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A founder *MLH1* mutation in Lynch Syndrome families from Piedmont, Italy, is associated with an increased risk of pancreatic tumours and diverse immunohistochemical patterns

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Abstract
The *MLH1* c.2252_2253delAA mutation was found in 11 unrelated families from a restricted area south-west of Turin among 140 families with mutations in the mismatch repair (MMR) genes. The mutation is located in the highly conserved C-terminal region, responsible for dimerization with the PMS2 protein.

Twenty-five tumour tissues from 61 individuals with the c.2252_2253delAA mutation were tested for microsatellite instability (MSI) and protein expression. We compared the clinical features of these families versus the rest of our cohort and screened for a founder effect.

All but one tumours showed the MSI-High mutator phenotype. Normal, focal and lack of MLH1 staining were observed in 16%, 36% and 48% of tumours, respectively. PMS2 expression was always lost. The mutation co-segregated with Lynch syndrome-related cancers in all informative families. All families but one fulfilled Amsterdam criteria (AC), a frequency higher than in other *MLH1* mutants. This was even more evident for AC II (72.7% versus 57.5%). Moreover, all families had at least one colon cancer diagnosed before 50 years and one case with multiple LS-related tumours. Interestingly, a statistically significant (p=0.0057) higher frequency of pancreatic tumours was observed compared to families with other *MLH1* mutations: 8.2% of affected individuals vs 1.6%. Haplotype analysis demonstrated a common ancestral origin of the mutation, which originated about 1550 years ago.

The mutation is currently classified as having an uncertain clinical significance. Clinical features, tissue analysis and co-segregation with disease strongly support the hypothesis that the *MLH1* c.2252_2253delAA mutation has a pathogenic effect.

Keywords Lynch syndrome; *MLH1* mutations; MLH1-PMS2 dimerization; focal immunohistochemical expression; founder effect

Introduction

Lynch syndrome (LS) is an inherited autosomal dominant cancer predisposition caused by deleterious germline mutations in any of four mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6* and *PMS2*.

Individuals affected by LS are at high risk of developing colorectal and endometrial cancer: the most common manifestation of this syndrome are tumours in the proximal colon (from the cecum to the splenic flexure), often synchronous and/or metachronous with an early age at onset (frequently younger than 45-50 years of age). LS also confers an increased risk of developing cancer in several other organs, including ovary, stomach, small bowel, urothelium and central nervous system (CNS) [1,2]. As a consequence of loss of MMR function, neoplastic cells from individuals with LS show an absent or altered expression of the protein corresponding to the mutated gene and a generalized genomic instability, which is particularly evident at microsatellite loci. Both features can be demonstrated on tumour specimens by immunohistochemisty and microsatellite analysis.

The MMR proteins work as heterodimers: MLH1 dimerizes with PMS2 and MSH2 with MSH6. In the absence of the usual partner, MLH1 can dimerize with PMS1 or MLH3 and MSH2 with MSH3 [3,4].

The majority of Lynch families have constitutional mutations in either *MSH2* or *MLH1* while *MSH6* and *PMS2* are less frequently involved. Recently, a 5-tiered classification scheme to constitutional variants in *MLH1*, *MSH2*, *MSH6* and *PMS2*, ranging from non pathogenic / low clinical significance (Class 1) to pathogenic (Class 5), has been proposed by The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) [5].

Germline mutations in *MLH1* have been identified throughout the entire gene, and are frequently located in the ATP binding domain and in the C-terminal region which is responsible for constitutive dimerization with the PMS2 protein [6-9].

Founder mutations, i.e. inherited by numerous descendants of a common ancestor, have been reported in the MMR genes associated with LS [10-21].

We characterized a cohort of patients belonging to 11 LS families carrying the c.2252_2253delAA terminal mutation in the *MLH1* gene. Aim of this work was to evaluate the clinical effect of this mutation and its possible founder origin given the high frequency in LS patients from Piedmont (Italy), particularly in the Turin area.

Patients and methods

Subjects and samples

The c.2252_2253delAA in *MLH1* exon 19 was found in 11 apparently unrelated families among 140 families harbouring germline MMR mutations (51 in *MLH1*, 74 in *MSH2* and 15 in *MSH6*) identified in index cases undergoing genetic analysis for suspected LS in the Turin Medical Genetics Unit from 2001 to middle 2013. Two hundred and seventy-eight control subjects from Piedmont were tested for the presence of the c.2252_2253delAA mutation.

Signed informed consent was obtained from all subjects included in this study, which fulfilled the policies of the local ethical committee. For each family, geographical origin was collected and pedigrees were reconstructed collecting information about the relatives' surnames going back three to five generations: no evidence that the 11 families are closely related was found.

The families carrying the c.2252_2253delAA mutation are listed in Table 1.

Histological samples from 23 bowel and 2 endometrial tumours (13 from probands and 10 from family members) were retrieved for microsatellite analysis and immunohistochemistry. All tumours were revised for classification according to the WHO recommendations [22,23].

DNA extraction

Constitutional DNA of probands, family members and controls was obtained from peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Paraffin embedded tumour sections 7-10 μm thick were manually microdissected to obtain samples with tumour cells purity over 80%. After deparaffinization, DNAs from both tumour and normal mucosa were isolated following an overnight digestion with lysis buffer and proteinase K (Qiagen GmbH).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tissues. Slides were incubated with mouse monoclonal antibodies against MLH1 (clone G168-15; BD PharMingen, San Diego, CA, USA), MSH2 (clone G219-1129; BD PharMingen), MSH6 (clone 44; BD Transduction Laboratories, San Jose, CA, USA), and PMS2 (clone A16-4; BD PharMingen) proteins. Visualization

was performed by application of HRP-linked secondary antibody (EnVision DakoCytomation, Glostrup, Denmark) and diaminobenzidine [24-26]. Immunohistochemistry analysis results have been confirmed on a different sample from the same cancer.

Detection of Microsatellite Instability

Microsatellite Instability (MSI) status was tested matching the length of three mononucleotide microsatellite markers (BAT25, BAT26 and BAT40) between tumour and normal DNA from all 25 available cancers. A tumour was considered to have high microsatellite instability (MSI-H) if at least 2/3 of the amplified markers were unstable [24,27].

DHPLC, MLPA and sequencing

Screening for point mutations was performed by DHPLC analysis (Transgenomic, Inc., Omaha, NE, USA). PCR products with heteroduplex profiles were sequenced on an ABI 3100 Avant sequencer (Applied Biosystems, Foster City, CA, USA). Large deletions were screened using SALSA MLPA KIT P003 MLH1/MSH2 [28], according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). Genotyping of the 278 controls was performed by DHPLC analysis.

RNA extraction and transcript analysis

The RNA of selected cases (M1/19-4, M1/19-6 and M1/19-7) was extracted from blood collected in PAXGene RNA blood tubes (PreAnalytics GmbH, Hombrechtikon, Switzerland) using the PAXgene Blood RNA kit (Qiagen GmbH). The cDNA was generated with the Transcripter first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). Reverse Transcription-PCR amplification was performed using forward (M1-iex18F: AATTGGGACGAAGAAAAGGAA) and reverse (M1-3UTR2: AAAGGAATACTATCAGAAGGCAAG) primers for exon 18 and exon 19 (3'UTR), respectively.

Mutation Nomenclature and reference sequence

Mutations were named following HGVS recommendations [29]. The *MLH1* gene and mRNA reference sequences are NG_007109.2 and NM_000249.3, respectively. Markers positions on chromosome 3 are derived from the UCSC Genome Browser, GRCh37/hg19 assembly (http://genome.ucsc.edu/)

Haplotype analysis

Data analysis from HapMap (release 24, CEU population) and Haploview v. 4.11 [30] led to the identification of six Tag SNPs (Table 2). The rs193922366 ins/del polymorphism in the 3'UTR region of *MLH1* was also analyzed. Ten additional microsatellites were retrieved from UCSC (GRCh37/hg19) to complete the study. In total, 17 polymorphic markers spanning 7.8 Mb on chromosome 3 were tested. Markers' location on chromosome 3 and physical distance are shown in Fig. 1.

Statistical Analysis

Adjusted chi-squared statistic [31] was used to test homogeneity of proportions between groups from family-clustered data.

The age of the *MLH1* c.2252_2253delAA mutation was estimated using DMLE+2.3, which uses Markov Chain Monte Carlo algorithm for Bayesian estimation [32]. For the population growth a rate of 1.05 fold per generation was considered according to Risch et al [33]. Genotyped markers included 7 SNPs and 10 microsatellites.

Results

Eleven apparently unrelated index cases undergoing genetic analysis for suspected LS were found to carry the same mutation in the last exon of *MLH1*, c.2252_2253delAA (p.Lys751Serfs*3). The mutation leads to a frameshift in the mRNA and a hypothetical shorter protein due to the generation of an early stop codon after the insertion of two novel amino acids at codons 751 and 752. The c.2252_2253delAA mutation was not found in 278 controls from the Piedmont population and is not reported in the Exome Variant Server (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, http://evs.gs.washington.edu/EVS/, accessed March 2014, release ESP6500SI-V2 including 6503 samples).

cDNA analysis, performed on 3 of 11 index cases, showed in all samples the balanced presence of both the wild-type and the r.2252_2253delAA transcript (as shown in Online Resource 1).

We subsequently extended the analysis to 61 relatives: the mutation was present in all the 12 subjects affected by LS related cancers. Among 49 healthy relatives the predictive test led to the identification of 14 additional mutation carriers, all under 50 years of age at the moment of testing (Table 1).

Clinical features of the 11 families are summarized in Table 1 and pedigrees are available as Online Resource 2. Ten of the 11 families fulfilled the Amsterdam criteria (AC) while one family met the Bethesda criteria. Compared to the rest of the cohort (140 mutated families overall), *MLH1* c.2252_2253delAA mutant families fulfilled AC more frequently than other *MLH1* mutants (90.9% versus 80%), and this was more evident for AC II (72.7% versus 57.5%). All *MLH1* c.2252_2253delAA mutant families had at least one colon cancer diagnosed before 50 years of age and at least one case with multiple LS related primary tumours (the latter were present in 70% of other *MLH1*, 77% of *MSH2* and 66.6% of *MSH6* mutant families). It is noteworthy that 7/11 families (63.6%) had more than one case with multiple LS related primary tumours, compared to 35% of other *MLH1*, 33.8% of *MSH2* and 13.3% of *MSH6* mutant families (see Online Resource 3).

Altogether, the median age at onset of the first LS related cancer in *MLH1* c.2252_2253delAA mutant families was 49 years (from 23 to 80 years): at diagnosis 30/56 subjects (53.6%) were younger than 50, fifteen of which were below 40 years of age. Among all LS related tumours, 59/86 (68.6%) were colorectal cancers; 37.7% of affected individuals showed LS related extra-colonic cancers at onset from the endometrium, pancreas, stomach, small bowel, biliary tract and central nervous system (CNS). In particular, pancreatic cancer was more frequent (5 tumours among 61 affected individuals, 8.2%) compared to affected subjects from families with other *MLH1* (1.6%, p=0.0057), *MSH2* (2.4%, p=0.04) and *MSH6* (3.8%, p=n.s.) mutations. Overall, pancreatic cancer was present in 5/11 (45.4%) of *MLH1* c.2252_2253delAA families. No tumours of the upper urinary tract or the ovary nor sebaceous skin neoplasias were observed.

Table 3 describes the histological features of available tumour specimens from carriers of the c.2252_2253delAA germline mutation. Of the 25 reviewed tumours, 23 were adenocarcinomas of the bowel and 2 of the endometrium (endometrioid type). Twelve out of 25 (48%) were moderately differentiated, 8 (32%) poorly differentiated and 5 (20%) well differentiated. Among bowel tumours, 11

out of 23 (47.8%) had a mucinous component (<50%), 6 (26.1%) were mucinous, and the remaining 6 (26.1%) had no mucinous component.

High MSI was found in all but one analyzed tumours (96%), in which low MSI was found, a proportion similar to *MSH2* carriers in our cohort (94.6%) but slightly higher than carriers of other *MLH1* mutations (91.3%) or *MSH6* mutations (82.3%). Immunohistochemistry (Fig. 2) showed complete loss of the MLH1 protein in only 12/25 (48%) cancers; 9/25 cancers (36%) showed a focal staining with or without weakened intensity, whereas in 4/25 specimens (16%) MLH1 was normally expressed. For comparison purpose, analysis of 43 tumour tissues from individuals with other *MLH1* mutations, all located outside exon 19, showed a normal MLH1 expression in 7 (16.2%) and a focal expression in 1 (2.3%) cancers.

Five of the seven cancers with normal MLH1 expression were from carriers of splicing mutations. In particular, 3 cancers were from carriers of the c.545+3A>G mutation, for which cDNA analysis was not performed by us but has been shown to cause aberrant splicing leading to the creation of a premature stop codon [34] and 2 from carriers of the c.589-2A>G mutation, for which cDNA analysis showed the skipping of the first 4 bases of exon 8 leading to a frameshift and the creation of a premature stop codon. The remaining mutations in which a normal MLH1 expression was observed were an *in frame* deletion in exon 16 and a truncating mutation in exon 18. The colon cancer with focal MLH1 expression was from a female carrier of the splicing defect c.589-17T>A in which RNA-cDNA analysis showed the *in frame* retention of the last 15 nucleotides of intron 7.

Normal MLH1 expression was found in normal mucosa adjacent to cancer in all samples. In the 23 tumours from individuals with the c.2252_2253delAA mutation tested for PMS2 expression, the protein was always lost. MSH2 and MSH6 were normally expressed in all tested cancers. A second sample from the same tissue was tested for each of the 25 analysed tumours, with no discordant results.

Haplotypes were reconstructed by genotyping all available informative relatives from 8 families, excluding families M1/19-7, M1/19-8 and M1/19-9 in which only the index case was available. A unique haplotype segregates with the c.2252_2253delAA mutation in all fully informative families spanning 1.7 Mb from marker 16xTG to 18xGA, while markers centromeric and telomeric to this region are not shared by all mutated families (Table 4). This haplotype belongs to a single CEU haplotype as defined by data analysis of 6 SNPs from HapMap and Haploview (marked as haplotype number 2 in Table 2) and includes the CTT deletion polymorphism in the 3'UTR of *MLH1* (c.*35_*37delCTT, rs193922366).

In family M1/19-8, for which only the index case was available, the markers' phase was deducted on the basis of the CEU haplotypes: only two SNPs' phases were possible, one of which coincided with the "mutated" haplotype. This was not possible for the index case of family M1/19-7 in which different phases were possible, while M1/19-9 was homozygote for the SNPs defining CEU "haplotype 2".

It is noteworthy that the genotypes of the 4 families that aren't fully informative are compatible with the same 1.7 Mb haplotype mentioned above (Table 4).

The age of the c.2252_2253delAA mutation predicted by the DMLE+2.3 software is 62 generations (95% CI: 28-96), corresponding to about 1550 years.

The wild-type haplotypes identified in our families are listed in Online Resource 4 and match 6 out of 8 CEU haplotypes as defined by tag SNPs. Taking into consideration microsatellites, 23 different haplotypes were defined. The shared region in wild-type haplotypes spans about 0.1 Mb, compared to 1.7 Mb in the "mutated" haplotype. Only two haplotypes (2.pol), both belonging to CEU "haplotype 2", carry the rs193922366 delCTT polymorphism and overlap with the "mutation-haplotype" for the same 0.1 Mb region mentioned above, but differ for the majority of microsatellites.

Discussion

The *MLH1* c.2252_2253delAA mutation is the most frequently recurring LS mutation found in our laboratory: apart from a *MSH2* exon 8 deletion with founder effect identified in 8 Sardinian families [21], the other recurrent mutations were found in 2 to 4 different families only. Overall, 21.6% of the families with a *MLH1* mutation identified in our laboratory carried the c.2252_2253delAA mutation. The six other recurrent *MLH1* mutations found in our cohort have not been tested for a founder effect.

The c.2252_2253delAA mutation has previously been reported in the InSiGHT LOVD database (http://www.insight-group.org/variants/database/) in a Korean family [35-37], a Danish family [38], and in three other occurrences as unpublished data from the UK, Germany and Australia. No details about families nor tumour tissue analysis are available.

Two different mutations involving the same nucleotides are also reported in the LOVD database. The missense variant c.2252A>G (p.Lys751Arg) has been classified as likely not pathogenic (Class 2) . The duplication c.2252_2253dupAA, which leads to a frameshift in the mRNA and is predicted to result in a protein longer by 26 amino acids, has been reported in three different families either with MSI-H and lack

of MLH1 expression [39], MSS (no information on MLH1 expression) [40] or in which tumour tissue had not been tested [41] and has been classified as uncertain (Class 3). The finding of both deletions and duplications of the same nucleotides in different cohorts may be explained by presence of three consecutive Adenines in the position c.2251_2253.

Many founder mutations associated with LS have been reported in the *MLH1* gene [10-15]. In Italy, a founder *MLH1* mutation was found in six Lynch families originating from a relatively small geographic area of Northern Italy [42,43] and three other have been described in Southern Italy [44].

Haplotype analysis of the 11 families with the c.2252_2253delAA mutation showed a shared haplotype spanning 1.7 Mb in all 8 fully informative families. It is of note that the heterozygous markers in the three remaining families are compatible with this "mutation-haplotype". The shared haplotype includes the CTT deletion in the 3'UTR region, which is present in only two wild-type haplotypes (see Online Resource 4). As these matched the "mutation-haplotype" for some 0.1 Mb, we can assume that the c.*35_*37delCTT deletion was the first mutational event on the ancestral "CEU 2" haplotype, followed by further recombination or microsatellite mutations, and lastly by the occurrence of the c.2252_2253delAA mutation, about 1550 years ago.

The evidence of a common ancestor obtained by haplotype analysis is coherent with the geographical origin of the 11 families, all from a 45 km-wide area south of Turin, Piedmont.

Clinical data showed a high cancer incidence in families carrying the c.2252_2253delAA mutation. A higher proportion of families fulfilled AC compared to other *MLH1* mutants, and this was more evident for AC II. All families had at least one colon cancer diagnosed before 50 years of age and at least one case with multiple LS related primary tumours; almost two-thirds of the families had more than one case with multiple LS related primary tumours.

A high frequency of extra-colonic cancer was observed in subjects with the c.2252_2253delAA mutation. Interestingly, pancreatic cancer was significantly more frequent compared to carriers of other *MLH1* mutations. Overall, 45.4% of families reported the presence of pancreatic cancer. This is higher than the frequencies reported up to date in other surveys, none of which was over 25% [45-47]. Our data are evocative of an increased pancreatic cancer risk in families with the c.2252_2253delAA mutation. Since haplotype analysis demonstrated a common origin of the 11 families, we cannot rule out that the observed increased frequence of pancreatic cancer is actually due to another shared factor of genetic

predisposition. However, to our knowledge no genes with a demonstrated role in pancreatic cancer are located inside the haplotype shared by the families carrying the mutation.

No tumours of the upper urinary tract and the ovary were observed. This is consistent with published data, as they are more frequently observed in *MSH2* [48] and *MSH6* [49] mutated families, respectively.

Tissue analysis showed high microsatellite instability in all but one tested tumours from subjects with the c.2252_2253delAA mutation.

In contrast, complete loss of MLH1 expression was found in only about half of the analyzed tumours, whereas about one third had focal immunostaining and the remaining had normal expression. Unusual staining patterns have been previously reported and do not seem to depend on the anti-MLH1 antibody used [50]. It has been suggested that different kinds of second hits can result in variable MLH1 immunostaining patterns in tumours of individuals carrying the same germinal mutation [51]. The possible association of the c.2252_2253delAA mutation with different somatic mutations in the wild type allele may lead to different expression patterns, from the complete absence of the MLH1 protein to the complete expression of a non-functional protein product. Immunohistochemistry analysis results support this hypothesis, considering that in all three cases in which distinct tumours from the same patient were analyzed, clearly different immunostaining patterns were observed (Table 3).

A positive IHC staining may be explained by the presence of a mutant MLH1 protein catalytically inactive but antigenically intact. This has been shown for some missense mutations in the N-terminal ATPase pocket or in the C-terminal PMS2 binding region, which show an impaired MMR activity and no decrease in protein expression [52-54]. Normal MLH1 expression, however, has also been described in tumours from subjects with small and large in-frame deletions and truncating mutations [51,55]. As the c.2252_2253delAA mutation is located in the last exon of *MLH1*, it is unlikely that the mutant mRNA is degraded by *nonsense-mediated decay (NMD)* [56]. Accordingly, Sanger sequencing of cDNA by 3 different carriers showed the balanced presence of both the wild-type and mutated transcripts (as shown in Online Resource 1). It is of note that Sanger sequencing is not a quantitative method and may therefore miss small imbalances that might be detected using more sensitive techniques (i.e. primer extension). The

presence of the mutated transcript, however, does not necessarily imply that the mutant MLH1 protein is correctly expressed nor stable.

It has been previously reported that terminal MLH1 defects prevent the formation of a stable complex with PMS2, resulting in an impaired DNA mismatch repair function [6,9]. Although dimerization is not required for nuclear localization, the MLH1-PMS2 heterodimer is imported in the nucleus more efficiently than either MLH1 or PMS2 monomers [57]. While the MLH1 protein is stable when expressed alone, PMS2 is quickly degraded if not bound to MLH1 [9].

The c.2252_2253delAA mutation is predicted to abolish 6 of the last 8 MLH1 amino acids, which are highly conserved in the evolution of eukaryotes (as shown in Online Resource 5) and therefore, even if the mutant MLH1 protein is expressed, it is likely that binding to PMS2 is impaired. Accordingly, PMS2 expression was lost in all analyzed tumour samples from individuals carrying the c.2252_2253delAA mutation.

In our cohort, a normal MLH1 expression was not limited to tumours from individuals with the c.2252_2253delAA mutation as it was observed with the same frequency in tumours from individuals with other *MLH1* mutations, all of which located outside exon 19 and all but one resulting in a stop codon. Unfortunately, data about IHC testing for PMS2 is not available for these cases as it was not routinely performed years ago.

Although the mechanism leading to MLH1 protein focal expression has not been clarified, it is noteworthy that in our experience this abnormal pattern has not been identified in patients with other *MLH1* mutations, with the exception of one tumour from a subject with a different *MLH1* mutation, a splicing defect leading to the *in frame* retention of the last 15 nucleotides of intron 7.

The antigenic epitopes for the four anti-MMR antibodies are particularly sensitive to fixation and therefore immunostaining patterns should only be assessed in well-fixed regions of the tissue section [26]. In our experience of immunohistochemistry analysis, only few tumour tissues have shown weak, focal or weak focal MMR protein staining. These expression patterns, however, were usually seen for more than one protein in the same specimen and were interpreted as inadequacy of the sample. In the present study we did exclude that focal staining was due to tissue poor preservation, since normal MLH1 expression was found in the normal mucosa adjacent to cancer in all samples, or to an erroneous interpretation due to the mucinous component of the tumour; moreover MSH2 and MSH6 proteins were normally

expressed in the nuclei of both cancer and normal mucosa of all samples. The focal MLH1 expression was unrelated to histological features of the analyzed tumours (cancer site, grading, mucinous component). We can speculate that this very terminal frameshift mutation leads to a MLH1 protein not only unable to hetero-dimerize with PMS2 but also less stable and/or with a less efficient nuclear import as a monomer, which would explain its proper expression in only a few nuclei.

The c.2252_2253delAA mutation has been classified as Class 3 (uncertain) by the Variant Interpretation Committee of the InSiGHT, mainly due to insufficient evidence. No familial or clinical data about previously reported cases is available.

Our data strongly support the hypothesis that the *MLH1* c.2252_2253delAA mutation has a pathogenic effect. The mutation is predicted to abolish 6 of the highly conserved last 8 amino acids of MLH1, in the C-terminal region which is responsible for constitutive dimerization with the PMS2 protein. Accordingly, all tested tumours from individual with the mutation lack PMS2 expression and all but one show MSI-H.

The mutation co-segregates with disease in all informative families: it is noteworthy that all healthy subjects who were over 50 years of age at the moment of testing, including four aged 61-77, do not carry the mutation. The mutated families showed clinical features typical of LS including tumours at young age and multiple primary tumours in the same subject. Moreover, a high frequency of pancreatic cancer was observed.

The c.2252_2253delAA mutation was absent in control subjects from the same Italian region and has not been reported as a normal polymorphism in the general population.

Since haplotype analysis demonstrated a single origin of the mutated allele, we cannot rule out that the cause of LS in the 11 tested families is actually an unidentified *MLH1* mutation residing on the same haplotype. This however is unlikely considering that the c.2252_2253delAA mutation has been reported in other two families ascertained for LS in different populations.

Conflict of interest

The authors declare no conflict of interest

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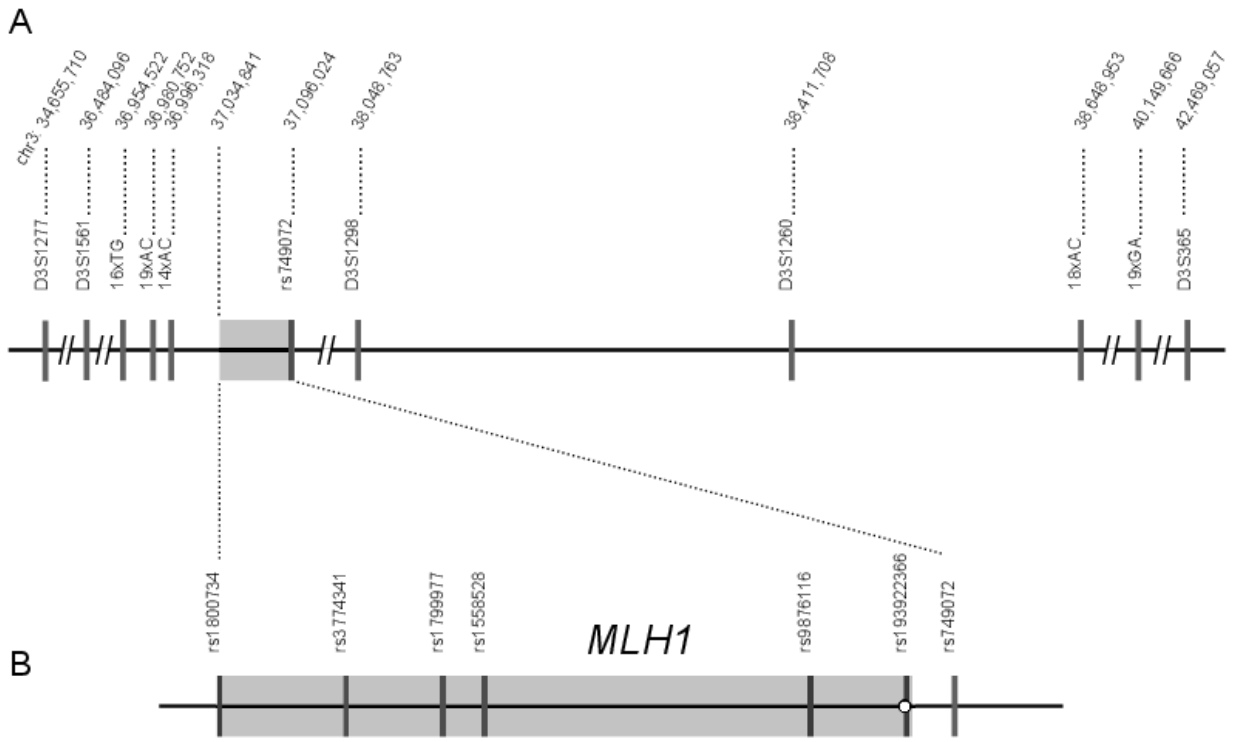
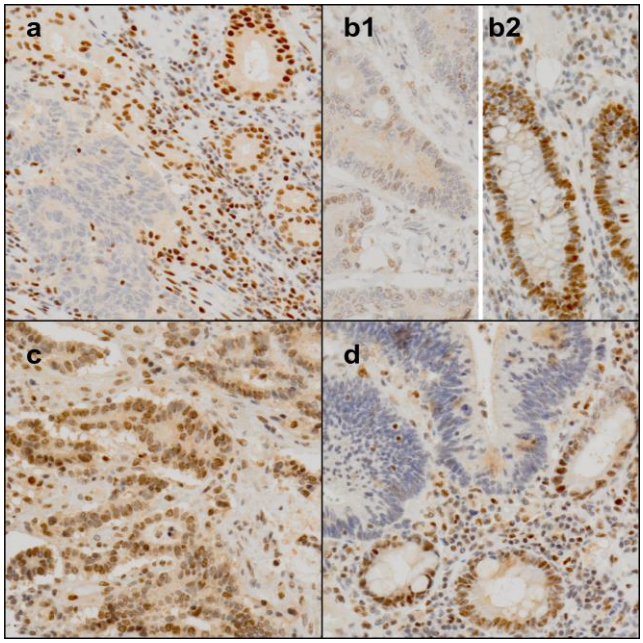
Fig. 1 Markers used to define the haplotypes carrying the *MLH1* c.2252_2253delAA mutation



a) Map of the markers surrounding the *MLH1* gene

b) Zoom-in of the region in which the tag SNPs are located. The position of rs193922366 (a three-nucleotide ins/del: CTT/-) is also shown. The mutation is shown as a *hollow circle*

Fig. 2 Immunohistochemical staining of mismatch repair proteins



a) absent MLH1 staining in colon cancer cells (left) and positive nuclear staining in normal colon mucosa (right); b1) focal MLH1 nuclear staining in colon cancer cells and b2) positive intense MLH1 staining in adjacent normal mucosa; c) positive nuclear MLH1 staining in colon cancer.

d) absent PMS2 nuclear staining in colon cancer expressing MLH1 (left, same tumour of panel c) and positive staining in normal mucosa (right)

(x 200 magnification)

Table 1 Clinical features of Lynch Syndrome families with the *MLH1* c.2252_2253delAA mutation

Family ID	Clinical criteria[a]	No. individuals affected by LS cancers[b]	Median age at onset of the first LS cancer (min-max age)	No. Individuals with Colorectal cancers	No. Colorectal cancers	No. Endometrial cancers	No. Gastric (Small bowel) cancers	No. Pancreatic (Biliary tract) cancers	CNS cancers[c]	No. individual with synchronous tumours (No. of tumours)	No. individuals with multiple primary tumours	No. individuals affected by non-LS cancers or unknown site	No. positive for the mutation / total affected relatives for confirmatory testing.	No. positive for the mutation / total healthy relatives with predictive testing.
M1/19-1	AC-I	5	68 (46-79)	5	7	0	0 (0)	0 (0)	0	1 (2)	2	0	0	1/4
M1/19-2	AC-II	4	51.5 (38-69)	4	4	1	0 (0)	0 (1)	0	0 (0)	2	4	0	0/3
M1/19-3	AC-II	3	43 (35-44)	1	1	1	1 (1)	1 (0)	0	0 (0)	2	0	1/1	3/4
M1/19-4	AC-II	13	56 (26-80)	6	6	3	2 (1)	1 (0)	2	0 (0)	2	3	5/5	8/24
M1/19-5	AC-II	7	44.5 (30-53)	6	8	2	1 (0)	0 (0)	0	1 (3)	2	0	3/3	1/4
M1/19-6	AC-II	8	46.5 (35-61)	7	12	0	0 (0)	1 (0)	0	2 (3 and 4)	2	0	1/1	0/4
M1/19-7	AC-I	3	66 (23-67)	3	4	0	0 (0)	0 (0)	0	0 (0)	1	1	0	0
M1/19-8	AC-II	5	43 (37-60)	4	4	0	0 (0)	1 (0)	1	0 (0)	1	4	0	0
M1/19-9	AC-II	7	51 (39-76)	4	5	1	1 (0)	0 (0)	1	0 (0)	1	2	0	0
M1/19-10	AC-II	4	40 (36-59)	3	3	1	0 (0)	1 (0)	1	0 (0)	1	3	1/1	1/3
M1/19-11	BR	2	(38-49)	2	5	0	0 (0)	0 (0)	0	0 (0)	2	0	1/1	0/3

[a] Clinical criteria: AC-I (Amsterdam criteria type 1), AC-II (Amsterdam criteria type 2), BR (revised Bethesda criteria)
[b] Number of individuals affected by Lynch syndrome (LS) tumours: colorectal, endometrial, gastric, small bowel, pancreatic, biliary tract, central nervous system (CNS). No ovarian and upper urinary or sebaceous cancers were present
[c] CNS tumours have not been included in the median calculation because medical records were not available for confirmation

Table 2 CEU haplotypes defined by selected tag SNPs

		CEU Haplotypes							
SNPs	**Gene**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
rs1800743	*MLH1*	G	G	A	G	G	A	A	G
rs3774341	*MLH1*	C	A	A	C	A	A	A	C
rs1799977	*MLH1*	G	A	A	A	A	A	G	A
rs1558528	*MLH1*	A	C	C	A	C	C	C	C
rs9876116	*MLH1*	G	A	A	G	A	A	A	G
rs749072	*LRRFIP2*	T	T	C	T	C	T	C	T
Haplotype frequencies: %		33.3	26.6	17.5	15.0	4.1	1.6	1.6	0.8

Table 3 Histological features of 25 cancers from individuals with the *MLH1* c.2252_2253delAA mutation

		WHO Classification of Tumours				IHC			
Subject ID[a]	**Cancer site**	**Histological diagnosis**	**Differentiation grading**	**Mucinous component: %**	**MS-status**	**MLH1**	**MSH2**	**MSH6**	**PMS2**
M1/19-1	rectum	adenoma with invasive carcinoma	well	5	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-1	caecum	adenocarcinoma	moderately	40	MSI-H	No loss	No loss	No loss	**loss**
M1/19-1.2	caecum	adenocarcinoma	moderately	>50	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-1.3	transverse	adenocarcinoma	moderately	25	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-2	caecum	adenocarcinoma	moderately	0	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-3	small bowel	adenocarcinoma	moderately	0	MSI-H	**loss**	No loss	ND[b]	ND
M1/19-4	caecum	adenocarcinoma	poorly	0	MSI-H	**loss**	No loss	ND	**loss**
M1/19-4.2	descending	adenocarcinoma	moderately	10	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-4.3	transverse	adenocarcinoma	moderately	30	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-4.4	Vater ampulla	adenocarcinoma	well	>50	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-5	caecum	adenocarcinoma	moderately	20	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-5	ascending	adenocarcinoma	moderately	0	MSI-H	No loss	No loss	No loss	**loss**
M1/19-5	endometrium	adenocarcinoma	well	0	MSI-L	No loss	No loss	No loss	**loss**
M1/19-5.2	splenic flexure	adenocarcinoma	poorly	10	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-5.3	ascending	adenocarcinoma	well	5	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-5.3	endometrium	endometrioid adenocarcinoma	well	0	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-5.4	hepatic flexure	adenocarcinoma	poorly	0	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-6	splenic flexure	adenocarcinoma	poorly	>50	MSI-H	**loss**	No loss	ND	ND
M1/19-7	caecum	adenocarcinoma	moderately	signet ring cells	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-8	caecum	adenocarcinoma	poorly	>50	MSI-H	No loss	No loss	No loss	**loss**
M1/19-9	ascending	adenocarcinoma	poorly	<5	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-10	rectum	adenocarcinoma	poorly	45	MSI-H	**loss**	No loss	No loss	**loss**
M1/19-10.2	ascending	adenocarcinoma	moderately	10	MSI-H	weak focal staining	No loss	No loss	**loss**
M1/19-11	transverse	adenocarcinoma, signet ring cells	poorly	>50, signet ring cells	MSI-H	focal staining	No loss	No loss	**loss**
M1/19-11.2	sigmoid	adenocarcinoma	moderately	0	MSI-H	**loss**	No loss	No loss	**loss**

[a] Probands were coded as M1/19 followed by a number from 1 to 11. The .2, .3, etc. subcodes indicate different affected individuals belonging to the same family. [b]ND = not determined

Table 4 Haplotypes identified in 11 probands with the MLH1 c.2252_2253delAA mutation

Markers on chromosome 3:	**M1/19-1**		**M1/19-2**		**M1/19-3**		**M1/19-4**		**M1/19-5**		**M1/19-6**	
D3S1277	**285**	297	**283**	281	**283**	291	**283**	291	**283**	285	**285**	291
D3S1561	**246**	246	**246**	262	**246**	246	**258**	248	**260**	248	**246**	262
16xTG	***243***	*243*	***243***	*247*	***243***	*249*	***243***	*249*	***243***	*243*	***243***	*249*
19xAC	***230***	*226*	***230***	*224*	***230***	*224*	***230***	*224*	***230***	*224*	***230***	*222*
14xAC	***178***	*180*	***178***	*184*	***178***	*180*	***178***	*180*	***(178)***	*(184)*	***178***	*180*
rs1800734	***G***	*G*	***G***	*A*	***G***	*G*	***G***	*G*	***G***	*G*	***G***	*G*
rs3774341	***A***	*A*	***A***	*A*	***A***	*C*	***A***	*C*	***A***	*A*	***A***	*C*
rs1799977	***A***	*A*	***A***	*A*	***A***	*G*	***A***	*G*	***A***	*A*	***A***	*G*
rs1558528	***C***	*C*	***C***	*C*	***C***	*A*	***C***	*A*	***C***	*C*	***C***	*A*
rs9876116	***A***	*A*	***A***	*A*	***A***	*G*	***A***	*G*	***A***	*A*	***A***	*G*
c.2252_2253delAA	***mut***[a]	*wt*[b]	***mut***	*wt*	***mut***	*wt*	***mut***	*wt*	***mut***	*wt*	***mut***	*wt*
*rs193922366**	**–**	*CTT*	**–**	*CTT*	**–**	*CTT*	**–**	*CTT*	**–**	*CTT*	**–**	*CTT*
rs749072	***T***	*T*	***T***	*C*	***T***	*T*	***T***	*T*	***(T)***	*(C)*	***T***	*T*
D3S1298	***225***	*229*	***225***	*231*	***225***	*213*	***225***	*219*	***225***	*219*	***225***	*227*
D3S1260	***286***	*284*	***286***	*290*	***286***	*284*	***286***	*286*	***286***	*274*	***286***	*286*
18xAC	***212***	*222*	***212***	*210*	***212***	*214*	***212***	*206*	***212***	*218*	***212***	*210*
19xGA	**172**	172	**172/178**		**170**	168	**172**	172	**172**	172	**172**	168
D3S3685	**236**	238	**220**	228	**220**	234	**236**	226	**238**	236	**238**	228

M1/19-7		**M1/19-8**		**M1/19-9**		**M1/19-10**		**M1/19-11**	
283/291		283/285		283/285		**283**	291	**283**	285
246/266		260/264		260/266		**262**	246	**246**	246
(243)	*(249)*	***(243)***	*(249)*	***243***	*243*	***243***	*249*	***243***	*249*
(230)	*(224)*	***(230)***	*(224)*	***(230)***	*(226)*	***230***	*224*	***230***	*222*
(178)	*(180)*	***(178)***	*(180)*	***(178)***	*(180)*	***178***	*180*	***178***	*180*
(G)	*(A)*	***G***	*G*	***G***	*G*	***G***	*G*	***G***	*G*
A	*A*	***A***	*C*	***A***	*A*	***A***	*C*	***A***	*C*
A	*A*	***A***	*G*	***A***	*A*	***A***	*G*	***A***	*A*
C	*C*	***C***	*A*	***C***	*C*	***C***	*A*	***C***	*A*
A	*A*	***A***	*G*	***A***	*A*	***A***	*G*	***A***	*G*
mut	*wt*	***mut***	*wt*	***mut***	*wt*	***mut***	*wt*	***mut***	*wt*
(–)	*(CTT)*	***(–)***	*(CTT)*	***(–)***	*(CTT)*	–	*CTT*	–	*CTT*
(T)	*(C)*	***T***	*T*	***T***	*T*	***T***	*T*	***T***	*T*
225	*225*	***225***	*225*	***225***	*225*	***225***	*231*	***225***	*217*
286	*286*	***286***	*286*	***286***	*286*	***286***	*286*	***286***	*286*
212	*212*	***212***	*214*	***(212)***	*(222)*	***212***	*206*	***212***	*222*
172	172	**172**	172	172/174		**172**	172	**168**	168
222/230		**236**	238	230/236		**230**	236	**232**	238

*rs193922366 is a three-nucleotide ins/del: CTT/-; [a]mut = mutated allele, [b]wt = wild-type allele.
The haplotype carrying the mutation is shown in bold. The 1.7 Mb region defined by the haplotype shared by all mutation carriers is marked in italics.
For non informative markers inside the shared region, alleles have been attributed based on the haplotype carrying the mutation identified in informative families and are shown in brackets.
For non informative markers outside the shared region, alleles could not be attributed and are separated by a "/".