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MethyQESD, a robust and fast method for quantitative methylation analyses in HNPCC diagnostics using formalin-fixed and paraffin-embedded tissue samples

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Promoter hypermethylation occurs in various tumors and leads to silencing of tumor-relevant genes. Thus, promoter methylation analysis (MA) has been established as an important tool in cancer research and diagnostics. Here we present MethyQESD (methylation-quantification of endonuclease-resistant DNA) as a fast, easy, precise and reliable method for quantitative MA without the need of bisulfite-treatment or fluorescent probes. Though MethyQESD principally works with any gene promoter we established MethyQESD for the mismatch repair gene *MLH1* and tested its utility to differentiate between sporadic microsatellite unstable (MSI-H) colorectal cancer and hereditary nonpolyposis colorectal cancer (HNPCC) by quantitative *MLH1* MA. We investigated formalin-fixed and paraffin-embedded tissue samples from a previously published, well-characterized tumor collective comprising 25 HNPCC, 14 sporadic MSI-H CRC and 16 sporadic microsatellite stable (MSS) CRC. We found a high accuracy of MethyQESD by spiking experiments with dilution series of methylated (SW48 cancer cell line) and unmethylated (blood) DNA (Pearson's $r = 0.9997$ (proximal MLH1 promoter region), $r = 0.9976$ (distal MLH1 promoter region)). MethyQESD and conventional quantitative MA using of 96 formalin-fixed and paraffin-embedded CRC showed a high degree of concordance of both methods (Pearson's $r = 0.885$). HNPCC tumors showed either null *MLH1* methylation or a significantly lower degree of *MLH1* methylation than sporadic MSI-H CRC ($P < 0.001$). *MLH1* methylation was negative in all MSS tumors. Receiver operating characteristic (ROC) curve analyses defined a cutoff value of 16.5% *MLH1* methylation for specific and sensitive identification of sporadic MSI-H CRC (area under ROC curve: 1.000; asymptotic significance: $P < 0.001$). Thus, quantitative *MLH1* MA by MethyQESD provides a simple, fast and valuable tool to identify HNPCC candidates. Furthermore, MethyQESD works reliably with formalin-fixed paraffin-embedded tissue and simplifies DNA MA both for research and diagnostic purposes.

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KEYWORDS: BRAF mutation; HNPCC; microsatellite instability; *MLH1*; MSI; quantitative methylation analysis

Aberrant CpG methylation patterns have been reported to occur frequently in cancer,[1,2] where hypermethylation of regulatory CpG islands can lead to the inactivation of a number of tumor-relevant genes.[3,4] Methylation analysis of defined marker genes is therefore a promising molecular tool with a broad range of application in diagnostics and prediction of response of chemotherapies,[5–7] but none of the current quantitative techniques of DNA methylation analysis (MA) are suited for routine-diagnostic purposes because of their laborious, complicated and time-consuming procedure.

Promoter hypermethylation of the mismatch repair gene *MLH1* leads to a high level of microsatellite instability (MSI-H)[8,9] and can be found in approximately 15% of sporadic CRC.[10–15] On the other hand, MSI-H is a hallmark of hereditary nonpolyposis colorectal cancers (HNPCCs)[10–12,14,15] and caused by germline mutations rather than by promoter hypermethylation.[16,17] For diagnostic purposes, objective quantitative methods with proven cutoff methylation values are required which are validated in well-defined patient collectives.

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Recently, we established a quantitative MA technique for HNPCC diagnostics[7] and showed that *MLH1* methylation quantification can help to identify HNPCC patients by detection of null or weak *MLH1* methylation and to distinguish them from sporadic MSI-H CRC showing virtually constantly high *MLH1* methylation levels. Thus, quantitative examination of *MLH1* promoter hypermethylation can detect HNPCC candidates who should undergo a *MLH1* germline mutation search through DNA sequencing and MLPA analysis.[8,9,18–20]

Here we present methylation-quantification of endonuclease-resistant DNA (MethyQESD) as a new, robust, fast and easy quantitative MA technique which works reliably with formalin-fixed and paraffin-embedded tissue and is best suited as a molecular tool both in routine diagnostic practice and in cancer research.

MATERIALS AND METHODS

Surgical Specimens and Cell Lines

In a multicentric study 96 formalin-fixed paraffin-embedded tumor samples were obtained from the pathology departments of the University of Regensburg ($n = 55$), the Ludwig-Maximilians-University of Munich ($n = 29$), and the Technical University of Munich ($n = 12$) and analyzed for *MLH1* promoter methylation, *BRAF* V600E mutation analysis and immunohistochemistry (IHC) of MLH1, MSH2 and MSH6 mismatch repair proteins. A reference group of 55 CRC patients were studied according to microsatellite status, *BRAF* V600E mutation, *MLH1* germline mutation and Amsterdam-1 criteria: (1) HNPCC patients ($n = 25$) were defined by (i) positive Amsterdam-1 criteria and/or pathogenic *MLH1* germline mutations, (ii) microsatellite instability (MSI-H) and (iii) negative MLH1 IHC (median age: 37.0 years); (2) sporadic MSI-H CRC patients ($n = 14$) were defined by (i) no evidence of fulfilled Amsterdam criteria or *MLH1* germline mutations, (ii) *BRAF* V600E mutation, (iii) age ≥ 75 years and (iv) negative MLH1 IHC (median age: 80.5 years); (3) sporadic microsatellite stable (MSS) CRC patients ($n = 16$, median age: 76.5) showing MSS and intact expression of the mismatch repair proteins were used as control group.

The human colon cancer cell line SW48 (*MLH1* methylation positive) was obtained from ATCC. The cell line was maintained in DMEM with 10% fetal bovine serum at 37°C and 5% CO_2.

DNA Isolation

DNA was isolated from blood, archival FFPE tissues and cell lines using the PUREGENE™ DNA Purification Kit (Gentra, Minneapolis, MN, USA) according to the supplier's recommendation. DNA was quantified photometrically.

Microsatellite Analysis

Microsatellite analysis was performed as previously described.[21] Briefly, 50–100 ng genomic DNA was used for multiplex microsatellite PCR amplification of the recommended Bethesda standard panel using the HNPCC Microsatellite Instability Test kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. MSI-H was defined by a MSI frequency of $>30\%$[22,23] according to the Bethesda guidelines. Amplified PCR product (1 μl) was applied to the ABI PRISM™ 310 Genetic Analyzer using POP6 polymer. Automatic fragment analysis was performed by GeneScan™ 3.1.2 software (Applied Biosystems, Darmstadt, Germany).

BRAF Mutation Analysis

Mutation analyses of *BRAF* codon 600 were performed by sequencing exon 15 using an ABI PRISM 3100 Genetic Analyser. Following primers were used for *BRAF* V600E mutation: BRAF-600 up: 5′-TGTAAAACGACGGCCAGTTC ATAATGCTTGCTCTGATAGGA-3′ und BRAF-600 down: 5′-CAGGAAACAGCTATGACCCTTTCTAGTAACTCAGCAG C-3′. Amplifications were carried out according to[7,24] using 0.02 U/μl Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany) and a PCR profile consisting of a 3-min initial denaturation at 94°C followed by 35 cycles of 60 s at 94, 60 and 72°C, respectively. Final extension was performed by a 8-min incubation at 72°C.

Principle of MethyQESD

The MA is based on a combination of methylation-sensitive digestion and real-time PCR. The proportion of methylated DNA, which resists digestion by the methylation-sensitive endonuclease *Hin*6I is determined by real-time PCR and calibrated using a reference DNA that remains uncut.

Two restiction digestion batches are prepared for each sample, both of them containing equal amounts of DNA: (1) a methylation-quantification digestion (MQD) containing the methylation-sensitive restriction endonuclease *Hin*6I that cuts only unmethylated CGCG recognition sites, present within the amplicon. (2) A methylation independent calibrator digestion (CalD) with the endonucleases *Xba*I and *Dra*I whose recognition sites must not be present within the amplicon. The *Xba*I/*Dra*I as well as the *Hin*6I digests generates fragmented genomic DNA with increased PCR-accessibility and a higher PCR amplification efficiency. The *Xba*I/*Dra*I leads to an equal PCR amplification efficiency but does not prevent amplification of the calibrator DNA. The proportion of methylated DNA is determined by subsequent real-time PCR using the MQD and the CalD as templates. In order to investigate methylation of archival paraffinized tissue samples, the size of the amplicon should range between 80 and 150 bp. The difference of the C_t-values give the proportion of methylated DNA according to the formula: methylation $(\%) = E^{(C_t\,\mathrm{CalD} - C_t\,\mathrm{MQD})} \times 100$ (*E*: PCR efficiency). The principle of MethyQESD is illustrated in Figure 1.

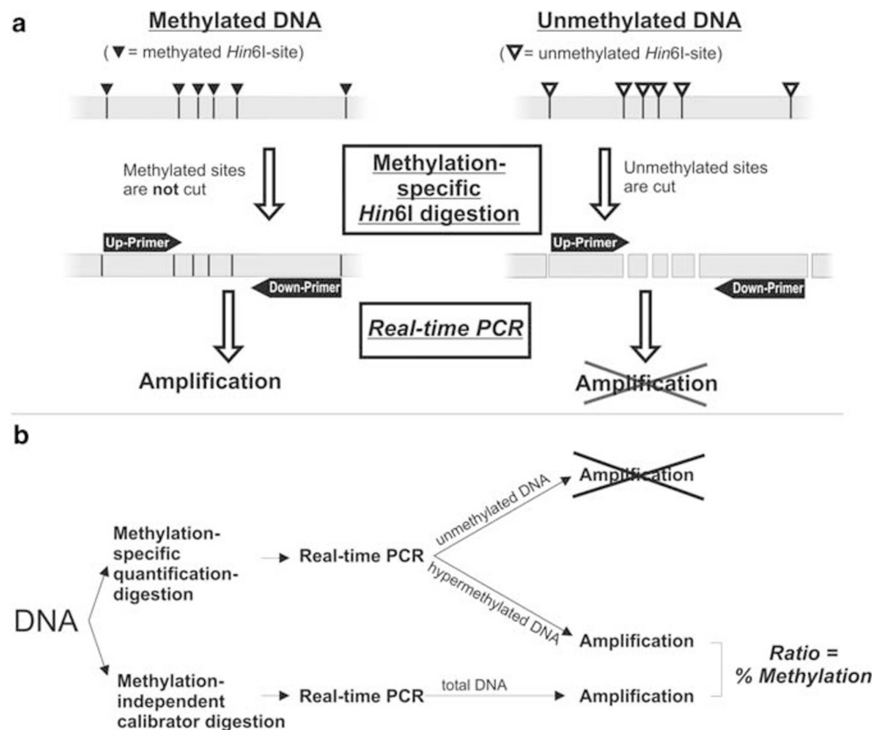

Figure 1 Principle of the MethyQESD. For each sample two digestions are prepared: a methylation-specific quantification digestion with *Hin*6I and a methylation independent calibrator digestion with methylation independent restriction endonucleases. (**a**) The restriction endonuclease *Hin*6I cuts DNA with unmethylated GCGC restriction sites, whereas hypermethylated DNA remain uncut and can be amplified by PCR using primers that flank the *Hin*6I sites of interest. The methylation independent endonucleases (ie *Xba*I and *Dra*I) have no restriction sites within the amplicon of the subsequent real-time PCR. The PCR efficiencies of both digestions are equal, as both digestions contain endonuclease treated genomic DNA. (**b**) The real-time PCR gives the proportion of methylated and therefore uncut DNA species by comparing the amount of amplicable DNA within the quantification digestion with those of the calibrator digestion.

Methylation-Specific Digestion

Two DNA samples each containing 5 μl DNA (1–300 ng/μl) were digested overnight at 37°C in a total volume of 20 μl 1xTango™-Buffer (Fermentas) with 30 U *Hin*6I and *Xba*I/*Dra*I (Fermentas; each 15 U), respectively. Subsequently, the endonucleases were inactivated by incubation at 70°C for 20 min and the samples were stored at 4°C.

Methylation-Specific Quantitative Real-Time PCR

The promoter methylation status was determined by relative quantitative real-time PCR using the LightCycler (Roche). Both, the methylation quantification batch and the calibrator DNA were used for PCR in duplicates.

Digested DNA of the *MLH1* methylation-positive CRC cell line SW48 served as control for positive methylation, digested DNA of nonmethylated blood DNA was used as a control for 0% methylation.

Real-time PCR was carried out using the QuantiTect SYBR Green I Kit (Qiagen, Hilden, Germany) with 2 μl digested DNA in a total volume of 20 μl, containing 0.5 μM of each primer and 10 μl 1 × QuantiTect™ SYBR Green I Kit. An initial denaturation for 15 min at 95°C was followed by 45 cycles of 95°C for 10 s, 60°C for 17 s and 72°C for 10 s. Melting point analyses were performed by heating the PCR products from 50 to 95°C with an increase of 0.2°C/s while fluorescence was monitored continuously. Primer sequences were ($5' \rightarrow 3'$): MLH1_prox_MS_up CGGCATCTCTGCTC CTATTG; MLH1_prox_MS_down TGCCCGCTACCTAG AAGGAT; MLH1_dist_MS_up 5′-AAGTCGCCCTGAC;GCA GAC; MLH1_dist_MS_down ACTACGAGGCTGAGCACGAA.

The proportion of methylated template was calculated from the differences of the Ct-values from the CalD and MQD ($=\Delta C_t$) according to the formula: methylation $(\%) = E^{\Delta C_t} \times 100$ (E: PCR efficiency).

RESULTS

Restriction Endonuclease Efficiency and Specificity

In order to test the minimum time needed for quantitative digestion of unmethylated DNA we treated 1.3 μg DNA from blood and 1.0 μg DNA from paraffinized normal tissue with *Hin*6I for different times. After 1 h 99.90% of the blood DNA was completely digested. The restriction efficiency of *Hin*6I was lower at the DNA from paraffinized tissue, thus complete restriction (0% undigested DNA) was achieved after an incubation time of 16 h (Figure 2). *Hin*6I has no intrinsic activity on methylated DNA as no degradation of methylated DNA was detectable after incubation with *Hin*6I for 30 h. We

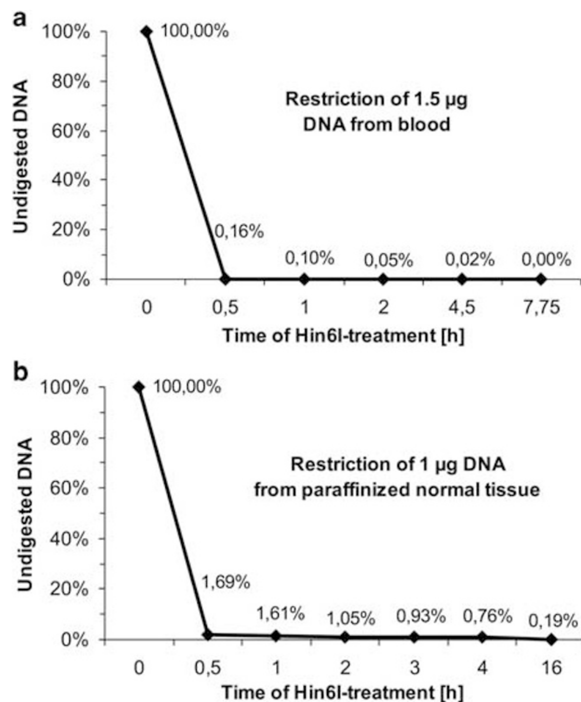

Figure 2 Restriction efficiency of the methylation-sensitive endonuclease *Hin*6I on unmethylated DNA from blood (**a**) and paraffinized normal colon mucosa (**b**).

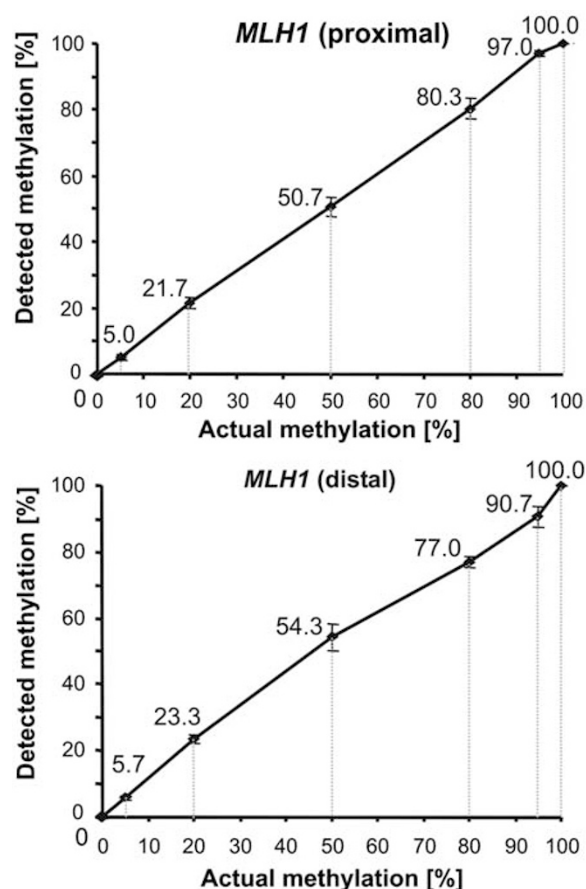

Figure 3 Curves of methylation values showing the methylation detected in percent of a spiking experiment with methylated DNA from SW48 cell line and unmethylated DNA from blood. The detected methylation amounts and the applied methylated DNAs were nearly identical (Pearson's $r = 0.9997$ (proximal), $r = 0.9976$ (distal)).

digested our DNA generally overnight (>16 h) to exclude false positive results from undigested, unmethylated DNA.

Quantitative Validation of MethyQESD

The linearity of the MethyQESD MA was tested by two different experiments. In a first experiment dilutions of hypermethylated DNA from the colon cancer cell line SW48 was mixed with unmethylated blood-DNA in a ratio of 1:1, 19:20, 4:5, 1:2, 1:5, 1:20 and 0:1, respectively (corresponding to 100, 95, 80, 50, 20, 5 and 0% methylated DNA). As shown in Figure 3 the methylation quantification analysis mirrored very exactly the ratios of the methylated templates in both the proximal and distal MLH1 promoter regions (Pearson's $r = 0.9997$ (proximal), $r = 0.9976$ (distal)) thereby demonstrating the high accuracy of the MethyQESD method.

To prove the reliability of the method using formalin-fixed and paraffin-embedded (FFPE) tissue samples we compared the methylation values of the proximal MLH1 promoter region using MethyQESD and quantitative MA using conventional bisulfite-modified DNA[5–7] of 96 formalin-fixed and paraffin-embedded CRC (Table 1). We found a high concordance of both methods (Pearson's $r = 0.885$).

Technical Sensitivity of the MLH1 MethyQESD

The sensitivity was checked by real-time PCR of *Hin*6I digested dilutions of defined concentrations of DNA from the hypermethylated cell line SW48. The *MLH1* MethyQESD was able to detect 3.2 pg of methylated DNA (Figure 4). The PCR efficiency of the *MLH1* MethyQESD PCR was 2.00. For detection of 0% methylation the difference of the C_t values must be at least 7.97 ($2^{-7.97} = 0.004$). Thus, the MQD- and CalD real-time PCR reactions must theoretically contain at least 800 pg DNA each to detect 0% methylation.

In addition, DNA was isolated from 10^6, 10^5, 10^4, 10^3, 10^2 and 10 SW48 cells, dissolved in 11 μl water and proceeded according to the QESD protocol. Reproducible results with C_t values below 32 were obtained with DNA isolated from 100 SW48 cells. Thus, for accurate quantification we recommend to use only results with crossing points (C_t values) of 32 or less in the CalD real-time PCR.

Distinction of HNPCC from Sporadic Colorectal Cancers with High Level of Microsatellite Instability by MLH1 MethyQESD

In order to evaluate the *MLH1* MethyQESD for HNPCC diagnostics we analyzed the methylation of the regulatory proximal *MLH1* promoter region in a published tumor reference group with HNPCC ($n = 25$) tumors, sporadic MSI-H CRC with loss of MLH1-expession ($n = 14$) and sporadic MSS tumors expressing MLH1 ($n = 16$). The overall

Table 1 Comparison of proximal *MLH1* methylation performed with MethyQESD and quantitative methylation PCR using bisulfite-modified DNA

Case no.	Age at diagnosis (years)	Microsatellite status	MLH1 expression by immunohistochemistry	MethyQESD	Quantitative *MLH1* methylation PCR with bisulfite-modified DNA
1	25	MSI-H	Negative	0	0
3	34	MSI-H	Negative	0	1
6	66	MSI-H	Negative	59	59
7	45	MSI-H	Negative	0	1
8	51	MSI-H	Negative	0	1
9	74	MSI-H	Negative	0	3
11	78	MSI-H	Negative	1	0
12	31	MSI-H	Negative	0	2
13	39	MSI-H	Negative	0	0
14	51	MSI-H	Negative	0	0
15	64	MSI-H	Negative	0	0
16	37	MSI-H	Negative	0	0
17	70	MSI-H	Negative	0	2
21	37	MSI-H	Positive	0	0
23	37	MSI-H	Negative	0	1
24	37	MSI-H	Positive	0	0
25	37	MSI-H	Negative	0	0
26	37	MSI-H	Negative	0	0
27	63	MSI-H	Positive	0	0
28	33	MSI-H	Negative	0	0
29	48	MSI-H	Negative	0	2
30	36	MSI-H	Negative	2	1
31	44	MSI-H	Negative	0	0
32	56	MSI-H	Negative	0	1
33	30	MSI-H	Negative	0	0
34	37	MSI-H	Negative	0	1
35	37	MSI-H	Negative	0	1
36	37	MSI-H	Negative	0	4
38	34	MSI-H	Negative	0	1
39	30	MSI-H	Negative	0	1
40	60	MSI-H	Positive	0	1
41	31	MSI-H	Negative	0	1
42	46	MSI-H	Negative	0	0
43	65	MSI-H	Negative	0	2
44	42	MSI-H	Negative	0	0
46	62	MSI-H	Negative	10	10
47	24	MSI-H	Negative	0	0
48	35	MSI-H	Negative	0	3
49	27	MSI-H	Negative	0	1
50	37	MSI-H	Negative	0	1
51	35	MSI-H	Negative	1	5
52	47	MSI-H	Negative	0	0

Table 1 Continued

Case no.	Age at diagnosis (years)	Microsatellite status	MLH1 expression by immunohistochemistry	MethyQESD	Quantitative *MLH1* methylation PCR with bisulfite-modified DNA
53	49	MSI-H	Negative	0	0
54	42	MSI-H	Negative	0	5
55	28	MSI-H	Negative	1	0
56	48	MSI-H	Negative	3	0
57	44	MSI-H	Negative	5	1
58	61	MSI-H	Negative	58	17
59	33	MSI-H	Negative	0	0
60	77	MSI-H	Negative	17	19
61	63	MSI-H	Negative	13	15
62	26	MSI-H	Negative	67	65
63	71	MSI-H	Negative	35	19
64	63	MSI-H	Negative	53	45
66	47	MSI-H	Negative	54	37
67	70	MSI-H	Negative	54	52
68	66	MSI-H	Negative	41	36
69	67	MSI-H	Negative	73	100
70	93	MSI-H	Negative	24	41
71	90	MSI-H	Negative	42	63
72	89	MSI-H	Negative	34	20
73	46	MSI-H	Negative	31	35
74	79	MSI-H	Negative	55	55
75	75	MSI-H	Negative	80	61
76	83	MSI-H	Negative	26	80
77	82	MSI-H	Negative	40	48
78	79	MSI-H	Negative	21	23
79	66	MSI-H	Negative	46	56
80	90	MSI-H	Negative	31	48
81	75	MSI-H	Negative	35	30
83	72	MSI-H	Negative	32	36
84	86	MSI-H	Negative	0	1
85	75	MSI-H	Negative	41	22
86	75	MSI-H	Negative	27	32
87	78	MSI-H	Negative	23	62
88	73	MSI-H	Negative	20	38
89	83	MSI-H	Negative	43	22
90	74	MSI-H	Negative	60	19
91	82	MSI-H	Negative	31	35
92	75	MSI-H	Negative	36	57
93	71	MSS	Positive	0	0
94	78	MSS	Positive	3	0
95	69	MSS	Positive	0	0
96	81	MSS	Positive	0	0
97	82	MSS	Positive	1	0

Table 1 Continued

Case no.	Age at diagnosis (years)	Microsatellite status	MLH1 expression by immunohistochemistry	MethyQESD	Quantitative *MLH1* methylation PCR with bisulfite-modified DNA
98	75	MSS	Positive	0	1
99	77	MSS	Positive	0	0
100	78	MSS	Positive	0	1
101	76	MSS	Positive	0	0
102	77	MSS	Positive	0	0
103	78	MSS	Positive	0	0
104	68	MSS	Positive	0	0
105	76	MSS	Positive	0	0
106	61	MSS	Positive	2	0
107	70	MSS	Positive	0	0
108	89	MSS	Positive	0	1

MSS, microsatellite stable; MSI-H, microsatellite unstable; MethyQESD, methylation-quantification of endonuclease-resistant DNA.
There is a significant correlation of MethyQESD and PCR using bisulfite modified DNA (Pearson's $r = 0.885$; $P < 0.01$; SPSS 15.0).

methylation values ranged from 0 to 55%. None of the 16 MSS CRC showed significant *MLH1* promoter methylation values (mean: 0.38%, median: 0.00%, range: 0–3%). All 14 sporadic MSI CRC showed methylation at the (proximal) *MLH1* promoter region (mean methylation values: 33.36%, median: 32.50%, range: 21–55%). 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 *MLH1* germline mutations. The median values of *MLH1* methylation in HNPCC was 0% (mean: 1.04%, range: 0–13%; two cases with values greater than 2%: 10, 13%). Receiver operating characteristic curve (ROC) analysis revealed an optimal cutoff value of 16.5% methylation of proximal MLH1 promoter region for sporadic MSI patients *vs* HNPCC cases (area under ROC curve: 1.000; asymptotic significance: $P < 0.001$). Using this methylation cutoff value both specificity and sensitivity of classification of HNPCC and sporadic MSI-H CRC with loss of MLH1 protein expression as well as MSS CRC was 100%, respectively (sensitivity: 25 true HNPCC cases/(25 true HNPCC cases + zero false sporadic case); specificity: 30 true sporadic cases/(30 true sporadic cases + zero false HNPCC case)). The positive predictive value was 100% (25 true HNPCC/(25 true HNPCC + 0 false sporadic CRC)) and the negative predictive value was 100% (30 true HNPCC/(30 true HNPCC + zero false sporadic CRC)).

Thus, in most of the HNPCC patients (92%, 23/25) no methylation (ie 0–2% methylation) was detected. However, 8% (2/25) of HNPCC tumors showed low-level methylation (10 and 13%).

The MethyQESD of proximal *MLH1* methylation could discriminate between the three tumor groups. A quantile box plot of the median methylation values demonstrates that methylation is significantly different in each tumor group ($P_{\text{Kruskal–Wallis}} < 0.001$; Figure 5). Sporadic MSI-H CRC showed strong *MLH1* promoter methylation ($\geq 21\%$), whereas HNPCC tumors do not show methylation at all or only low values (up to 13% at most). MSS sporadic tumors which were used as controls are consistently *MLH1* methylation negative.

DISCUSSION

The aim of this study was to establish an easy, fast and robust quantitative MA as a molecular tool in research and diagnostics. Generally, several difficulties can complicate methylation analyses. Stromal and inflammatory lymphocytes within tumor tissues can have other methylation patterns than the tumor and may cause false results if nonquantitative MA techniques like simple methylation-specific PCR (MSP) are applied. Moreover, nonquantitative methylation detection techniques principally cannot distinguish between low grade, partial or monoallelic and biallelic methylation.

Beside MSP, other quantitative methylation analyses[25,26] usually require a time consuming bisulfite modification step which generates unstable modified DNA impairing the speed and reproducibility of analyses. In addition the design of primers or fluorescent probes for the degenerated sequence of bisulfite-modified DNA can be difficult or even impossible for certain promoter regions. Methylation detection by pyrosequencing is a powerful technique, but it includes also a bisulfite modification step and requires a costly technical equipment.

These drawbacks could be overcome by the MethyQESD technique which is highly reproducible, easy and sensitive as it uses stable genomic DNA and quantification based on SYBR Green real-time PCR. The high sensitivity of SYBR Green real-time PCR ensures quantification even of very small amounts of DNA, eg DNA from fine-needle biopsies.

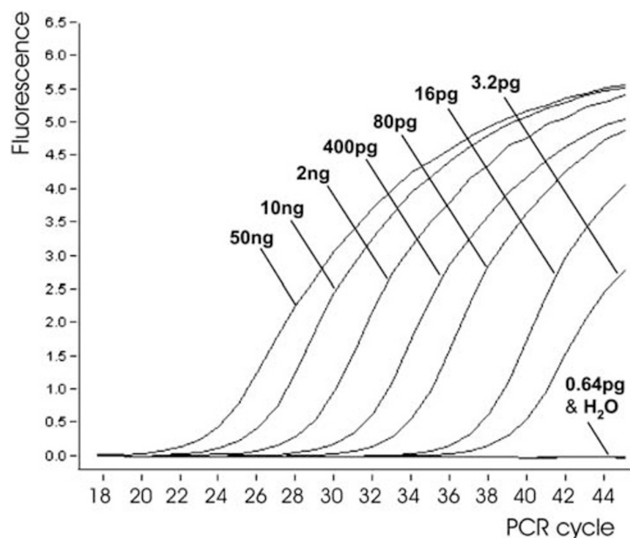

Figure 4 Sensitivity of the *MLH1* MethyQESD. The amount of *Hin*6I treated methylated DNA from SW48 which was added to the corresponding real-time PCR is indicated in the figure.

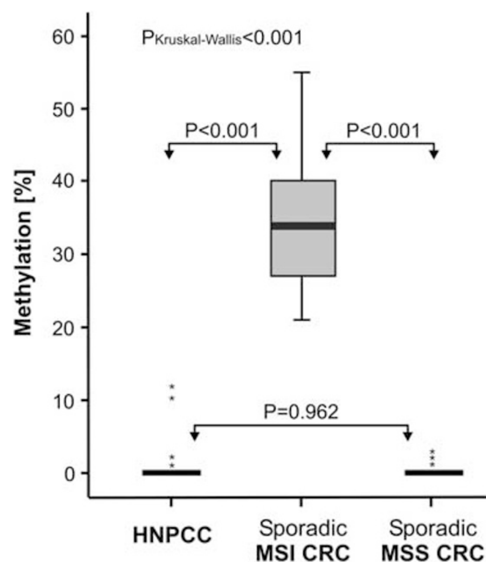

Figure 5 Comparison of *MLH1* promoter methylation in tumors from HNPCC, sporadic MSI-H CRC and sporadic MSS CRC. The box for each tumor group represents the interquartile range (25–75th percentile), the line within each box shows the median value. Bottom and top bars of the whisker indicate the 10th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

We show here that only few nanograms of DNA from as little as 100 cells are sufficient for a MA. However, for routine analysis of formalin-fixed paraffin-embedded tumor tissue we recommend to use 50–100 ng for DNA restriction and to take a 10th aliquot (ie 5–10 ng) of digested DNA for quantitative PCR. As higher C_t-values are accompanied with an increased standard deviation, we recommend the use of DNA triplicates in the real-time PCR reactions containing less than 2 ng DNA. The small size of the amplicons (80–150 bp) ensures reproducible results from fragmented DNA extracted from formalin-fixed and paraffin-embedded tissue samples. This analytical robustness makes MethyQESD best suited for retrospective studies of archival material.

A main advantage of MethyQESD is that no bisulfite modification step is required. Furthermore, the parallel use of a quantification and a calibrator batch of each DNA sample provides a self-calibrating real-time PCR with equal PCR efficiencies. This avoids the need of standard curves as well as the adjustment of DNA concentration. Furthermore, the digested DNA samples can be used for further MethyQESD MA of other genes than *MLH1*.

As PCR efficiency is enhanced by fragmentation of genomic DNA by endonucleases cutting outside the amplicon (data not shown) undigested DNA should not be used for creating a standard curve to avoid an overestimation of methylation. This issue is unfortunately not considered in another quantitative methylation detection methods.[27]

The accuracy of our technique was proven in two different ways. First, we performed spiking experiments of methylated and unmethylated template DNA from a colorectal cancer cell line and blood, respectively (Figure 3), and found a high accuracy (Pearson's $r = 0.9997$ (proximal MLH1 promoter region), $r = 0.9976$ (distal MLH1 promoter region)). Second, in order to evaluate MethyQESD with routine formalin-fixed and paraffin-embedded tissue samples, we compared the methylation values of the proximal MLH1 promoter region generated with MethyQESD and another quantitative MA using conventional bisulfite-modified DNA[7] of 96 formalin-fixed and paraffin-embedded CRC (Table 1). Both methods showed again a high concordance (Pearson's $r = 0.885$).

False positive signals from undigested unmethylated DNA can be excluded, as amounts of 1.5 μg DNA from blood are completely digested by *Hin*6I within 2 h. As *Hin*6I has no intrinsic activity on methylated restriction sites we digested the DNA overnight, although MethyQESD produces reliable results already within less than 5 h (3 h digestion, 1 h real-time PCR, approximately 20 min hands-on time).

We have established MethyQESD for *MLH1* MA as a molecular tool in HNPCC diagnostics as it has been shown that a strong MLH1 methylation occurs in sporadic MSI CRC whereas no or only low levels of MLH1 methylation are present in HNPCC patients.[7] The quantification of *MLH1* methylation by MethyQESD allows the distinction between low level methylation due to, eg partial or monoallelic methylation or epigenetic germline defects in HNPCC[28] and biallelic methylation. Thus, MethyQESD significantly improves the accuracy of HNPCC diagnostics as also HNPCC cases with low level of MLH1 methylation can be detected which were otherwise falsely assigned to sporadic MSI CRC.

The lowest value within the group of sporadic MSI-H tumors was 21% (Figure 5) and the highest value in HNPCC

tumors was 13%. A cutoff value of 16.5% was calculated by a ROC curve analysis. False positive methylation results could be excluded as no positive methylation was detected at any MLH1 expressing MSS CRC. Interestingly, two HNPCC patients (62 and 63 years old, respectively) with pathogenic *MLH1* germline mutations and loss of MLH1 expression showed weak DNA *MLH1* methylation (10 and 13%, respectively). We assume that these cases carry monoallelic *MLH1* promoter methylation, ie one allele is silenced by methylation, whereas the other allele carries the *MLH1* germline mutation. Unfortunately, we were not able to discriminate between maternal and paternal alleles to verify monoallelic methylation by cloning and sequence analysis as there was no sequence polymorphism within the promoter regions.

Although *MLH1* MA by MethyQESD is not a stand-alone test in HNPCC diagnostics, MethyQESD is a valuable tool to identify HNPCC candidates for further analysis like mutation analysis of MMR genes. In addition to *MLH1* methylation, a *BRAF* V600E mutation has been reported to occur specifically in about 70% of sporadic MSI-H CRC.[29,30] Although the detection of a *BRAF* V600E mutation may exclude HNPCC, wild-type *BRAF* V600 can occur in both sporadic CRC and HNPCC making a *BRAF* V600 wild-type result meaningless. However, for best diagnostic results we recommend to perform both *MLH1* MethyQESD and *BRAF* V600E analysis in all CRC with MSI-H and lack of MLH1 protein expression. Patients without significant MLH1 methylation and with wild-type BRAF V600 represent HNPCC candidates who should further clarified by genetic counseling and who might benefit from a tight surveillance program.

In summary, MethyQESD is a robust, fast, reliable and easy-to-handle method for quantitative MA. MethyQESD avoids a bisulfite modification step and can readily be established also for MA of other genes as we have performed it already for *MGMT*, *P16*, *GSTP1*, *RASSF1*, *SFRP1*, *PITX2*, *CA4*, *NEUROG1*, *CDH3* and *APC*. As MethyQESD works reliably with formalin-fixed and paraffin-embedded tissue MethyQESD is ideally suited for quantitative methylation analyses both in diagnostic purposes and for research studies using archival tissues.

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