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ORIGINAL ARTICLE

Co-occurrence of nonsense mutations in *MSH6* and *MSH2* in Lynch syndrome families evidencing that not all truncating mutations are equal

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The majority of pathogenic mismatch repair (*MMR*) gene mutations detected in Lynch syndrome patients are truncating (frameshift or nonsense). However, the classification of terminal truncating mutations is sometimes difficult and predictive testing based on non-deleterious variants can have very serious consequences. Here, we report eight probands that have two germline nonsense mutations, namely *MSH6* c.1030C>T, p.(Gln344Ter) and *MSH2* c.2785C>T, p.(Arg929Ter), and one additional patient who presented only the *MSH2* mutation previously reported as deleterious. The novel *MSH6* truncating mutation was classified as deleterious, as it is predicted to encode a protein with loss of 1017 amino acid residues. The *MSH2* mutation, which is expected to encode a protein lacking six amino acid residues, was considered a variant of unknown significance. Five tumors of the eight double-mutant individuals had normal MSH2 expression, whereas MSH6 immunoexpression was lost in all evaluable cases. None of the variants were detected in normal controls or associated with other *MMR* germline mutations in our series. This study emphasizes that not all truncating mutations are equal and that one must be cautious in the interpretation of the presumed deleterious effect of terminal frameshift or nonsense mutations.
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INTRODUCTION

Lynch syndrome, also known as Hereditary Nonpolyposis Colorectal Cancer (MIM# 120435), is the most common hereditary colon cancer syndrome, representing 2–5% of all colorectal cancer (CRC), including a large proportion of those with a young age of diagnosis.[1–3] Germline mutations in mismatch repair (*MMR*) genes (*MLH1*, MIM# 120436; *MSH2*, MIM# 609309; *MSH6*, MIM# 600678; and *PMS2*, MIM# 600259) are the major causes of Lynch syndrome, which is a highly penetrant autosomal dominant disease characterized by increased risk for CRC and extracolonic tumors of the endometrium, stomach, small bowel, ureter, renal pelvis, ovary and hepatobiliary tract.[4] The vast majority of Lynch syndrome-associated tumors presents microsatellite instability due to their DNA MMR deficiency. In addition, microsatellite instability tumors can be identified by abnormal MMR immunohistochemical staining as the *MMR* gene mutated in the germline usually loses the wild-type allele in the tumor cells.[5]

The detection of pathogenic MMR mutations in patients suspected of having Lynch syndrome confirms the diagnosis and allows genetic counseling and predictive testing in their relatives.[6] About 90% of germline mutations in Lynch syndrome are found in *MLH1* and *MSH2*, 7% in *MSH6* and 1% in *PMS2*.[7] The germline mutations in *MLH1* or *MSH2* are usually associated with clinically recognizable Lynch syndrome families, whereas mutations in the *MSH6* and *PMS2* genes appear to be more associated with atypical Lynch syndrome.[8–10] As molecular characterization of Lynch syndrome was established, the identification of gene carriers has become a critical issue with important clinical implications for cancer surveillance, incidence and mortality.[11] The identification of patients with Lynch syndrome can be facilitated in populations with founder mutations by targeting the mutational analysis to specific gene regions as a first step, improving the cost effectiveness of the genetic testing strategy.[12–14]

The majority of *MMR* gene variants that are detected in Lynch syndrome patients are truncating mutations, which are generally classified as pathogenic. However, there are examples in the literature that terminal nonsense mutations may be polymorphisms with no major functional consequences for the encoding protein, as is the case of the terminal *BRCA2* mutation c.9976A>T, p.(Lys3326Ter).[15] The classification of terminal truncating mutations may therefore be difficult and could have very serious consequences for cancer families if a non-deleterious variant is used for predictive genetic testing. We here present eight families that have two germline nonsense mutations, one novel mutation in *MSH6* exon 4 and a *MSH2* exon 16 mutation previously reported as deleterious.

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MATERIALS AND METHODS

Patients, samples and DNA extraction

This study includes eight Lynch syndrome families presenting two concomitant truncating mutations in the *MSH6* and *MSH2* genes, from a total series of 114 families with pathogenic *MLH1*, *MSH2*, *MSH6* or *PMS2* germline mutations (data not shown). Seven of these index patients were identified by routine genetic diagnosis during the period of 1997–2014 at the Genetics Department of the Portuguese Oncology Institute, Porto, Portugal, after genetic counseling and informed consent. The remaining index patient was initially sent to us as a familial case belonging to a family with the *MSH2* mutation identified in France. In addition to these eight families, we also identified one family that only presented the *MSH2* germline mutation. Seven families were followed up at the Portuguese Oncology Institute and two at São João Hospital. Three families fulfilled the Amsterdam criteria, whereas the remaining presented the Bethesda criteria for genetic testing (Table 1 and Figure 1).[16,17] After written informed consent, DNA was isolated from peripheral blood samples from the nine index individuals and subsequently from 16 family members, using standard procedures. The geographic origin of these families was inferred from the birthplace of the oldest carrier or of the oldest affected family member most likely to be a carrier.

Screening for MMR germline alterations

Genomic DNA from the eight patients was screened for MMR germline mutations. *MSH2* and *MSH6* coding exons and flanking regions were studied by denaturing gradient gel electrophoresis using primers and conditions as described by Wu and co-workers and Ingeny (Goes, The Netherlands) and/or by direct sequencing in an ABI PRISM 3500 automatic sequencer using Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations.[18] Whenever necessary, *MSH6* exon 7 was re-sequenced using a different set of primers to exclude or confirm the presence of a polymorphism at the initial primer annealing site.[19] *MSH2* and *MSH6* exonic rearrangements were screened by multiplex ligation-dependent probe amplification, according to the SALSA MLPA P003 MLH1/MSH2 and P072 MSH6 kits (MRC-Holland, Amsterdam, The Netherlands) instructions. The variants described are according to the Human Genome Variation Society guidelines and we used the LRG_218 (NM_000251.2) for the *MSH2* gene and the LRG_219 (NM_000179.2) for the *MSH6* gene. Relatives were screened for the MMR germline mutations identified in the index patients by direct sequencing. In addition, KASPar SNP genotyping (LGC, Herts, UK) was used to screen the *MSH2* and *MSH6* mutations in 100 controls from the blood donors bank of the Portuguese Oncology Institute, Porto.

Table 1 Clinical data of the families with germline *MSH6* and *MSH2* mutations (families 1 to 8) or with only the *MSH2* variant (family 9)

Family	*Criteria*	*Index cancer (age)*	*Family history (age)*
1	Amsterdam	CA (26)[a]	EC (50), EC (49); EC (59); CRC (53)
2	Bethesda	CRC (38)	CRC (70)
3	Amsterdam	CA (31)	CRC (70); CRC (58); CRC (70); CRC (40); IBL (34)
4	Bethesda	CRC (48)	CRC (32); CRC (65)
5	Bethesda	CRC (40)	CRC (49); PP (55)
6	Amsterdam	CRC (47)	CRC (45); CRC (67); BC (49); BT (56); EC (42)+PaC (?); CRC (67); LC (70)
7	Bethesda	OC (46)	CRC (37)
8	Bethesda	EC (47)	EC (44); CRC (47)
9	Bethesda	CRC (36)	No family history

Abbreviations: BC, breast cancer; BT, brain tumor; CA, colon adenoma; CRC, colorectal cancer; EC, endometrial cancer; IBL, intestinal Burkitt lymphoma; LC, lung cancer; OC, ovarian cancer; PaC, pancreatic cancer; PP, polyposis.
[a]Patient initially studied as a familial case belonging to a family with the *MSH2* mutation identified in France.

MMR immunohistochemical analysis

Assessment of MLH1, MSH2, MSH6 and PMS2 immunoexpression was performed as previously described in the eight tumors from the patients harboring the two truncating mutations in *MSH2* and *MSH6* (the tumor from the patient with isolated *MSH2* mutation was not available).[20]

Microsatellite typing and haplotype analysis

A total of 9 probands and 16 family members were genotyped for a subset of 10 microsatellite markers (D2S391, D2S2227, Clen33, Clen30, Clen29, D2S2156, D2S123, Clen43, Clen44 and D2S378) flanking *MSH6* and *MSH2*. The physical distances of the genetic markers were derived from the National Center for Biotechnology Information (NCBI) Map Viewer (http://www.ncbi.nlm.nih.gov/projects/mapview/). The primer sequences for amplification of the markers D2S391, D2S123 and D2S378 were derived from the Human Genome database; primers for markers D2S2227 and D2S2156 were designed using the online Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and markers Clen33, Clen30, Clen29, Clen43 and Clen44 were derived from Clendenning and co-workers.[21] All 10 markers were assayed by PCR using fluorescently end-labeled primers. PCR products were run on an ABI PRISM 310 Genetic Analyzer together with the fluorescence-labeled DNA fragment size standard TAMRA (Applied Biosystems). Haplotype construction was performed manually based on the genotypes obtained from index cases and family members.

Statistical analysis

Comparison of mean age of CRC diagnosis was performed by Mann–Whitney test and *P*-values <0.05 were considered statistically significant.

RESULTS

Mutation analyses

Analysis of the constitutional DNA by denaturing gradient gel electrophoresis and Sanger sequencing in eight index cases initially studied in Porto revealed, in seven of them, two germline mutations, the *MSH6* exon 4 c.1030C>T, p.(Gln344Ter) and the *MSH2* exon 16 c.2785C>T, p.(Arg929Ter), whereas the eighth presented only the latter (Figure 2 and Table 2). In addition, one individual with a colon adenoma referred to our institution as a familial patient belonging to a Lynch syndrome family with the *MSH2* mutation c.2785C>T, p.(Arg929Ter), detected in a relative living in France, was included in this study. Given the co-occurrence of this *MSH2* mutation with the *MSH6* c.1030C>T mutation in seven of our families, both mutations were screened, and we observed that this patient also presented the two variants (Table 2). Furthermore, of the 16 family members studied, 5 were wild-type and 11 presented the two mutations concomitantly. Moreover, the *MSH6* c.1030C>T and *MSH2* c.2785C>T mutations were not found in the 100 healthy controls screened, nor in the remaining families tested by our group (251 probands tested for *MSH2* and 118 probands tested for *MSH6* germline mutations; data not shown).

Clinical and phenotype analyses

Three of the eight double-mutant families presented Amsterdam criteria for Lynch syndrome genetic analysis and three of these presented endometrial cancer in their family history (Table 1). The proband presenting only the *MSH2* mutation had no family history of cancer (Table 1). The mean age of CRC onset in the double-mutant families was 52.7 years, which is closer to what has been reported for *MSH6* mutated families, but does not significantly differ from that of the remaining Lynch syndrome families with pathogenic mutations studied by us (43.7 for *MLH1*, 44.2 for *MSH2* and 43.5 for *MSH6* mutation carriers; $P=0.992$, $P=0.992$ and $P=0.9522$, respectively).

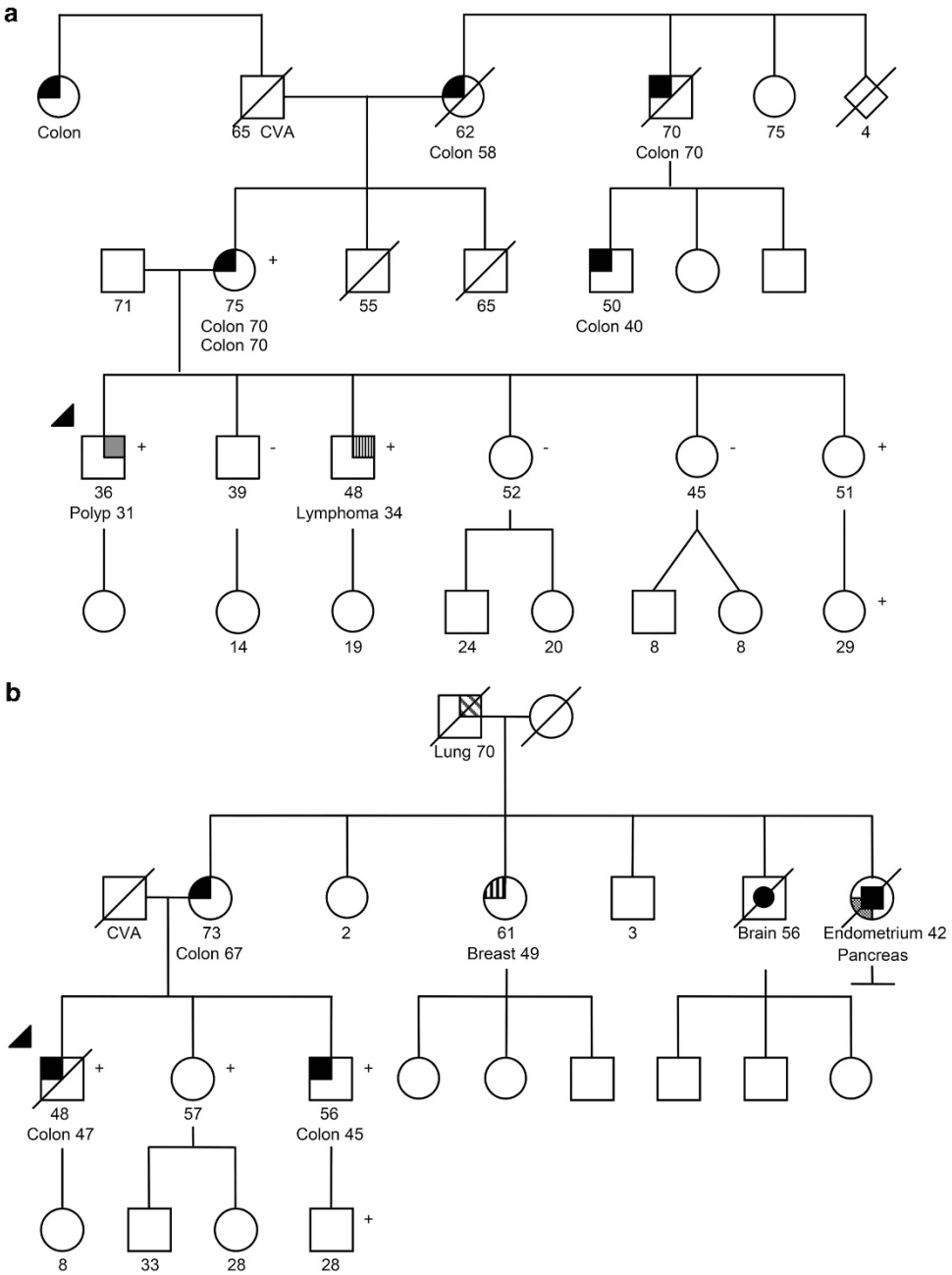

Figure 1 Two examples of pedigrees having co-occurrence of both *MSH6* and *MSH2* nonsense mutations: (**a**) family number 3; (**b**) family number 6.

Immunohistochemistry

Immunohistochemistry analysis of tumor tissue revealed loss of MSH6 protein immunoexpression in seven of the eight double-mutant families, whereas the analysis of the eighth was not interpretable (due to the absence of MSH6 immunoreactivity also in the adjacent normal tissue). Regarding the MSH2 protein, five of these families presented normal expression, one presented decreased expression, and in two there was complete loss of expression in concomitance with the loss of MSH6 expression. All tumors presented normal MLH1 and PMS2 protein expression (Table 2 and Figure 3).

Haplotype analysis

Microsatellite haplotypes for all markers were phased in two out of the eight double-mutant Lynch syndrome families analyzed and two different haplotypes were observed. However, these two haplotypes present a conserved region of ~1.5 Mb encompassing the most telomeric markers (from the *MSH6* gene to the D2S391 microsatellite marker). The remaining six double-mutant families and the index patient with only the *MSH2* mutation presented unphased haplotypes, but harbored alleles consistent with the conserved region (Table 3).

All families presented in this study were apparently unrelated, but were originated from a restricted area, north to the city of Porto.

DISCUSSION

Molecular characterization of Lynch syndrome families is a fundamental step for their adequate clinical management. Knowing the deleterious constitutional mutation in the proband makes possible the identification of the family members that have the same risk as the

general population and the relatives at high risk of developing Lynch syndrome associated cancers, allowing for cost-effective screening and/or prophylactic measures in the latter.

We, here, describe a set of eight families presenting nonsense mutations in two different *MMR* genes, namely *MSH6* and *MSH2*. As all probands and mutation carrier relatives presented the two mutations in concomitance and both *MSH2* and *MSH6* are located in chromosome 2, we can infer that the two mutations are in *cis*, being, therefore, transmitted together unless a recombinational event occurs in chromosome 2 between the two *MMR* genes. These mutations were not detected in the remaining families suspected of Lynch syndrome studied at our Department (251 probands tested for *MSH2* and 118 probands tested for *MSH6* germline mutations) nor in 100 normal controls. To the best of our knowledge, the *MSH6* mutation c.1030C>T, p.(Gln344Ter), has not previously been reported, and we classified it as deleterious because it is predicted to result in a protein lacking 1017 amino acid residues. The *MSH2* mutation c.2785C>T, p.(Arg929Ter), on the other hand, occurs in the final 3′ part of the gene, encoding a protein that is only six amino acids shorter and with no interruption of any known functional protein domain. This mutation has only been described previously in one individual who also presented the pathogenic c.942+3A>T *MSH2* mutation, being classified as pathogenic in the InSiGHT database.[22–24] Given that the *MSH2* mutation c.2785C>T, p.(Arg929Ter), occurs in concomitance with a pathogenic mutation both in the eight families we here report and in the single family previously reported, that there is a lack of family history of cancer in the proband with only this *MSH2* variant, together with the demonstration that MSH2 protein expression was normal in tumors of five double-mutant patients (Table 2), we consider this mutation as a variant of unknown significance, further studies being necessary to fully characterize this mutation. It should be noted that there are examples in the literature of nonsense variants classified as common polymorphisms, for instance, the *BRCA2* c.9976A>T, p.(Lys3326Ter), which occurs in the last exon of the *BRCA2* gene and is predicted to encode a functional, shorter protein lacking the last 93 amino acids.[15]

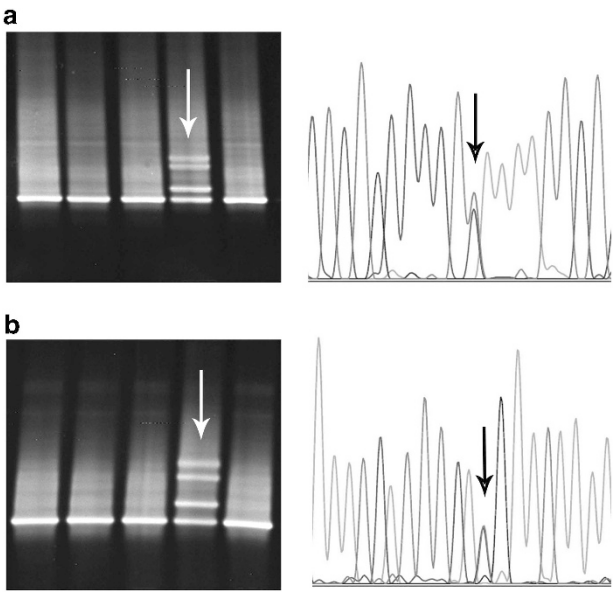

Figure 2 Germline mutations identified in *MSH6* and *MSH2*. (**a**) *MSH6* exon 4c.1030C>T, p.(Gln344Ter). (**b**) *MSH2* exon 16c.2785C>T, p.(Arg929Ter). Denaturing gradient gel electrophoresis (DGGE) gels are shown to the left, and sequence electropherograms are shown to the right; arrow indicates the mutations. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Our data does not totally exclude the remote hypothesis that the two nonsense mutations in *MSH6* and *MSH2* may act cooperatively to confer an increased CRC risk. For instance, Martinez and Kolodner proposed that weak *MMR* gene alleles are capable of polygenic interactions with other *MMR* gene alleles and thereby rising cancer risk.[25] On the other hand, it is widely described in the literature that *MSH6* mutation carriers develop CRC at a significantly older age than reported for *MLH1* and *MSH2* mutation carriers, and it is also associated with an increased risk of developing endometrial carcinoma.[10,26] The double-mutant families we, here, present have no atypical clinical features: the mean age of CRC diagnosis in the eight families (52.7 years) was higher than that of the *MSH2* (44.2 years) or the *MSH6* (43.5 years) mutation carrier families studied at our institution (although the difference is not statistically significant) and two families presented endometrial carcinoma. These data are consistent with the phenotype classically associated with *MSH6* mutation carrier families, suggesting that the *MSH2* variant does not increase the cancer risk in these families, supporting the idea that the pathogenicity of this mutation is uncertain.

Table 2 Molecular and immunohistochemistry data of the families with germline *MSH6* and/or *MSH2* mutations

	MSH6		*MSH2*		*IHC*			
Family	*Mutation*[a]	*Protein effect*	*Mutation*[b]	*Protein effect*	*MLH1*	*PMS2*	*MSH2*	*MSH6*
1[c]	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	No expression	No expression
2	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	Normal	No expression
3[d]	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	Normal	No expression
4	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	Normal	Not interpretable
5	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	No expression	No expression
6[d]	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	Normal	No expression
7	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	Diminished expression	No expression
8	c.1030C>T	p.(Gln344Ter)	c.2785C>T	p.(Arg929Ter)	Normal	Normal	Normal	No expression
9	Normal	Normal	c.2785C>T	p.(Arg929Ter)	NA	NA	NA	NA

Abbreviations: IHC, immunohistochemistry; MMR, mismatch repair; NA, tumor not available.
[a]According to NM_000179.2, nucleotide numbering starts with the A of the start codon.
[b]According to NM_000251.1, nucleotide numbering starts with the A of the start codon.
[c]MMR immunohistochemical analysis performed in the adenoma of the index patient.
[d]MMR immunohistochemical analysis performed in one carcinoma from a relative of the index case.

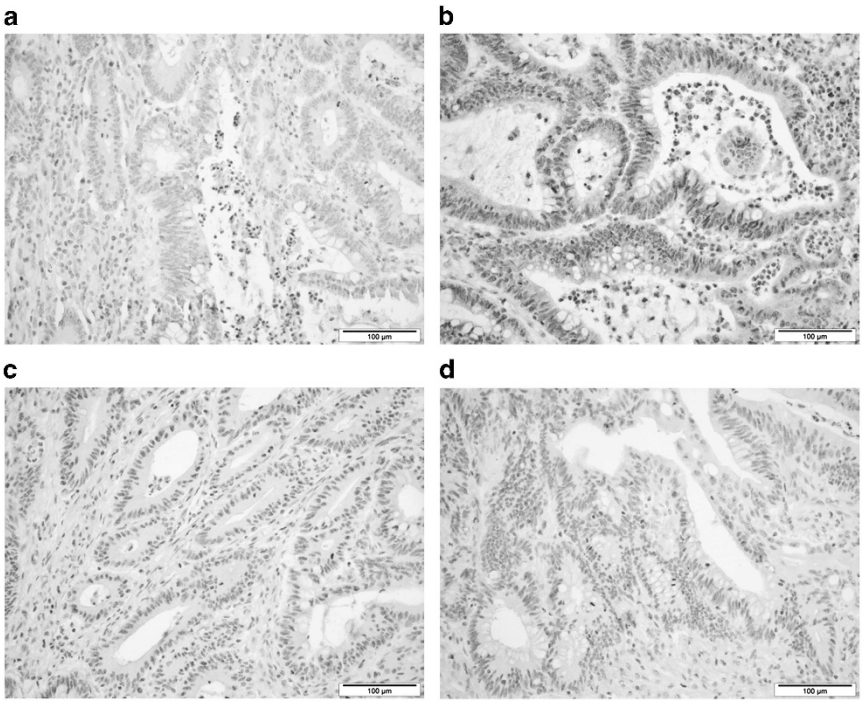

Figure 3 Immunohistochemistry analyses of a tumor from a carrier of both *MSH6* and *MSH2* truncating variants, showing loss of expression of MSH6 (**a**) and normal expression of MSH2 (**b**), MLH1 (**c**) and PMS2 (**d**). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 3 Microsatellite marker haplotypes of the Portuguese Lynch syndrome families presenting the germline *MSH6* and/or *MSH2* mutations

	Family[a]								
Marker	*3*	*5*	*2*	*6*	*4*	*7*	*8*	*1*	*9*
tel									
D2S391	142	142	142	142	142	142	140/**142**	142	**142**/146
D2S2227	129	129	129	117/**129**	117/**129**	117/**129**	117/**129**	117/**129**	117/**129**
Clen33	162	162	162	162	158/**162**	160/**162**	158/**162**	158/**162**	158/**162**
MSH2	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*
Clen30	272	272	272	272	272	266/**272**	272	**272**/274	270/**272**
MSH6	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	*wt*
Clen29	184	186	184	184	146/**184**	146/**184**	172/**184**	172/**184**	146/180
D2S2156	123	123	123	123	119/125	123	123	121/**123**	123
D2S123	209	209	209	209	209	209	209	**209**/211	**209**/225
Clen43	179	173	177/**179**	179	159/177	**173/179**	169/**179**	175/**179**	**173/179**
Clen44	114	114	114	114	116	118	**114**/118	**118**/120	**118**/120
D2S378	211	201	211	209	**211**/213	**211**/213	197/**211**	**209/211**	203/**211**
cen									

Abbreviations: mut, mutated; wt, wild type.
[a]The families are organized by a grade of informative haplotype. In the unphased haplotypes, the alleles that are consistent with the phased haplotypes (families 3 and 5) are indicated in bold.

The finding of double *MSH2/MSH6* variants in eight families originated from a confined geographic area, north to the city of Porto, indicates that they might have a common ancestor. This is in accordance with the haplotype analysis showing that all families harbored alleles consistent with a conserved region. On the other hand, the available data does not allow us to infer the mechanism behind the co-occurrence of these two mutations in eight families and the presence of the isolated *MSH2* variant in another family. Hypothetically, the mutation in the *MSH6* gene could have occurred in a haplotype previously carrying the *MSH2* variant. Alternatively, both could have occurred in the same haplotype of chromosome 2 and a subsequent recombination event could have separated the two variants. Finally, considering that the *MSH2* mutation has been also described in one family outside Portugal, we cannot exclude that this mutation may have occurred independently due to a recurrent mutational event. Be that as it may, this study emphasizes that not all truncating mutations are equal and that one must be cautious in the interpretation of the presumed deleterious effect of terminal frameshift or nonsense mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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