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Jürgen Schlegel · Tina Bocker · Hubert Zirngibl
Ferdinand Hofstädter · Josef Rüschoff

Detection of microsatellite instability in human colorectal carcinomas using a non-radioactive PCR-based screening technique

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Abstract The aim of the present study was to establish a rapid, non-radioactive screening method for the detection of microsatellite instability (MIN). MIN is the primary characteristic of the mutator phenotype in tumours constituting hereditary non-polyposis colon cancers (HNPCC). We investigated 30 patients suffering from colorectal cancer using a non-radioactive PCR-based technique. MIN was present in 7 of 30 (23%) of the cases. There was a statistically significant correlation between MIN and localization of the tumour. Five of 7 (72%) tumours with MIN but only 4 of 23 (17%) tumours without MIN were localized in the proximal colon ($P<0.01$). There was a tendency to higher MIN frequency in tumours of patients with familial clustering of cancers. However, this was statistically not significant ($P>0.05$). In addition, no correlation between MIN and tumour grade and stage was found. For the investigations in the present study we used a non-radioactive PCR-based method followed by denaturating polyacrylamide gel electrophoresis and silver staining. This method is highly sensitive and reproducible. Thus, PCR-based analysis using a non-radioactive staining technique represents a comprehensive tool for MIN screening in diagnostic pathology.

Key words Colon cancer · Hereditary non-polyposis colon cancer · Microsatellite · Polymerase chain reaction

Introduction

Genomic instability due to spontaneous errors in DNA replication has been implicated in neoplastic transformation and as a mechanism to explain chromosomal alterations in cancer cells [4]. It has been shown that gene amplifications and deletions of putative tumour suppressor genes accumulate during carcinogenesis of many human malignant tumours [5]. However, the mechanisms leading to genomic instability are poorly understood. Widespread alterations in simple repeated sequences have been found in several tumour types [7, 11, 13, 16]. Such alterations are most easily observed as changes in the lengths of microsatellite sequences between tumour DNA and DNA from non-neoplastic tissue of the same individual. This type of genomic sequence consists of one- to six-nucleotide motifs, which are tandemly repeated numerous times. The dinucleotide repeat $(CA)_n$ constitutes one of the most abundant classes of repetitive DNA sequences in the human genome, with an estimated occurrence of 10^5 loci [21]. Recently, it has been shown that microsatellite instability (MIN), that is expansion or reduction of the number of dinucleotide repeats, is the major characteristic of the mutator phenotype in tumours constituting hereditary non-polyposis colon cancers (HNPCC) [1, 8, 17]. In these tumours MIN is due to aberrant DNA repair enzymes. Mutations have been reported in the human *mut*S and *mut*L homologous genes *hMSH2* and *hMLH* in patients suffering from HNPCC [6, 12]. In some HNPCC families there is also a linkage to the chromosomal locations of the two genes on chromosome 2 and 3, respectively [14]. Thus, detection of MIN in colorectal tumours would be of interest in diagnostic pathology and may allow a risk assessment for disease. However, in all investigations regarding MIN radioactive detection systems were used, with clear limitations for routine diagnosis since they require special laboratory equipment and are expensive and time consuming. Here we report a rapid, non-radioactive screening method which allows the detection of MIN in diagnostic pathology.

J. Schlegel (✉) · T. Bocker · F. Hofstädter · J. Rüschoff
Universitätsklinik, Institut für Pathologie, D-93042 Regensburg, Germany

H. Zirngabel
Chirurgische Universitätsklinik, Universität Regensburg, Germany

Materials and methods

Biopsy specimens of surgically removed colorectal carcinomas from 30 consecutive patients operated at the Department of Surgery, University of Regensburg (20 male, 10 female) were snap-

Table 1 Comparison of microsatellite instability and clinicopathological data

	n	Number of loci showing MIN			*P*
		0	1	≥2	
Total	30	23	3	4	
Age and Sex					
Male	20	17	2	1	
Female	10	6	1	3	n.s.
Mean age	62.6	62.4	52.0	67.5	n.s.
(standard deviation)	(12.8)	(10.2)	(30.6)	(17.4)	
Localization					
Proximal to splenic flexure	9	4	1	4	
Distal to splenic flexure	21	19	2	0	<0.01
Familial history					
0	22	19	2	1	
1/2[a]	3	2	0	1	
3/4[b]	4	2	0	2	
FAP	1	0	1	0	n.s.
Tumour grade					
G1	1	1	0	0	
G2	25	19	2	4	
G3	4	3	1	0	n.s.
pTNM					
T1	2	2	0	0	
T2	7	5	1	1	
T3	12	10	1	1	
T4	9	6	1	2	n.s.
N0	21	17	2	2	
N1	5	4	0	1	n.s.
N2-4	4	2	1	1	
M1	4	3	0	1	

[a] Amsterdam criteria [19],
[b] according to Lothe et al. [10], as described in " Materials and Methods"

frozen in liquid nitrogen immediately after resection and stored at –80°C until analysis. In addition, formalin-fixed and paraffin-embedded material from 5 tumours was used to compare the results with those obtained from frozen material. The patients' mean age was 62 years (range: 20–84 years). Tumour localization, grading and staging are summarized in Table 1. Whole blood from the same patient was collected as a source of non-neoplastic leucocyte DNA.

Information concerning cancer in first- and second-degree relatives has been obtained for all patients investigated in the present study. On the basis of these data different subgroups were defined according to Lothe et al. [10]. Briefly, subgroups 1 and 2 consisted of patients suffering from Lynch I and II syndrome, respectively, in agreement with the Amsterdam criteria for HNPCC [19]. Subgroups 3 and 4 were defined by the criteria of Lothe et al. [10] as 2 colorectal cancer patients, first-degree relatives, 1 being diagnosed before age 50 (group 3), and 3 cancer patients, first- or second-degree relatives, suffering from colon carcinoma, upper gastrointestinal tract cancer or endometrial cancer (group 4). In addition, the tumour of 1 patient with familial adenomatous polyposis (FAP) was investigated in the present study (Table 1).

For PCR amplification, genomic DNA was isolated by standard methods [15]. For DNA isolation from paraffin-embedded material a modified protocol was used [9]. Extracted DNA was examined for genetic alterations at five separate $(CA)_n$ repeats localized on chromosome 5q (APC), 10 (D10S89, Mfd28), 15q (635/636), 17p (p53), and 18q (D18S34, Mfd26). Oligonucleotides used as primers for the PCR were APC [7], Mfd28 [22], 635/636 [7], P53 [7], and Mfd26 [23]. Primer sequences are summarized in Table 2.

PCR amplifications were performed with 100 ng purified genomic DNA in a reaction mixture containing 0.3 µM each primer, 0.2 mM each dNTP, 50 mM KCl, 10 mM Tris-Cl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 1 mM DTT, 2.5 U taq polymerase (Promega, Madison, Wis.) in a final volume of 50 µl. In addition, PCR amplification for two $(CA)_n$ loci (Mfd28, Mfd26) using matched genomic DNA from 8 patients was performed in the presence of 5 µCi [^{35}S]-dATP each reaction. The PCR reactions were carried out in a MJ Research Thermocycler (MJ Research, Watertown, Mass.) for 30 cycles of amplification (94°C, 1 min, 55–64°C 1 min, dependent on primer, 72°C, 2 min; first cycle 94°C, 5 min; last cycle 72°C, 10 min).

In polyacrylamide gel electrophoresis, 2.5 µl of each reaction was added to 5 µl of formamide-dye mixture and subsequently run

Table 2 DNA sequences of the primers used in the present study

APC [7]	
Sense	5′-ACT CAC TCT AGT GAT AAA TCG -3′
Antisense	5′-AGC AGA TAA GAC AGT ATT ACT AGT T -3′
Mfd28 [22]	
Sense	5′-AAC ACT AGT GAC ATT ATT TTC A -3′
Antisense	5′-AGC TAG GCC TGA AGG CTT CT -3′
635/636 [7]	
Sense	5′-TTG ACC TGA ATG CAC TGT GA -3′
Antisense	5′-TTC CAT ACC TGG GAA CGA GT -3′
P53 [7]	
Sense	5′-AGG GAT ACT ATT CAG CCC GAG GTG -3′
Antisense	5′-ACT GCC ACT CCT TGC CCC ATT C -3′
Mfd26 [23]	
Sense	5′-CAG AAA ATT CTC TCT GGC TA -3′
Antisense	5′-CTC ATG TTC CTG GCA AGA AT -3′

on 6.7% polyacrylamide (acrylamide/bis-acrylamide 19:1)/50% urea gels (3 h, 2000 V, 50°C) in a Sequigene sequencing gel chamber (BioRad, Hercules. Calif.). Silver staining was performed according to the method of Budowle et al. [3] in a staining chamber previously described by Vogt et al. [20]. Briefly, gels were placed in 10% ethanol (5 min), oxidized in 1% nitric acid (3 min), placed in 0.012 M silver nitrate (20 min) and reduced in a solution containing 0.28 M sodium carbonate and 0.019% formalin until bands developed. Reduction was stopped with 10% glacial acetic acid (2 min) and gels were placed in distilled water (2 min). Subsequently gels were dried in vacuum and stored for permanent record. For autoradiography of radioactive PCR amplifications, gels were dried in vacuum and exposed to Kodak XAR-5 films overnight at room temperature.

Differences in the frequency distribution between two or more samples were tested using Fisher's exact test. A Mann–Whitney test (U-test) was performed as a non-parametric test for differences in the central tendency between two samples. A Kruskal–Wallis test (H-test) was used for more than two samples.

Results

Investigation of MIN in colorectal tumours was performed by PCR amplification of five different $(CA)_n$ repeats. Subsequently PCR fragments were resolved by denaturating polyacrylamide (PAA) gel electrophoresis and visualized with silver staining. For the staining procedure a specific frame was used that allows an easy handling of large PAA gels during multiple incubation and washing steps without damaging. Using this approach we detected PCR fragments within 45 min after termination of the gel run. Silver staining usually revealed two bands per allele and a variable number of shadow bands due to taq polymerase slippery (Fig. 1). The size of the PCR fragments corresponded with those obtained by radioactive labelling (Fig. 2). Identical results were obtained when DNA from frozen and paraffin-embedded material of the same patient was used (data not shown).

Using matched genomic DNA from 30 colon carcinomas and blood of the same patients we observed MIN in 7 of the 30 tumours (23%). MIN consisted in expansion and contraction of $(CA)_n$ repeats. Of these 7 tumours, 1 showed MIN at all five loci investigated in the present study, 3 tumours at three or four loci, and 3 tumours at one $(CA)_n$ repeat. Among the 4 tumours showing MIN at more than one locus, 3 exhibited the same type of MIN, that is expansion or contraction, at all loci whereas only 1 tumour showed different types of MIN. In addition, we also detected LOH of chromosome 5q (APC), 17p (p53) and 18q (DCC) in some tumours using this approach. As expected, there was no correlation between LOH and MIN.

There was no statistically significant correlation between MIN and tumour grade or stage. The 7 patients suffering from colorectal carcinomas showing MIN included 3 who had a family history of cancer with at least three relatives with carcinomas. Tumours from these patients exhibited MIN at more than two of the loci investigated in the present study. However, these results were not statistically significant ($P>0.05$ in Fisher's exact test). One case with familial adenomatous polyposis (FAP)

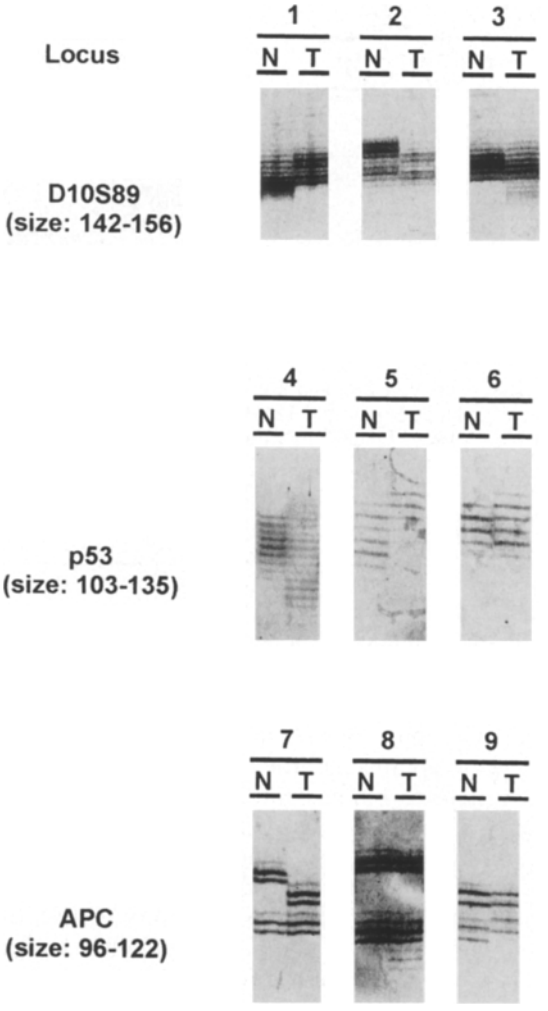

Fig. 1 Silver-stained denaturating polyacrylamide gels of PCR ampification from colorectal carcinomas (*N* normal DNA from lymphocytes, *T* tumour-DNA) using primer pairs for three different microsatellite loci demonstrating microsatellite instability (MIN) in tumour DNA. MIN consists in new alleles, i.e. contraction (No. 2,3,4,7,8) and/or expansion (No. 1,5,6,9) of PCR fragments. Samples 3 and 8 exhibit a shorter allele in addition to the normal PCR fragments (loci and sizes indicated in the *left column*)

demonstrated LOH5q and MIN at one locus. Three patients suffering from tumours containing MIN had no family history, whereas 4 patients with family history for carcinomas showed no MIN phenotype. There was a statistically significant correlation between MIN and tumour localization ($P<0.01$). Of 7 tumours containing MIN, 5 (72 %) were localized in the proximal colon, and 4 of these tumours showed MIN at more than one locus. Two tumours with MIN at one locus were found in the distal colon. Only 4 out of 23 tumours (17%) without MIN at the five loci investigated in the present study were localized in the proximal colon. There was no correlation between familial history and localization of the tumour. Only 4 out of 11 patients (36%) with familial background suffered from tumours in the proximal colon. These results are summarized in Table 1.

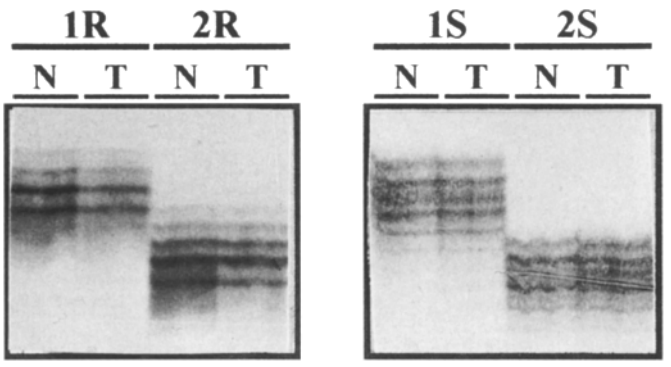

Fig. 2 Radioactive (1,2R) and non-radioactive (1,2S) PCR amplification of matched genomic DNA (*N* normal, *T* tumour) using primer pair Mfd28 (D10S89) exhibit identical allele sizes. By internal labelling using [^{35}S]-dATP and and silver-staining both strands of the two alleles and variable numbers of shadow bands are stained

Discussion

Genetic changes in the genomes of susceptible cells have been implicated as the cause of neoplastic transformation and progression of cancer cells, and genomic screening methods may thus improve the diagnostic repertoire and provide new approaches for cancer therapy. Using molecular genetic techniques the activation of dominant acting genes (oncogenes) and loss of function of recessive genes (tumour suppressor genes) have been demonstrated in correlation with different cancers and grades of malignancy [5]. Microsatellite instability (MIN), the expansion or reduction of the number of short tandem repeats, is a newly discovered mechanism involved in the tumorigenesis of hereditary non-polyposis colon cancers (HNPCC) [1, 8, 17], and detection of MIN would thus be of high interest as a screening technique for hereditary cancer syndromes in diagnostic pathology of colorectal tumours.

It has been shown in different studies that colon carcinomas exhibiting the MIN phenotype are mainly localized in the right colon [10, 17]. There are other biological features associated with tumours containing MIN. The great majority of tumours exhibiting the MIN phenotype show a diploid DNA status [10], whereas a high proportion of colon carcinomas without MIN are aneuploid. It has also been shown that patients suffering from tumours with MIN seem to have a more favourable clinical course [17]. These data might indicate different aetiologies for colon tumours from the proximal and the distal colon, but more data are needed to confirm this hypothesis.

Data concerning the occurrence of MIN in colon cancer in correlation with a familial background are contradictory. Although first identified in tumours constituting HNPCC (Lynch syndromes I and II), several reports demonstrated that the MIN phenotype is not restricted to hereditary tumours [2, 10]. One critical point for the verification of hereditary syndromes are the criteria used for the diagnosis. They are based on clinical data and have been formulated in the Amsterdam criteria for HNPCC [19]. Clinical definitions do not allow the identification of cases due to new germline mutations, and sometimes the number of family members is too low to fulfil these criteria. To date, there is no way of verifying the classification on the basis of objective pathological features.

Therefore, rapid and efficient genomic screening methods are necessary for the investigation of large colon cancer collectives. On the basis of these data it will be possible to identify patients at risk for further genetic inquiry of alterations in the responsible repair enzymes. In the present study we investigated 30 patients suffering from colon cancer in order to establish a rapid non-radioactive method for MIN detection. Using this approach we found MIN in 7 of the 30 (23%) cases. In accordance with previously published results [2, 10] we observed a tendency to increased presence of MIN in tumours of patients with familial background. However, this was not statistically significant. In addition, we found a close correlation between localization and MIN. Five out of 7 (72%) tumours with MIN but only 4 of 23 (17%) tumours without MIN were localized proximal to the splenic flexure of the colon. There was no correlation between tumour grade or stage and demonstration of MIN. However, in the present study we investigated a small number of 30 patients with only 7 showing a hereditary background of cancer, and it is possible that the number was too small for statistically significant correlations between all clinical variables and MIN to be apparent.

The demonstration of MIN in colon cancer emphasizes the potential of genomic screening methods in diagnostic pathology. The use of PCR-based analysis has introduced several advantages. The high sensitivity of the PCR reaction means that only nanogram amounts of genomic DNA are necessary, in contrast to the standard Southern analysis, which requires 10 μg of high-molecular-weight genomic DNA. In contrast, DNA from paraffin-embedded tissue can be used for PCR amplification. Microsatellites have been increasingly used as markers for genetic mapping and are highly polymorphic, widely distributed and easy to assay by PCR. Recently, non-radioactive PCR methods have been introduced for LOH studies using microsatellite markers [3]. However, in all previously published investigations regarding MIN in colon cancer and other tumour types radioactive detection procedures were used. The prerequisite for these methods may hamper their applicability in diagnostic pathology, since they require special laboratory equipment and are expensive and time consuming. In the present study we used a non-radioactive PCR-based method for the demonstration of MIN in colon cancer. The results obtained by silver-staining were sensitive and highly reproducible. Our findings corroborate data from several studies which have demonstrated that this non-radioactive technique is comparable to radioactive detection in sensitivity [3, 18]. It has been shown that signals obtained by silver staining are more complex owing to staining of both strands of the two alleles. However, we found almost identical results using internal radioactive labelling

and silver staining. By internal labelling radioactive nucleotides should be incorporated selectively in one strand (CA or GT) during PCR, but most PCR fragments generated using primer pairs for microsatellite mapping contain small sequences adjacent to the $(CA)_n$ repeats and these flanking sequences also incorporate the radioactive nucleotide, resulting in labelling of both strands of the two alleles.

In accordance with previously published results our data demonstrate the implications of MIN for diagnostic pathology of colorectal tumours. This applies to the ability to identify patients of hereditary risk as well as the possibility to define aetiologically different subgroups in colorectal carcinoma. We have demonstrated a rapid, non-radioactive PCR-based detection system which can supplement genomic screening techniques in diagnostic pathology.

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