

Label-free analysis of microsatellite instability in colorectal carcinoma by on-chip electrophoresis

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Abstract

Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system and occurs frequently in various types of cancer. Since sporadic MSI are associated with approximately 10 to 15% of colorectal, gastric or endometrial carcinoma and impact clinical prognosis, MSI analysis is an important tool in clinical research and molecular diagnostics. Given that conventional techniques used for MSI detection - e.g. polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis - turned out to be laborious or expensive, this study aimed to develop a simple and efficient procedure of MSI detection. Forty cases were selected out of a panel of 150 patients with colon carcinoma. Of the forty cases selected, 26 cases had no MSI, while 14 cases had a positive MSI. Amplicons derived from areas of non-tumor (N) and tumor (T) were analyzed by microfluidic based on-chip electrophoresis on the Agilent 2100 bioanalyzer. The results presenting a pattern of five microsatellite loci were compared with the findings obtained by fluorochrome-associated PAGE technology. In all cases, label-free microfluidic separation of the PCR amplicons resulted in highly resolved, distinct patterns for each of the five microsatellite loci. Detection of MSI was demonstrated by microsatellite loci-associated, well defined deviations in the electropherogram profiles of tumor and non-tumor material, in agreement with the classification of the MSI cases performed by conventional technology (95% concordance rate). The presented data demonstrate that microfluidic chip technology is a simple, reliable and robust technology for MSI detection, which allows label-free analyses of microsatellite amplicons within 30 minutes.



Introduction

Microsatellites are short tandem repeat sequences, located in non-coding genomic DNA. Presumably up to 100,000 microsatellite loci are widely distributed throughout the human genome, being unique to each individual. Alterations in these microsatellite loci are caused by base pair mismatches frequently appearing during the DNA replication process that are not repaired by the proofreading DNA mismatch repair process because of enzymatic failure. Therefore, a defect in the mismatch repair results in microsatellite instability (MSI) which manifests as a deviant pattern of the tandem repeats in the respective loci (for reviews see [1-3]). The neoplastic pathway of MSI has been studied best in the setting of colorectal carcinoma (CRC). Most CRC cases are caused by a well established step-by-step course from adenoma to carcinoma based on failure of tumor suppressor genes, DNA hypomethylation or loss of heterozygosity (LOH), described by Fearon and Vogelstein in 1990 [4]. In 10-20% of CRC cases, however, colon carcinogenesis is due to genomic defects in the mismatch repair machinery. In addition to their sporadic occurrence in CRC or in other carcinoma, a defective DNA repair due to germ-line mutations has been linked to hereditary non-polyposis colorectal carcinoma syndrome (HNPCC). In HNPCC or in sporadic CRC, the mutations and promoter hypomethylation occur mainly in the genes of the *hMLH1* and *hMSH2* resulting in loss of their expression [1, 2]. Expression loss indicates that the failure of the repair system is the main cause of genomic instability and carcinogenesis, both associated with better prognosis and a higher therapeutical achievement [5, 6].

Immunostaining (as shown in Figure 1) which has a 70 to 90 % sensitivity, is insufficient for prognostic diagnosis [7], implicating that additional direct molecular testing of the MSI status is of great importance. Many different microsatellite loci have been used to identify MSI in tumors. In a broad study by Dietmaier et al. in which 32 loci were tested for diagnostic usability, five loci were shown to be at least necessary for MSI diagnosis. In an attempt to provide uniformity in clinical diagnosis an international meeting at National Cancer Institute (NCI) picked up the results from different studies and research trials and recommended primary microsatellite markers for use in CRC MSI testing in both clinical and research settings. The recommended MSI set comprises the microsatellite loci, Bat25, Bat26, D2S123, D5S346, and D17S250 [8]. Tumors with MSI at two or more of the recommended loci are defined as high frequency MSI (MSI-H), tumors with MSI at only one locus are defined as low frequent MSI (MSI-L), and tumors with no instability are referred as stable (MSS).

While many different technologies have demonstrated their ability to detect MSI, many of them are very complex and laborious. Recently, Banerjea et al. demonstrated that the microsatellite locus BAT26 can be microfluidically analyzed with the Agilent 2100 bioanalyzer [9]. This application note presents that data, demonstrating how the 2100 bioanalyzer can be used as an analytical platform for MSI detection at all five loci known as the Bethesda panel.

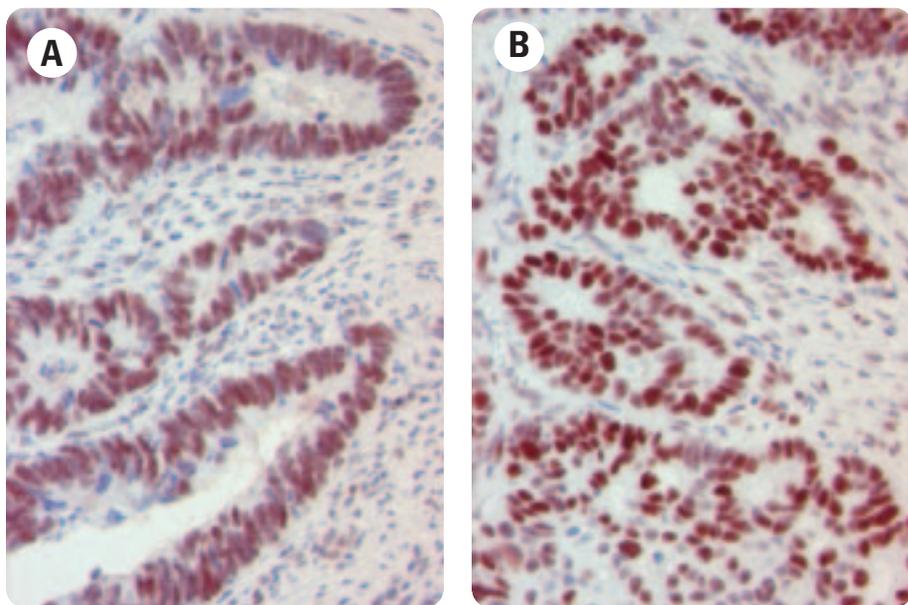


Figure 1: Colon carcinoma immunostained for the mismatch repair enzymes hMLH1 (A) or hMSH2 (B).

In both cases, positive immunostaining (red) shows nuclear localization of the respective repair enzyme.

Material and Methods:

Tissue materials

Tissues, fixed in formalin and embedded in paraffin, were collected at the Institute for Pathology of the University Clinic Cologne (GER). Forty (15 MSI positive and 25 MSI negative) cases of a panel of 150 colorectal carcinoma, prediagnosed by PCR assays according to the Bethesda guidelines [8], followed by PAGE resolution, were chosen to estimate the usage of on-chip electrophoresis for MSI diagnosis. Using two 3 µm-sections cut from paraffin blocks, deparaffinised and stained with hematoxylin, two pathologists evaluated morphology and subsequently tumor and non-tumor areas of tissues were taken by manual microdissection for further analysis.

DNA extraction and PCR

The deparaffinised sections were lysed overnight at 56°C by proteinase K digestion (500 µg/ml proteinase K, 5 mM EDTA, 20 mM Tris pH 8.0). Then, DNA was extracted from lysates using the DNA extraction kit from Qiagen (Hilden, GER) according to the manufacturer's instructions. For amplification of the microsatellite loci, which are recommended by the Bethesda guidelines (Bat25, Bat26, D2S123, D5S346 and D17S250), primers shown in table 1 were used [8]. 2 µl of the 50 µl-DNA extracts were applied in the Multiplex-PCR approach of Qiagen (Hilden, GER) according to manufacturer's instructions using an annealing T_m of 60°C. Amplification of the Bat25 and the D2S123 loci, and of the APC and the MFD15 loci were combined in duplex assays.

MSI analysis by fluorescence-linked PCR

Fluorescence-linked PCR was carried out to compare the conventional MSI analysis method to the results from the *lab-on-a-chip* technology [10, 11]. The a-primer (primer MS1a-MS5a) of each primer set was IRD-800-fluorescence end-labeled and used for PCR amplification. Fluorescent IRD-800 amplicons were resolved by electrophoresis using denaturing 6 %-polyacrylamide gels and analyzed by the LI-COR DNA-Analyzer Gene Reader 4200.

MSI analysis by on-chip electrophoresis on the Agilent 2100 bioanalyzer

For the separation of microsatellite PCR products we used DNA 1000 LabChip Kits and the Agilent 2100 bioanalyzer according to the manufacturer's instructions. In brief, the chips were prepared with gel-dye mix and then pressurized. Marker solution and DNA 1000 ladder were added. 1 µl of each PCR reaction was pipetted into one of the twelve sample wells of the prepared chip. After vortexing, the chip was placed in the Agilent 2100 bioanalyzer and run using the DNA 1000 assay. Electrophoresis of the 12 samples took 30 - 40 minutes. Fragment analysis was carried out using Agilent's 2100 expert software. To identify MSI in the colorectal carcinoma patients an overlay of two electropherograms was used to compare PCR patterns derived from tumor and non-tumor tissues. Differences in the PCR pattern of the overlaid electropherograms were evaluated as MSI. Per patient, three overlays were used to identify instabilities in microsatellite loci Bat25 and D2S123 (MS1 and MS3), D5S346 and D17S250 (MS4 and MS5) and Bat26 (MS2), respectively (table1).

primer name	microsatellite	locus	sequence	length of amplicates [bp]
1-a	BAT 25	1p13.1	TCG CCT CCA AGA ATG TAA GT	around 125
1-b	BAT 25	1p13.1	TCT GCA TTT TAA CTA TGG CTC	around 125
2-a	BAT 26	2p	TGA CTA CTT TTG ACT TCA GCC	around 120
2-b	BAT 26	2p	AAC CAT TCA ACA TTT TTA ACC C	around 120
3-a	D2S 123	2p16.	AAA CAG GAT GCC TGC CTT TA	210-230
3-b	D2S 123	2p16.	GGA CTT TCC ACC TAT GGG AC	210-230
4-a	APC-D5S346	5q21/22	ACT CAC TCT AGT GAT AAA TCG	110-130
4-b	APC-D5S346	5q21/22	AGC AGA TAA GAC AGT ATT ACT AGT T	110-130
5-a	MFd15 D17S250	17q11.2-q12	GGA AGA ATC AAA TAG ACA AT	150-160
5-b	MFd15 D17S250	17q11.2-q12	GCT GGC CAT ATA TAT ATT TAA ACC	150-160

Table 1: Primers and characteristics of microsatellite loci analyzed.

Results and Discussion

High resolution of the amplified microsatellite loci by the 2100 bioanalyzer

All five microsatellite loci, Bat25, Bat26, D2S123, D5S346, and D17S250, amplified by label-free PCR, could be well resolved by microfluidic-based on-chip electrophoresis on the 2100 bioanalyzer. In each case, 1 µl of the amplicons were required for analyses and the electrophoresis of 12 samples took only 30 minutes. Since electropherograms distinctly revealed the individual pattern of the five microsatellite loci (Bethesda panel), which are recommended for CRC analyses by the conference at NCI [8], the resolution should also be sufficient to prove the MSI status. Therefore, we analyzed tumorous and non-tumorous DNA from colon cancer patients pre-classified by fluorescence-linked PCR.

The overlay of two corresponding electropherograms derived from tumor material and from non-tumor material of one patient allowed the comparison of the loci-specific patterns. All microsatellite loci are represented in a distinct distribution. Since the electropherogram pattern of microsatellite amplicons from tumor DNA exactly corresponds to the pattern obtained from non-tumor DNA, the microsatellite status of this case was declared as stable (MSS). Figure 2 shows an overlay of the electropherograms of the microsatellite loci Bat25 and D2S123 (Fig. 2a). In Fig. 2a both patterns show an exact match indicating stability of both loci in this patient. In contrast, the overlaid electropherograms derived from another patient show highly variable patterns of the same loci in tumorous versus non-tumorous DNA the overlaid electropherograms derived from another patient. These distinguishable results allow a first classification of the five different MSI loci based on differences in the PCR fragment pattern.

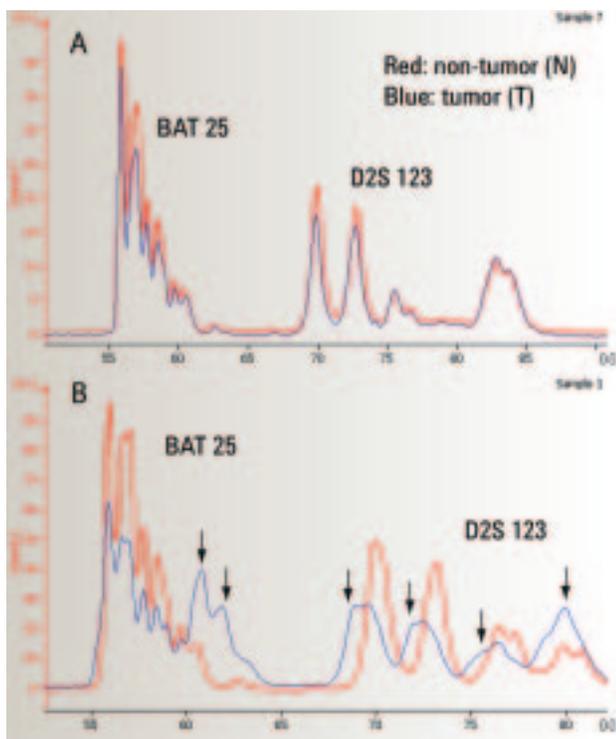


Figure 2: Overlay of electropherograms to classify the status of microsatellite loci BAT25 and D2S123.

The electropherogram represents the pattern of the separated PCR fragments (MS1 and MS3). No MSI could be determined due to the perfectly matching pattern of both electropherograms (A). Significant differences in the pattern of two overlaid electropherograms derived from tumor and non-tumor material of the same patient strongly point to MSI status (B).

Sample	Prediagnosis by flu- PAGE	MSI analysis by on-chip electrophoresis
1	MSI-H	MSI-H
2	MSI-H	MSI-H
3	MSI-H	MSI-H
4	MSI-H	MSI-H
5	MSI-H	MSI-H
6	MSI-H	MSI-H
7	MSI-H	MSI-H
8	MSI-H	MSI-H
9	MSI-H	MSI-H
10	MSI-H	MSI-H
11	MSI-H	MSI-H
12	MSI-H	MSI-H
13	MSI-L	MSI-H
14	MSI-L	MSI-L
15	MSI-S	MSS
16	MSS	MSI-L
17	MSS	MSS
18	MSS	MSS
19	MSS	MSS
20-40	MSS	MSS

Table 2: Comparison of MSI states of 40 colorectal carcinoma patients based on the results obtained by PAGE and on-chip electrophoresis on the bioanalyzer.

(MSS) Stability in all microsatellite loci; (MSI-L) Instability in one microsatellite locus; (MSI-H) Instability in more than one microsatellite loci. The comparison of MSI-detection by PAGE and on-chip electrophoresis resulted in a 95% concordance rate. Deviating results were observed in 2 out of 40 samples.

High reliability of MSI analysis based on on-chip electrophoresis

Most of the conventional methods used for MSI detection depend on the labeling of PCR products with either fluorochromes or radioactive nucleotides. In both approaches the detection is time-consuming and dependent upon specialized equipment such as automated laser-based sequences or specialized laboratories. Alternatively, the pattern of the label-free amplicons can be visualized by Maldi TOF [12] or by silver staining, which is unfortunately a time intensive procedure. In comparison to these approaches, the analysis of microsatellites by on-chip electrophoresis is a straightforward method [9].

To prove the reliability of this technique, we compared the results obtained by the 2100 bioanalyzer with the fluorescence-based PAGE resolution of labeled PCR amplicons. The amplified microsatellite loci of 40 colon carcinoma samples were separated and the results were visually inspected and classified. Comparing the results of both methods, the classification of the MSI status shows a 95% concordance rate (table 2). Demonstrating this

technology, Fig. 3 presents the results of a patient with stable microsatellite loci (MSS), whereas Fig. 4 shows differences in the PCR pattern of three MSI loci classifying the tumor status as MSI-H. However, the MSI status of two cases disagreed and the MSI analyses performed by the 2100 bioanalyzer detected an additional locus of instability in each case. In particular, when PCR-amplification by Taq-polymerase results in stutter bands, interpretation of the microsatellite pattern might be difficult. In such cases, additional clinical information or data concerning the immunostaining of repair enzymes is helpful for the decision about further clinical proceeding. In one discrepant case, negative immunostaining of the MLH1 repair enzyme supported the MSI status proposed by the microfluidic-based electrophoresis of the 2100 bioanalyzer.

In summary, on-chip electrophoresis is a reliable procedure fulfilling the Bethesda criteria for MSI detection while saving time and technical efforts.

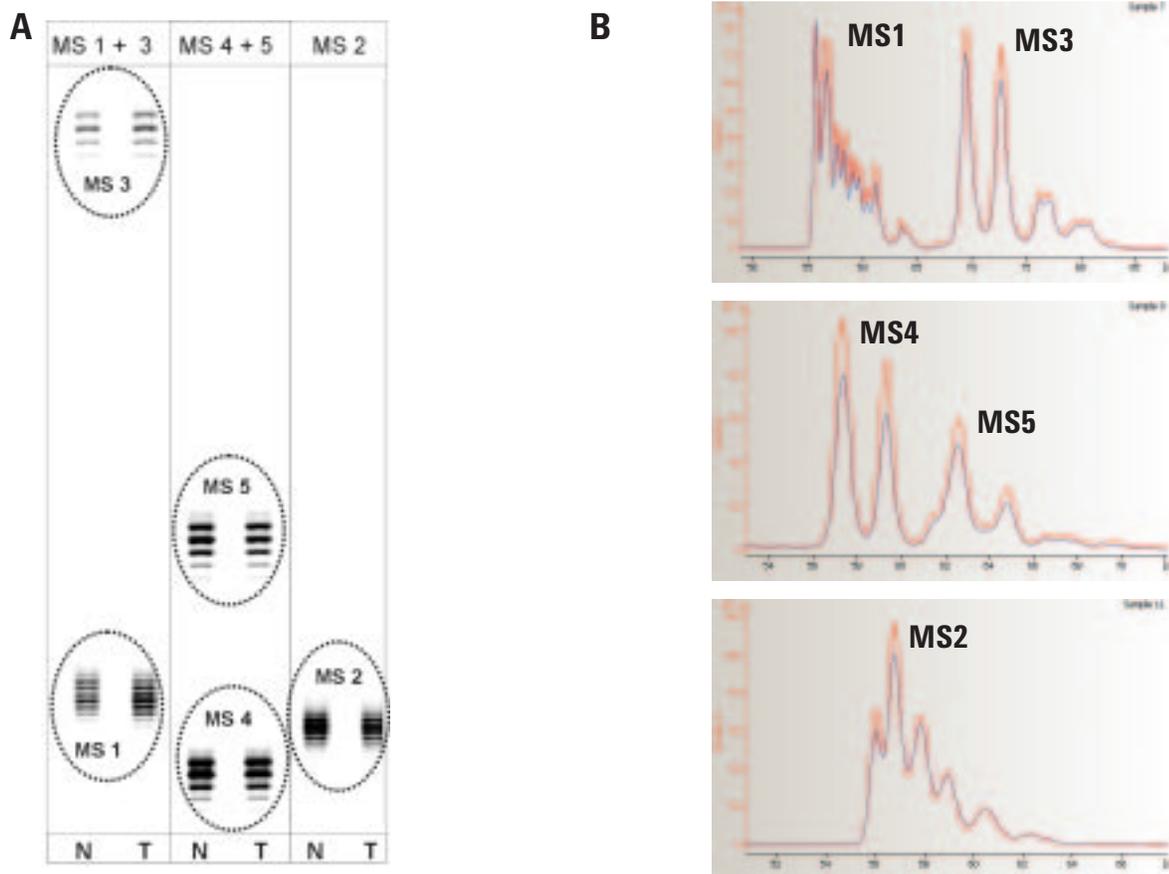


Figure 3: Comparative analysis of five microsatellite loci with MSS status by PAGE and on-chip electrophoresis.

Fluorescently labeled PCR products of all five microsatellite loci (MS1 + MS3; MS4 + MS5; MS2) of one colorectal carcinoma patient were separated by PAGE and on-chip electrophoresis. Similar results could be obtained by both techniques clearly pointing to a MSS patient status.

3a) Fluorescently labeled PCR products of all five microsatellite loci (MS1 + MS3; MS4 + MS5; MS2) were separated by PAGE using a denaturing 6% polyacrylamide gel. The patterns derived from normal tissue (N) and separated tumorous tissues (T) are perfectly matching.

3b) Microfluidic separation of unlabeled PCR products on the Agilent 2100 bioanalyzer. The figure shows three electropherograms each of with presenting an overlay of the PCR pattern derived from normal tissue and tumorous tissue. The patterns of all electropherogram overlays are perfectly matching similar to the results obtained by PAGE.

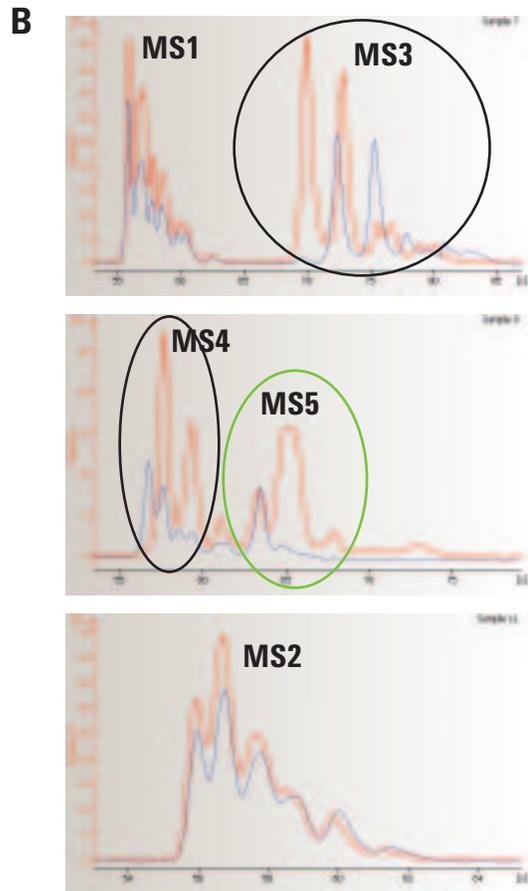
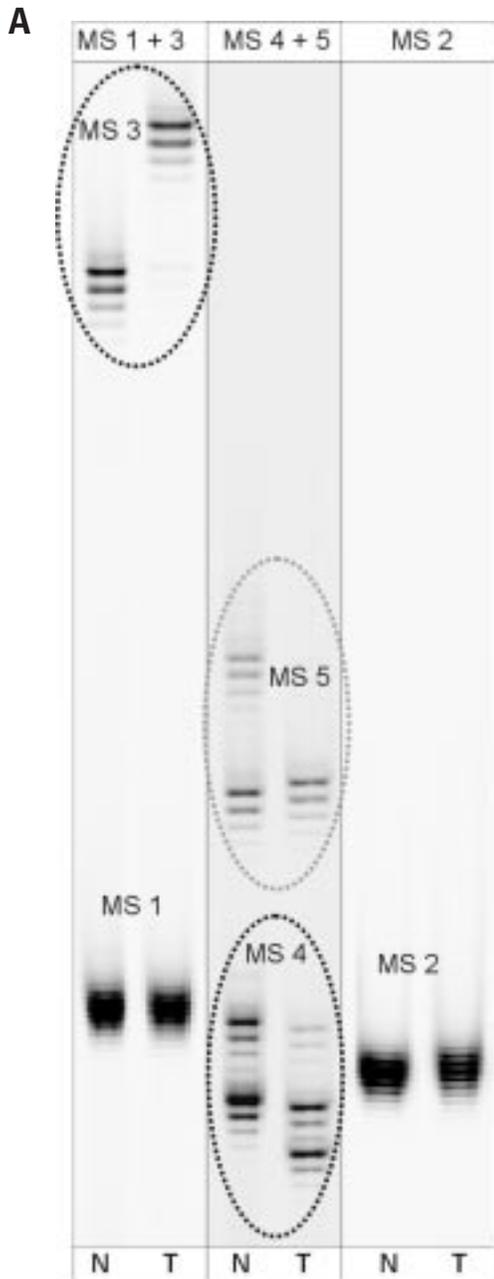


Figure 4: Comparative analysis of five microsatellite loci with MSI status by PAGE and on-chip electrophoresis.

Fluorescently labeled PCR products of all five microsatellite loci (MS1 + MS3; MS4 + MS5; MS2) of one colorectal carcinoma patient were separated by PAGE and on-chip electrophoresis. Both techniques show significant differences in the microsatellite loci MS3, MS4 and MS5 clearly indicating a MSI patient status. Circles highlight differences in the pattern of the amplified microsatellite patterns. 4a) Fluorescently labeled PCR products of all five microsatellite loci (MS1 + MS3; MS4 + MS5; MS2) were separated by PAGE using a denaturing 6% polyacrylamide gel.

4b) Microfluidic separation of unlabeled PCR products on the Agilent 2100 bioanalyzer. The figure shows three electropherograms, each with an overlay of the PCR pattern derived from normal tissue and tumorous tissues. The electropherogram overlays show significant deviations in the electrophoretic patterns MS3 - 5.

Conclusions:

In this study we demonstrated that microfluidic MSI elucidation is a highly timesaving procedure, with the added benefit that it is not dependent on a labeling. The results of the MSI detection were comparable to conventional techniques showing that the quality of analysis was maintained, while providing a superior edge in terms of speed, usability and data management.

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