time consuming.

The use of a cross-sectional visual representation in FCE facilitates the interpretation of microsatellite instability making it also less subjective. The appearance of accuracy-limiting stutterbands was overcome in this technique. FCE is so sensitive that only a limited amount of the amplification product is sufficient to detect MSI-status. The results obtained with FCE were in complete agreement with the results of DHPLC for the quasimonomorphic Bat markers.

We therefore recommend the use of FCE to analyze MSI status. This technique is sensitive, reproducible and user-friendly because it leads to an easy interpretation of the results and it also has a high-throughput. We are currently using this technique for MSI analysis in a larger population of colorectal cancer patients.

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REFERENCES

1. Grady WM, Rajput A, Lutterbaugh JD, Markowitz SD. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. *Cancer Res* 2001;61:900–902.
2. Edmonston TB, Cuesta KH, Burkholder S, et al. Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. *Hum Pathol* 2000;31:1506–1514.
3. Nash GM, Gimbel M, Shia J, et al. Automated, multiplex assay for high-frequency microsatellite instability in colorectal cancer. *J Clin Oncol* 2003;21:3105–3112.
4. Atkin NB. Microsatellite instability. *Cytogenet Cell Genet* 2001;92:177–181.
5. Gryfe R, Kim H, Hsieh ET, et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000;342:69–77.
6. Samowitz WS et al. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. *Cancer Epidemiol Biomarkers Prev* 2001;10:917–923.
7. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 1998;58:5248–5257.
8. Schlegel J, Vogt T, Munkel K, Ruschoff J. DNA fingerprinting of mammalian cell lines using nonradioactive arbitrarily primed PCR (AP-PCR). *Biotechniques* 1996;20:178–180.
9. Carethers JM, Smith EJ, Behling CA, et al. Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. *Gastroenterology* 2004;126:394–401.
10. Lindfors U, Papadogiannakis N, Zetterquist H, Lindberg G, Olivecrona H. Distribution of genetic variants in preneoplastic areas of colorectal tumours. *European J Surgical Oncol* 2003;29:491–496.
11. Kim IJ, Shin Y, Kang HC, et al. Robust microsatellite instability (MSI) analysis by denaturing high-performance liquid chromatography (DHPLC). *J Hum Genet* 2003;48:525–530.
12. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Ruschoff J. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. *Cancer Res* 1997;57:4749–4756.
13. Thibodeau SN, French AJ, Cunningham JM, et al. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res* 1998;58:1713–1718.
14. Cawkwell L, Li D, Lewis FA, Martin I, Dixon MF, Quirke P. Microsatellite instability in colorectal cancer: improved assessment using fluorescent polymerase chain reaction. *Gastroenterology* 1995;109:465–471.
15. Loukola A, Eklin K, Laiho P, et al. Microsatellite marker analysis in screening for hereditary nonpolyposis colorectal cancer (HNPCC). *Cancer Res* 2001;61:4545–4549.
16. Pedroni M, Tamassia MG, Percesepe A, et al. Microsatellite instability in multiple colorectal tumors. *Int J Cancer* 1999;81:1–5.
17. Ward R, Meagher A, Tomlinson I, et al. Microsatellite instability and the clinicopathological features of sporadic colorectal cancer. *Gut* 2001;48:821–829.
18. Smyrk TC, Watson P, Kaul K, Lynch HT. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. *Cancer* 2001;91:2417–2422.
19. Prall F, Duhrkop T, Weirich V, et al. Prognostic role of CD8+ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. *Human Pathol* 2004;35:808–816.
20. Guda K, Upender MB, Belinsky G, et al. Carcinogen-induced colon tumors in mice are chromosomally stable and are characterized by low-level microsatellite instability. *Oncogene* 23:3813–3821.
21. Halford S, Sasieni P, Rowan A, et al. Low-level microsatellite instability occurs in most colorectal cancers and is a nonrandomly distributed quantitative trait. *Cancer Res* 2002;62:53–57.
22. Pan K, Liu W, Lu YY, et al. High throughput detection of microsatellite instability by denaturing high-performance liquid chromatography. *Hum Mutat* 2003;22:388–394.