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Extended Microsatellite Analysis in Microsatellite Stable, MSH2 and MLH1 Mutation-Negative HNPCC Patients: Genetic Reclassification and Correlation with Clinical Features

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Key Words

Hereditary nonpolyposis colorectal cancer · Mismatch repair genes · Microsatellite stable tumor phenotype · Microsatellite instability

Abstract

Background: Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant disorder predisposing to predominantly colorectal cancer (CRC) and endometrial cancer frequently due to germline mutations in DNA mismatch repair (MMR) genes, mainly MLH1, MSH2 and also MSH6 in families seen to demonstrate an excess of endometrial cancer. As a consequence, tumors in HNPCC reveal alterations in the length of simple repetitive genomic sequences like poly-A, poly-T, CA or GT repeats (microsatellites) in at least 90% of the cases. ***Aim of the Study:*** The study cohort consisted of 25 HNPCC index patients (19 Amsterdam positive, 6 Bethesda positive) who revealed a microsatellite stable (MSS) – or low instable (MSI-L) – tumor phenotype with negative mutation analysis for the MMR genes MLH1 and MSH2. An extended marker panel (BAT40, D10S197, D13S153, D18S58, MYCL1) was analyzed for the tumors of these patients with regard to three aspects. First, to reconfirm the MSI-L phenotype found by the standard panel; second, to find minor MSIs which might point towards an MSH6 mutation, and third, to reconfirm the MSS status of hereditary tumors. The reconfirmation of the MSS status of tumors not caused by mutations in the MMR genes should allow one to define another entity of hereditary CRC. Their clinical features were compared with those of 150 patients with sporadic CRCs. ***Results:*** In this way, 17 MSS and 8 MSI-L tumors were reclassified as 5 MSS, 18 MSI-L and even 2 MSI-H (high instability) tumors, the last being seen to demonstrate at least 4 instable markers out of 10. Among all family members, 87 malignancies were documented. The mean age of onset for CRCs was the lowest in the MSI-H-phenotyped patients with 40.5 ± 4.9 years (vs. 47.0 ± 14.6 and 49.8 ± 11.9 years in MSI-L- and MSS-phenotyped patients, respectively). The percentage of CRC was the highest in families with MSS-phenotyped tumors (88%), followed by MSI-L-phenotyped (78%) and then by MSI-H-phenotyped (67%) tumors. MSS tumors were preferentially localized in the distal colon supposing a similar biologic behavior like sporadic CRC. MSH6 mutation analysis for the MSI-L and MSI-H patients revealed one truncating mutation for a

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patient initially with an MSS tumor, which was reclassified as MSI-L by analyzing the extended marker panel. ***Conclusion:*** Extended microsatellite analysis serves to evaluate the sensitivity of the reference panel for HNPCC detection and permits phenotype confirmation or upgrading. Additionally, it confirms the MSS status of hereditary CRCs not caused by the common mutations in the MMR genes and provides hints to another entity of hereditary CRC.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant disease characterized by early onset of colorectal, particularly right-sided, cancer (CRC). It occurs with an incidence of up to 5% of all CRCs [1, 2]. Tumors in HNPCC reveal alterations in the length of simple repetitive genomic sequences like poly-A, poly-T, CA or GT repeat (microsatellites) in at least 90% of the cases [3]. This so-called microsatellite instability (MSI) is a consequence of germline mutations within the DNA mismatch repair (MMR) genes, mainly MSH2 [4], MLH1 [5], and MSH6 [6], thus resulting in DNA replication errors. In 1997, 126 mutations have been documented in a database of the International Collaborative Group on HNPCC in Leiden, The Netherlands [7]. Meanwhile, other novel mutations in the MMR genes have been detected [8–11], so that more than 300 different predisposing mutations are known to date. Genetically predisposed individuals carry a defective copy of an MMR gene in every cell. Somatic inactivation of the remaining wild-type copy in a target tissue, typically colon, gives rise to a profound repair defect, progressive accumulation of mutations and cancer. The absence of MMR gene proteins due to these mutations is also detectable by immunohistochemical analysis with monoclonal antibodies [12, 13].

MSI, however, is not specific for HNPCC, but also occurs in up to 16% of sporadic CRC [14, 15]. Additionally, MMR gene mutations preferentially occur in cases of MSI with at least 2 out of 5 markers [40%, so-called MSI-high phenotype (MSI-H)], whereas they have rarely been found in individuals with 1 out of 5 markers [20%, MSI-low phenotype (MSI-L)] [16]. The most valid clinical parameters for the prediction of an MSI-H phenotype and occurrence of MMR gene mutations are the fulfilled Amsterdam criteria (AC) [17].

In our present study, we investigated 25 patients from HNPCC families (19 Amsterdam positive, 6 Bethesda positive), who revealed microsatellite stable (MSS) or MSI-L tumor phenotype in the 5 markers of the international reference panel [18] with negative mutation analysis for the common MMR genes MSH2 and MLH1 and with normal immunohistochemical expression of the corresponding proteins.

Our purpose was, first, to reconfirm the MSI-L phenotype found by the standard panel, second, to find minor MSIs which might point towards a MSH6 mutation, and third, we wanted to reconfirm the MSS status of hereditary tumors not caused by mutations in the MMR genes and, in this way, define another entity of hereditary CRC.

Table 1. AC for HNPCC [19]

1	At least 3 family members with CRC, 2 of whom are first-degree relatives
2	At least 2 consecutive generations affected
3	At least 1 individual less than 50 years old at diagnosis
4	Exclusion of familial adenomatous polyposis

Table 2. Bethesda criteria for HNPCC [20]

1	Individuals with cancer in families that meet the AC
2	Individuals with 2 HNPCC-related cancers, including synchronous and metachronous CRCs or associated extracolonic cancers
3	Individuals with CRC and a first-degree relative with CRC and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; 1 of the cancers diagnosed at age <45 years, and the adenoma diagnosed at age <40 years
4	Individuals with CRC or endometrial cancer diagnosed at age <45 years
5	Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age <45 years
6	Individuals with signet-ring-cell-type CRC diagnosed at age <45 years
7	Individuals with adenomas diagnosed at age <40 years

Patients and Methods

Patients

DNA samples were obtained from 25 consenting patients fulfilling the AC or Bethesda criteria shown in tables 1 and 2 [19, 20]. Patients were recruited by genetic counseling or from the Departments of Gastroenterology and Surgery of the Universities of Mu-

Table 3. Microsatellite markers of the expanded panel with their chromosomal location, repeat motif, primer sequences, annealing temperature and length of the amplification products

Name	Chrom. loc.	Repeat motif	Primer sequence (5′–3′)	PCR temperature, °C	Length, bp
BAT40	1p13.1	$(T)_7...(T)_{40}$	ATT AAC TTC CTA CAC CAC AAC GTA GAG CAA GAC CAC CTT G	55	80–100
D10S197	10p12	$(CA)_7...(CA)_{12}$	ACC ACT GCA CTT CAG GTG AC GTG ATA CTG TCC TCA GGT CTC C	58	161–173
D13S153	13q14	$(CA)_{17}$	AGC ATT GTT TCA TGT TGG TG CAG CAG TGA AGG TCT AAG CC	58	190–230
D18S58	18q22	$(GC)_5GA(CA)_{17}$	GCT CCC GGC TGG TTT T GCA GGA AAT CGC AGG AAC TT	58	144–160
MYCL1	1p32	$(GAAA)_2...(GAAA)_{14}$	TGG CGA GAC TCC ATC AAA G CTT TTT AAG CTG CAA CAA TTT C	55	140–209

BAT40 is a mononucleotide repeat, D10S197, D13S153 and D18S58 are dinucleotide repeats and MYCL1 is a tetranucleotide repeat. Chrom. loc. = Chromosome location.

nich, Germany. In routine testing, their corresponding tumors were analyzed with 5 markers of the international reference panel and were judged to be MSS or MSI-L. Immunohistochemistry and mutation analysis for MLH1 and MSH2 did not reveal a loss of expression of the corresponding protein nor a disease-causing mutation in these patients.

The clinical features like age at onset and tumor localization were compared with 150 patients with sporadic CRC who are under regular surveillance in our gastroenterologic department.

Tumors

Tumors were judged macroscopically and microscopically by two pathologists (G.B. and M.M.) including histologic examination (hematoxylin and eosin staining). Carcinomas were staged following the TNM classification. They were graded in regard to their differentiation (G1–4).

DNA Extraction

For the isolation of tumor DNA, blocks of surgically resected cancerous tissue that had been fixed in formalin and embedded in paraffin were used. Regions of invasive cancer with the highest proportion of neoplastic cells were microdissected (10-μm sections). DNA was extracted by proteinase K digestion followed by repeated ethanol precipitation with gradual declining ethanol concentrations [21] using the QIAmp Tissue Kit (Quiagen, Hilden, Germany). From the same patients, DNA was isolated out of peripheral blood leukocytes by using standard methods. Purified DNA was then quantified spectrophotometrically before polymerase chain reaction (PCR).

Marker Selection

At first, the standard panel of 5 microsatellite markers (BAT25, BAT26, D2S123, D5S346 and D17S250) was studied, which revealed an MSI-L or MSS phenotype for all selected patients. Five other microsatellite markers (BAT40, D10S197, D13S153, D18S58 and MYCL1) were then chosen. This panel is composed of 1 mononucleotide, 3 dinucleotide and 1 tetranucleotide repeats. The sequences of the microsatellite PCR primers were taken as described [22]. One member of each primer pair was labeled with a fluorescent dye to permit detection by using an automated fluorescent DNA fragmenting apparatus. Primer sequences are given in table 3.

Polymerase Chain Reaction

PCR was performed in a final volume of 20 μl containing 100 ng DNA, 10 × PCR buffer, 20 μ*M* of each dNTP, 3 m*M* Mg^{2+}, 30 pmol of each primer and 1 U of Taq DNA polymerase (Master Mix, Quiagen). The DNA was amplified in a thermocycler (Biometra, Göttingen, Germany) using a hot-start approach. PCR initially denatured at 94°C for 30 s, annealed at 55°C for 25 s for BAT40 and MYCL1, at 58°C for 25 s for D10S197, D13S153 and D18S58, with extension at 72°C for 1 min. The final extension after 35 cycles was performed at 72°C for 5 min and was followed by cooling to 4°C. Amplification products were visualized by ethidium bromide staining in a 1.5% agarose gel electrophoresis.

Fragment Analysis

PCR products were analyzed by a polyacrylamide/formamide gel electrophoresis in a sequencing gel chamber (ABI PRISM 377 Genetic Analyzer). Analysis was performed by a special software (PE Applied Biosystems) and included determination of the length of the PCR products and the height of the peaks. Amplification products ranged between 80 and 230 base pairs.

MSI was defined by the presence of novel bands following PCR amplification of tumor DNA, which were not present in PCR products of the corresponding normal DNA. To determine those markers in which band shifts are difficult to interpret, all gels were evaluated independently by two different observers (U.S. and E.H.-F.). Every locus was scored as MSI or MSS.

Interpretation of MSI Phenotypes

A tumor was considered as having an MSI-H phenotype if at least 4 out of 10 markers (40%) exhibited band size shifts. Samples were qualified as MSI-L phenotype if only up to 3 markers demonstrated genetic alteration. Tumors without band shifts were reclassified as MSS.

Mutation Analysis for MSH2, MLH1 and MSH6

All exons of the MLH1 and MSH2 genes were amplified using primers published previously [23, 24] with a touch-down PCR program: 94°C for 5 min and first annealing at 63°C for 30 s, then a progressive decrease in the annealing temperature by 1°C each cycle until the lowest annealing temperature of 50°C, followed by synthesis at 72°C for 30 s and then denaturation at 94°C for 30 s with another 15 cycles at an annealing temperature of 50°C.

DHPLC analysis was carried out on an automated DHPLC device equipped with a DNA separation column (WAVE: Transgenomic, San Jose, Calif., USA). Four to 7 µl of each PCR product (containing 50–100 ng of DNA) was denatured at 95°C for 3 min. The DNA strands were gradually reannealed by decreasing the sample temperature from 95 to 65°C over a period of 30 min. PCR products were then separated through a linear acetonitrile gradient (flow rate 0.9 ml/min). The column mobile phase consisted of a mixture of 0.1 *M* triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Gradient parameters were determined based on the size and G-C content of the amplicon. Generally, analysis took approximately 7 min, including column regeneration and re-equilibration to starting conditions. The temperature for the successful resolution of heteroduplex molecules was determined by running fragment-specific melting curves and by using the DHPLC melting algorithm WAVE-MAKER of the wave instrument. Melting curves were determined as follows: the elution time of a specific fragment was determined under standard conditions. This specific gradient was then tested with the same PCR product and temperatures ranging from 48 to 70°C, and the retention time versus temperature was plotted to yield a fragment-specific melting curve. These combined results revealed an analysis temperature for each melting domain of the fragment, which is optimal for 80–90% of the α-helical fraction of each domain.

Immunohistochemistry for MSH2 and MLH1

MSH2. Monoclonal mouse antihuman MSH2 antibody (Calbiochem Oncogene Research Products, Heidelberg, Germany) was used at a dilution of 1:200. Two-micrometer sections of the paraffin-embedded, formalin-fixed tissue blocks were pretreated by microwaving with target unmasking fluid (TUF, DAKO, Hamburg, Germany) for 15 min at high power. The tissue was then incubated with the primary antibody overnight at 4°C. Staining was visualized using avidin-biotin complex (Vectastain Elite ABC-Kit, Vektor Labs, Wertheim-Büttingen, Germany) with 3-amino-9-ethyl-carbazol (AEC, Sigma, St. Louis, Mo., USA) and counterstaining was performed with hematoxylin.

MLH1. Monoclonal mouse antihuman MLH1 antibody (Zymed, Berlin, Germany) was used at a dilution of 1:80. Pretreatment was done by microwaving with target unmasking fluid (TUF, DAKO) for 30 min at high power. Incubation was performed overnight at room temperature, visualization was carried out using the Envision+ peroxidase complex (DAKO).

The staining pattern for both antibodies was nuclear. A negative staining reaction in tumor cells was only regarded as a loss of protein expression in the presence of a positive staining reaction in stromal cells and lymphocytes which served as internal controls. Specificity of staining was verified by replacement of the primary antibody with the mouse IgG isotype (Southern Biotechnology Associates Inc., Birmingham, UK), resulting in a negative staining reaction.

Statistics

Statistical evaluation between the tumor subgroups was performed using the paired Student t test; $p < 0.05$ was considered as significant.

Results

The study published here was set out to further analyze HNPCC families without MSI-H tumors and without disease-causing mutations in MLH1 and MSH2. An extended marker panel was analyzed, first, to receive additional information of a putative involvement of other MMR genes, possibly resulting in a low degree of MSI that was not detected by the standard panel; second, to reconfirm the MSI-L phenotype for patients without mutations in MLH1 and MSH2, and third, to reconfirm the MSS status in order to gather hints towards another entity of hereditary CRCs.

Extended MSI Analysis

The mean age of the 25 MSS, MLH1 and MSH2 mutation-negative HNPCC patients (14 females, 11 males) was 46.1 ± 12.9 years. An extended marker panel (BAT40, D10S197, D13S153, D18S58, MYCL1) was analyzed for the tumors of these patients regarding two aspects. First, to confirm MSS and MSI-L tumors with an additional marker set and second, to find MSI-L tumors, initially judged as MSS, which then underwent MSH6 mutation analysis. The extended marker panel includes one mono-A-run (BAT40), 3 dinucleotide repeats (D10S197, D13S153 and D18S58) and 1 tetranucleotide repeat.

The results of the microsatellite analysis with the extended marker panel are summarized in table 4. In total, 35 MSIs were found. From 17 original MSS tumors, 11 could now be reclassified as MSI-L (65%) and 1 even as MSI-H (6%), whereas 5 were still seen to have an MSS phenotype (29%). From 8 MSI-L tumors, 7 were still considered to be MSI-L (87%), but 1 was also found to be MSI-H (13%). Both reclassified MSI-H tumors came from Amsterdam-positive families. In summary, 17 MSS and 8 MSI-L tumors were analyzed with an extended marker panel and reclassified as 5 MSS, 18 MSI-L and 2 MSI-H tumors.

As an example, figure 1 shows the setting for the marker BAT40 in a 55-year-old male HNPCC patient. In his tumor DNA (lane below), novel bands in the amplification product could be detected compared to the corresponding genomic DNA (lane above).

The relative percentage of instability found for the single markers was distributed fairly evenly with 9 tumors

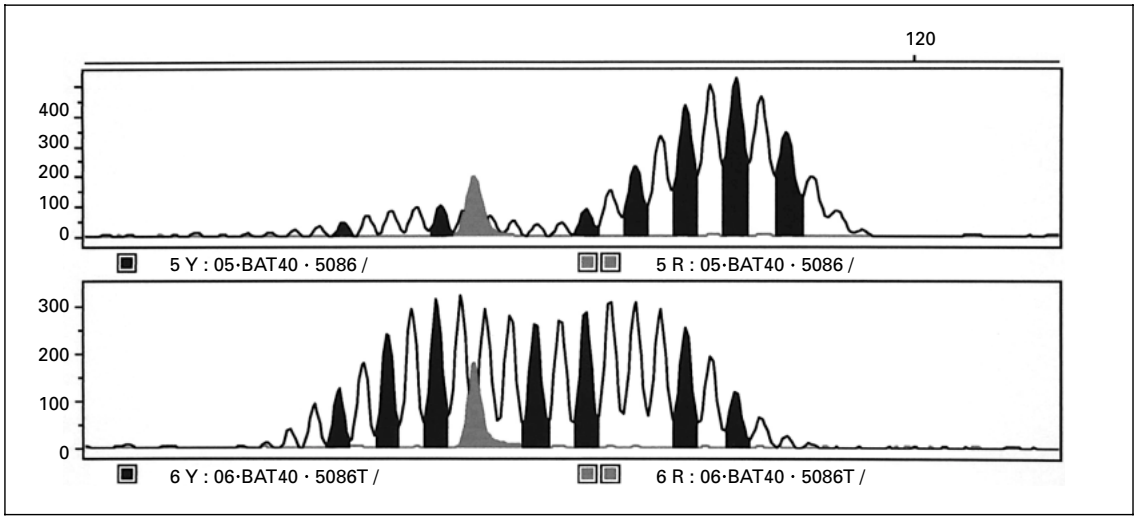

Fig. 1. Microsatellite analysis of the marker BAT40 (monocleotide repeat) in a 55-year-old male HNPCC patient. Tumor DNA (lane below) reveals new bands and a band shift compared with the corresponding genomic DNA (lane above).

Table 4. Age of onset, gender, tumor localization, clinical diagnosis and microsatellite status for the 5 reference markers BAT25, BAT26, D5S346, D2S123, and D17S250 and 5 additional markers BAT40, D10S197, D13S153, D18S58 and MYCL1 in 25 MMS, MSH2 and MLH1 mutation-negative HNPCC patients

Patient No.	Age	Sex	Tumor	Diag-nosis	BAT25	BAT26	D5S346	D2S123	D17S250	BAT40	D10S197	D13S153	D18S58	MYCL1	Status
1	57	m	CRC	AC	s	i	s	s	s	i	s	s	i	s	MSI-L
2	35	f	CRC	AC	s	s	s	s	s	s	s	s	s	s	MSS
3	46	f	CRC	AC	s	s	s	s	s	s	s	s	s	s	MSS
4	42	f	CRC	AC	s	s	s	s	s	i	s	s	s	s	MSI-L
5	51	f	CRC	AC	s	i	s	s	s	i	LOH	s	i	s	MSI-L
6	50	m	CRC	AC	s	s	s	s	s	s	s	s	i	s	MSI-L
7	38	m	CRC	BC	s	s	s	s	s	i	s	s	i	i	MSI-L
8	37	f	CRC	AC	s	s	i	s	s	i	i	i	s	i	MSI-H
9	45	m	CRC	AC	s	s	s	s	s	s	s	s	s	s	MSS
10	47	m	CRC	AC	s	s	s	s	s	s	s	i	s	i	MSI-L
11	44	f	CRC	AC	s	s	s	s	s	i	i	i	s	i	MSI-H
12	44	f	CRC	AC	s	s	s	s	s	s	s	i	s	s	MSI-L
13	57	m	CRC	AC	s	s	s	s	s	s	s	s	s	s	MSS
14	39	f	CRC	BC	s	s	s	s	s	i	i	s	s	s	MSI-L
15	44	m	CRC	BC	s	s	s	s	s	s	i	s	i	i	MSI-L
16	60	f	CRC	AC	s	s	s	s	s	s	s	s	i	s	MSI-L
17	5	f	brain	AC	s	s	i	s	s	s	s	s	s	s	MSI-L
18	68	f	sm. bow.	BC	s	s	s	s	i	i	s	s	s	s	MSI-L
19	38	m	CRC	BC	s	s	s	s	i	LOH	s	i	s	LOH	MSI-L
20	66	m	CRC	BC	s	s	s	s	s	s	s	s	s	s	MSS
21	48	f	CRC	AC	s	s	s	s	s	s	s	s	s	i	MSI-L
22	44	f	CRC	AC	s	s	s	s	s	s	s	s	i	i	MSI-L
23	64	f	CRC	AC	s	s	s	s	s	s	s	s	s	i	MSI-L
24	38	m	CRC	AC	s	i	s	s	s	s	i	s	s	s	MSI-L
25	69	f	CRC	AC	s	s	s	i	s	s	s	LOH	i	i	MSI-L

BC = Bethesda criteria; s = stable; i = instable; sm. bow. = small bowel; LOH = loss of heterozygosity.

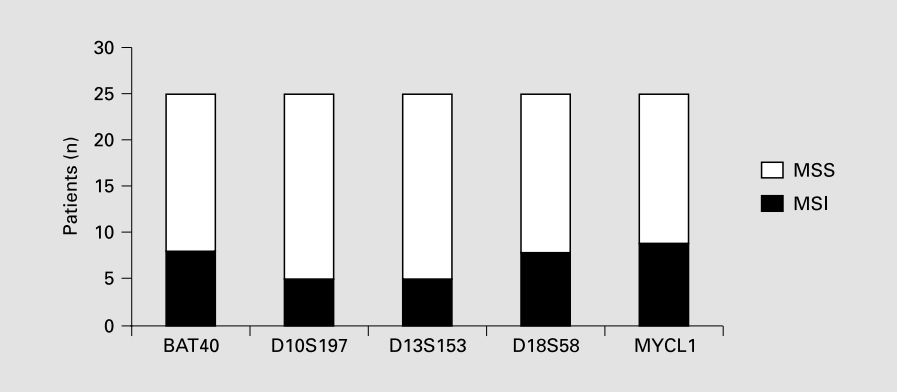

Fig. 2. Distribution of MSI and MSS of each additional microsatellite marker among MSH2 and MLH1 mutation-negative HNPCC tumors.

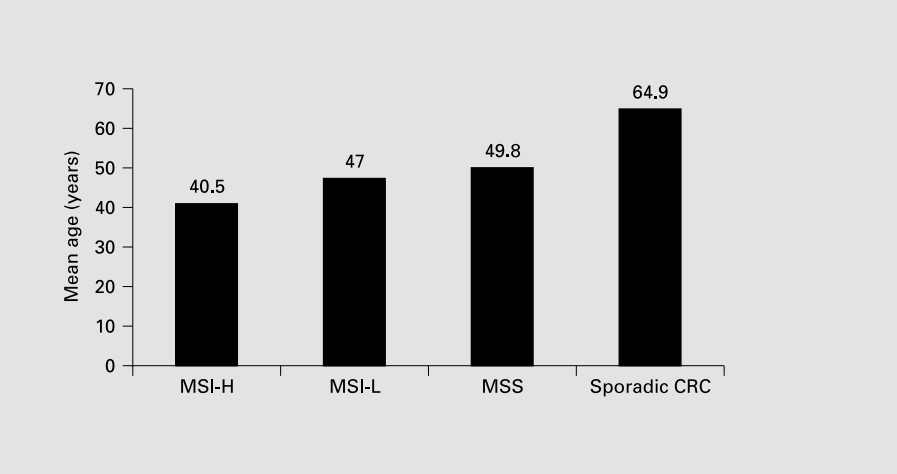

Fig. 3. Mean age of HNPCC patients in dependence on their microsatellite phenotypes according to the extended panel of 10 markers in total. Patients with MSI-H tumors were younger than patients with MSI or MSS tumors. Mean age of patients with sporadic CRC was significantly higher than in all subgroups of HNPCC patients ($p < 0.01$).

(36%) being instable for MYCL1, 8 tumors (32%) for BAT40 and D18S58, and 5 tumors (20%) for D13S153 and D10S197 (fig. 2).

Immunohistochemistry

All investigated tumors revealed a positive immunohistochemical reaction for MLH1 and MSH2 protein.

Mutation Analysis in MSH6

MSH6 mutation analysis for all patients with MSI-L and MSI-H tumors revealed 1 truncating mutation in exon 4 (c.1190–1191 del AG) for patient No.10 who was diagnosed with CRC at the age of 47 years. His tumor was judged as MSS with the reference panel and judged as MSI-L after reevaluation with the extended marker panel. Interestingly, his tumor did not show MSI for 1 of the 3 mono-A-runs analyzed here. The dinucleotide repeat D18S58 and the complex repeat MYCL1 however showed MSI for his tumor. Seven CRC cases were diagnosed among his first-degree relatives between 48 and 70 years of age. Six of the affected family members were females, none of them had endometrial cancer.

Clinical Features: Tumor Localization and Age at Onset

Subdividing the patients into three groups depending on their reclassified MSI status, with 10 microsatellite markers in total, some interesting clinical findings could be revealed by evaluating 87 malignancies for the index patients of the study cohort and their familiy members.

The mean age at onset was lowest in MSI-H tumors, with 40.5 ± 4.9 years, whereas it was higher in MSI-L tumors, with 47.0 ± 14.6 years, and was the highest in MSS tumors with 49.8 ± 11.9 years (fig. 3). There was no difference in age between Amsterdam-positive (45.2 ± 12.8 years) and Bethesda-positive (45.4 ± 12.9 years) families. The mean age at onset in sporadic CRC was significantly higher with 64.9 ± 11.1 years ($p < 0.01$).

Among the MSI-H and MSI-L tumors, no clearly dominant localization of the CRCs in regard to the splenic

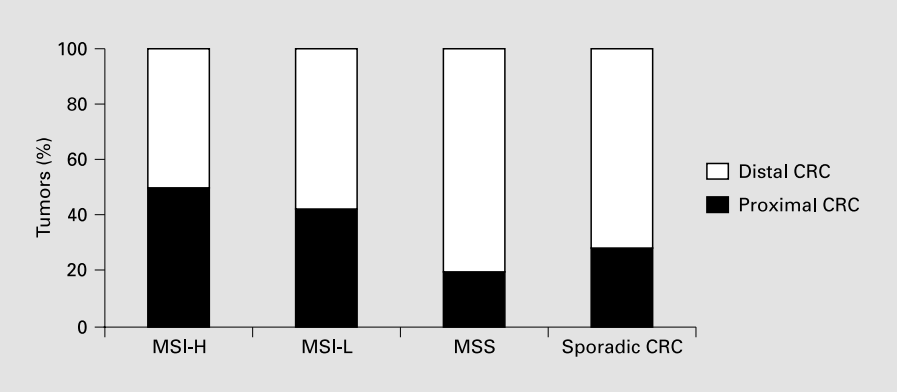

Fig. 4. The distribution of CRC localization in dependence on the reclassified MSI status of the MSH2 and MLH1 mutation-negative HNPCC tumors. There was no clearly dominant localization in regard to the splenic flexure among the MSI-H and MSI-L tumors, whereas MSS HNPCC tumors and sporadic CRC occurred preferentially in the distal colon.

Table 5. Occurrence of malignancies among all index patients and family members of the 25 MSS, MSH2 and MLH1 mutation-negative HNPCC patients

Tumor	MSI-H	MSI-L	MSS
Colorectal	6	47	16
Endometrial	0	4	0
Gastric	0	2	0
Brain	0	2	0
Urinary tract	0	1	1
Small bowel	0	1	0
Breast	0	1	0
Lung	0	0	1
Laryngeal	1	1	0
Leukemia	2	1	0
Total	9	60	18

flexure could be revealed (fig. 4). One of the MSI-H CRCs was proximally, while the other was distally localized (50/50%). Among the MSI-L tumors, 7 were recognized to be proximal (43%), and 9 to be distal (57%) CRCs. However, only 1 of the MSS tumors was proximal (20%), but 4 were distal (80%) CRCs so that a preferential localization in the distal colon of these tumors can be assumed (fig. 4). Among sporadic CRC, tumors are seen to have occurred proximal to the splenic flexure in 44 cases (29%) and in the distal colon in 106 cases (71%).

Among the families of the MSS, MSH2 and MLH1 mutation-negative index patients, 87 affected family members, including index patients, could be evaluated. Examples of typical pedigrees are shown in figure 5a and b. The occurrence of malignancies within these families is given in table 5 (absolute numbers) and figure 6 (percent-

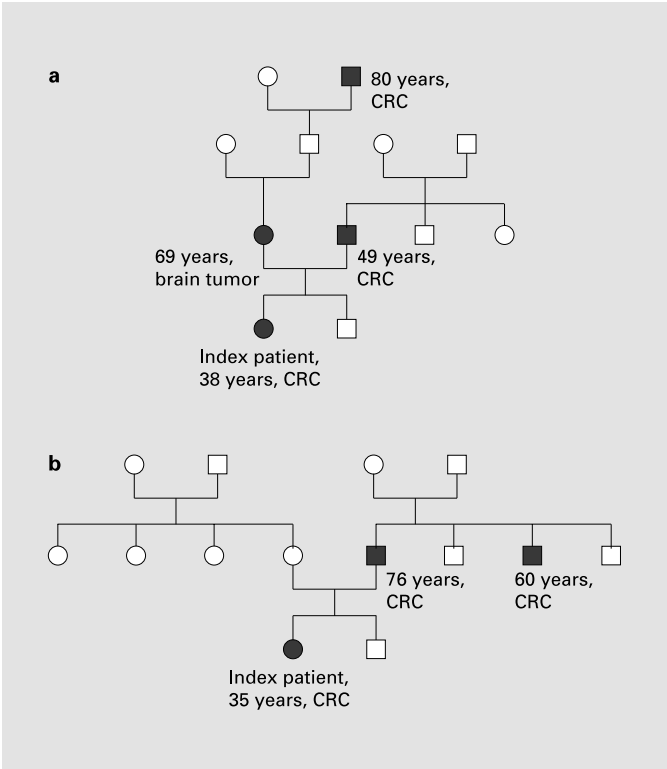

Fig. 5. a Pedigree of a 38-year-old female patient fulfilling the Bethesda criteria without detectable MSH2 or MLH1 mutation. An extended microsatellite analysis revealed an MSI-L phenotype. **b** Pedigree of a 35-year-old female patient fulfilling the AC without detectable MSH2 or MLH1 mutation. Even an extended microsatellite analysis revealed an MSS phenotype. CRC was the only kind of malignancy in this family.

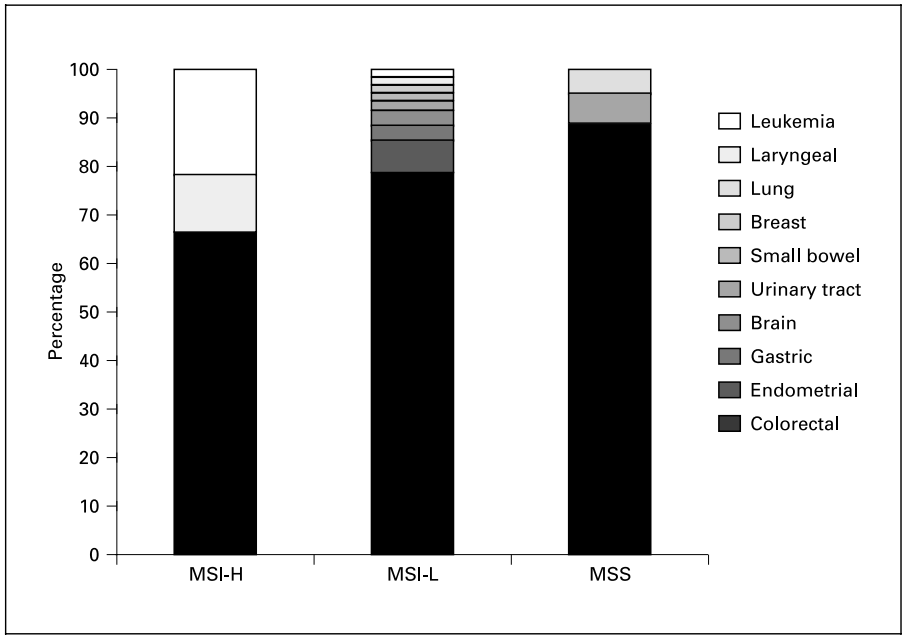

Fig. 6. Occurrence of malignancies among all family members of the MSS, MSH2 and MLH1 mutation-negative HNPCC patients. The percent incidence of CRC was higher in families of MSI-L- than MSI-H-phenotyped patients (78 vs. 67%), and was highest in the families with an MSS-phenotyped index patient (88%).

age). The percent incidence of CRC was higher in families of MSI-L-phenotyped than in MSI-H-phenotyped patients (78 vs. 67%), and the highest in families with an MSS-phenotyped index patient (88%), although the differences between the tumor subgroups were not significant ($p = 0.19$, 0.35 and 0.46, respectively).

Among Amsterdam-positive families, the percent incidence of CRC was higher than in Bethesda-positive families, but also without significance (81 vs. 74%).

Discussion

It is widely accepted that MSI assessment is a suitable and cost-effective method in the diagnosis of HNPCC [25]. In combination with immunohistochemistry, this strategy has the potential to diagnose more than 90% of tumors that involve alterations of the MSH2 and MLH1 gene [22]. Following the recommendation of the International Collaborative Group on HNPCC [18], we first examined the reference panel of 5 microsatellite markers in the 25 patients. Although in most cases even the AC for HNPCC were fulfilled, which are considered to be highly predictive for both, tumor instability and MMR gene mutations [26], these index patients did not show mutations in MSH2 and MLH1, nor did their tumors show a loss of expression for the corresponding proteins. Microsatellite analysis revealed MSS tumors in 68% and low instable tumors in 32% of the cases. To reconfirm the MSS status or to reveal putative instabilities in other than the recommended markers, we performed an extended microsatellite analysis with 1 mononucleotide (BAT40), 3 dinucleotide (D10S197, D13S153, D18S58), and 1 tetranucleotide (MYCL1) repeats. These 5 microsatellites were chosen since they had been shown to also be useful in HNPCC screening [27–29]. Among them, especially MYCL1 has been shown to have a relatively high instability rate [22].

Due to an extended panel of microsatellite markers, our data show that 2 tumors revealed an MSI-H phenotype (8%), which, in combination with a positive family history (AC), is a very strong sign for cancerogenesis on the MIN pathway.

Large genomic deletions, which occur in up to 12.6% of patients meeting the AC [30], have not been excluded for MSH2 and MLH1, as the tumors did not show aberrant immunohistochemical patterns for these proteins. No MSH6 mutation was found for these 2 patients. This points towards the possible involvement of other MMR genes in the tumorigenesis within these families with MSI-H tumors.

In fact, we detected 1 mutation in the MSH6 gene of a 47-year-old male patient with an initial MSS tumor that was reclassified as MSI-L by analyzing the extended marker panel. In this connection, several authors already described MSH6 mutations and variants among patients

with suspected HNPCC and MSI-L or MSS tumors, with the restriction that these MSH6 mutations are rare in families fulfilling the strong AC [16, 31]. The penetrance of CRC appears to be significantly lower among MSH6 mutation carriers when compared with MSH2 and MLH1 mutation carriers (32 vs. 80%, by the age of 80 years) [32, 33]. An explanation may be that the loss of MSH6 causes only a partial MMR defect leading to no or only low MSI [34]. In addition, in vitro investigations revealed that certain MSH6 mutations do not affect MMR function [35]. All these findings underline the important value of the MSH2 and MLH1 genes, and reveal that disease-causing mutations are only rarely identified in any other MMR genes in HNPCC [36].

Furthermore, 18 tumors could be reclassified as MSI-L phenotype (72%), showing instability for 1–3 markers out of 10, including tumors (16%) with 3 instable markers. With regard to the international reference panel of 5 markers defining 1/5 instabilities (20%) as MSI-L, these tumors with 3/10 instabilities (30%) were already marginally of an MSI-H phenotype.

With the extended marker panel of microsatellites, 5 tumors were reconfirmed as being of an MSS phenotype (20%). This finding hints to the supposition that these tumors do not follow the MIN pathway, as they reveal neither MSI nor causative mutations in the common MMR genes MSH2 and MLH1. Genes, other than the MMR genes might be disease-causing in these HNPCC tumors.

With regard to the distribution of all malignancies in HNPCC families, CRC has been shown to be the most frequent tumor entity with up to 70%, while endometrial carcinoma is observed in only 9% and gastric cancer in only 6% of the cases [37]. Among the family members of our selected patient group, CRC was even more frequent with 69 of the 87 tumors (79.3%), whereas endometrial carcinoma (4/87 = 4.6%) and gastric cancer (2/87 = 2.2%) had a lower incidence as usual (table 4). Other tumors which are not generally associated with HNPCC (laryngeal carcinoma, lung cancer, leukemia) occurred in quite a considerable number of cases (6/87 = 6.9%; table 4). Among CRCs of our patients, the total percentage of proximal tumors (40%) was seen to be lower compared to HNPCC data in the literature by up to 68% [37–39]. Consequently, a higher frequency of distal CRC was detected. Interestingly, this preferential localization was found among the reclassified MSS tumors, whereas no dominance of either proximal or distal CRC could be revealed among the MSI-H- and MSI-L-phenotyped tumors. Therefore, we suppose that these MSS tumors have a similar biologic behavior to that of sporadic CRC, which is known to be predominantly localized in the distal colon. This phenomenon is emphasized by the fact that the tumor spectrum in their families was clearly smaller (88% CRC) than in the families of reclassified MSI-L patients, which included different cancer manifestations.

The latter finding agrees with data of a recently published study comparing Amsterdam-positive HNPCC patients with and without detectable MMR gene mutations [40]. Among the mutation-negative patients, the authors described a major subgroup with a later age at onset (mean of 49.6 years), an abundance of distal CRC and fewer HNPCC-related cancers. Similar results were presented by others revealing that HNPCC patients with MMR gene mutations or MSI show an earlier age of onset, more proximal CRC and the whole spectrum of tumor manifestations known for MSI tumor disease as compared to MSS, mutation-negative patients [41–43].

MSI-L tumors are difficult to define. On the one hand, their tumor spectrum of affected family members points to 'classic HNPCC', but the few MSIs and their mean age at onset are difficult to be judged (47.0 ± 14.6 vs. 40.5 ± 4.9 years in MSI-H tumors). The detection of 1 MSH6 mutation for these patients underlines the sometimes impaired MSI phenotype caused by proteins that do not work in the 'front line', like MSH2 and MLH1. Immunohistochemistry and mutation analysis for these 'second line' proteins and genes, like MLH3 and PMS2, need to be performed.

In summary, we detected additional MSIs in an extended panel of markers among MSS, MSH2 and MLH1 mutation-negative HNPCC patients, including patients who revealed MSS for BAT26, which is described to be the most sensitive marker for MSI assessment [44, 45]. MSS or MSI-L phenotypes should not be considered as an exclusion criterion for additional marker analysis or mutation testing of MMR genes in general, above all if the patients fulfill the strict AC, which are known to be the best predictive clinical features for HNPCC due to mutations in DNA MMR genes [46, 47].

Conclusions

(1) The upgrading of 2 tumors to MSI-H, which were initially classified as MSI-L, points towards the involvement of other MMR genes.

(2) The MSI-L tumors are difficult to define, however, they might have a low penetrance instability not caused by the analyzed MMR genes. These tumors need further

immunohistochemical evaluation and mutation analysis by including other tumors in these families as well.

(3) Five tumors (20%) did not show MSI. Their presumably hereditary tumors are not caused by mutations in DNA MMR genes. Other genes that might classify another entity of hereditary CRC may be involved. A first indication for this is given by the clinical phenotype. These families show a higher age at onset and mainly distally localized CRC with rare cancer occurrence outside the gastrointestinal tract.

(4) One MSH6 mutation would have been missed by the reference panel. In the extended marker panel, this mutation did not result in an instability for mono-A-runs either, in spite of the fact that this is the supposed function of MSH6. MSS tumors should be analyzed with an extended marker panel, and in case of instability, should be analyzed for MSH6.

To summarize, the analysis of an extended marker panel, which is more accessible and available than MMR genotyping, helps in the further classification of hereditary CRC. Reclassification of phenotypes indicates the clinical and biologic heterogeneity of HNPCC tumors. Further research work to identify the disease-causing genes will be necessary.

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