Concomitant mutation and epimutation of the MLH1 gene in a Lynch syndrome family

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Abstract

Lynch syndrome (LS) is an inherited predisposition cancer syndrome, typically caused by germline mutations in the mismatch repair genes MLH1, MSH2, MSH6 and PMS2. In the last years, a role for epimutations of the same genes has also been reported. MLH1 promoter methylation is a well known mechanism of somatic inactivation in tumors, and more recently, several cases of constitutional methylation have been identified. In four subjects affected by multiple tumors and belonging to a suspected LS family, we detected a novel secondary MLH1 gene epimutation. The methylation of MLH1 promoter was always linked in cis with a 997 bp-deletion (c.-168_c.116+713del), that removed exon 1 and partially involved the promoter of the same gene. Differently from cases with constitutional primary MLH1 inactivation, this secondary methylation was allele-specific and CpGs of the residual promoter region were totally methylated, leading to complete allele silencing. In the colon tumor of the proband, MLH1 and PMS2 expression was completely lost as a consequence of a pathogenic somatic point mutation (MLH1 c.199G>A, p.Gly67Arg) that also abrogated local methylation by destroying a CpG site. The evidences obtained highlight how MLH1 mutations and epimutations can reciprocally influence each other and suggest that an altered structure of the MLH1 locus results in epigenetic alteration.

Introduction

Lynch syndrome (LS), or hereditary non poliposys colon cancer, is an inherited syndrome (OMIM #120435, http://www.ncbi.nlm.nih.gov/omim/) responsible for ~3% of all colorectal cancer (CRC) cases, with a population incidence of ~1 in 4–500 (1,2). It is caused by germline mutations in the mismatch repair (MMR) genes MSH2, MSH6, MLH1 and PMS2, and is associated with high-level microsatellite instability (MSI-H) (3–9).

Patients with LS have an elevated risk of developing CRC and endometrial cancer (up to 80% and 60%, respectively) and a moderate risk for other types of cancer like stomach, small bowel, urinary bladder, other urothelial and ovarian cancer (2,10). Mutations of MSH2 and MLH1 genes account for the most part of LS with a frequency of 40–60% and 40–50%, respectively, while minor percentages have been found for MSH6 (10–20%) and PMS2 (2%) (11). According to the Leiden Open Variation Database of MMR gene variants (http://insight-group.org/variants/database/), most mutations are single nucleotide nonsense or missense substitutions and small frameshift deletions/insertions, less frequently large deletions/duplications.

In recent years, in patients with LS phenotype and no germline mutation in MMR genes, a role for epimutations in these genes was identified. Epimutations are defined as stable and inherited changes that do not involve primary gene sequence, but include biochemical modifications that finally alter the transcription of the same gene. The principal epigenetic mechanisms are histone acetylation/deacetylation and cytosine methylation/demethylation in CpG sites (12).

CpG dinucleotides may be found in CpG islands (CGIs), short DNA regions (~200bp) with >60% of dinucleotide CpG, or
developed a CRC at the age of 30 years, and an endometrial cancer at 46 years. Immunohistochemistry of both cancers was negative for MLH1 and PMS2, and it correlated with a MSI-H phenotype. Loss of MLH1 protein and MSI-H were also displayed by the tumors of her dead brother (CF559) and of two cousins of her father (CF658 and CF659), who were enrolled at the ‘Ospedale di Circolo e Fondazione Macchi Polo Universitario’ in Milan. Informed consent for genetic testing and research was obtained from the three living subjects.

DNA sequencing, MLPA and MS-MLPA analyses

Screening for point mutations of the MLH1 gene was carried out on blood DNA by standard procedures of exon-by-exon amplification and bidirectional Sanger sequencing (primers and PCR conditions available upon request). The reference sequences are NM_000249.3 and NG_007109.1. Multiplex ligand-dependent probe amplification (MLPA) and methylation-specific MLPA (MS-MLPA) (p003, p248 and MEO1 SALSA® Probemixes kits by MRC-Holland®, Amsterdam, Holland) were used to detect large gene deletions/duplications in MLH1 and the other MMR genes and to investigate their methylation status. Data were analyzed with GeneMapper® software (Applied Biosystem/Life Technologies, Foster City, CA) and Coffalyser.Net software (MRC-Holland).

Long-range PCR

MLH1 region flanking the deletion was amplified using Expand Long Range dNTPack (Roche, Mannheim, Germany), according to manufacturer’s protocol, using the following conditions: 94°C 2’, 94°C 10’, 50°C 30’, 68°C 5’ (10 cycles); 94°C 10’, 50°C 30’, 68°C 30’→20’ each cycle (25 cycles). Long-range PCR product was purified with illustra™ ExoStar™ (GE Healthcare, Little Chalfont, UK) and then directly sequenced by standard Big Dye Terminator v3.1 protocols (Applied Biosystems/Life Technologies).

Bisulfite conversion

Five hundred nanograms of genomic DNA was treated with EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA) according to manufacturer’s alternative protocol 1 (98°C 10’, 58°C 4h). Bisulfite conversion is used to deaminate unmethylated cytosine to produce uracil in DNA. Methylated cytosines are protected from the conversion to uracil, allowing the use of direct sequencing to determine the locations of unmethylated cytosines and 5mC at single-nucleotide resolution.

Bisulfite PCR and sequencing

Two different pairs of primers for bisulfite PCR in the promoter region were designed with MethPrimer avoiding regions comprising CpG (25). The wild-type allele (PCR 1) was amplified using a forward primer annealing to 5’UTR (Fw: GATTTTTATTTTGTTTTTTTTGGG) and a reverse primer annealing to exon 1 (Rev1: CATCTCCTAAATAAAGATCAAATACC), giving an amplicon of 611 bp. The deleted allele (PCR 2, expected size 508 bp) was amplified using the same forward primer and a reverse primer annealing in intron 1 (Rev2: CTTATCTCCTAATCCATTAACCT). Exon 2 from both wild-type and deleted alleles (PCR 3) was amplified using primers ML2-T7 (TAATTACATCTACTATAGGG-TGATTGTUTAAGATAAAAGAAAAATAAT) and ML2-M13 (CAGAAAACACGCT-ATGCC-AAAAATGCTACTACTTTGATCCAGCA), which incorporated the T7 and M13 sequencing primers, respectively. PCR were performed with GoTaq® Green Master Mix (Promega Corporation Madison, WI) with 40 cycles of amplification and decreasing annealing temperature at the following conditions: 95°C 45’, 62°C→54°C 90’, 72°C 90’ (PCR 1) and 95°C 45’, 58°C→50°C 90’, 72°C 90’ (PCR 2) and 95°C 45’, 58°C→54°C 90’, 72°C 90’ (PCR 3).

After purification with Illustra™ ExoStar™ 1-Step (GE Healthcare), bidirectional sequencing reaction was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems/Life Technologies) and a modified protocol: 94°C 5’, 50°C 10’, 55°C 2’30’ for 25 cycles.

Real-time qPCR

The relative quantity of MLH1 exon 1 was evaluated on gDNA using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc, Hercules, CA), with a qPCR assay performed on a CFX36 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). Exon 1 was normalized to exon 13 of the same gene. Both exons were amplified with primer pairs mapping on

Material and methods

Patients

Patient CFS279 is a woman with a strong family history of CRC and other tumors, throughout at least three generations. She was counseled at the ‘Ospedale di Circolo e Fondazione Macchi Polo Universitario’ in Varese. The pedigree of this family, A-VA8, is shown in Figure 1. The proband interspersed in the gene body at relative low density. In normal tissues, CGIs, which often overlap the transcription starting sites and promoter regions in mammalian genes, are mostly unmethylated. In contrast, interspersed CpGs usually show cytosine methylation (5 methylcytosine, 5mC) (13).

In sporadic CRC, as in other human cancers, many tumor suppressor genes are inactivated by promoter methylation (14). Methyl-CpG binding domain proteins bind methylated CGIs and recruit histone deacetylases with consequent chromatin compaction and gene silencing (13). The first evidence of epigenetic regulation for MMR genes was the somatic methylation of the MLH1 promoter in four CRCs that did not express MLH1 (15). It has been demonstrated that methylation of CpG sites in the C and D ‘Deng’ region of MLH1 promoter (respectively from c.→248 to c.→178, and from c.→109 to c.115) correlates with loss of MLH1 protein expression (16). Nowadays it is widely known that ~15% of CRCs with MSI presents somatic MLH1 methylation (17).

MLH1 promoter methylation was also found as a constitutive event in patients with LS and no other MMR mutations (18). Acting like a classic ‘first hit’ mutation on genomic DNA, MLH1 methylation confers elevated risk of CRC and other typical LS cancers. The exact frequency of this event has not been established yet, varying between 3% and 9% in suspected LS patients with no germline MMR mutation, MSI-H and immunohistochemical loss of MLH1 (19). These subjects usually show severe LS-related cancer phenotypes with respect to tumor site, age of cancer onset and molecular pathology (20).

In principle, constitutional MLH1 methylation may be a primary or a secondary epimutation, with different patterns of inheritance. Primary epimutation is a modification that arises de novo in a patient, reversible between generations and then not necessarily heritable. The secondary epimutation is caused by a cis-acting genetic-based alteration, and shows a classical Mendelian autosomal dominant inheritance pattern. To date, about 50 cases of constitutional MLH1 promoter methylation are known, recently reviewed by Peltonäki (20), but only very few cases of secondary epimutations, with or without demonstrated transmission among generations, have been reported (21–24).

Here, we present a family with LS history, in which a large genomic deletion centered on MLH1 exon 1 coexists with constitutional methylation of CGIs in the MLH1 promoter and nearest region. Both deletion and methylation have been found in four subjects of different generations in the same family.

Abbreviations:

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SmC</td>
<td>5 methylcytosine</td>
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<tr>
<td>CGI</td>
<td>CpG island</td>
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<tr>
<td>CRC</td>
<td>colorectal cancer</td>
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<td>LS</td>
<td>Lynch syndrome</td>
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<td>MLPA</td>
<td>multiplex ligation-dependent probe amplification</td>
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<tr>
<td>MMR</td>
<td>mismatch repair</td>
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<td>MSI-H</td>
<td>high-level microsatellite instability</td>
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<td>MS-MLPA</td>
<td>methylation-specific MLPA</td>
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intrinsic flanking sequences: ML1N (AGGCCACTGAGGTGATTGC) and ML1C (TCGTACCTTAATGAGC), ML13N (TGCAACCACAAAATTGGC) and ML13C (CTTTCTCCATTTCCAAACCC), respectively.

MLH1 expression was estimated on cDNA with a similar qPCR assay, but using a couple of primers mapping on exon 11 (MLH-1026, GGGCTCCAATTCCTCCAG) and exon 12 (MLH-1219, TGGACAGGGTTGTGCTCG). MLH1 transcript levels were normalized to the average of three housekeeping genes: GAPDH, β2-microglobulin, β-actin.

Somatic analyses

Genomic DNA was extracted from manually microdissected paraffin-embedded tissues of CRC and endometrial cancer. Standard and bisulfite DNA sequencing, MLPA and MS-MLPA were carried out as for constitutional DNA obtained from blood samples.

The BRAF p.Val600Glu mutation analysis was performed in duplicate by pyrosequencing using BRAF Anti-EGFR MoAb response (BRAF status) kit (Diatech Pharmacogenetics, Jesi, Italy) according to the manufacturer’s instructions and using the pyroMarkQ96 ID system (Qiagen GmbH, Hilden, Germany).

Interphasic FISH analysis was performed as described previously (26) on 3-5-µm-thick paraffin sections and using orange-labeled RP11-491D6 BAC probe by Blue-Gnome (Technogenetics, Milan, Italy). This BAC probe mapping in 3p22.2 region contained the MLH1 gene only.

Results

MLPA assay of patient CFS279 showed a probable constitutive deletion of exon 1 and part of intron 1 of the MLH1 gene (Figure 2A). The same deletion was also present in blood DNA of her fourth-degree relatives (CFS658 and CFS659) and in tumor DNA of her brother (CFS597). In all cases, the MS-MLPA test performed with the ME-011 probemix confirmed the presence of a heterozygous deletion, comprising exon 1 and part of the promoter. Indeed, two out of the five probes that detect the MLH1 promoter, mapping respectively, 13 bp upstream ATG (Deng D-region) and 93 bp after exon 1, showed a normalized ratio of 0.5. Surprisingly, after HhaI digestion, the three retained probes (mapping on Deng A, B and C-region) produced peaks that indicated an average methylation rate of 50%, compatible with promoter methylation in one allele (Figure 3A).

Deletion was then further confirmed and characterized by Long-range PCR and direct sequencing of the amplicon surrounding the deletion. Control DNA sample yielded only the wild-type fragment of 3634 bp; instead, DNAs of three patients of family A-VA8, in addition to the wild-type fragment, produced a shorter amplicon of about 2500 bp from the deleted allele (Figure 2B). Sequencing of the breakpoint defined the mutation as MLH1 c.-168_c.116+713del, corresponding to a 997 bp deletion comprising the entire exon 1 and part of intron 1 and removing part of the MLH1 promoter (Figure 2C). The breakpoint did not involve repeated elements such as Alu or Sine/Line.

Methylation of MLH1 promoter was further investigated using bisulfite sequencing. Wild-type and deleted alleles were selectively amplified using specific sets of primers (PCR

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**Figure 1.** Pedigree of the family with current or death age, tumors site and age of onset. In bold, patient ID of carriers. Arrow indicates the proband CFS279.

**Figure 2.** Characterization of large MLH1 deletion in blood DNA of carriers. (A) MLPA electropherogram of proband DNA (upper panel) compared with a wild-type control (lower panel). Red arrows show deletions, blue circles show corresponding wild-type exon/intron. (B) Long-range PCR of surrounding deleted region. Lane1: 1kb size marker; lanes 2–4: CFS279, CFS658, CFS659 (3634 and ~2500 bp); lane 5: wild-type control (3634 bp). (C) Sequence of the deleted amplicon comprising breakpoint.
1 and PCR 2, respectively). PCR 2 amplified only the deleted allele, yielding an amplicon of 508 bp that comprises the breakpoint, while the larger wild-type amplicon (expected size 1505 bp) was not amplified under the PCR conditions used (Figure 3B). The deleted allele showed total and homogeneous methylation on CpG along all residual Deng regions and extended to CpG in intron 1. Conversely, the wild-type allele was totally unmethylated (Figure 3C). The same results were also confirmed on CFS658 and CFS659 relatives (data not shown).

Levels of the MLH1 transcript were estimated by real-time qPCR on the lymphoblastoid cell line obtained from peripheral blood of proband CFS279. This cell line, which retained both deletion and promoter methylation, displayed MLH1 expression levels of ~50% compared to the wild-type control, indicating that the mutated allele was switched off by the concurrent mutations. A similar 50% signal reduction was obtained by testing exon 1 on gDNA of three carriers (Figure 4).

DNAs from the two tumors of the proband, not expressing MLH1 protein, were investigated to evaluate presence of a second MLH1 somatic hit. The MLPA and FISH approaches failed to reveal allelic losses in both CRC and endometrial cancers (data not shown). MS-MLPA and pyrosequencing analysis of Deng C promoter region of tumor DNAs did not show substantial differences of methylation pattern compared to DNA from blood (Supplementary Figure S1, available at Carcinogenesis Online). Direct sequencing of CRC DNA also excluded the presence of the somatic p.Val600Glu mutation of the BRAF gene (data not shown).

However, MLH1 mutation scanning revealed the presence of the somatic mutation c.199G>A (p.Gly67Arg) in CRC (Figure 5A). This G>A mutation occurs within a CpG site within exon 2 (c.198_199) and corresponds to a C>T transition on the opposite strand. Since this site is usually methylated in normal tissues (27), we assessed its methylation status in CRC DNA. Bisulfite sequencing (PCR 3) evidenced a partial reversion of methylation in exon 2 involving c.198C, the position immediately preceding the somatic c.199G>A mutation (Figure 5B). The only somatic variant detected in DNA from endometrial cancer was c.90C>T, in the 3’UTR (Figure 5C).

**Discussion**

In this article, we report on a case with a heritable large genomic deletion of MLH1, never described before, which is associated with constitutional promoter methylation of the same gene. MLH1 gene methylation is present in four affected subjects of different generations in the LS family, and it is always linked in cis to the deletion of promoter and exon 1. At molecular level, the cause-effect relationship of this association is presently unknown.

Previously, it was reported of a Finnish woman with CRC at 22 and 45 years, and endometrial cancer at 40 years, who displayed methylation of the MLH1 promoter together with a large deletion, comprising exon 1 and 2 of the same gene, but no data on relatives were available (21). Another interesting case of secondary MLH1 epimutation was described in three members of a family in which the presence of a large duplication involving the complete MLH1 gene correlated with low levels of constitutional promoter methylation (22). A secondary methylation event leading to transgenerational inheritance has also been suspected in the case of a woman with LS and two of her children, but mutational screening of the MLH1 gene was negative and the influence of a trans-acting factor could not be excluded (28). Finally, a dominantly transmitted constitutional MLH1 epimutation has been recently linked to an MLH1 haplotype bearing two single-nucleotide variants, c.-27C>A and c.85G>T, in five LS Caucasian families from Western Australia (24). This haplotype has been reported only by Hitchins and coworkers (24,29), thus its worldwide frequency and its impact on LS need to be determined. In our experience on over 100 LS cases tested, these variants have never been detected.

**Figure 3.** Methylation of MLH1 promoter in blood DNA of the proband. (A) Electropherogram of proband sample (upper) analyzed with the ME-011 MLPA kit compared with a wild-type control (lower). On the left, undigested reaction: red arrows show deleted probes; on the right, digested reaction: green arrows show methylated probes, not digested by HhaI. (B) Allele specific bisulfite PCR: schematic representation of PCR for wild-type allele (upper, PCR 1, expected size 611 bp) and deleted allele (lower, PCR 2, expected size 508 bp); gel electrophoresis shows PCR 1 (lanes 1-2) and PCR 2 (lanes 3-4) products of CFS279 (lanes 1, 3) and wild-type control (lanes 2, 4); lane 5, PhiX-Hae III size marker. (C) Sequence from allele specific bisulfite-PCR of wild-type (upper) and deleted (lower) allele. Arrows highlight the difference between unmethylated (red) and methylated (blue) CpG sites.
The case reported here represents a new example of secondary MLH1 epimutation, linked and likely caused by a cis-acting genetic-based alteration. Methylation was detected in both blood and tumor samples, supporting the notion that it is probably constitutively present in all cellular types, including colon. Moreover, it is inherited as an autosomal dominant...
character in four family members. We have demonstrated that the deleted allele is fully and homogeneously methylated on all residual CpG of MLH1 promoter, whilst the wild-type allele is totally unmethylated. qPCR assays on cDNA evidenced halved transcription levels and suggested that the mutated/methylated allele is completely silenced. Unfortunately, no heterozygous SNPs were identified in the promoter or in the coding sequence, preventing us to address this hypothesis by measuring the allele-specific expression.

In the tumors of the proband, MLH1 protein expression is absent, as well as PMS2, but the wild-type MLH1 allele does not appear to be inactivated by either allelic loss or somatic methylation. According to this, the BRAF p.Val600Glu mutation, usually associated with somatic methylation in sporadic CRC, is absent in proband’s CRC. Conversely, we have obtained evidence of somatic MLH1 point mutations in both proband’s tumors. The c.‘90C>T variant of endometrial tumor has never been reported before and its functional meaning remains questionable, although a miRNA-mediated mechanism of gene silencing could be hypothesized. Instead, the c.199G>A (p.Gly67Arg) variant found in CRC DNA is also a frequent constitutional mis-sense change with a well established pathogenic role in LS. We previously described it in a LS patient who developed an MSI-H tumor in which the wild-type allele was inactivated by loss of heterozygosity (30). c.199G>A (p.Gly67Arg) is reported in Leiden Open Variation Database (85 entries) and it has been associated with loss of MLH1 protein expression in tumors and in different in vitro models (31).

Noteworthy, the somatic c.199G>A (p.Gly67Arg) second hit, which is expected to inactivate MLH1 gene in CRC tissue, occurs in a CpG site within exon 2 (c.198_199). This site is not part of a CGI and, as expected (27), is methylated in both alleles in proband blood DNA, as well as in wild-type control samples (data not shown). The presence of 5mC in gene body may induce C to T transition, due to spontaneous hydrolytic deamination of 5mC to thymine, as reported for hotspot mutations of the tumor suppressor p53 and other genes (32). Since cytosine methylation is maintained symmetric in CpG by DNA methyl transferase 1 (32), c.199G corresponds to a 5mC in the opposite strand. Then, it is not surprising to find the c.199G>A transition (5mC>T in the complementary strand) on tumoral DNA. As a consequence of the c.199G>A somatic change, cytosine in position 198, losing its context of CpG, is no longer methylated by DNA methyl transferase 1. As a support of this view, we found that methylation status of this CpG was similarly altered in two subjects carrying the same c.199G>A mutation in germline DNA (data not shown). Again, mutation and epimutation appear strictly interconnected. According to You and Jones (33), they are two sides of the same coin.

Another important mechanism of constitutional epimutation involved in the pathogenesis of LS is the methylation of MSH2 promoter, secondary to the deletion of EPCAM gene (34). In these cases methylation of an MMR gene coexists with (and is the consequence of) a mutation in cis. However, this mutation involves the neighboring EPCAM gene leaving intact the MSH2 locus. EPCAM-MSH2 read-through transcripts are unable to generate a proficient MMR protein and the concomitant methylation of the MSH2 promoter downstream the EPCAM locus prevents transcription of a normal full-length MSH2 transcript. Conversely, in our MLH1 family the primary in cis-genetic alteration involves the MLH1 gene itself, namely part of the promoter and the entire exon 1 including the ATG start site, and is therefore predicted to cause complete allelic inactivation on its own. Nevertheless, an epigenetic event is superimposed and this may appear as a redundant mechanism of allele inactivation. It is worth of noting that, differently from cases carrying EPCAM deletion and showing variable methylation levels with mosaic distribution, the MLH1 epimutation secondary to the MLH2 deletion is fully manifest in both epithelial tissues and blood, with important consequences in terms of penetrance and genetic testing.

In conclusion, our results, in line with recent evidences (21–24), corroborate the concept that an altered structure or sequence of MLH1 could be a cause of epigenetic alteration. These findings disclose novel input for the diagnostic algorithm of LS. In fact, the screening for abnormal constitutional MLH1 methylation is currently recommended only for patients with immunohistochemical loss of MLH1 and MSI-H tumors, but no germline MLH1 mutation (23,35–37). We recently screened 15 suspected LS patients with these features, but we failed to find altered constitutional methylation pattern in any of them (unpublished data). Based on our evidence, in presence of large structural changes or point mutations involving promoter region, analysis of methylation pattern should also be recommended.

Supplementary material
Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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