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ARTICLE

Further evidence for heritability of an epimutation in one of 12 cases with *MLH1* promoter methylation in blood cells clinically displaying HNPCC

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Germline mutations in mismatch repair (MMR) genes, tumours with high microsatellite instability (MSI-H) and loss of MMR protein expression are the hallmarks of HNPCC (Lynch syndrome). While somatic *MLH1* promoter hypermethylation is generally accepted in the tumorigenesis of sporadic tumours, abnormal *MLH1* promoter methylation in normal body cells is controversially discussed as a mechanism predisposing patients to HNPCC. In all 94 patients suspected of HNPCC-syndrome with a mean age of onset of 45.5 years, MLH1-deficiency in their tumours but no germline mutation, underwent methylation-specific PCR-screening for *MLH1* promoter methylation. In peripheral blood cells of 12 patients an *MLH1* promoter methylation, in seven informative cases allele-specific, was found. Normal colonic tissue, buccal mucosa, and tumour tissue available from three patients also presented abnormal methylation in the *MLH1* promoter. The heredity of aberrant methylation is questionable. Pro: *MLH1* promoter methylation was found in a patient and his mother giving evidence for a familial predisposition for an epimutation in *MLH1*. Contra: a *de novo* set-up of methylation in one patient, a mosaic or incomplete methylation pattern in six patients, and no evidence for inheritance of *MLH1* promoter methylation in the remaining families. Our findings provide strong evidence that *MLH1* promoter methylation in normal body cells mimics HNPCC and constitutes a pathogenic pre-lesion in *MLH1*. The identification of hypermethylation as an epigenetic defect has important implications for surveillance recommendations, as these patients should be treated like Lynch syndrome patients, whereas the heritability of methylation is still under investigation.
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Introduction

The hereditary non-polyposis colorectal cancer (HNPCC) syndrome is a familial predisposition to early-onset colorectal cancer (CRC) and tumours of the endometrium, stomach, small intestine, hepatobiliary system, ureter, renal pelvis,

ovary, brain and skin (MIM No 114500).[1] Germline mutations in the DNA mismatch repair (MMR) genes *MLH1, MSH2, MSH6* or *PMS2* are frequently found in combination with high microsatellite instability (MSI-H) and loss of MMR protein expression in tumours.

Suspicion of HNPCC is raised according to the Amsterdam criteria[2,3] or the less stringent Bethesda guidelines.[4] MMR deficiency due to loss of MLH1 in tumour tissue may be due to a germline mutation – but may also result from gene inactivation or silencing by DNA methylation or promoter mutation.

Methylation of cytosines in single CpG dinucleotides located in the promoter region of the *MLH1* gene can epigenetically modify gene expression.[5,6] The *MLH1* CpG islands D and B/C (Figure 1b) are normally unmethylated[7] while biallelic hypermethylation in the tumour tissue was found to be a causative mechanism for 15% of sporadic endometrial,[8,9] gastric[10,11] and colorectal[10,12–14] cancers showing MSI-H and immunohistochemical (IHC) MLH1-deficiency.

The transcriptional activity of the *MLH1* promoter is strongly dependent on two CAAT boxes at c.1-282 and c.1-145 bp[15] and on transcriptional enhancers in a region from c.1-250 to c.1-151 bp[16] (Figure 1a). Another possibility for silencing would be promoter mutations, which have so far not been discovered.[16–18]

The CpG island methylator phenotype (CIMP) describes the hypermethylation of many CpG islands and the silencing of multiple genes, which is a result of the imbalance between methylation protection and *de novo* methylation activity.[19,20] CIMP cancers include a majority of tumours with sporadic MMR deficiency through hypermethylation of the *MLH1* promoter, but also include tumours without microsatellite instability.[21]

Besides methylation of the *MLH1* promoter in tumour cells, epigenetic silencing of *MLH1* in normal body cells is discussed as a novel predisposition to *MLH1* deficiency and CRC. Eleven cases of hemiallelic hypermethylation in the *MLH1* promoter region in peripheral blood of patients with early-onset of CRC or HNPCC-associated endometrial cancer have been reported in the literature.[22–27]

We add the so far largest number of a further 12 patients to this list, which not only helps to prove that aberrant methylation in the *MLH1* promoter is an alternative pathomechanism for the HNPCC phenotype but also helps to better define the specific clinical features of these cases, including heritability. Furthermore, this is the second report of a maternal transmission of an *MLH1* promoter methylation, excluding a *de novo* methylation by chance and pointing towards a familial and heritable predisposition for an epimutation in *MLH1*.

Materials and methods

Patients and material

Patients were recruited in six centers of the German HNPCC consortium. All patients gave written informed consent for the study, approved by the ethics committees.

94 CRC patients (51 males, 43 females, mean age 45.5, SD 33.5 years) with IHC loss of MLH1 expression and MSI-H tumours were included in this study. Of those, 16 fulfilled the Amsterdam criteria and the others met at least one of the revised Bethesda criteria. Tumour localisation was right-sided (proximally of the splenic flexure) in 75% of the cases. The *BRAF* mutation p.V600E which is associated with sporadic *MLH1* inactivation via methylation was excluded in the tumours of all 27 patients available.[28]

DNA from peripheral blood cells was extracted using the Flexigene kit (Qiagen), DNA from buccal mucosa was extracted using the QIAamp DNA Blood Mini kit (Qiagen). DNA extraction of tumour tissue and normal

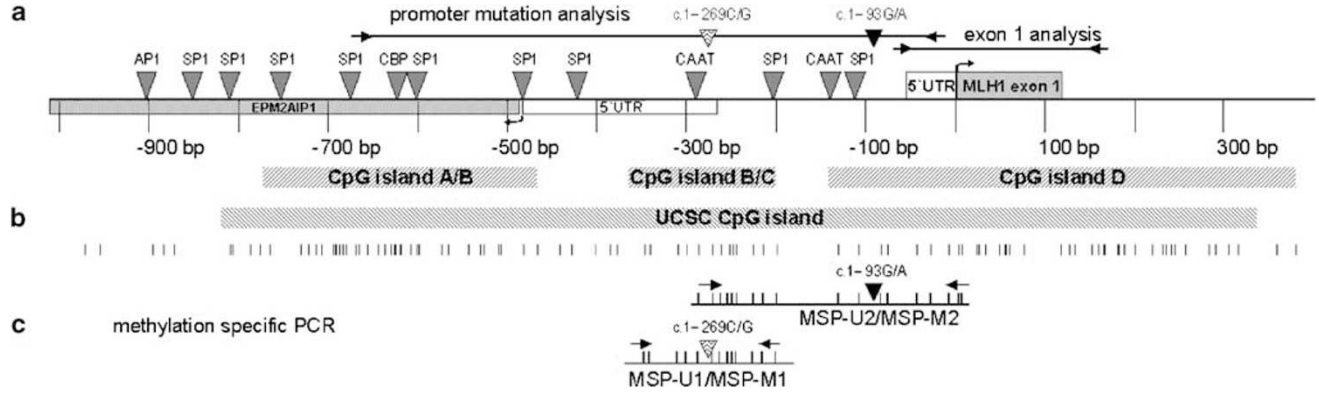

Figure 1 Promoter region of the MLH1 gene. (**a**) Genomic region of *MLH1* and *EPM2AIP1* with translation start sites (bowed arrows), coding regions (filled boxes), promoter and transcription factor-binding sites. The black lines above represent fragments used for mutation analysis and show the localisation of two SNPs. (**b**) Prediction of three CpG islands with prediction programs MethPrimer and EMBOSS CpGplot (depicted in grey bars) or, alternatively, one large CpG island by the UCSC software. (**c**) Localisation of methylation-specific primers for amplification of bisulphite-converted DNA. CpG dinucleotides are presented as vertical bars. Base positions are given relative to the *MLH1* translation start site.

tissue from paraffin-embedded material was done by microdissection.[29]

Microsatellite analyses and immunohistochemical staining

MSI-analysis and IHC-staining was done as described.[4,30]

Mutation and deletion screening of the *MLH1* gene and promoter

Mutation screening of the *MLH1* gene was done by DHPLC analyses[31] or by direct sequencing. Deletion or duplication analysis of the *MLH1* and *MSH2* gene with an MLPA assay was published previously.[32]

The *MLH1* core promoter is located between c.1-300 and c.1-140 bp.[15,16,33] For amplification and sequencing of the region including c.1–667 to c.1–26 bp (Figure 1a and Supplementary Table 1), a standard touchdown PCR protocol[34] was performed.

Bisulphite treatment and methylation analyses

Sodium bisulphite treatment was performed as published,[35] followed by methylation status-specific PCR (MSP) (Figure 1c).[34,36] The MSP primer pairs for either methylated (MSP-M1/MSP-M2) or unmethylated (MSP-U1/MSP-U2) templates in CpG islands D and B/C were designed to match for bisulphite-converted DNA covering 2-3 CpG dinucleotides (Figure 1c and Supplementary Table 1). After amplification, sequencing of MSP-M1 and MSP-M2 was performed.

MS-MLPA

The methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA kit ME011) method can quantify CpG methylation in five different CpG-dinucleotides in the *MLH1* promoter (c.1-637, c.1-401, c.1-244/-250, c.1-7, c.166 + 105 bp, control probe c.1-517 bp). In MS-MLPA, the ligation of MLPA probes is combined with digestion of the genomic DNA with the methylation-sensitive endonuclease *Hha*I and calculated against the undigested MLPA essay.[37,38]

cDNA expression analysis

Total RNA was extracted from peripheral blood by the PAX Gene Blood RNA and preparation kit (PreAnalytix), cDNA was generated with the first-strand cDNA-Synthesis kit (Amersham Biosciences).[35] Analysis of SNP rs1799977 in exon 8 was performed with cDNA primers 7/8forward and 9reverse (Supplementary Table 1) in a standard procedure[34] followed by sequencing.

Haplotype analyses

To determine the haplotypes 4 markers D3S1260, D3S1283, D3S3518, and D3S1611 and SNPs rs1800734, rs35032294, and rs1799977 were investigated in DNA of all family members available.

Primer sequences are available in the Supplementary Table 1 or upon request.

Results

The study cohort consisted of 94 unrelated Bethesda-positive patients with MSI-H tumours, loss of MLH1 protein expression and a negative result in mutation- and deletion-screening in *MLH1*. The *BRAF* mutation p.V600E, frequently associated with sporadic *MLH1* methylation, was excluded in the tumours available from 27 patients, including patients 1, 2, 3, 4, and 12 of Table 1.

Sequence analysis of the *MLH1* promoter

Potential transcription factor-binding sites are located in a region of up to c.1-900 bp according to TESS (www.cbil.upenn.edu/cgi-bin/tess/tess) (Figure 1). To screen for functional mutations in the *MLH1* promoter, sequence analysis of a fragment from c.1–667 to c.1–26 bp relative to the translation start of *MLH1* was performed. The heterozygous sequence alteration rs35032294 at c.1-269C > G[18] was found in individual 6 (Table 1). A common SNP rs1800734[39] at c.1-93G/A relative to the translation start site of *MLH1*, was found to be heterozygote in 41 cases (44%), homozygote A in 11 cases (12%) and homozygote G in 41 cases (44%). 90 control individuals showed an allelic distribution for the SNP c.1-93G/A similar to that of our HNPCC cohort, 3% showed the heterozygote c.1-269C/G SNP.

CpG island prediction

Analysis of the *MLH1* promoter with the prediction programs MethPrimer[40] and EMBOSS CpGplot[41] resulted in three CpG-islands depicted in grey boxes in Figure 1b. Alternatively, one large CpG island of 1128 bp was described by the UCSC Genome Browser.[42]

CpG islands D and B/C, both downstream of c.1-500 bp are unmethylated in normal cells.[7,43] Abnormal methylation of these CpG dinucleotides causes transcriptional silencing, whereas in the upstream CpG island A/B (c.1–755 to c.1–574 bp), methylation is not relevant for *MLH1* transcription regulation.[5,7,43]

Methylation analysis of bisulphite-converted DNA

Screening for methylation in the functionally important regions of the *MLH1* promoter, which includes CpG islands D and B/C (Figure 1b), identified 12 individuals (13%) with a strong amplification of MSP-M1 and MSP-M2 (Figure 1c), indicating an aberrant *MLH1* promoter methylation in peripheral blood cells in these patients. Sequence analyses of MSP-M1 and MSP-M2 showed complete methylation of all 22 CpG dinucleotides (Figure 1c). Sequences of MSP-U1 and MSP-U2 did not reveal any methylation.

Seven methylation-positive patients were heterozygous in the promoter SNP c.1–93G/A or in the SNP c.1-269G/A and allowed an allelic differentiation (Table 1, patients 1, 5,

Table 1 Results of mutation and methylation analyses in the *MLH1* promoter region

Patient number gender	*Age at diagnosis of tumour*	*Affected family member (age)*	*MLH1 coding SNP*	*MLH1 promoter sequencing*	*MSP-U1/MSP-U2 sequencing*	*MSP-M1/MSP-M2 sequencing*	*Methylation in other tissue*	*cDNA expression*	*Methylation status*
1 Male	33 Ascending c 47 Sigmoid c 47 Rectum	Brother gastric 51	c 655G/A	c 1-93G/A	U1 NI U2 c 1-93G monoallelic	M1 NI M2 c 1-93A monoallelic	Colon mucosa, tumour	Complete silencing of MLH1 c 655A	Clonal methylation in different tissues
2 Female	58 Sigmoid c 59 Transversal c	Son hyperplastic polyp 34	None	c 1-93G/G	U1 NI U2 NI	M1 NI M2 NI	Buccal, colon mucosa, tumour	NI	Clonal methylation in different tissues
3 Male	41 Rectum	Mother CC 59 brother polyps 44	None	c 1-93G/G	U1 NI U2 NI	M1 NI M2 NI	Buccal, colon mucosa, tumour	NI	Methylation in different tissues
4 Male	39 Transversal c	Father polyps 50	None	c 1-93G/G	U1 NI U2 NI	M1 NI M2 NI	—	NI	Not defined
5 Female	40 Rectum 41 sigmoid c	Mother cervical 33 CC 64	None	c 1-93G/A	U1 NI U2 c 1-93A monoallelic	M1 NI M2 c 1-93G monoallelic	—	NI	Clonal methylation in blood
6 Male	40 Ascending c 44 Rectum	Father polyps 50	None	c 1-269C/G	U1 c 1-269C>T monoallelic U2 NI	M1 c 1-269G monoallelic M2 NI	—	NI	clonal methylation in blood
7 Male	33 Transversal c		None	c 1-93G/A	U1 NI U2 c 1-93A monoallelic	M1 NI M2 c 1-93G monoallelic	—	—	Clonal methylation in blood
8 Male	35 Ascending c 42+48 Skin 49 Cyst seb gland	Uncle CC 72 grandmother CC 84	c 655G/A	c 1-93G/G	U1 NI U2 NI	M1 NI M2 NI	—	partial silencing of MLH1 c 655G	Mosaic methylation in blood
9 Female	37 Ascending c	Father renal 41	None	c 1-93G/A	U1 NI U2 c 1-93 A/G biallelic	M1 NI M2 c 1-93G monoallelic	—	NI	Mosaic methylation in blood
10 Female	30 Left flexure		None	c 1-93G/A	U1 NI U2 c 1-93G monoallelic	M1 NI M2 c 1-93A monoallelic	—	NI	Clonal methylation in blood
11 Male	46 Ascending c 46 Transversal c		None	c 1-93A/A	U1 NI U2 NI	M1 NI M2 NI	—	NI	Not defined
12 Female	35 Ascending c		c 655G/A	c 1-93G/A	U1 NI U2 c 1-93A/G biallelic	M1 NI M2 c 1-93G monoallelic	—	—	Mosaic methylation in blood

Abbreviations: CC, colon cancer; c, colon; Cyst seb, cystic sebaceous; NI, not informative; —, no material available.
Of 94 HNPCC-suspected *MLH1* mutation-negative patients with MSI-H and loss of MLH1 protein expression in their tumours, 12 patients were methylation-positive.

7, 9, 10, 12). Sequencing of MSP-M2 showed only one allele, either G (four cases) or A (two cases), indicating an allele-specific methylation, which is not restricted to either the G- or A-allele. MSP-U2 represented exclusively the other allele in four cases (Table 1, patients 1, 5, 7, 10, Figure 2a) but was still heterozygous in two cases (Table 1, patients 9 and 12, Figure 2b). This finding denotes an allele-specific methylation, which might not be complete in all hematopoietic cells – indicating a mosaic methylation pattern in two patients.

Patient 6, carrying the heterozygous sequence alteration c.1-269C>G, showed a complete and allele-specific methylation of only the variant c.1-269G allele in MSP-M1, while MSP-U1 monoallelically represented the c.1-269C allele (Table 1).

Methylation was also found in buccal mucosa and normal colonic tissue in three methylation-positive cases analysed. Analysis of the tumour tissues of these patients also revealed methylated and unmethylated alleles. No methylation of the *MLH1* promoter was found in 82 patients and 75 healthy control subjects.

The methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA ME011) assay was used for quantification of aberrant methylation in single CpG-dinucleotides in the *MLH1* promoter, data are displayed in detail in Supplementary Figure 2. The average

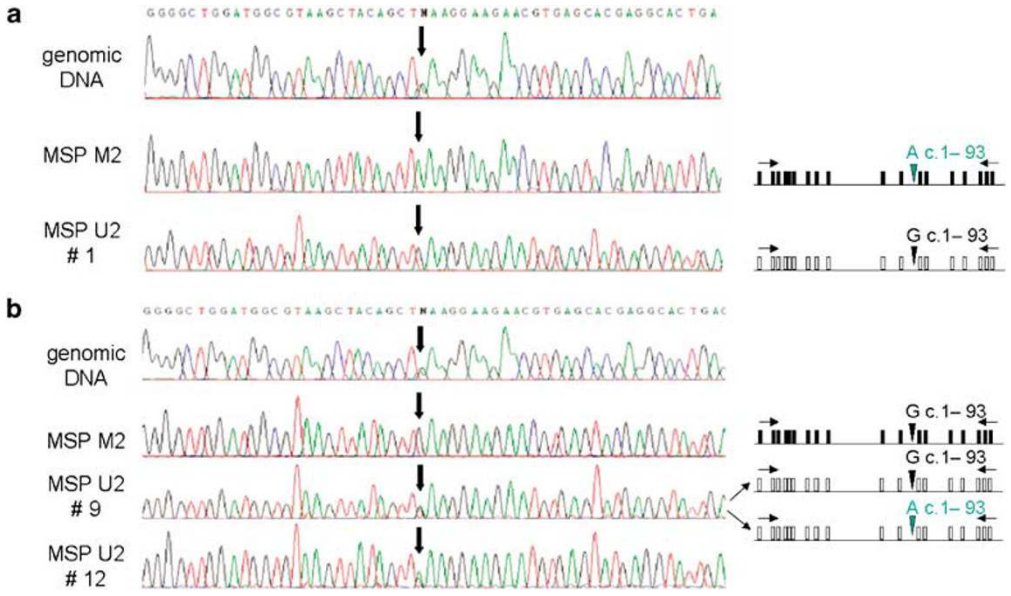

Figure 2 Bisulphite sequences of the methylated fragments revealed allele-specific methylation in all seven genomically heterozygous patients allowing allelic discrimination by promoter SNPs. Five cases showed complete methylation profiles by monoallelic presentation also in the unmethylated fragment (**a**). However, in two patients (9, 12) the methylation of the G-allele was incomplete, as the unmethylated fragment represented two alleles (**b**).

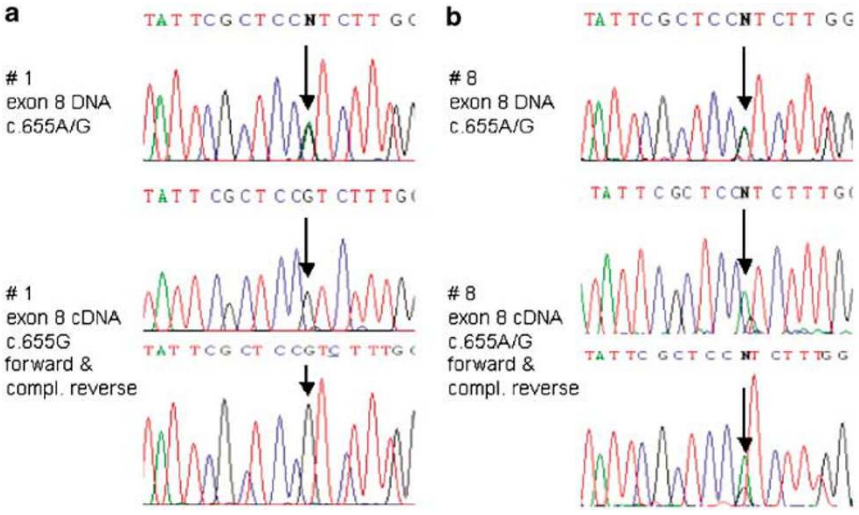

Figure 3 Allelic expression of *MLH1* in two methylation-positive patients genomically heterozygous in *MLH1* exon 8 (top lanes). Bottom lanes: cDNA sequences showed monoallelic expression of the c.655G-allele and complete transcriptional silencing of the c.655A-allele in patient 1 (**a**). Only partial silencing was found in patient 8 (**b**) showing a significant expression reduction of the c.655G-allele.

percentage of methylation was 45–55% in the five patients (1, 5, 6, 7, and 10) with complete methylation of one allele found by allele-specific sequencing of promoter SNPs. For those three patients (8, 9, and 12) with supposed mosaic methylation, MS-MLPA analysis revealed methylation of 19–31% supporting our previous data. The data for four patients without SNPs revealed full methylation in patient 2 and methylation of 10–20% in patients 3, 4, 11 and the mother of patient 4, which are therefore also assumed to be mosaic.

MLH1 cDNA expression

The allelic expression of *MLH1* cDNA was analysed in two methylation-positive patients, both heterozygous for the SNP (c.655A>G; I219V; rs1799977) in *MLH1* exon 8. CDNA-sequencing detected a monoallelical expression of the c.655G allele of *MLH1* for patient 1 (Figure 3a), indicating a complete transcriptional silencing of the c.655A allele, whereas patient 8 showed a signal reduction of approximately 50%, indicating an incomplete transcriptional silencing of the c.655G allele (Figure 3b).

Family studies

Blood samples of eight first degree relatives of five methylation carriers revealed methylation in the *MLH1* promoter in one case (for details see Supplementary Figure 1). The mother of patient 4 showed methylation in blood cells and no external hint to be affected at age 64 but was sent to colonoscopy.

For patient 3, the methylation could not be linked to one allele but his three healthy sons inherited different paternal alleles and were all methylation negative. The methylated allele of patient 9 was derived from her healthy mother who is methylation negative. The son of patient 6 showed no methylation but had inherited the allele unmethylated in his father.

The methylated allele of patient 5 was inherited from her affected mother (cervical cancer at age 33, colon cancer at age 64 without MSI and without loss of MMR protein expression in the CRC), but in the mother the allele was unmethylated. The healthy brother had an unmethylated *MLH1* promoter and did not carry the allele methylated in his sister.

Clinical phenotypes of methylation-positive patients

The clinical phenotypes of the patients are summarised in Table 1. None of the 12 methylation-positive patients fulfilled the Amsterdam criteria, but four patients had one or two family members with gastrointestinal malignancies (Table 1). Eight CRCs were right-sided (proximal to the splenic flexure) and four left-sided primary tumours. Syn- or metachronous CRCs were diagnosed in 50% of the patients. The mean age at tumour diagnosis was 39 ± 19 years SD.

Discussion

We report the so far largest cohort of unrelated HNPCC-suspected individuals with hypermethylation of the proximal promoter region of the *MLH1* gene in peripheral blood cells. This doubles the number of cases reported to date.[22–27]

Seven of the 12 patients had promoter SNPs, which enabled us to prove allele-specific hemiallelic methylation in peripheral blood cells (mesodermal). For three patients endodermal tissues (colonic or buccal mucosa) were available and also methylation-positive. A pan-cellular, allele-specific hypermethylation of the *MLH1* promoter in different germ cell lineages was previously described for nine patients.[22–27] Monoallelic *MLH1* expression could be shown in one of our patients, which was also found in three cases in the literature.[22,24,27]

For the first time, we report two patients with evidence of a mosaic methylation pattern on one allele for the *MLH1* promoter using a SNP at position c.1-93. This incomplete methylation might be due to a clonal expansion of methylated and unmethylated cells, or to a loss or gain of methylation as a somatic event in hematopoietic cells. One further patient revealed an incomplete silencing of a coding SNP in cDNA exon 8, again indicating an inconsistent methylation pattern. The results of methylation extent gained by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) supported our previous findings by MSP-sequencing. Further three patients without SNPs analysed by MS-MLPA revealed a mosaic methylation pattern. A possible explanation might be a *de novo* methylation event after fertilisation, which leads to a clonal or mosaic methylation.

Furthermore, we could show in three patients that inactivation of the second allele in the tumour tissue is due to a mechanism independent of methylation, as no methylation for the second allele was detectable in the three tumour tissues analysed.

Methylation is an epigenetic modification of the DNA which is passed on in somatic mitosis, but it is reversible, considering the comprehensive remodification processes of genomic methylation that occur between primordial germ cell formation and the preimplantation embryo.[44–46]

The family history of methylation carriers was negative in eight of our cases as well as in nine of 11 cases reported in the literature.[22,24,25] Inheritance was analysed in literature in 11 families, but so far only in one family a maternal transmission of the methylation in the *MLH1* promoter could be proven.[22,24,27] For the patients studied here, we were able to analyse inheritance in eight family members. The finding of *MLH1* promoter methylation in a mother and her son implicates a maternal transmission of methylation, while methylation erasure was found in the three methylation negative sons with different paternal alleles of a methylation-positive father. In two families a *de novo* set up of methylation in maternal alleles was found. One daughter had a mosaic or incomplete methylation, while the daughter of the other family showed a complete methylation of the *de novo* methylated allele.

We suspect a methylation event by chance without familial segregation and a low risk of transmission as four allele-sharing siblings published previously and six family members described here, were discordant in respect to *MLH1* promoter methylation.[22,27]

Strikingly, we found incomplete methylation (8%) in the 64-year-old mother of patient 4 (10% methylation) hinting towards a heritable predisposition to an epimutation in *MLH1* eventually due to (1) a maternally transmitted germline methylation or (2) a methylation-inducing mechanism *in cis* caused by the genetic environment.

A striking transmission of mosaic methylation in the promoter of *MSH2* has been reported in one family over three generations and further investigations are necessary to clarify the cause of inheritance of only mosaic and low-level methylation in blood.[47] Here, one could argue for a possible partial erasure of methylation in highly

proliferative cells, accompanied by a consistent methylation in germ cells. We could not find mosaic and low-level methylation in blood of siblings.

Nevertheless, the negative or weak family history in the majority of our cases, the consideration that six patients display a mosaic methylation in hematopoietic cells and the fact that *de novo* methylation was shown in two cases, underlines that inheritance of a predisposition to an epimutation for *MLH1* promoter hypermethylation is a rare event. The erasure of methylation in gametogenesis found in the sons of methylation-carrier 3 is supported by methylation patterns of either 0 or 1% in the spermatozoa of two methylation carriers.[22] The incomplete erasure of methylation (1%) might be an artefact as well.[48]

CpG islands of some genes, among them *MLH1*, are more prone to methylation in several types of cancers.[7,9,11,49] In this respect it is noteworthy that the methylated alleles traced back were of maternal origin in three cases in the literature[24,27] and in one case published here. An increased maternal age at pregnancy can be excluded, as all pregnancies came prior to a maternal age of 34 years, as well as methylation errors due to assisted reproductive techniques.

The clinical phenotypes of 12 *MLH1* methylation-positive cases described here are similar to patients with pathogenic *MLH1* germline mutations.[50,51] The mean age of tumour onset was 39 years (range 30–58 years), however, we found a high incidence of syn-/metachronous tumours in 50%. In the current literature of *MLH1* promoter methylation cases[22–27] clinical features of 11 patients revealed a mean age of onset of 32 years (range 17–46 years) and syn-/metachronous disease in 50%. The clinical features of the six patients with a mosaic methylation pattern did not differ and were not in any sense milder; one showed the Muir-Torre phenotype and was 35 years old at tumour manifestation, the others were 35, 37, 39, 41 and 46 years old at tumour manifestation.

There is increasing evidence that aberrant methylation in the promoter region of one *MLH1* allele is functionally equivalent to a pathogenic *MLH1* germline mutation and mimics the clinical phenotype of Lynch syndrome. To which extent the tumour risk is influenced by the mosaic methylation has to be determined. With the few data we have generated here, there is no difference in age of onset in correlation to the methylation status, mosaic or not mosaic, in blood cells. Nevertheless, there is increasing evidence that these seemingly 'sporadic' HNPCC-patients need to be treated the same way as Lynch syndrome patients in regard to clinical aspects.

Of all patients with specified pre-lesion in the *MLH1* gene 2% have an aberrant *MLH1* promoter methylation in their normal cells, the others are carriers of pathogenic germline point mutations or deletions in *MLH1*. Aberrant methylation of promoters has hitherto been rarely found and investigated, but in the thus far largest cohort analysed constitutes 13% of patients with suspected HNPCC-syndrome without *MLH1* germline mutation but with *MLH1*-deficient and MSI-H tumours. Therefore, methylation analysis of the *MLH1* promoter should be performed for all early-onset or multiple colorectal cancer patients with MSI-H tumours, and loss of IHC MLH1 protein expression of unknown cause. At least first degree relatives of methylation-positive should be offered the *MLH1* promoter methylation analysis to exclude the rare event of inheritance of a familial epimutation, as found in one of our cases.

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Ethics approval
Local ethics committees of all university hospitals involved in the study approved the study protocol.

Conflict of interest
Authors and co-authors declare that there are no competing interests related to the presented article.

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