Detection of Microsatellite Instability (MSI) and Loss of Heterozygosity (LOH) in Colorectal Tumors by Fluorescence-based Multiplex Microsatellite PCR

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Introduction

Colorectal cancer (CRC) is one of the most frequent causes of cancer deaths in the western world [1]. About 15% of CRC cases occur as dominantly inherited patterns [2]. The two best defined familial forms of CRC are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). The key characteristic of the HNPCC syndrome is microsatellite instability (MSI), which is caused by a defective mismatch repair (MMR) system [3, 4, 5, 6, 7]. As a consequence, errors that occur during replication of DNA cannot be repaired and lead to nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences.

In a recent study, we defined a set of five microsatellite markers for the highly sensitive specific detection of MSI in CRC [8]. This marker panel has been established as the "reference panel" recommended by the international guidelines for evaluation of microsatellite instability in CRC [9, 10]. In this article we report on the evaluation of a HNPCC Microsatellite Instability Test (Roche Molecular Biochemicals) based upon these markers as a routine HNPCC screening assay.

Material and methods

One hundred sixty-five primary colorectal adenocarcinomas were analysed for microsatellite instability (MSI). Genomic DNA was prepared from 5 µm-thick paraffin-embedded tissue sections according to the protocol supplied with the HNPCC Microsatellite Instability Test and in combination with the High Pure PCR Template Preparation Kit (both Roche Molecular Biochemicals). In brief, histological sections were deparaffinized by a one-hour incubated step at 65°C and subsequently incubation in xylene at room temperature (RT) for 2x 15 min. The sections were rehydrated in a series of decreasing ethanol concentrations (100%, 96%, 70%) for 2x 10 min each. After a 5 min incubation in H_2O, specimens were stained with hematoxylin/eosin. By microdissection, approximately 0.5 cm^2 tumor and normal tissue were prepared from each section. DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals). In brief, the microdissected tissue samples were each transferred to one 1.5 ml reaction tube containing 200 µl lysis buffer and 20-40 µl proteinase K from the High Pure PCR Template Preparation Kit. Incubation was performed for 3 h or overnight, both at 50°C with continuous shaking, until a clear solution was obtained. Any insoluble material was removed by centrifugation (13000 rpm, 5 min). The clear supernatant was used for DNA purification using the High Pure PCR Template Preparation Kit.

Multiplex PCR

The multiplex PCR was performed according to the HNPCC Microsatellite Instability Test. In brief, 1-4 µl (approx. 20-100 ng) purified genomic DNA from a total elution volume of 100 µl from normal and tumor tissue in each case, was mixed with 5 µl Multi Primer Mix (5x conc.), containing all 5 PCR primer pairs for the selected microsatellite markers, and 5 µl Enzyme Master Mix (5x conc.) from the HNPCC Microsatellite Instability Test. Water was added to make up to a final volume of 25 µl. PCR was performed using a Perkin Elmer 9600 thermocycler. The PCR conditions were as follows: after an initial 2 min denaturation step at 94°C, 30 amplification cycles were performed, each consisting of a 10 s step at 94°C, a 30 s step at 55°C, and a 30 s elongation step at 72°C. Amplification was completed with a final incubation step at 72°C for 7 min.

Detection of microsatellites

The amplified PCR products were analyzed using the automated ABI PRISM sequencer model 310 Genetic Analyzer (PE Applied Biosystems). In brief, 12 µl deionized formamide were combined with 0.5 µl GeneScan-350 [TAMRA] size standard (PE Applied Biosystems) and 1 µl PCR product in a Genetic Analyzer sample tube. The tubes were closed with Genetic Analyzer septa and, after short mixing (vortex), the samples were denatured in a heat block for 2 min at 90°C, chilled on ice, and spun briefly in a microcentrifuge in order to collect the contents. The samples were loaded on the ABI PRISM 310 Genetic Analyzer and the run started in accordance with the supplier's protocol.

MSI was defined by the presence of novel peaks, following the PCR amplification of tumor DNA, that were not present in normal DNA. A tumor was defined as high-MSI (MSI-H) if more than one of the five examined loci showed unequivocal instabilities (at least 40% unstable loci; 8, 10). Tumors were classified as microsatellite stable (MSS) if no microsatellite instability was found. Tumors were declared as low microsatellite instable (MSI-L) if only one marker showed instability (20% unstable loci).

Results and discussion

Of 165 primary colorectal adenocarcinomas under investigation, it was possible to amplify and analyze DNA from both tumor and normal tissue successfully in 148 cases (90%). Amplification in either tumor or normal tissue failed in the remaining 17 cases (10%). Nor could these samples be amplified in control reactions for these microsatellite markers using non-labeled primers in non-multiplex PCR, followed by silver staining for detection. This indicates that the target DNA in these samples had degraded due to prolonged or unsuitable fixation of the tissue samples before embedding in paraffin.

- 148 cases were successfully analyzed using the HNPCC Microsatellite Instability Test, whereas PCR with unlabeled primers and detection by silver staining failed in 12 of these 148 cases. This demonstrates that the sensitivity of the standard silver staining method is not sufficient for analyzing certain cases, in contrast with the HNPCC Microsatellite Instability Test, which succeeded even in cases where only minor amounts of intact genomic DNA could be isolated.

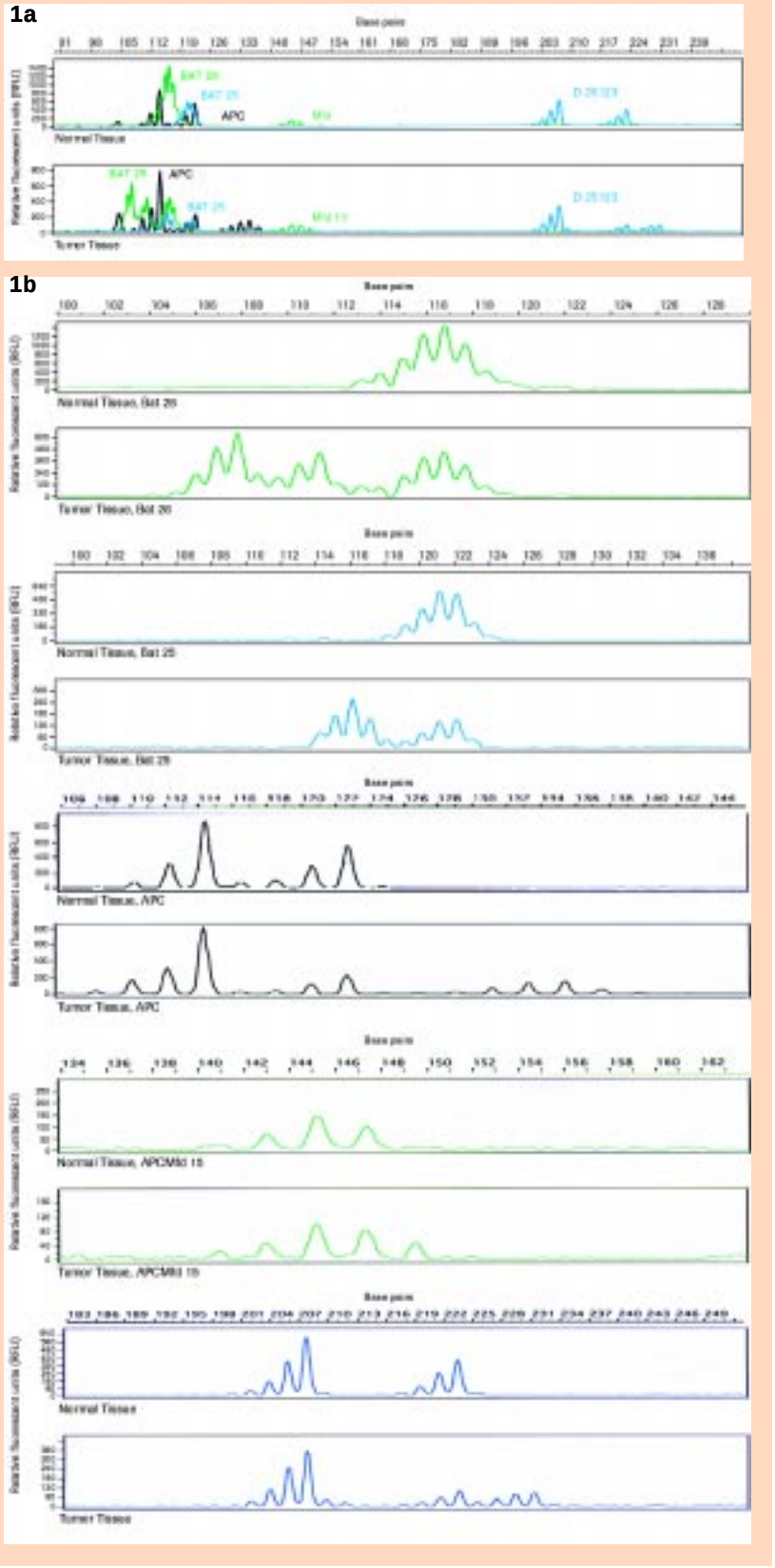

Figure 1: Fragment pattern of a MSI-H case showing microsatellite instability at all five loci analyzed.

Figure 1A: *Combined display of the fluorescence of all three different labels. Order in increasing fragment size: Bat 26 (green), Bat 25 (blue), APC (black), Mfd15 (green), D2S123 (blue).*

Figure 1B: *Detailed display of separate electropherograms of each label.*

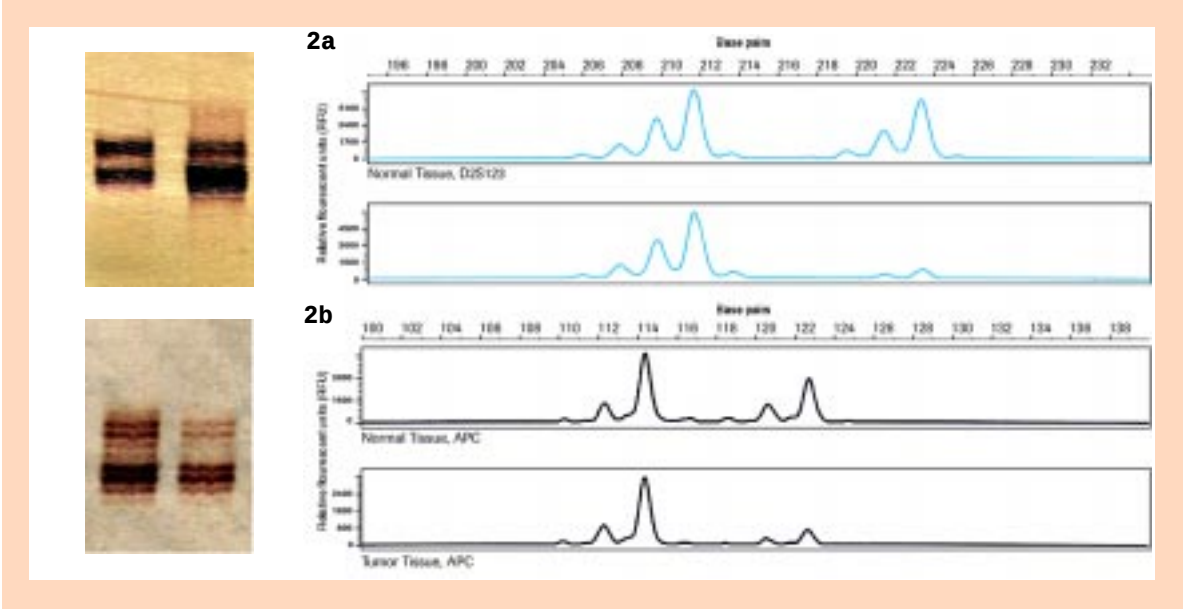

Figure 2: Comparison of data analysis using monoplex PCR with silver staining and multiplex PCR with the HNPCC Microsatellite Instability Test, respectively. *Data for an LOH case with two loci (D2S123 and APC) showing loss of heterozygosity are displayed.* **Figure 2A:** *D2S123 locus. An LOH value of 6.6 was calculated based on the height of the major peak of each allele being displayed in the GeneScan Analysis software.* **Figure 2B:** *APC locus. An LOH value of 3.0 was calculated based on the height of the major peak of each allele being displayed in the GeneScan Analysis software.*

Of the 148 cases analyzed successfully with the Microsatellite Instability Test, high microsatellite instability (MSI-H) was found in 18 cases (11%) and low microsatellite instability (MSI-L) in 8 cases (5%). The MSI status could easily be detected by alterations that were interpreted unambiguously.

Figure 1A shows the fragment pattern of all five microsatellite loci being investigated by multiplex PCR in a case of MSI-H. A typical phenomenon of microsatellite amplification was the generation of several peaks ("stutter bands") per allele during PCR amplification as a consequence of polymerase slippage. Since the HNPCC Microsatellite Instability Test has the advantage of using different colors to resolve PCR products of comparable size from different loci, all markers could be identified and analyzed unambiguously by different colors and/or different sizes. Although BAT25 (blue), BAT26 (green), and APC (black) fragment patterns display the same size range of 100-130 bp, these markers can be distinguished from each other by the different colors. Mfd15 (green) and D2S123 (blue) are markers with unique size ranges of 140-160 bp and 200-230 bp, respectively. A more detailed and easier analysis of the fragment patterns is possible when each color is displayed separately (**Figure 1B**).

- As expected from the results of previous studies [8], all analyzed MSI-H cases showed clear instability in at least two loci. Nearly 70% (n=12) of all MSI-H tumors (n=18) showed MSI in each of the five markers. This shows the simplicity of dividing tumors into MSS and MSI types after multiplex microsatellite PCR.

- In addition to MSI, loss of heterozygosity (LOH) can be detected in the cases analyzed with the HNPCC Microsatellite Instability Test. In contrast to MSI, where new alleles are generated, LOH is correlated with the loss of a wild type allele in tumor DNA (i.e., in a certain subpopulation of analyzed cells). Therefore, LOH detection is especially problematic if tumor cells are not accurately separated from adjacent normal cells by microdissection. A major advantage of the fluorescence-based HNPCC Microsatellite Instability Test is that the loss of alleles can be determined precisely by calculation of the ratio of the peak heights of normal and tumor alleles according to the following formula:

$$\text{LOH} = \frac{(\text{peak height of normal allele 2}) / (\text{peak height of normal allele 1})}{(\text{peak height of tumor allele 2}) / (\text{peak height of tumor allele 1})}$$

If microdissection is accurately performed, LOH is strongly indicated by ratios less than 0.5 or higher than 2.0. LOH analysis should generally be performed by methods allowing quantification of both size and extent of amplified products. As fluorescence-based PCR using the HNPCC Microsatellite Instability Test

optimally fulfills this criteria, it is clearly the method of choice, particularly when compared to non-quantitative methods such as analysis based on silver staining following denaturing polyacrylamide gel electrophoresis.

Of the colorectal adenocarcinomas analyzed, LOH was detected in 47% (n=77) of cases. Most LOHs were found at the APC locus (32%), while significantly fewer LOHs were detected at the Mfd15 locus (8%) and the D2S123 locus (5%). Interestingly, there was no case with both MSI and LOH.

■ **Figure 2** shows a comparison of silver staining analysis and fluorescence-based quantitative analysis using the HNPCC Microsatellite Instability Test. In fluorescence-based PCR, the ratio of fluorescence intensities from normal and tumor alleles can be determined precisely, thereby enabling validation of the LOH status. Early saturation of silver staining and low resolution of "stutter" bands in manual gel electrophoresis make the determination of the LOH status more difficult.

Conclusion

We analyzed 165 colorectal tumors in order to determine the microsatellite instability status using the HNPCC Microsatellite Instability Test. This kit allows the fluorescence-based simultaneous amplification of five microsatellite markers (i.e., BAT25, BAT26, D5S346 [APC\, D2S123, and Mfd15 [D17S250])*. These markers have been proven capable of detecting MSI sensitively and specifically [8], and are recommended as the first-choice panel for defining MSI status by the American Joint Commission on Cancer [10], the International Collaborative Group on HNPCC [10], and the HNPCC Cancer Study Group in Germany. Both MSI and LOH status can clearly be defined by use of the HNPCC Microsatellite Instability Test and subsequent automatic fragment analysis using an ABI PRISM 310 Genetic Analyzer. Thus, this assay is ideally suited to the screening of colorectal tumors for selection of HNPCC candidates before examination of mutations in mismatch repair genes in the germline DNA.

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* These microsatellite loci (BAT25, BAT26, D5S346 [APC], D17S250 [Mfd15CA], and D2S123) have been recommended as a "reference" panel by the American Joint Commission on Cancer, the International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (HNPCC), and the HNPCC Cancer Study Group in Germany. This panel should be used for microsatellite instability status determination in further research studies of suspected HNPCC tumors (Bethesda guidelines, NCI workshop 1996 [9], and NCI workshop on microsatellite instability, Dec. 1997[10])

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