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ARTICLE

MLH1 methylation screening is effective in identifying epimutation carriers

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Recently, constitutional *MLH1* epimutations have been identified in a subset of Lynch syndrome (LS) cases. The aim of this study was the identification of patients harboring constitutional *MLH1* epimutations in a set of 34 patients with a clinical suspicion of LS, *MLH1*-methylated tumors and non-detected germline mutations in mismatch repair (*MMR*) genes. *MLH1* promoter methylation was analyzed in lymphocyte DNA samples by MS-MLPA (Methylation-specific multiplex ligation-dependent probe amplification). Confirmation of *MLH1* constitutional methylation was performed by MS-MCA (Methylation-specific melting curve analysis), bisulfite sequencing and pyrosequencing in different biological samples. Allelic expression was determined using heterozygous polymorphisms. Vertical transmission was evaluated by MS-MLPA and haplotype analyses. MS-MLPA analysis detected constitutional *MLH1* methylation in 2 of the 34 individuals whose colorectal cancers showed *MLH1* methylation (5.9%). These results were confirmed by bisulfite-based methods. Both epimutation carriers had developed metachronous early-onset LS tumors, with no family history of LS-associated cancers in their first-degree relatives. In one of the cases, the identified *MLH1* constitutional methylation was monoallelic and results in *MLH1* and *EPM2AIP1* allele-specific transcriptional silencing. It was present in normal somatic tissues and absent in spermatozoa. The methylated *MLH1* allele was maternally transmitted and methylation was reversed in a daughter who inherited the same allele. *MLH1* methylation screening in lymphocyte DNA from patients with early-onset *MLH1*-methylated LS-associated tumors allows the identification of epimutation carriers. The present study adds further evidence to the emerging entity of soma-wide *MLH1* epimutation and its heritability.

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INTRODUCTION

Lynch syndrome (LS) is characterized by an autosomal dominant inheritance of early-onset colorectal cancer (CRC) and increased risk of other cancers.[1,2] It is caused by germline mutations in DNA mismatch repair (*MMR*) genes. *MLH1* or *MSH2* are the most commonly mutated *MMR* genes in LS, whereas mutations in *MSH6* or *PMS2* are significantly less common.[3,4] Occasionally, the presence of constitutional epimutations in *MSH2* and *MLH1* has been reported (reviewed in Hitchins and Ward[5] and Kuiper *et al*[6]).

Constitutional epimutations are those stable changes in gene expression that do not affect DNA sequence and that are present in normal tissues of a given individual.[7] An epimutation that occurs in the germline or early embryo can affect all or most of the soma, and phenocopy genetic disease. *MSH2* epimutations, associated with a strong heritability, have been shown secondary to the presence of deletions in the neighboring *EPCAM* gene.[6] The mutations lead to mosaic methylation of *MSH2* in EPCAM-expressing cells.[8]

Approximately 40 index cases of constitutional *MLH1* methylation have been reported.[9–23] However, the prevalence of *MLH1* constitutional epimutations is still unknown. Most studies addressing this issue have enriched their sampling with patients affected with CRC showing loss of MLH1 protein expression.[13,17,20,22] In other cases, series were enriched for patients with CRC at an age of onset below 50 years.[9,14,17,23]

In a very few cases genetic alterations in *cis* (gross rearrangements and variants in the promoter region) have been identified as responsible for the methylation.[13,16,19] In these cases, an autosomal dominant pattern is readily observed. However, in most cases no genetic cause for the epimutation has been identified (reviewed in Hitchins and Ward[5]). In this context, the inheritance of the epimutation has only been experimentally confirmed in three cases.[10,17,20] The functional impact of these epimutations seems clear. In the few cases analyzed, methylation has been linked to allele-specific silencing of *MLH1* and *EPM2AIP1*.[12,13,15,17] This associates with an allele-specific methylation pattern.[11,17,20,21] In these cases, methylation seems to be widespread affecting all embryonic layers being mosaicism reported.[10,12,20]

The aim of our study was to investigate the prevalence of *MLH1* epimutations in a series of 34 patients with *MLH1*-methylated

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CRC and no detected germline *MLH1* mutations. We identified two *bona fide MLH1* epimutation carriers and extensively characterized one of them. The epimutated allele is maternally transmitted, methylation is present in all embryonic layers, erased in spermatozoa and not transmitted to the next generation.

MATERIALS AND METHODS

Patients and samples

Patients were assessed through Cancer Genetic Counselling Units of the Institut Català d'Oncologia (ICO) and the University of Michigan (UM) from 1998 to 2010. A total of 34 individuals (30 ICO, 4 UM) presenting *MLH1*-methylated tumors (methylation levels above 20% in C or D regions) were included in this study (Table 1). The ICO patients were selected from a series of 56 individuals with *MLH1*-deficient CRC and no germline mutations identified in *MLH1*.[24] In all, 29 patients met Bethesda criteria, 1 case met Amsterdam criteria and 4 cases showed other types of CRC familial aggregation. Clinico-pathological information was recorded. Informed consent was obtained from all individuals, and ethics committee approved this study. Sample processing is detailed in Supplementary Methods.

MLH1 promoter methylation analyses

DNA from RKO colorectal tumor cell line (American Type Culture Collection, Manassas, VA, USA) was used as a biallelic *MLH1* methylation control. To generate unmethylated DNA, peripheral blood lymphocyte (PBL) DNA was amplified using the REPLI-g kit (Qiagen, Valencia, CA, USA). A sample of CEPH DNA from the Coriell Institute was used as an unmethylated control in pyrosequencing analyses.[25]

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

SALSA MS-MLPA ME011 kit (MRC Holland, Amsterdam) is based on the use of probes that contain a digestion site specific for the methylation-sensitive *Hha*I enzyme. All reactions were carried out using 100 ng of DNA. The kit includes five probe pairs in *MLH1* promoter (with the respective *Hha*I sites

Table 1 Clinical and molecular features of patients with *MLH1*-methylated CRC

										% somatic MLH1 methylation		
Case	*Gender*	*Clinical criteria*	*CRC age of onset*	*CRC location*	*TNM*	*Grade*	*Mucinous component*	*Other tumors (age of onset)*	*BRAF*	*C region (−246)*	*D region (−13)*	*MLH1 rs1800734 (c.−93G/A)*
1	M	BC	32	L	T3N0M0	G1	No	CRC (34)	wt	57.6	59.7	GA
2	F	BC	49	R	T2N2M0	G3	Yes		V600E	24.9	36.9	GA
3	M	BC	37	L	T3N0M0	G2	No		wt	29.3	31.7	na
4	F	BC	73	R	T4aN0M0	na	Yes		wt	73.5	70.5	GA
5	M	BC	50	R	T3N1M0	G2	No		wt	28.6	33.6	na
6	F	FA	62	R	T3N0M0	G3	No		wt	61.5	78.5	AA
7	M	BC	42	R	T4N2M0	G2	No	CRC (synch)	wt	24.1	25.2	GG
8	M	BC	29	R	T3N0M0	G2	No		wt	25.1	27.6	na
9	F	BC	47	L	T3N1M0	G2	Yes		wt	38.5	34.9	na
10	F	BC	77	R	T3N0M0	na	Yes		wt	38.2	24.1	GG
11	M	BC	52	R	T3N0M0	G2	No		V600E	35.4	4.4	AA
12	F	BC	62	L	T3N0M0	G2	No		wt	53.7	76.7	GA
13	F	BC	59	R	T3N0M0	G2	No		V600E	39.4	45.8	GG
14	F	BC	77	R	T3N0M0	G2	No		V600E	34.5	28.4	GG
15	F	BC	52	R	T4aN0M0	G2	Yes		V600E	22.9	41.4	GG
16	F	BC	24	R	T3N0M0	G3	No		V600E	57.5	75.1	GG
17	M	FA	78	R	T3N0M0	G2	No		wt	12.5	24.0	GG
18	M	BC	48	R	na	na	No		wt	32.8	34.8	GA
19	M	FA	73	R	T3N0M0	G3	Yes		V600E	19.4	31.2	GA
20	F	BC	50	R	T3N0M0	G2	Yes		V600E	35.8	27.0	GG
21	F	BC	58	R	T3N0M0	G2	No	3 CRC (synch)	V600E	40.6	66.6	GG
22	M	FA	85	R	T4bN0M0	G3	No		V600E	41.4	42.5	GA
23	F	BC	47	L	T3N0M0	G3	Yes		V600E	20.3	39.3	AA
24	F	BC	59	R	T1N0M0	G2	No	CRC (29)	V600E	11.4	20.6	AA
25	M	BC	69	R	T4N0M0	G3	Yes	CRC (synch)	wt	50.3	43.1	GA
26	F	BC	75	R	T2N0M0	G2	No	CRC (64)	V600E	27.1	30.3	GA
27	M	BC	47	L	T3N0M0	G1	No		wt	40.1	21.6	AA
28	M	BC	31	L	T4N0M0	G2	Yes		wt	26.2	32.7	GG
29	F	BC	23	L	T4N1M0	G2	No	GC (26)	wt	79.8	50.4	GA
30	M	BC	86	R	T3N0M0	na	na	BrC (69); RC (78)	wt	na	na	na
31	M	AMS	68	R	T3N0M0	na	na	M (80)	wt	na	na	na
32	F	BC	55	R	T2N0M0	na	na		wt	na	na	na
33	F	BC	52	R	T3N1M0	G3	na		wt	na	na	na
34	F	BC	47	R	T1N0M0	na	No	CRC (29), EC (49)	wt	26.1	37.3	GG

Abbreviations: AMS, Amsterdam criteria; BC, Bethesda criteria; FA, Familial aggregation; M, male; F, female; R, right; L, left; CRC, colorectal cancer; EC, endometrial cancer; GC, gastric cancer; BrC, breast cancer; RC, renal cancer; M, mesothelioma; synch, synchronous; wt, wild-type; na, not available.

located at −659, −383, −246, −13 and +208 relative to the start codon; GenBank accession number U26559) that cover five independent regions: regions A to D of the promoter and intron 1.[26]

Methylation-specific melting curve analysis

Methylation-specific melting curve analysis method consists in a real-time PCR followed by temperature dissociation on DNA previously treated with sodium bisulfite,[27] using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). Experimental conditions and primers are detailed in Supplementary Methods, Figure S1 and Table S1.

Bisulfite sequencing

A total of 1 μl of bisulfite-converted DNA was used in a PCR reaction for the amplification and subsequently sequencing of *MLH1* promoter regions C and D.[26] Experimental conditions and primers are detailed in Supplementary Methods, Figure S1 and Table S1.

Clonal bisulfite sequencing

A total of 1 μl of bisulfite-modified DNA was amplified, cloned and sequenced. Experimental conditions and primers are detailed in Supplementary Methods, Figure S1 and Table S1.

Pyrosequencing

In all, 2 μl of bisulfite-converted DNA were used in a PCR reaction for the amplification of regions C and D of the *MLH1* promoter[26] using HotStarTaq master mix (Qiagen) and biotinylated primers (Supplementary Table S1 and Figure S1). Primers were designed using the Pyromark Assay Design Software 2.0 (Qiagen). Experimental conditions are detailed in Supplementary Methods.

MLH1 allelic expression analyses

For allelic expression analyses at the c.655A>G SNP (rs1799977) within *MLH1* exon 8, the relative levels of the A/G alleles were determined in genomic DNA and cDNA by single-nucleotide primer extension (SNuPE) and pyrosequencing, as described in Supplementary Methods.

EPM2AIP1 allelic expression analysis

Amplification and sequencing of rs9311149 flanking region, within *EPM2AIP1* gene, was performed as previously described.[12] For allelic expression analysis at rs9311149, the relative levels of G/T alleles were determined in genomic DNA and cDNA by SNuPE as described in Supplementary Methods, using primers listed in Supplementary Table S1.

Direct sequencing of *MLH1* promoter

Screening for mutations within the *MLH1* promoter was performed by PCR amplification and sequencing as described.[28] One reverse amplification primer has been modified (Supplementary Table S1).

Haplotype analysis

Haplotype analysis was performed using four intragenic *MLH1* single-nucleotide polymorphisms (rs1800734, rs9876116, rs1799977 and rs4234259) and seven microsatellite markers (D3S1609, D3S1612, D3S2369, D3S1611, D3S3623, D3S1298, D3S3564) covering 12 Mb around *MLH1*, as previously described.[29] To deduce the methylation-associated haplotype, intrafamilial segregation analysis was performed under the assumption that the number of crossovers between adjacent markers was minimal.

Second hit analysis

Loss-of-heterozygosity (LOH) analysis was performed on DNA extracted from paraffin-embedded tumor tissue and compared with PBL DNA at informative microsatellites (see haplotype analysis) and SNP rs1799977, either by genotyping or SNuPE (see Supplementary Methods), respectively. *MLH1* somatic mutation status was assessed in tumor DNA by direct sequencing and multiplex ligation-dependent probe amplification (SALSA MLPA P003-B1; MRC Holland).

BRAF V600E screening

A 196-bp region of human *BRAF* gene spanning the hotspot mutation c.1799T>A (V600E) was amplified by PCR (Supplementary Table S1) as described.[24] The PCR products were purified using Illustra GFX DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK). *BRAF* V600E mutation detection was performed by SNuPE using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) and a specific primer.

RESULTS

Clinical and molecular features of patients with *MLH1*-methylated CRC

In all, 34 patients (15 males; 19 females) were analyzed (Table 1). Mean age at diagnosis was 55 (range 23–86 years). Twenty-six tumors (76%) were located in the right colon and ten (33%) were classified as mucinous. Only six patients (18%) had lymph node involvement and none of them had distal metastasis. *BRAF* mutations were detected in 13 tumors (38%). A common SNP rs1800734 (c.−93G>A) within the *MLH1* promoter was found to be heterozygote in 10 cases (38%) and homozygote A in 5 (19%). In eight individuals (24%), additional LS-associated tumors were diagnosed, three synchronous and five metachronous (Table 1). Molecular characterization of these additional tumors (Table 2) allowed demonstrating the existence of two *MLH1*-methylated tumors in four individuals (cases 1, 7, 29 and 34).

Identification of new LS cases harboring a constitutional *MLH1* epimutation

The methylation status of *MLH1* promoter was analyzed by MS-MLPA in DNA extracted from PBLs. Constitutional methylation was only detected in 2 individuals (cases 1 and 34) of the 34 patients included (5.9%). It represented 2 out of 100 LS cases in ICO series (2%). In both cases, methylation in *MLH1* promoter was detected in the five regions analyzed, including C and D promoter regions, which was correlated with transcriptional silencing[26] (Table 3).

Sequencing analysis of the whole *MLH1* promoter (from c.−1469 to intron 1) in PBL DNA from cases 1 and 34 did not detect any variant affecting the binding of MLPA probes nor *Hha*I restriction sites. Likewise, it revealed that case 1 was heterozygous for SNP rs1800734 (c.−93G>A) and case 34 was heterozygous for SNP rs34566456 (c.−607G>C). No other variants – including c.−27C>A and c.85G>T[16] – were identified within the promoter region.

Case 1 is a 47-year-old male who underwent urgent sigmoidectomy due to intestinal occlusion secondary to a sigmoid adenocarcinoma (pT3N0M0, stage II) at the age of 32. After 2 years, the patient was diagnosed with an adenocarcinoma of the hepatic flexure (pT3N0M0, stage II) and a subtotal colectomy was carried out. Microsatellite analysis showed MSI, loss of MLH1 and PMS2 expression, absence of *BRAF* V600E mutation and somatic *MLH1* methylation in both tumors (Table 2). The patient had no family history of cancer in his first-degree relatives as it is shown in his pedigree (Figure 1a).

Case 34 is a 55-year-old female who was diagnosed of a sigmoid adenocarcinoma (pT3N1M0, stage III) at the age of 29 years and underwent a sigmoidectomy. After 15 years, the patient was diagnosed with an adenocarcinoma of the hepatic flexure (pT1N0M0, stage I). At the age of 49 years, she was diagnosed of an endometrial adenocarcinoma (pT1N0M0). Microsatellite analysis showed instability of the five analyzed markers in the second CRC, and instability of bat26 and MONO-27 in the endometrial cancer. Both second colorectal and endometrial tumors showed loss of MLH1 and

Table 2 Molecular features of tumors from patients affected by multiple LS-associated tumors

				IHC					% somatic MLH1 methylation	
Case	Tumor type	Age of onset	MSI analysis	MLH1	MSH2	MSH6	PMS2	BRAF	C region (−246)	D region (−13)
1	CRC[a]	32	+	−	+	+	−	wt	57.6	59.7
	CRC	34	+	−	+	+	−	wt	60.5	62.8
7	CRC[a]	42	+	−	+	+	ND	wt	24.1	25.2
	CRC	42	+	−	+	+	−	wt	28.9	24.2
21	CRC[a]	58	+	−	+	+	ND	V600E	40.6	66.6
	CRC	58	−	+	+	+	+	ND	ND	ND
	CRC	58	−	+	+	+	+	ND	ND	ND
	CRC	58	−	ND	ND	ND	ND	ND	ND	ND
24	CRC	29	NA	NA	NA	NA	NA	NA	NA	NA
	CRC[a]	59	+	−	+	+	ND	V600E	11.4	20.6
25	CRC[a]	69	+	−	+	+	ND	wt	50.3	43.1
	CRC	69	NA	NA	NA	NA	NA	NA	NA	NA
26	CRC	64	NA	NA	NA	NA	NA	NA	NA	NA
	CRC[a]	75	+	−	+	+	−	V600E	27.1	30.1
29	CRC[a]	23	+	−	+	+	ND	ND	79.8	50.4
	GC	26	+	−	+	+	ND	ND	63.0	73.0
34	CRC	29	NA	NA	NA	NA	NA	NA	NA	NA
	CRC[a]	47	ND	−	ND	ND	−	wt	55.5	48.8
	EC	49	+	−	+	+	ND	wt	26.1	37.3

Abbreviations: NA, not available; ND, not done.
[a]Tumors included in the initial series listed in Table 1.

Table 3 Analysis of *MLH1* methylation using MS-MLPA in samples from the proband and relatives

			% MLH1 methylation				
Family	Individual	Sample	A region (−659)	B region (−383)	C region (−246)	D region (−13)	Intron 1 (+208)
A	I.1	PBL	0	0	0	0	0
	II.1 (case 1)	CRC 1	**61.2**	**83.7**	**57.6**	**59.7**	**60.9**
		CRC 2	**62.3**	**86.9**	**60.5**	**62.8**	**63.5**
		PBL	**60.5**	**76.7**	**56.0**	**56.2**	**60.2**
		fibroblasts	**55.8**	**53.2**	**64.0**	**52.4**	**63.0**
		colonic mucosa	**52.3**	**78.9**	**58.3**	**48.5**	**62.6**
		sperm	0	0	0	0	0
	II.2	PBL	0	0	0	0	0
	III.1	PBL	0	0	0	0	0
	III.2	PBL	0	0	0	0	0
B	case 34	EC	**33.6**	**59.4**	**26.1**	**37.3**	**28.5**
		CRC	**58.0**	**56.3**	**55.5**	**48.8**	**56.4**
		PBL	**35.9**	**45.3**	**25.1**	**27.6**	**27.7**
		RKO	**110.1**	**113.2**	**103.0**	**88.2**	**103.4**

Peripheral blood lymphocytes (PBL), skin fibroblasts, colorectal tumors (CRC 1 and 2), normal adjacent mucosa and sperm from case 1 (II.1), PBL from his relatives, and PBL, CRC and endometrial cancer (EC) from case 34, were analyzed. DNA from RKO cell line (methylated in *MLH1*) is used as a positive control. Representative data from two independent experiments is shown. Methylation levels above 20% are shown in bold.

PMS2 expression, absence of *BRAF* V600E mutation and somatic *MLH1* methylation (Table 2). Patient's mother was affected by a breast cancer at the age of 77 years (Figure 1b).

Methylation-specific melting curve analysis confirmed the presence of a methylated peak in C and D promoter regions in both cases (Figure 2a and Supplementary Figure S2). Likewise, bisulfite sequencing showed the presence of both methylated C as well as non-methylated T (bisulfite-converted non-methylated C) alleles at each CpG site in the samples of interest (Figure 2a and Supplementary Figure S2). Average methylation levels in PBL of the case 1 were 34% and 39% in C and D regions, respectively, as assesed by pyrosequencing (Figure 2a; Table 4). Clonal bisulfite sequence analysis confirmed hemiallelic methylation in PBL DNA confined to allele A of the rs1800734 (Figure 2b). In case 34, average methylation levels in PBL were 20% and 19% in C and D regions, respectively (Supplementary Figure S2; Table 4).

Functional impact of the *MLH1* epimutations

The *MLH1* promoter is bi-directional for transcription of *MLH1* and *EPM2AIP1* genes. In case 1, the neutral heterozygous polymorphism c.655G>A (rs1799977) within *MLH1* exon 8 was used to determine the effect of the epimutation on *MLH1* transcriptional activity. Monoallelic expression of *MLH1* transcript, associated to G allele, was demonstrated by pyrosequencing and SNuPE (Figure 3). ASE (allele-specific expression) values obtained in patient and control sample were 0.05 and 1.17 when analyzed by pyrosequencing, and 0.02 and 0.98 by SNuPE, respectively. In case 34, the absence of coding heterozygous polymorphisms in *MLH1* prevented its transcriptional analysis.

SNuPE analysis at rs9311149 of *EPM2AIP1* evidenced complete silencing of *EPM2AIP1* G allele in case 1 (Figure 3b, right panel) and partial silencing of the same allele in case 34 (Supplementary Figure S2b), further reinforcing the functional impact of the constitutional methylation. The obtained ASE values were 0.02 in case 1, 0.48 in case 34 and 1.00 in control sample.

Characterization of the *MLH1* epimutation

MLH1 methylation pattern. Follow-up of case 34 and her family has proved difficult. Thus, for the purpose of detailed characterization, we

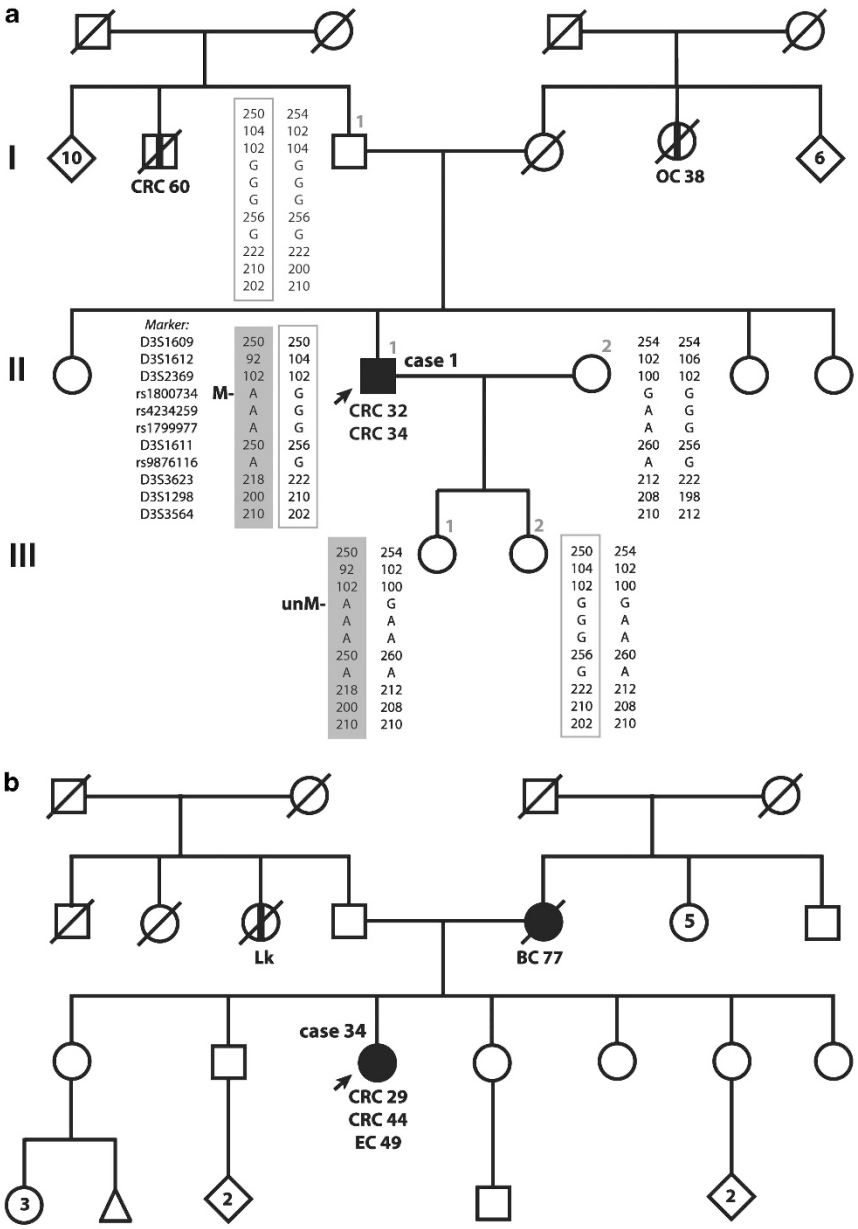

Figure 1 Family pedigree of the epimutation carriers. Circles, females; squares, males; filled, cancer affected; vertical line at center, non-confirmed cancer affected. Cancer localization (CRC, colorectal cancer; OC, ovarian cancer; EC, endometrial cancer; BC, breast cancer; Lk, leukemia) and age at diagnosis are indicated. (**a**) Pedigree and haplotypes of case 1. The epimutation carrier (II.1) is indicated by an arrow. Generations are indicated on the left margin in Roman numerals and analyzed relatives are identified by numbers. Haplotypes, generated by analyzing SNP and microsatellite markers flanking or within *MLH1*, are detailed according to the key indicated in individual II.1. The paternally inherited allele in II.1 is in a square and the maternally derived allele is highlighted in dark gray. The presence of methylation (M) or its absence (unM) is indicated. (**b**) Pedigree of case 34. The epimutation carrier is indicated by an arrow.

have focused in the characterization of case 1. First, we wanted to explore whether methylation was present in all embryonic layers and in the germline of case 1. MS-MLPA analysis in skin fibroblasts (ectoderm) and colorectal mucosa (endoderm) revealed similar levels of *MLH1* methylation than in PBL (Table 3), indicating hemiallelic methylation in all embryonic layers. In contrast, no methylation was detected in patient sperm as evidenced by MS-MLPA and pyrosequencing analyses (Tables 3 and 4). Direct sequencing of the PBL and sperm for *MLH1* promoter C region evidenced the presence of both alleles at rs1800734 in both samples (data not shown). These results indicate the reversion of the epimutation in patient spermatozoa.

Inheritance pattern of the epimutant allele. To further investigate the inheritance pattern of the allele harboring the epimutation, we analyzed the *MLH1* promoter methylation status as well as a haplotype of 12 Mb around *MLH1* in available PBL DNA from patient's first-degree relatives. MS-MLPA analysis showed no evidence of *MLH1* methylation in relatives (Table 3). Haplotype analysis revealed that the

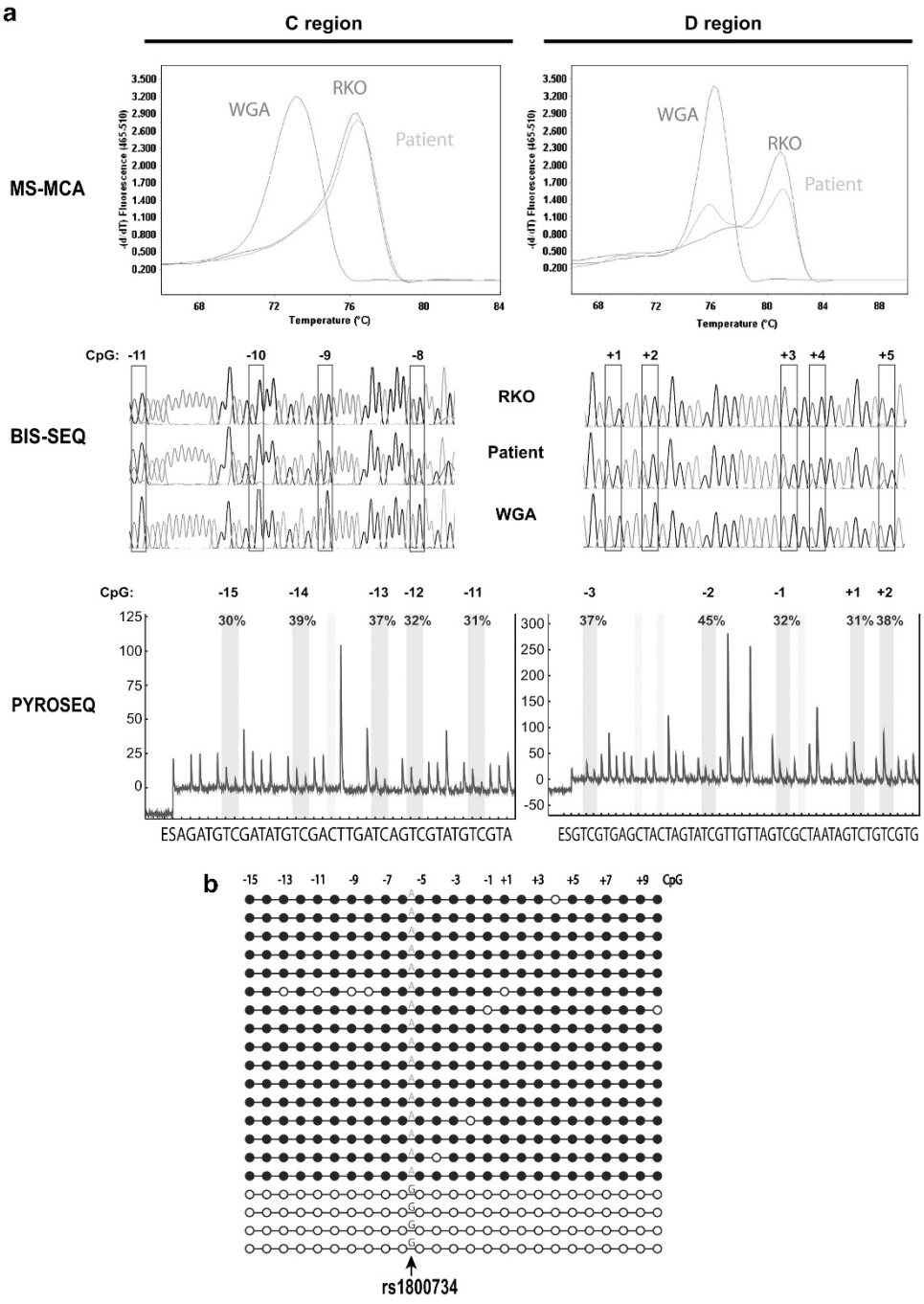

Figure 2 Confirmation of the constitutional *MLH1* epimutation of case 1. (**a**) Analysis of the *MLH1* promoter C and D regions by methylation-specific melting curve analysis (MS-MCA), bisulfite sequencing (BIS-SEQ) and pyrosequencing (PYROSEQ). Top panel: MS-MCA of *MLH1* promoter. In the analysis of C region, WGA DNA (unmethylated control) and RKO DNA (methylated control) show single melting peaks at 73 and 77°C, respectively. In D region, WGA and RKO melting peaks temperature are 76 and 82°C, respectively. Analysis by MS-MCA in PBL DNA from the patient 1 (green line) shows the presence of the methylated peak in both regions. Middle panel: sequence analysis of bisulfite-converted DNA. WGA DNA shows T at each CpG analyzed, consistent with complete modification of the DNA. RKO DNA shows C at each CpG. Patient DNA shows a mixture of T and C at CpG sites, attributable to partial methylation. Bottom panel: representative pyrograms obtained in the analysis of C and D *MLH1* promoter regions in PBL DNA from the patient. The peaks within the shaded area of the pyrogram correspond to the CpG interrogated. Percentage methylation at each site is calculated as the C:T ratio of peak heights (representing methylated:unmethylated cytosine). *x* axis represents the nucleotide dispensation order. *y* axis units are arbitrary representing light intensity. (**b**) Clonal bisulfite sequencing of the *MLH1* promoter in PBL DNA from the epimutation carrier 1. Each horizontal line represents a single allele. CpG dinucleotides are depicted by circles. Black and white circles indicate methylated and unmethylated CpG, respectively. The allele at rs1800734 (c.−93G>A) is indicated as A or G. Methylation is confined to the A allele. Each CpG analyzed is numbered according to its position relative to the translation initiation codon.

epimutated allele is only shared by the patient and one of his daughters (Figure 1a). The lack of availability of biological material from the mother has precluded us from analyzing the presence of the epimutation in her. These results confirmed that the epimutated allele is maternally inherited in the patient, and that methylation is erased in the patient's daughter who inherited the same allele.

Table 4 Quantification of *MLH1* promoter methylation by pyrosequencing

MLH1 *promoter C region*

			CpG position								
Family	*Individual*	*Sample*	*−15*	*−14*	*−13*	*−12*	*−11*	*Mean*	*SD*	*Min*	*Max*
A	II.1 (case 1)	PBL	32.0	38.1	36.1	31.7	33.6	34.3	2.8	31.7	38.1
		sperm	2.1	0.0	3.8	2.1	1.4	1.9	1.4	0.0	3.8
B	case 34	PBL	22.1	21.6	20.1	17.1	17.7	19.7	2.3	17.1	22.1
		RKO	95.5	96.5	94.2	92.6	95.9	94.9	1.6	92.6	96.5
		CEPH	2.2	2.15	3.6	2.55	2.3	2.6	0.6	2.2	3.6

MLH1 *promoter D region*

			CpG position											
Family	*Individual*	*Sample*	*−6*	*−5*	*−4*	*−3*	*−2*	*−1*	*1*	*2*	*Mean*	*SD*	*Min*	*Max*
A	II.1 (case 1)	PBL	39.0	50.0	38.9	36.4	43.8	33.3	32.1	39.4	39.1	5.8	32.1	50.0
		sperm	0.0	5.3	0.0	1.6	6.5	2.9	0.0	1.7	2.3	2.5	0.0	6.5
B	case 34	PBL	19.7	20.5	19.1	19.2	17.2	18.3	19.8	19.2	19.1	1.0	17.2	20.6
		RKO	95.5	92.6	84.0	90.2	76.1	72.7	81.0	93.7	85.7	8.6	72.7	95.5
		CEPH	3.4	5.7	0.0	0.0	9.4	3.8	2.7	2.9	3.5	3.0	0.0	9.4

Each sample was run in triplicates. Methylation at each specific CpG was calculated as the mean of the triplicates. Values for each specific CpG within the region are given in percentage. Average percentage of methylation of the whole region was calculated as the mean for the five CpGs analyzed in C region and the eight CpGs in the D region. Both peripheral blood lymphocytes (PBL) and sperm from the proband (II.1) were analyzed. DNA from the colorectal cancer cell line RKO was used as positive control. CEPH DNA was used as negative control. Each CpG analyzed is numbered according to its position relative to the translation initiation codon.

Inactivation of the non-methylated allele in tumor tissue. We explored the nature of the putative second hit in the patient's sigmoid colon cancer. Full exonic sequencing of the *MLH1*-coding region did not identify any additional mutation. LOH was evidenced at *MLH1* rs1799977 and D3S1611 (data not shown). Retention of heterozygosity was observed at the distal marker D3S3564, whereas LOH was not evaluable at markers D3S1612, D3S3623 and D3S1298 due to their instability. These results point to the loss of the wild-type *MLH1* allele in tumor DNA. MLPA analysis in tumor DNA was not conclusive, probably owing to the poor quality of tumor FFPE-DNA.

DISCUSSION

We identified two *bona fide MLH1* epimutations and one of them has been extensively characterized. In previous reports, *MLH1* epimutations were detected in 8–13% of patients with tumors showing MLH1 loss of expression.[13,17,20,22] We have detected this alteration in 2 out of 30 patients with *MLH1*-methylated CRC meeting Bethesda or Amsterdam criteria (6.7%) and in 2 of 14 patients with an age of onset below 50 years (14.2%), in whom no germline *MLH1* mutation was identified. This is in line with the prevalence reported by van Roon *et al*[23] in patients with *MLH1*-methylated tumors enriched for cases with an early age of onset. If we take into consideration only the ICO series, *MLH1* epimutations represent so far 2% of all LS cases.

In accordance with previous reports (reviewed in Hitchins and Ward[5]), the cases identified in this study had developed multiple LS tumors at an early age. This may not only reflect the phenotype associated with the epimutation but also the selection criteria used so far in most studies. Of note, methylation was not only detected in metachronous colon tumors but also in endometrial carcinomas as well. *BRAF* mutation was absent in four analyzed tumors from the identified epimutation carriers. However, the presence of somatic *BRAF* V600E mutation has been previously reported in tumors from three epimutation carriers,[10,12,23] representing 15.8% (3/22) of the reported cases. In our set of cases, the degree of *MLH1* methylation is highly variable among tumors from both epimutation carriers and the remaining patients. Epimutations have been detected in two of four cases where multiple tumors showed somatic *MLH1* hypermethylation.

PBL methylation levels correlated with the observed transcriptional silencing, suggesting the presence of mosaicism in case 34. Dosage of the methylated allele is important. In line with previous observations, approximately 50% of the alleles were methylated in case 1.[10,11,14,17,20,21] As reported, the functional impact of the epimutation seems clear, as it associates with monoallelic expression of *MLH1* and *EPM2AIP1* transcripts[12,13,15,17] and an allele-specific methylation pattern.[14,17,20,21] LOH in an intragenic *MLH1* microsatellite marker was detected, consistent with somatic loss of the unmethylated allele. In fact, LOH has been found to be the most frequent mechanism of inactivation of wild-type allele in tumors from epimutation carriers.[12]

So far, in all cases identified but one, the methylated allele was of maternal origin.[10,12,15,17,20] The epimutation was found in the maternally inherited allele. Although we were unable to definitively demonstrate whether the epimutation was inherited or *de novo*, this may further support the notion that this type of aberration is more likely to accumulate during the oogenesis. We were able to perform a more detailed study of the index case and descendants. While *MLH1* methylation was present in every embryonic layer of the index case, a complete erasure was observed in the spermatozoa, as reported by Hitchins *et al*.[17,30] The lack of methylation in spermatozoa does not necessarily mean that inheritance cannot occur. In fact, this was clearly demonstrated in one descendant who inherited the epimutation out of three harboring the same allele.[17] In our case, the epimutated allele was transmitted unmethylated to one of his daughters.

In spite of an extensive search, we have not been able to identify a genetic alteration underlying the epimutated allele. Genetic aberrations in *cis* (gross rearrangements in two cases (one deletion of *MLH1* exons 1 and 2, and one duplication involving the whole gene) and in a third one the variant c. −27C>A within the promoter region) have been identified as responsible for *MLH1* methylation.[13,16,19] Dominant transmission pattern is observed in these cases.

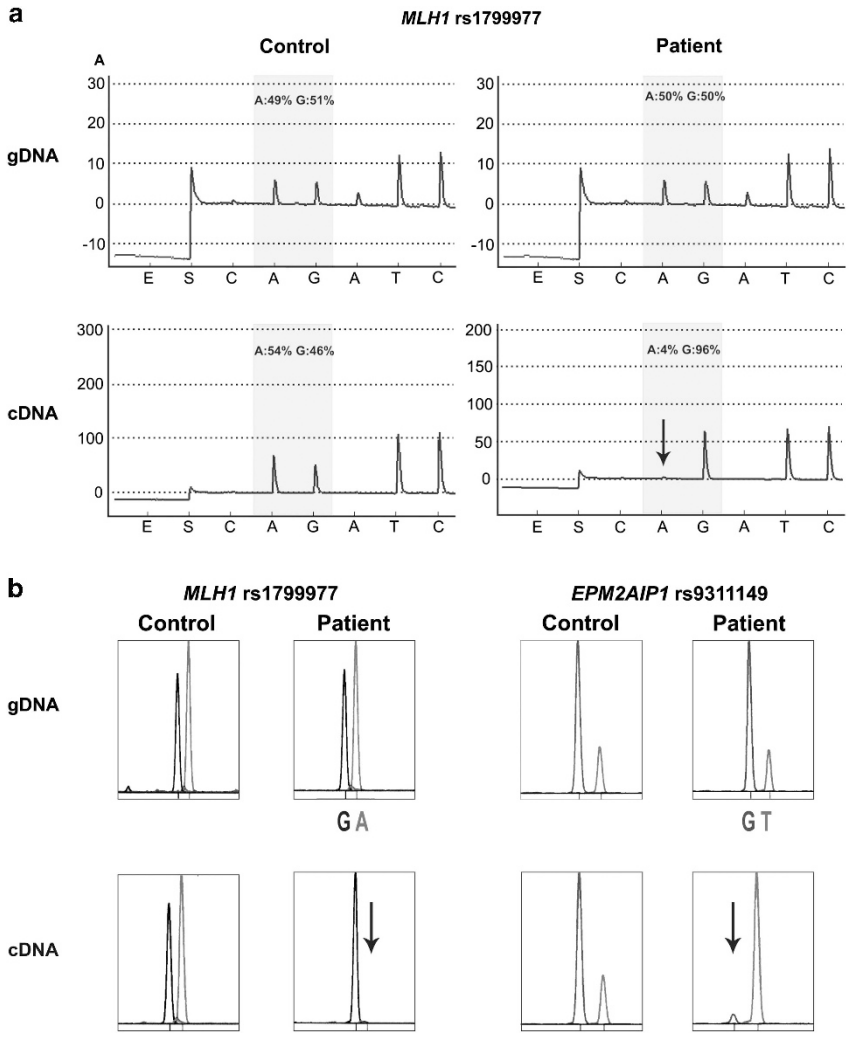

Figure 3 Transcriptional inactivation of *MLH1* and *EPM2AIP1* alleles. (**a**) Illustrative example of the pyrogram across the expressible *MLH1* rs1799977 (c.655A>G) in genomic DNA (gDNA) (top panels) and cDNA (bottom panels) derived from a heterozygous healthy control (left panels) and the epimutation carrier (right panels). The peaks within the shaded area of the pyrogram are the nucleotides at the SNP site, quantified with respect to neighboring nucleotides. Their relative values are given as percentage values above the pyrogram trace. There was a transcriptional inactivation of the 'A' allele (indicated with a downward arrow) in the cDNA of the patient with the *MLH1* epimutation. *x* axis represents the nucleotide dispensation order. *y* axis units are arbitrary representing light intensity. (**b**) Representative results of the SNuPE analysis at *MLH1* rs1799977 (c.655A>G) (left panel) and *EPM2AIP1* rs9311149 (right panel) in gDNA and cDNA derived from a heterozygous control and the epimutation carrier. Transcriptional silencing of the A allele at *MLH1* rs1799977 and T allele at *EPM2AIP1* rs9311149 in the cDNA of the patient was observed.

Dominant inheritance has been also observed in cases where no genetic alterations are detected.[10,12,15,17,20] In these cases, methylation was mosaic and associated to a shared haplotype.

Although we cannot completely rule out that aberrations have been missed, the lack of family history and the lack of vertical transmission are compatible with a *de novo* methylation occurred in the early embryo, where there is no apparent predisposing genetic mechanism that would allow for the restoration of methylation after the gametogenesis. However, this is an unsettled issue. The epimutation carrier identified in this study showed methylation confined to the A allele at rs1800734, although allele-specific methylation is not restricted to either A or G allele in other reported cases.[14,17,20,21] It is intriguing that the A allele at rs1800734 associates with somatic *MLH1* promoter methylation and increased risk of MSI CRC.[23,31–35] In addition, it has been shown that this polymorphism modifies the efficiency of *MLH1*/*EPM2AIP1* transcription.[36]

It is difficult to translate these findings into specific recommendations for these patients and their relatives. At this time caution is mandatory. In the presence of a detected constitutional epimutation, genetic screening of descendants is important. However, in the presence of an inherited non-methylated allele in lymphocyte DNA, two options are available. On the one hand, descendants can be counseled as relatives of a LS case where direct genetic testing has been non-informative. In this setting, it is assumed that lack of methylation in the inherited allele does not rule out that a mosaic status is present in the patient or that a non-detected genetic alteration predisposing to a late acquisition of methylation is present in this family. Alternatively, recommendation can be made based on the degree of personal and familial history of cancer. Further knowledge is needed to translate these research findings into useful information for management of patients and families.

The increasing detection of epimutations has lead to the suggestion that the diagnostic algorithm of LS might be improved. So far, the detection of somatic *MLH1* hypermethylation is often used to exclude patients from further MMR mutation analysis, based on cost effectiveness considerations.[24,37] The patients with somatic *MLH1* hypermethylation could now be considered as candidates to screen for constitutional *MLH1* epimutations. Based on the clinical presentation of the reported cases[5] and our experience, this screening could be restricted to those diagnosed earlier than 50 years or with multiple tumors the first one before the age of 60. If this was the case, MS-MLPA could be a good methodological approach. The robustness and informativeness already shown for paraffin-embedded tissues[24] has been confirmed when being used in the germline. In any case, confirmation with at least another technique (ie, pyrosequencing) would be mandatory.

In summary, *MLH1* methylation screening in PBL from patients with early-onset *MLH1*-methylated CRC allows the identification of epimutation carriers. Using this strategy we have identified two *bona fide MLH1* epimutations. In one of them, the methylated allele is from maternal origin, is present in all embryonic layers and is absent in spermatozoa. The characterization of these cases provides further evidence of the emerging entity of soma-wide MLH1 epimutation and its heritability.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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