Distinction of Hereditary Nonpolyposis Colorectal Cancer and Sporadic Microsatellite-Unstable Colorectal Cancer through Quantification of MLH1 Methylation by Real-time PCR

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Abstract

Purpose: Promoter hypermethylation occurs frequently in tumors and leads to silencing of tumor-relevant genes like tumor suppressor genes. In a subset of sporadic colorectal cancers (CRC), inactivation of the mismatch repair gene *MLH1* due to promoter methylation causes high level of microsatellite instability (MSI-H). MSI-H is also a hallmark of hereditary nonpolyposis colorectal cancer (HNPCC) in which mismatch repair inactivation results from germ-line mutations. For differentiation of sporadic and hereditary MSI-H tumor patients, *MLH1* promoter methylation analysis is a promising tool but is not yet used in daily diagnostics because only qualitative techniques without standardization are available. The aim of this study is to establish a reliable and quantitative *MLH1* methylation analysis technique and to define valid *MLH1* methylation cutoff values for HNPCC diagnostics.

Experimental Design: We developed a new real-time PCR–based technique to detect and quantify methylation of both proximal and distal *hMLH1* promoter regions. We established and validated this technique in a cohort of 108 CRCs [94 MSI-H and 16 microsatellite stable (MSS) cases] comprising a reference ($n = 58$) and a tester tumor group ($n = 50$).

Results: The reference tumor group contained 28 HNPCC with proven germ-line mutations or positive Amsterdam I criteria (median age, 37 years) and loss of MLH1 expression, 14 sporadic MSI-H CRC tumors with loss of MLH1 expression and BRAF V600E mutation (median age, 80.5 years), and 16 sporadic MSS CRC (median age, 76.5 years). No MLH1 promoter methylation could be found in any MSS tumors. HNPCC patients showed no or low level of *MLH1* promoter methylation. A cutoff value of 18% methylation extent could be determined in this study to define MLH1 hypermethylation specific for sporadic MSI-H cases. Methylation could also be verified qualitatively by melting point analysis. BRAF V600E mutations were not detected in any HNPCC patients ($n = 22$ informative cases).

Conclusion: According to the present data, quantitative *MLH1* methylation analysis in MSI-H CRC is a valuable molecular tool to distinguish between HNPCC and sporadic MSI-H CRC. The detection of a BRAF V600E mutation further supports the exclusion of HNPCC.

Aberrant CpG methylation patterns have been reported in various diseases (1), especially in cancer (2, 3), wherein hypermethylation of regulatory CGI (CpG islands) can lead to inactivation of tumor suppressor genes (4, 5). Promoter hypermethylation of the mismatch repair gene *MLH1* leads to a high level of microsatellite instability (MSI-H; refs. 6, 7), which can be found in ~15% of sporadic colorectal cancer (CRC; refs. 8–13). On the other hand, MSI-H is a hallmark of hereditary nonpolyposis colorectal cancers (HNPCC; refs. 8–10, 12, 13) and can arise by mismatch repair inactivation caused by germ-line mutations rather than by promoter hypermethylation (14, 15). Thus, examination of *MLH1* promoter hypermethylation may be a promising tool to distinguish between sporadic MSI-H CRC and HNPCC candidates (6, 7, 16, 17). The *MLH1* promoter methylation analysis could be combined with BRAF V600E mutation detection because BRAF V600E mutations are very frequent in MSI-H sporadic CRC but virtually not found in tumors of HNPCC patients (18, 19).

A variety of nonquantitative methods for the analysis of CpGs have been reported (15, 20–22), which are based on digestion of DNA with methylation-sensitive restriction enzymes or bisulfite modification of DNA and methylation-specific PCR primers. Because bisulfite treatment of genomic DNA converts all nonmethylated cytosines to uracils, whereas

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methylated cytosines remain unchanged, the methylation status of the genomic DNA is represented by the sequence of the bisulfite-modified DNA. DNA methylation can be determined by direct sequencing or sequencing of subclones (23, 24), methylation-specific PCR (25), combined bisulfite restriction analysis (26), single-strand conformational polymorphism (27), melting curve analysis (28, 29), or denaturing gel electrophoresis (30).

However, for diagnostic purposes, objective quantitative methods are required, which include clear validated cutoff values for methylation determination proven in a well-defined patient collective. Few quantitative techniques have been described [MethyLight (31), ConLight-MSP (32), MethylQuant (33)] which are not established for *MLH1* methylation detection nor are they tested in a patient cohort of HNPCC cases with germ-line mutations in mismatch repair genes, sporadic MSI-H CRC, and microsatellite stable (MSS) CRC. In addition, these methods have several drawbacks. They use unstable bisulfite-modified DNA directly as template impairing reproducibility and robustness, or expensive and optimized internal fluorescent probes are required. In MethylQuant, only single methylated nucleotides are investigated (33), which is not sufficient for a loss of MLH1 expression (34, 35). We designed a quantitative method detecting only complete methylation of several CpGs in the regulatory regions of *MLH1* without the need of fluorescence probes and studied a patient cohort of MLH1-negative HNPCC patients, sporadic MSI-H patients with loss of MLH1 expression and BRAF mutation V600E, and sporadic MSS CRC patients. The aim of the present study was to establish a robust, reliable, and cost-effective real-time PCR–based method to quantitatively and qualitatively analyze DNA methylation and to develop cutoff values of methylation extent, which can be used in the daily practice of HNPCC diagnostics.

Materials and Methods

Surgical specimens and cell lines

In a multicentric study, 108 formalin-fixed paraffin-embedded tumor samples were obtained from the pathology departments of the University of Regensburg (n = 56), the Ludwig-Maximilians-University of Munich (n = 40), and the Technical University of Munich (n = 12) and then analyzed for *MLH1* promoter methylation, BRAF V600E mutation analysis, and immunohistochemistry of MLH1, MSH2, and MSH6 mismatch repair proteins. A reference group of 58 CRC patients were studied according to microsatellite status, BRAF V600E mutation, MLH1 germ-line mutation, and Amsterdam I criteria: (*a*) HNPCC patients (n = 28) were defined by (*i*) positive Amsterdam I criteria and/or pathogenic hMLH1 germ-line mutations, (*ii*) microsatellite instability (MSI-H), and (*iii*) negative hMLH1 immunohistochemistry (median age, 37 years); none of the 21 of 28 successfully BRAF codon 600 tested HNPCC cases showed a V600E mutation. (*b*) Sporadic MSI-H CRC patients (n = 14) were defined by (*i*) no evidence of fulfilled Amsterdam criteria (36) or *hMLH1* germ-line mutations, (*ii*) BRAF V600E mutation, (*iii*) age ≥75 years, and (*iv*) negative hMLH1 immunohistochemistry (median age, 80.5 years). (*c*) Sporadic MSS-CRC patients (n = 16; median age, 76.5 years) showing MSS and intact expression of the mismatch repair proteins were used as control group. The institutional ethics committee approved the study.

The human colon cancer cell lines SW48 (*MLH1* methylation positive) and SW480 (no *MLH1* methylation) were obtained from American Type Culture Collection. All cell lines were maintained in DMEM with 10% fetal bovine serum at 37°C and 5% CO_2.

DNA isolation

DNA was isolated from tissues and cell extracts using the High Pure PCR Template Preparation Kit (Roche) according to the supplier's recommendation. DNA was quantified photometrically.

Microsatellite analysis

Microsatellite analysis was done as previously described (37). Briefly, 50 to 100 ng of genomic DNA were used for multiplex microsatellite PCR amplification of the recommended Bethesda standard panel using the HNPCC Microsatellite Instability Test kit (Roche) according to the manufacturer's instruction. MSI-H was defined by a MSI frequency of >30% (38, 39) according to the Bethesda guidelines. One microliter of amplified PCR product was applied to the ABI PRISM 310 Genetic Analyzer using POP6 polymer. Automatic fragment analysis was done with GeneScan 3.1.2 software (Applied Biosystems).

Bisulfite modification

Bisulfite treatment was done to convert unmethylated cytosines into uracils whereas methylated cytosines remain unchanged. One hundred nanograms of genomic DNA were treated with sodium bisulfite using the EZ DNA Methylation Kit (ZYMO Research) according to the supplier's instructions. In brief, 5 μL of M-Dilution Buffer were added to the DNA sample and a volume of 50 μL was adjusted with sterile, nuclease-free water. After incubation at 37°C for 15 min, 100 μL of CT Conversion Reagent were added and the sample was incubated in the dark at 50°C for 16 h, and then chilled on ice for 10 min. Four-hundred microliters of M-Binding Buffer were added and then the sample was loaded into a Zymo-Spin I Column and centrifugated at 16,000 × g for 30 s. The modified DNA was washed with 200-μL M-Wash Buffer, treated with 200-μL M-Desulfonation Buffer, and washed twice with 200-μL M-Wash Buffer. Finally, the cleaned modified DNA was eluted with 12-μL M-Elution Buffer. Because bisulfite-modified DNA is very unstable, it was used immediately as template for PCR or stored at -80°C.

PCR preamplification of bisulfite-modified DNA

Principle. Bisulfite-modified DNA is very unstable, and results from methylation-specific quantitative real-time PCR with a bisulfite-modified DNA template are poorly reproducible. Therefore, we did an initial 15-cycle preamplification PCR using bisulfite-modified DNA and primers flanking the CpG stretches of interest. These *flank* primers contain preferably no CpGs and anneal therefore independently from methylation pattern (Fig. 1). *flank* primers may contain a wobble base if the sequence makes it unavoidable to include an eventually methylated cytosine within the primers. The preamplified promoter fragment generated with the *flank* primers is a stable DNA product and is used at 10-fold dilution as template in the subsequent methylation quantification.

MLH1 *methylation analysis.* Methylation of both proximal and distal located CpG islands of *MLH1* promoter was analyzed. Each promoter region was separately preamplified by 15 PCR cycles using methylation-independent *flank* primers [*Proxflank-up* + *Proxflank-down* (proximal region), *Distflank-up* + *Distflank-down* (distal region)]. PCR was done in a total reaction volume of 20 μL, containing 5% (v/v) DMSO, 2.0 mmol/L Mg^{2+}, 0.2 mmol/L deoxynucleotide triphosphates, 0.3 μmol/L of each *flank* primer, 10% (v/v) 10× PCR reaction buffer, 0.5 unit of Taq-Polymerase (Fermentas), and 4 μL (20 ng) of bisulfite-modified DNA. The sequences of primers are *Proxflank-up*, 5′-TTTT-AAAAAYGAATTAATAGGAAGAG-3′; *Proxflank-down*, 5′-AAATACCAAT-CAAATTTCTCAACTC-3′; *Distflank-up*, 5′-GAGTGAAGGAGGTTA-YGGGTAAGT-3′; and *Distflank-down*, 5′-AACRATAAAACCCTATACC-TAATCTATC-3′.

An initial denaturation step of 20 s at 94°C was followed by 15 cycles of 20 s at 94°C, 1 s at 60°C, a temperature ramp-down of 0.2°C/s to 55°C, 1 min at 55°C, 1 min at 72°C, and an final elongation step at 72°C for 3 min. PCR products were purified from salts, primers, nucleotides, and enzyme with the MinElute PCR Purification Kit

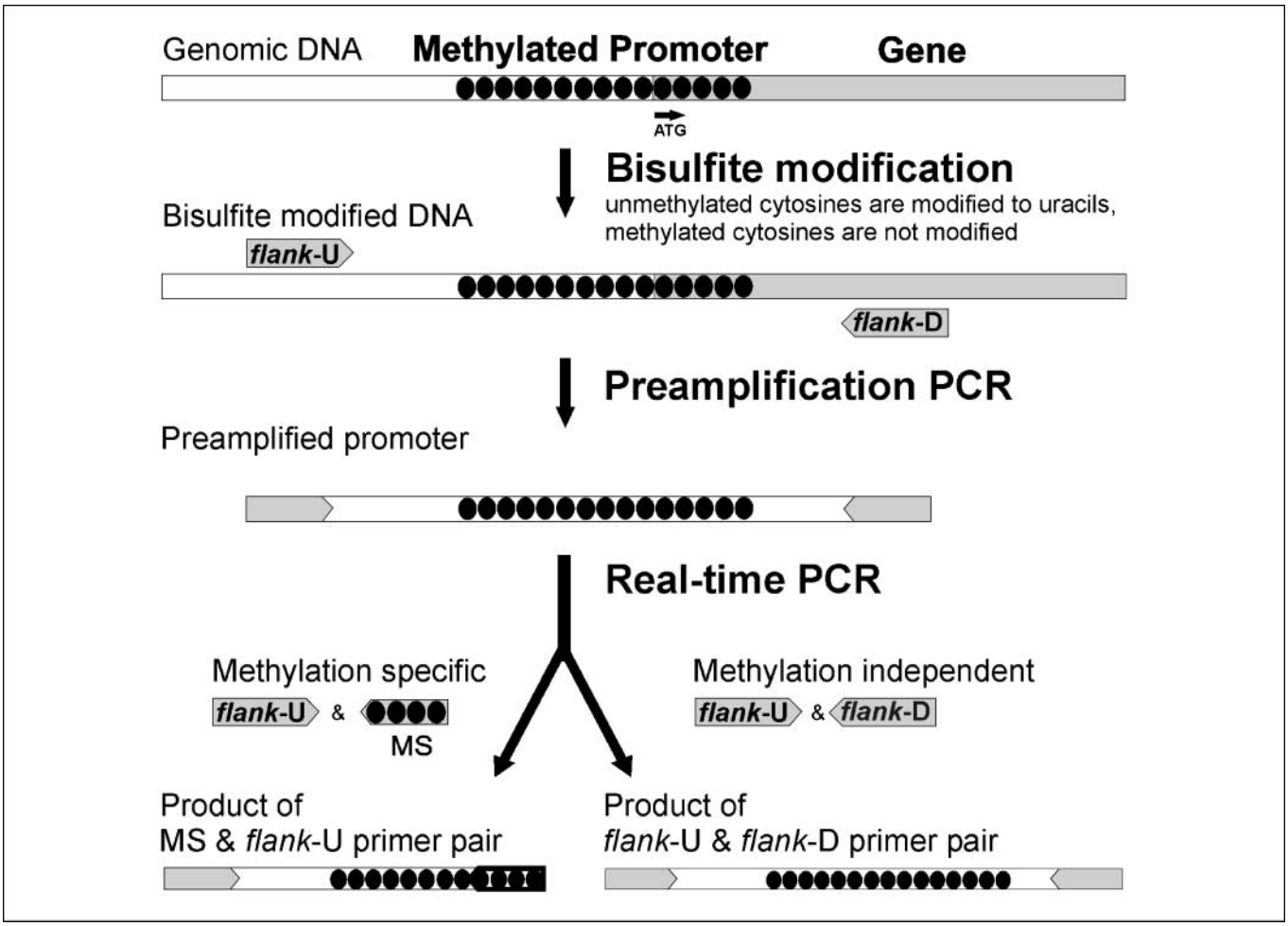

Fig. 1. Scheme of the methylation quantification. Bisulfite modification converts unmethylated cytosines into uracils whereas methylated cytosines remain unchanged. The CpG island of interest is preamplified using the bisulfite-modified DNA as template by the methylation-independent specific *flank* primer pair adjacent to the CpG island. The preamplified promoter is used as template in the methylation-specific real-time PCR: one reaction uses the methylation-specific (*MS*)-*flank* primer pair and amplifies only methylated template species whereas the other methylation-independent primer pair (*flank*-U-*flank*-D) amplifies the CpG-containing calibrator fragment.

(Qiagen) according to the supplier's instructions and eluted in 12 μL of Tris-Cl (10 mmol/L, pH 8.5). The preamplified DNA was diluted by 1:10 with sterile, nuclease-free water and stored at 4°C.

Methylation-specific relative quantitative real-time PCR

The methylation status was determined by relative quantitative real-time PCR (LightCycler System, Roche). For each promoter region, two real-time PCR reactions with diluted preamplified regions as template were done: (*i*) a methylation-independent PCR using CpG flanking primers as a 100% amplification reference, and (*ii*) a methylation-specific PCR using a flank primer and a methylation-specific primer located inside the preamplified promoter fragments [for the proximal region: *Proxflank-up* and *ProxMSr* (5′-CGATTTTTAACGCGTAAGC-3′); for the distal region: *Distflank-up* and *DistMSf* (5′-GAACGAC-GAACGCGCG-3′)]. The methylation-specific primers cover at least four eventually cytosines and have a 3′ specificity for a methylated cytosine.

The preamplified promoter regions from the *MLH1* methylation–positive CRC cell line SW48 (35) were used as calibrators and controls for 100% methylation in the methylation-specific PCR. The non-methylated preamplified promoter regions from the CRC cell line SW480 served as controls for 0% methylation.

LightCycler PCR was carried out using the LightCycler FastStart DNA Master^{Plus} SYBR Green I Kit (Roche) with 2 μL of 1:10 diluted preamplified promoter fragments in a total volume of 20 μL, containing 0.5 μmol/L of each primer and 4 μL of 5× LightCycler-FastStart DNA Master^{Plus} SYBR Green I Mix (Roche). An initial denaturation for 10 min at 95°C was followed by 40 cycles of 95°C for 2 s, 55°C for 6 s, and 72°C for 8 s. Melting point analysis was done by heating the PCR products from 50°C to 95°C with an increase of 0.2°C/s whereas fluorescence was monitored continuously.

For relative quantification, the methylation values of samples were normalized to the methylation value of the calibrator, which is defined as 100%. The mathematical analysis was done with the RelQuant 1.01 software (Roche) using PCR efficiency correction, giving the proportion of methylated template.

BRAF mutation analysis

Mutation analysis of BRAF codon 600 was done by sequencing exon 15 using an ABI PRISM 3100 Genetic Analyser (Fig. 2). The following primers were used for BRAF V600E mutation: BRAF-600 up, 5′-TGTAAAACGACGGCCAGTTCATAATGCTTGCTCTGATAGGA-3′; BRAF-600 down, 5′-CAGGAAACAGCTATGACCCTTTCTAGTAACT-CAGCAGC-3′. Amplifications were carried out according to ref. 40 using 0.02 unit/μL Taq DNA polymerase (Fermentas) and a PCR profile consisting of a 3-min initial denaturation at 94°C followed by 35 cycles of 60 s at 94°C, 60°C, and 72°C, followed by a 8-min final extension at 72°C.

Results

Quantitative validation of MLH1 methylation analysis. The accuracy of the methylation quantification was tested by spiking experiments with purified preamplified promoter fragments from the *MLH1* hypermethylated colon cancer cell line SW48 and the MLH1 unmethylated cell line SW480 in ratios of 1:1, 19:20, 4:5, 1:2, 1:5, 1:20, and 0:1 (corresponding to 100%, 95%, 80%, 50%, 20%, 5%, and 0% methylated DNA, respectively). Both distal and proximal *MLH1* promoter regions were tested (Fig. 3). The methylation quantification analysis mirrored the ratios of the methylated templates in both promoter regions, thus showing the high accuracy of the method described.

Qualitative methylation detection by melting point analysis. Quantification analyses were also verified by melting point analysis. The median melting point of distal *flank* PCR products was 85.0°C for methylated and 79.0°C for unmethylated DNA species. Proximal *flank* PCR products showed melting points of 78.5°C (methylated *MLH1*) and 73.0°C (unmethylated *MLH1* promoter). If the sample contained both methylated and unmethylated promoters, both corresponding melting peaks appeared in the melting point analyses (Fig. 4).

Evaluation of quantitative MLH1 methylation in known HNPCC tumors and sporadic CRC (reference tumor group). We assessed methylation status of the distal and proximal regions of the *hMLH1* promoter in three tumor cohorts: sporadic MSS CRC patients (n = 16), sporadic MSI-H CRC patients (n = 14), and HNPCC patients (n = 28). The methylation values ranged from 0% to 100%. None of the 16 MSS CRCs showed significant *MLH1* promoter methylation. The median methylation values were distal, 2.5% (range, 0-6%); proximal, 0% (range, 0-1%); and mean of distal/proximal, 1.6% (range, 0-3%). All 14 sporadic MSI CRCs showed methylation at both MLH1 promoter regions [median methylation values: distal, 56% (range, 31-100%); proximal, 38% (range, 20-80%); mean of distal/proximal, 50.4% (range, 33-81%)]. The MLH1 methylation-positive sporadic MSI-H CRC patients had a mean age of 80.5 years; all of them showed BRAF V600E mutations and none of them fulfilled the Amsterdam criteria or carried hMLH1 germ-line mutations. The median values of MLH1 methylation in HNPCC were distal, 0% (range, 0-16%; five cases with values greater than 2%: 3%, 5%, 14%, 15%, 16%); proximal, 1% (range, 0-15%; three cases with values greater than 2% methylation: 3%, 10%, 15%); and mean of distal/proximal, 0.7% (range, 0-15%; four cases with values greater than 2% methylation: 5%, 7%, 8%, and 15%). According to these data, we defined a cutoff for methylation positivity of median values +5 SD. That is, (*i*) 18.3%, mean of distal/proximal median values (= 1.9 + 16,4); (*ii*) 26.5%, distal methylation: (= 2.2 + 23.3); or (*iii*) 17.8%, proximal methylation (= 1.6 + 16.2). Thus, in most of the HNPCC

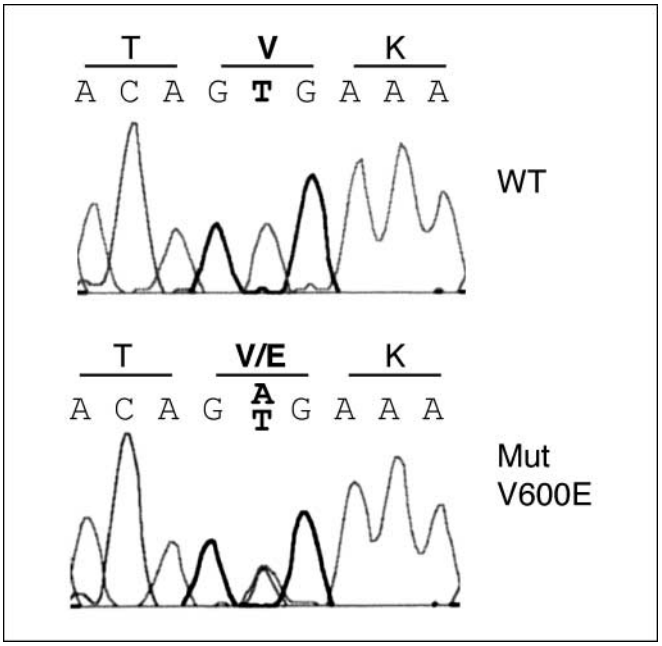

Fig. 2. DNA sequence analysis showing a representative BRAF V600E mutation.

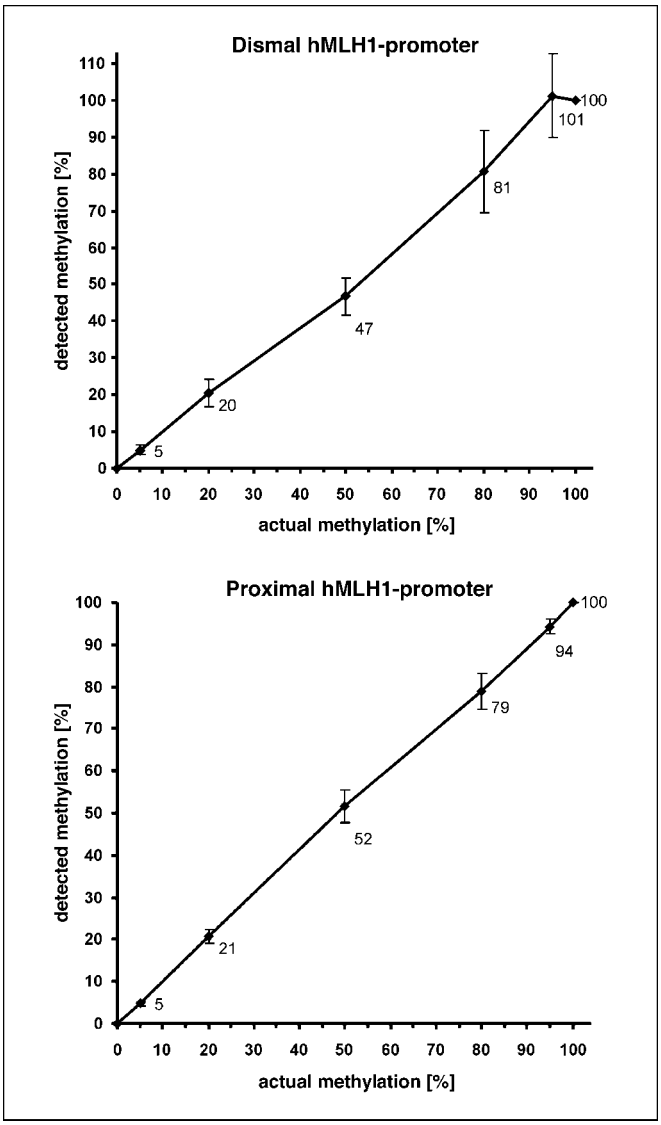

Fig. 3. Curves of methylation values showing the measured extent of methylation in percent of a spiking experiment with methylated and unmethylated samples using hMLH1 promoter fragments from CRC cell lines mixed in different ratios. The results of the methylation quantification mirror the real sample composition.

patients (86%; 24 of 28), no methylation [i.e., 0-2% methylation (mean of distal/proximal values)] was detected. However, 17% of HNPCC tumors show low-level methylation.

A quantile box plot of the median methylation values from the distal/proximal means shows that methylation is significantly different in each tumor group ($P_{\text{Kruskal Wallis}} < 0.001$; Fig. 5). Sporadic MSI-H CRCs show strong MLH1 promoter methylation (≥31%), whereas HNPCC tumors do not show methylation or only values up to 16% at most. MSS sporadic tumors, which are used as controls, are consistently MLH1 methylation negative.

Validation of quantitative methylation in a MSI-H CRC test group. We tested the MLH1 methylation analysis using the 18% cutoff in further 50 MSI-H CRCs (test group, Table 1) in which a complete set of clinical and molecular data were not available. All cases with positive Amsterdam I (n = 4) or Amsterdam II (n = 2) criteria were methylation negative. All tumors with BRAF V600E mutations (n = 10) were methylation positive, independently from the Bethesda criteria. In 8 of 19 (42%) methylation-positive cases, no BRAF mutation was found. One of these patients (case no. 65) had a hemiallelic germ-line MLH1 methylation in nuclear blood cells (data not shown) and showed a nearly 50% mean methylation value (48%) also in tumor DNA. Overall, in 40 cases with informative methylation and BRAF data, a significant correlation of methylation positivity and BRAF V600E mutation was found (P = 0.005). Bethesda-positive patients with both BRAF mutation and MLH1 promoter methylation but without evidence of positive Amsterdam criteria or *hMLH1* germ-line mutations (n = 4) showed a median age of 70 years (range, 66-74 years) and thus can be assigned to the sporadic MSI-H CRC group. Six cases with negative Amsterdam and positive (n = 4) or negative (n = 2) Bethesda criteria were methylation positive but did not carry a BRAF V600E mutation. Twenty-two

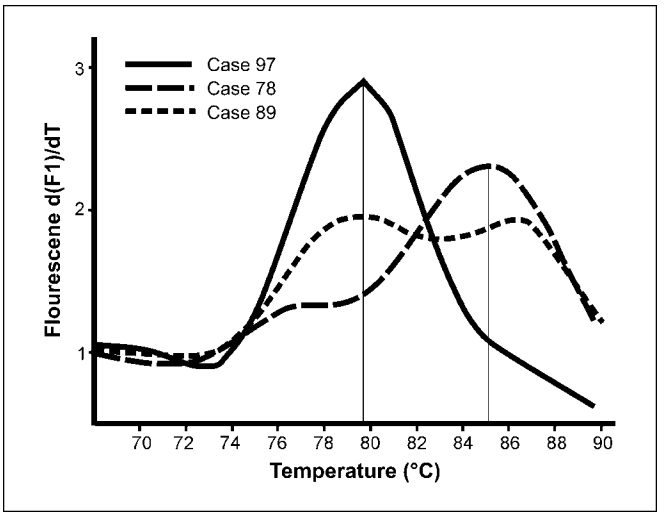

Fig. 4. Melting point analysis of *flank* PCR products. Representative melting curves of *flank* PCR products (distal promoter) from CRC patients. Case no. 97 shows a single peak at ~79°C resulting from unmethylated *hMLH1* promoter fragment. Case no. 78 shows a high melting (85°C) point in a *flank* PCR product of a 74% methylation-positive patient. Besides the slight slope resulting from the 26% unmethylated promoter fragments at 79°C, a main peak is visible at 85°C originated from methylated PCR products. Case no. 89 with 44% methylated promoter fragments shows a melting peak of unmethylated (79°C) and methylated DNA (85°C) species.

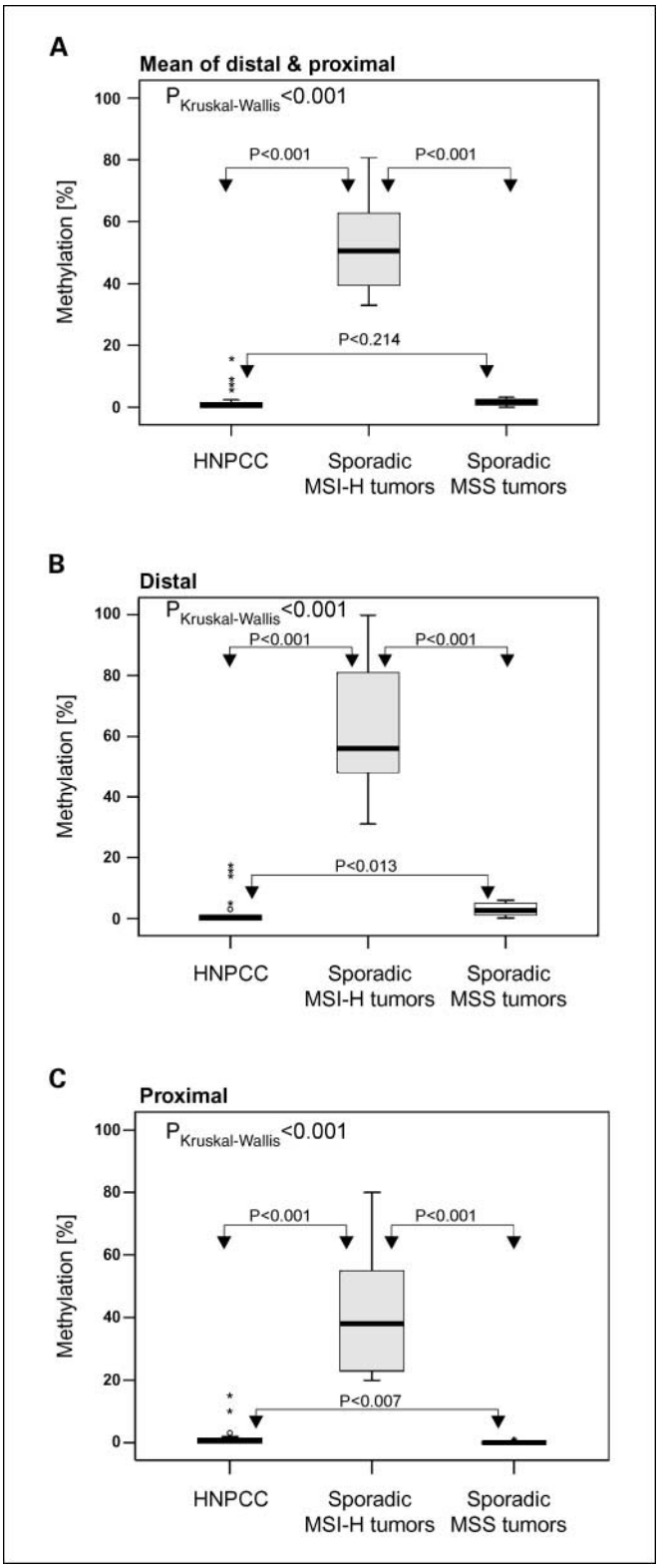

Fig. 5. Comparison of *MLH1* promoter methylation in tumors from HNPCC, sporadic MSI-H CRC, and sporadic MSS CRC. The box for each tumor group represents the interquartile range (25-75th percentile); the line within each box shows the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (*asterisks*). The nonparametric Kruskal-Wallis test was used to examine differences between the three independent tumor groups (HNPCC, sporadic MSI-H tumors, and sporadic MSS tumors). The Mann-Whitney *U* test was used in case of two tumor groups. Statistical analysis was done using the SPSS13 software.

Table 1. Clinical and molecular data of the tumor test group

Case no.	Age at onset (y)	Microsatellite status*	MLH1 expression [IHC]†	Fulfilled Amsterdam criteria I or II‡	Bethesda criteria§	Pathogenic mutation‖	BRAF V600E mutation¶	Distal methylation (%)	Proximal methylation (%)	Mean value of distal and proximal methylation (%)
2	36	1	0	—	1	N.a.	N.a.	0	0	0
4	44	1	0	—	1	Neg.	N.a.	0	0	0
5	38	1	0	—	1	N.a.	N.a.	0	0	0
6	66	1	0	—	—	N.a.	Pos.	49	59	54
7	45	1	0	—	1	Neg.	Neg.	1	1	1
9	74	1	0	—	—	N.a.	Neg.	2	3	3
10	64	1	0	—	—	N.a.	Neg.	3	0	1
11	78	1	0	—	—	N.a.	Neg.	3	0	1
12	31	1	0	—	1	N.a.	Neg.	3	2	3
13	39	1	0	—	1	N.a.	Neg.	2	0	1
15	64	1	0	—	1	Neg.	Neg.	5	0	3
16	37	1	0	—	1	Neg.	Neg.	6	0	3
17	70	1	0	—	—	N.a.	Neg.	3	2	3
20	46	1	0	—	1	Neg.	N.a.	0	0	0
21	37	1	0	—	1	Neg.	N.a.	0	0	0
24	37	1	0	—	1	Neg.	Neg.	0	0	0
25	37	1	0	—	1	Neg.	Neg.	0	0	0
27	63	1	0	—	1	Neg.	Neg.	0	0	0
28	33	1	0	II	1	Neg.	Neg.	0	0	0
29	48	1	0	—	1	Neg.	Neg.	0	2	1
31	44	1	0	—	1	Neg.	Neg.	0	0	0
32	56	1	0	I	1	Neg.	Neg.	0	1	1
36	37	1	0	I	1	Neg.	Neg.	0	4	2
37	47	1	0	II	1	Neg.	N.a.	0	0	0
40	60	1	0	—	1	Neg.	Neg.	0	1	1
45	31	1	0	—	1	N.a.	N.a.	1	0	0
51	35	1	0	—	1	Neg.	Neg.	2	5	3
52	47	1	0	I	1	Neg.	N.a.	2	0	1
54	42	1	0	—	1	Neg.	Neg.	3	5	4
56	48	1	0	—	1	Neg.	Neg.	3	0	2
57	44	1	0	—	1	Neg.	Neg.	12	1	6
58	61	1	0	II	1	Neg.	Pos.	26	50	38
60	77	1	0	I	1	Neg.	N.a.	14	19	16
62	26	1	0	—	1	Neg.	N.a.	21	65	43
63	71	1	0	—	1	Neg.	Pos.	24	19	21
64	63	1	0	—	1	Neg.	Neg.	28	45	36
65**	33	1	0	—	1	Neg.	Neg.	32	64	48
66	47	1	0	—	1	Neg.	Neg.	40	37	38
67	70	1	0	—	1	Neg.	Pos.	41	52	46
68	66	1	0	—	1	Neg.	Pos.	41	36	39
69	67	1	0	—	1	N.a.	Pos.	59	100	85
71	90	1	0	—	—	N.a.	Neg.	100	63	82
73	46	1	0	—	1	N.a.	Neg.	100	35	68
75	75	1	0	—	1	N.a.	Neg.	88	61	74
79	66	1	0	—	—	N.a.	Pos.	69	56	62
82	83	1	0	—	—	N.a.	Neg.	57	30	44
83	72	1	0	—	1	N.a.	Pos.	54	36	45
84	86	1	0	—	1	N.a.	Neg.	53	1	27
88	73	1	0	—	—	N.a.	Pos.	47	38	43
90	74	1	0	—	—	N.a.	Pos.	39	19	29

*1, MSI-H.
†0, negative. IHC, immunohistochemistry.
‡—, no criterion.
§1, fulfilled; -, negative.
‖Neg., negative (no sequence alteration and no deletions/amplifications detectable). N.a., not available.
¶Pos., positive; Neg., negative.
**Germ-line methylation detected.

patients showed neither methylation nor BRAF mutation, had a median age of 44.5 years (range, 31-78 years), and represent putative HNPCC patients. In 56% (28 of 50) of cases, the methylation level was higher in the distal than in the proximal promoter region. In one presumably sporadic tumor (case no. 84: BRAF mutation negative; age, 86 years), methylation positivity was detectable only in the distal but not in the proximal promoter region.

Discussion

In the daily routine practice, it is frequently difficult to identify likely candidates for HNPCC among CRC patients. To minimize the risk of overlooking HNPCC cases, the strict clinical criteria to diagnose HNPCC (Amsterdam criteria; ref. 36) were complemented by the Bethesda criteria (39, 41) to select patients for MSI analysis and immunohistochemical studies of the mismatch repair proteins. The drawback is that the higher sensitivity of the new clinical criteria is associated with a considerably lower specificity. In addition, although HNPCC patients are diagnosed with CRC at an earlier age, there are also studies showing that HNPCC-related CRC can also be diagnosed at age 60 years and older (42). Therefore, additional molecular methods are urgently needed to subdivide MSI-H–positive Amsterdam-negative patients into sporadic CRC or HNPCC cases.

The aim of this study was to establish a quantitative methylation analysis as a diagnostic tool that separates HNPCC candidates from sporadic MSI-H CRC. In this study, we analyzed the *MLH1* promoter methylation, hMLH1 protein expression, microsatellite instability, and BRAF V600E mutation status in 108 CRCs and in 2 CRC cell lines.

Generally, several difficulties can complicate methylation analysis. Stromal and inflammatory cells within tumor tissues with methylation patterns different from the tumor methylation pattern may easily lead to false results if nonquantitative methylation analysis techniques such as methylation-specific PCR are used. Moreover, nonquantitative methylation detection techniques cannot distinguish between partial or monoallelic and complete biallelic methylation. Methylation analyses usually require a bisulfite modification step, which provides unstable modified DNA (bisulfite-modified DNA), impairing the reproducibility of analyses.

These drawbacks could be overcome by the quantitative methylation analysis technique presented here, which is highly reproducible because it uses a stabilized bisulfite-modified DNA generated by a 15-cycle preamplification PCR, providing sufficient stable template DNA for numbers of methylation analyses, but minimizes the risk of cross contaminations. The accuracy of this technique was proved by spiking experiments of methylated and unmethylated template DNA for both proximal and distal *MLH1* promoter regions.

Because the melting point of double-stranded DNA increases with its CG content, melting points of the methylation-independent *flank* PCR products increase with each methylated cytosine because it resists bisulfite modification. Thus, methylation can be determined by melting point analysis (described here for the first time for *MLH1*; Fig. 4), which provides an additional valuable qualitative tool to control and verify the quantitative methylation analysis. Patients determined as methylation negative show only one melting peak (distal, ~80°C; proximal, ~72°C) whereas methylation-positive patients show melting peaks at a higher temperature (proximal, >78°C; distal, >85°C) dependent on the degree of methylation. Usually, the melting curves from methylation-positive CRC show two peaks (Fig. 4); the peak at the lower temperature (80°C) may be caused by nonmethylated promoter DNA from residual normal cells, unmethylated subpopulations of tumor cells, or unmethylated DNA in monoallelic methylated tumor cells.

In contrast to other methods like methylation-specific PCR, the methylation detection method described here allows the determination of methylation by using specific thresholds. Thus far, this is the first report providing cutoff values for *MLH1* methylation to discriminate between HNPCC and sporadic CRC. Because the lowest value within the group of sporadic MSI-H tumors was 33% (Fig. 5A) and the highest value in HNPCC tumors was 15%, a cutoff value of 18% (= mean of proximal and distal median methylation values plus 5-fold SD) was defined for positive scoring. However, this value depends basically on purity grade of microdissected tumor samples. False-positive results could be excluded because no positive methylation was detected at any hMLH1-positive MSS CRC. Interestingly, three HNPCC patients from the reference group (25, 33, and 63 years old) with pathogenic *MLH1* germ-line mutations and loss of MLH1 expression showed weak DNA *hMLH1* methylation (16%, 14%, and 15%, respectively) in the distal promoter region and shifted melting peaks (data not shown). For this reason, we regard the analysis of the proximal region as more reliable for HNPCC diagnostics. We assume that these tumors carry monoallelic *MLH1* promoter methylation (i.e., one allele is silenced by methylation), whereas the other allele carries the MLH1 germ-line mutation. Unfortunately, because there was no sequence polymorphism within the promoter regions, we were not able to discriminate between maternal and paternal alleles to verify monoallelic methylation by cloning and sequence analysis.

Within the MSI-H and MLH1 immunohistochemically negative tumor test group, we found a significant correlation of a BRAF mutation and *MLH1* methylation in tumors apparently representing sporadic MSI-H CRC and loss of MLH1 protein expression (mean age, 69 years). This finding confirms the results from others (43, 44). Some patients (20%; 8 of 40) show *MLH1* methylation but no BRAF mutation and may represent sporadic MSI-H tumors because there was no evidence for fulfilled Amsterdam criteria or pathogenic mutations and the median age of these patients was 69 years. However, in those cases, a hemiallelic germ-line *MLH1* methylation (45–48) must be excluded by analysis of blood cells or normal tissue (e.g., case no. 65).

One case (no. 84), a 86-year-old patient and therefore most likely a sporadic CRC patient, had methylation only in the distal promoter region, showing that distal *MLH1* promoter methylation alone is apparently sufficient for loss of MLH1 expression.

In summary, our data have a strong impact for HNPCC diagnostics. Accordingly, the analysis of both BRAF mutation and quantitative *MLH1* methylation analysis should be done in any MSI-H CRC with loss of MLH1 expression for the following reasons:

(*a*) First, nonquantitative *MLH1* methylation analysis detecting any *MLH1* methylation level is not suited to differentiate between sporadic MSI-H and HNPCC patients because *MLH1* methylation can occur not only in sporadic MSI-H CRC but also in some HNPCC patients (49).

(*b*) Second, quantitative *MLH1* methylation analysis described here shows that HNPCC tumors show no or weak *MLH1* methylation levels significantly lower than the high methylation levels in sporadic MSI-H CRCs. This difference can only be identified by quantitative *MLH1* methylation analysis. To detect also HNPCC patients from, for example, small families not fulfilling the Amsterdam criteria or HNPCC patients not meeting the Bethesda criteria due to an age of more than 60 years, any MSI-H tumor with loss of MLH1 expression should be

quantitatively analyzed for *MLH1* methylation. Furthermore, not only tumor DNA but also DNA from normal or nuclear blood cells should be analyzed to identify also a potential monoallelic germ-line methylation (45–48) with *MLH1* methylation values of ~50% both in tumor and blood cells.

(*c*) Third, BRAF V600E mutations virtually do not occur in HNPCC patients carrying pathogenic germ-line *MLH1* mutations as shown in our study as well as in other reports (18, 19, 43, 44, 50). Vice versa, BRAF V600E mutations occur specifically in a part of sporadic MSI-H CRC (40-74%; refs. 19, 44) as well as in 4% to 12% of MSS CRC (current study; refs. 19, 43). According to the present data, the detection of a BRAF V600E mutation is a highly specific molecular marker to exclude HNPCC and should be used in parallel with quantitative *MLH1* methylation analysis.

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