Article

Incomplete Segregation of *MSH6* Frameshift Variants with Phenotype of Lynch Syndrome

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Abstract: Background: Lynch syndrome, the most frequent form of hereditary colorectal cancer and involves mutations in mismatch repair genes. The aim of this study was to identify mutations in *MSH6* from 97 subjects negative for mutations in *MLH1* and *MSH2*. **Methods:** By direct sequencing, we identified 27 *MSH6* variants, of which, nine were novel. To verify the pathogenicity of these novel variants we performed *in silico* and segregation analyses. **Results:** Three novel variants were predicted by *in silico* analysis as damaging mutations and segregated with the disease phenotype. While, a novel frameshift deletion variant that was predicted to yield a premature stop codon, did not segregate with the LS phenotype in 3 of 4 cases in the family. Interestingly, another frame-shift variant identified in this study, already described in the literature, also did not segregate with the LS phenotype in 1 of 2 affected subjects in the family. In all affected subjects of both families, no mutation was detected in other *MMR* genes. Therefore, it is expected that within these families other genetic factors contribute to the disease either alone or in combination with *MSH6* variants. **Conclusion:** We conclude that caution should be exercised in counseling for *MSH6*-associated LS family members.

Keywords: Lynch syndrome; segregation analysis; *MSH6* gene; hereditary colorectal cancer; oligogenic model

INTRODUCTION

Lynch syndrome (LS) is the most common form of hereditary colorectal cancer (CRC) with an incidence of 3–5% among all sporadic cases, whereas its main genetic counterparts, familial adenomatous polyposis (FAP) and *MYH*-associated polyposis (MAP) syndromes, account for 1% of CRC diagnoses [1-3]. LS is characterized by a high lifetime risk for tumor development, especially CRC (20–70%), endometrial cancer (15–70%) and other extra-colonic tumors (15%), including carcinomas of the small intestine, stomach, pancreas and biliary tract, ovary, brain, upper urinary tract and skin. LS is caused by germline mutations in the DNA Mismatch Repair (*MMR*) genes [1]. Germline mutations in *MLH1* and *MSH2* account for approximately 40% and 39%, respectively, of all LS-associated mutations [4]. Approximately 10% of the mutations in *MMR* genes have been identified in *MSH6* gene, and *PMS2* and *MLH3* mutations contribute to a combined 5% [5, 6]. Only one study has investigated mutations in *MSH3* [7]. These mutations manifest as high levels of microsatellite

instability (MSI), which occurs in >90% of all LS carcinomas [8, 9]. Identification of families affected by LS occurs by the Amsterdam Criteria (AC) and Bethesda guidelines (BG) [10-12].

Identifying carriers of *MMR* mutations is critical for improving cancer surveillance and prevention. Usually, genetic testing is performed for *MLH1* and *MSH2*; when this is negative, possible mutations in *MSH6* are investigated [4, 13].

MSH6 is located on chromosome 2p16, near *MSH2* [14, 15], and can cause a LS phenotype, although germline mutations have been attributed to families with atypical clinical features, such as incomplete penetrance, delayed cancer onset and low MSI [16-18]. Detecting mutations in *MMR* genes has been mainly carried out in Lynch patients with tumors with high MSI, which may explain the lower frequency of observed mutations in *MSH6*. The analysis of mutations in *MSH6* is recommended particularly in Lynch families negative for mutations in *MLH1* and *MSH2* and in families in which there are several cases of endometrial tumors [19, 20]. The aim of this study was to investigate the contribution of mutations in *MSH6* in 74 Lynch families, negative for mutations in *MLH1* and *MSH2*. We report several molecular variants in our Lynch patients, of which nine are novel variants. Moreover, we describe two Lynch families in which *MSH6* variants do not fully segregate with the cancer phenotype and discuss the clinical implications of this finding.

RESULTS

All *MSH6* exons were analyzed on DNA extracted from 97 CRC patients belonging to 74 families selected by the AC and BG. As shown in Table 1, 27 germline variants were identified in *MSH6*, nine of which were novel variants not previously reported in the NCBI SNP database, the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), the International Society for Gastrointestinal Hereditary Tumors (InSight; http://www.insightgroup.org/) or the *MMR* variants database [21]. MSI analysis was performed for all patients with available paraffin-embedded tumor tissues; these results are listed in Table 1.

Missense mutations (30%) and intronic alterations (30%) were the most common aberrations. The other observed mutations were silent variants (26%), frame-shift variants (7%) and an in-frame insertion of three nucleotides (4%). Details on the prevalence of each variant are given in Table 1. The nine novel DNA variants were not detected in the 100 healthy controls (Table 2). To verify the pathogenicity of the novel variants, we used a combination of computational and segregation analyses, as described in our previous studies [7, 21]. The results are shown in Table 2.

Table 1: Variants identified in MSH6 gene in our study.

Exon	Nucleotide change	Aminoacid change	Frequency in hereditary CRC	Reference (reportage)	Other studies		
					Segregation analysis	MSI	IHC
1	c.116 G>A	p.Gly39Glu	42 families	dbSNP-rs1042821	ND	ND	ND
1	c.186 A>C	p.= (Arg)	40 families	Nicolaides et al. 1996 (29 times)	ND	ND	ND
1	c.260 +22 C>G		29 families	Kolodner et al. 1999 (8 times)	ND	ND	ND
2	c.261 -46 A>G		1 family	this study	ND	ND	ND
2	c.276 A>G	p.= (Pro)	20 families	dbSNP-rs1800932	ND	ND	ND
2	c.431 G>T	p.Ser144Ile	1 family	Wu et al. 1999 (26 times)	ND	MSI-H	ND
2	c.457 +33_+34insGTGT		1 family	this study	(+)	MSI-L	ND
2	c.457 +50 T>A		1 family	this study	ND	MSI-H	ND
2	c.457 +52 T>A		3 families	Plaschke et al. 2000 (25 times)	ND	ND, ND, MSI-H	ND
3	c.540 T>C	p.= (Asp)	11 families	dbSNP-rs1800935	ND	ND	ND
4	c.642 C>T	p.= (Tyr)	6 families	Wijnen et al. 1999 (26 times)	ND	ND	ND
4	c.663 A>C	p.Glu221Asp	1 family	Devlin et al. 2008 (7 times)	ND	ND	ND
4	c.990 A>T	p.= (Ser)	1 family	this study	ND	MSI-H	ND
4	c.1164 C>T	p.= (His)	1 family	Kolodner et al. 1999 (4 times)	(-)	MSI-H	ND
4	c.1395 A>T	p.= (Ala)	1 family	this study	(-)	MSI-H	ND
4	c.2049_2050insAGT	p.Ala683_Leu684insSer	1 family	this study	(+)	MSI-H	MSH6-MSH2+ MLH1+
4	c.2398 G>C	p.Val800Leu	1 family	Kolodner et al. 1999 (3 times)	(+)	MSI-H	ND
4	c.2633 T>C	p.Val878Ala	2 families	dbSNP-rs2020912	ND	MSI-H, ND	ND
4	c.2941 A>G	p.Ile981Val	1 family	this study	(+)	MSI-H	ND
5	c.3226 C>T	p.Arg1076Cys	1 family	Plaschke et al. 2000 (8 times)	ND	MSI-H	ND

5	c.3261dup	p.Phe1088Argfs*3	1 family	Bonk et al. (2 times)	(-)	MSI-L	MSH6- MSH2+ MLH1+
5	c.3296_3297delTT	p.Ile1099delinsAsnfs*8	1 family	this study	(-)	MSI-H	ND
5	c.3438 +14 A>T		15 families	dbSNP-rs2020911	ND	ND	ND
7	c.3639 T>A	p.Asp1214Glu	1 family	this study	ND	ND	ND
7	c.3646 +31_+34del		16 families	dbSNP-rs1805181	ND	ND	ND
8_9	c.3802-42insT		4 families	Plaschke et al. 2000 (2 times)	ND	ND ND ND ND	ND
8_9	c.3801 +54C>G		8 families	Kolodner et al. 1999 (10 times)	ND	ND	ND

NCBI accession number: NM000179.CRC, Colorectal Cancer; ND, Not Detected; MSI-L/H: Low/High Microsatellite Instability; IHC, Immunohistochemistry.

Table 2: Novel MSH6 variants identified in this study.

Family	ID	Mutation	Protein effect	In silico analysis			Frequency in healthy controls	PhenotypeMSI	Segregation analysis inaffected subjects
				PolyPhen (score)	SIFT (score)	HSF			
31	808	Ex2 c.261 -46 A>G		ND[3]	ND	+3′ss +BP	0/100	AM- ND	ND
33	409 425 426	Ex2 c.457+33_+34insGTGT		ND	ND	+2 3′ss +ESE	0/100	AM- MSI-L	3/3
10	9529	Ex2 c.457 +50 T>A		ND	ND	+3′ss	0/100	AM+ MSI-H	ND
34	410	Ex4 c.990 A>T	p.= (Ser)	ND	ND	-SRp55 -EIE	0/100	AM+ MSI-H	ND
26	210	Ex4 c.1395 A>T	p.= (Ala)	ND	ND	-SRp55 +2ESS +ESR	2/100	AM+ MSI-H	1/2

102	1445 1454	Ex4 c.2049_2050insAGT	p.Ala683_Leu684insSer	ND	ND	+2 3′ss +BP	0/100	AM- MSI-H	2/2
26	210 211	Ex4 c.2941 A>G	p.Ile981Val	Benign (0.181)	Tolerated (1)	+3′ss +2ESE -2EIE +ESS +9G8 +ESR	0/100	AM+ MSI-H	2/2
21	105	Ex5 c.3296_97delTT	p.Ile1099delinsAsnfs*8	ND	ND	ND	0/100	AM+ MSI-H	1/4
18	013	Ex7 c.3639 T>A	p.Asp1214Glu	Probably damaging (1)	Damaging (0)	+2 3′ss +5ESE +EIE +Tra2β -3IIE +ESR	0/100	AM+ ND	ND

ID: Identification number patient; AM: Amsterdam Criteria; MSI-L/H: Low/High Microsatellite Instability; ND: Not Detected; motifs identified (+) or broken (-) by HSF (Human Splicing Finder): 3′ss, acceptor criptic splice site; BP, Branch Point; ESE, Exonic Splicing Enhancer; EIE, Exon Identity Element; ESS, Exonic Splicing Silencer; ESR, Exonic Splicing Regulatory; IIE, Intron Identity Element; SRp55, 9G8 and Tra2β, splicing enhancer proteins.

Our results showed that the novel variant c.3296_97delTT in exon 5 of *MSH6*, identified in the index case n°105 (Table 2), did not segregate with disease in the family (figure 1.a). However, this variant was a frameshift mutation that produced a premature stop codon, resulting in a truncated protein; therefore, it would be considered a pathogenic variant. This variant was not identified in the other affected family members that meet the AC (Table 2); MSI testing performed on DNA extracted from tumor tissue of the index case showed a MSI-H status, but MSI-H was also identified in tumor DNA from subject 0414 who was not a carrier of this variant. The other affected subjects from this family II-2 and II-3 were not carriers of this variant (figure1.a).

Moreover, we identified another frameshift variant in in exon 5 of *MSH6*, c.3261dup, that also produced an early stop codon and a truncated protein. This variant was already reported in the literature [22] and was classified as Class 5 in the Insight database. In our study this variant was identified in the index case n°103 a patient that developed CRC at age 61 (figure 1.b). MSI analysis showed a MSI-L; IHC showed absence of MSH6 protein in tumor tissue (figure 2), but segregation analysis showed that the variant was not present in the sister of the index case, who also developed a colon adenocarcinoma (figure 1.b). We did not analyze other affected subjects in this family. For all affected individuals of these two families, carriers of *MSH6* frameshift mutations or not, the other *MMR* genes (*MLH3*, *MSH3* and *PMS2*) were sequenced but no mutations were identified. MLPA analysis of *MLH1*, *MSH2*, *MSH6* and *PMS2* in these individuals showed no deletions or duplications [23].

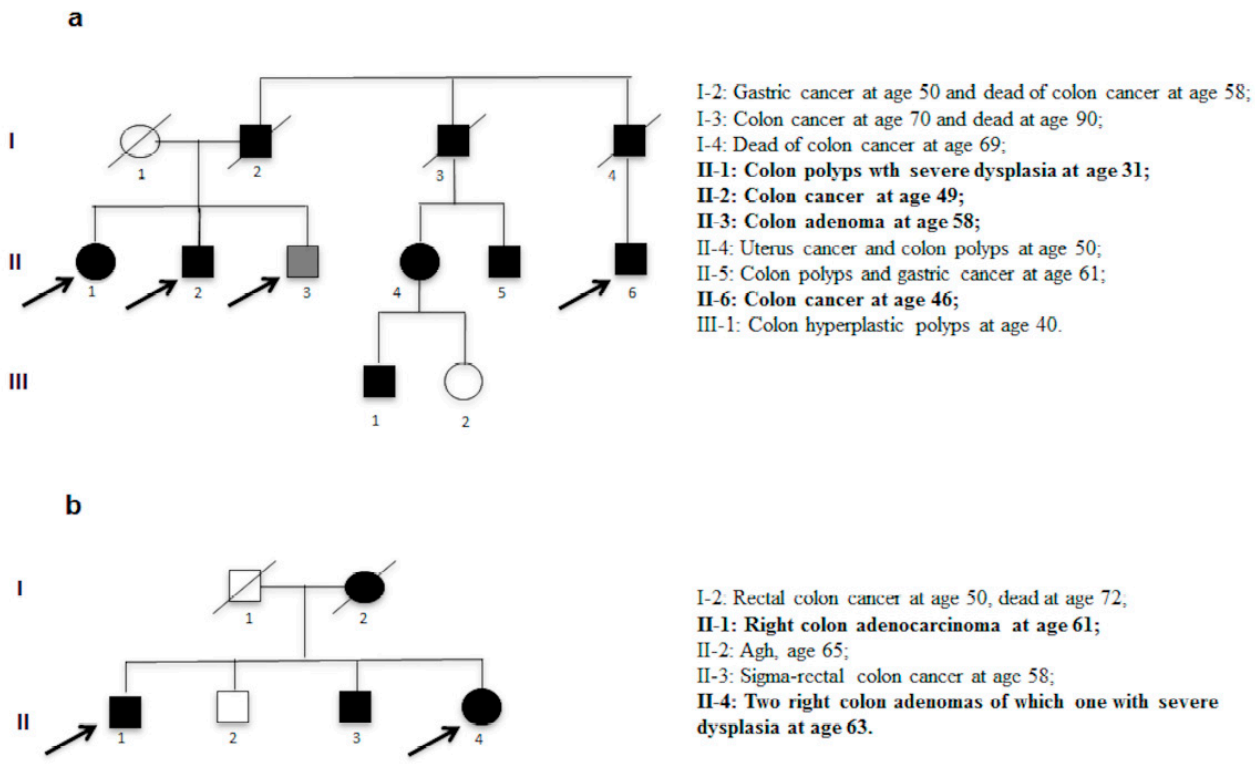

Figure 1: Pedigree of **(a)** 105 (II-6 index case) and **(b)** 103 (II-1 index case) families. Symbols and abbreviations used are denoted as fellow: arrows, analysed members of family; black symbol, colorectal cancer or cancer associated with HNPCC; gray symbol, adenoma or cancer not associated with HNPCC. The patient phenotypes and disease age of onset are reported next to pedigree. Agh, apparent good health.

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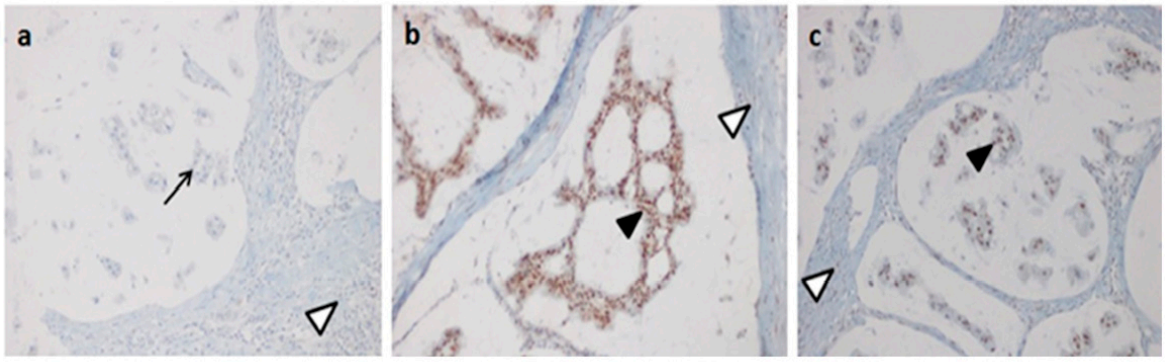

Figure 2: MSH6, MSH2 and MLH1 immunohistochemistry (IHC) results in the colon tumor section of patient carrier of the c.3261dup variant in MSH6 exon 5. (a) Absence of MSH6 protein in the tumor cells; (b) normal IHC for MSH2 protein in the tumor cells; (c) normal IHC for MLH1 protein in the tumor cells. Arrow point to IHC– tumor cells; filled arrows heads point to IHC+ tumor cells; open arrows heads point to blue nuclear staining of lymphocytes (positive internal control).

DISCUSSION

Data from the literature showed that approximately 10% of families with LS were associated with mutations in *MSH6* [5]. In this study we performed mutation analysis of *MSH6* in 97 patients with CRC belonging to 74 families selected by the AC and BG. For all patients that fulfilled the revised BG, an MSI analysis was performed using DNA extracted from tumor tissues. We identified 27 genetic variants, of which nine are novel. To identify the pathogenic effect of these novel variants, we used a multivariate analysis, as described in our previous studies [7, 21]. *In silico* analysis showed a possible pathogenic effect for seven of the nine novel variants identified *MSH6* (Table 2). Segregation analysis, which is critical to understanding the contribution of a mutation to disease, does not always confirm computational data. Where it was possible, the segregation analysis was performed not only for novel variants but for each variant identified in this study to verify the association with disease (Table 2). Interestingly, the novel variants, c.457+33_+34insGTGT, c.2049_2050insAGT and c.2941A>G, which were predicted to be pathogenic by *in silico* analysis, were also found to segregate with disease in their families. These three variants do not create a truncated protein but probably alter the MSH6 protein, making it nonfunctional. However, the two frame-shift variants (the novel variant c.3296_97delTT and the c.3261_62insC, both in exon 5 of the *MSH6* gene) that yield a truncated protein were not found to segregate with a Lynch phenotype in either of their families. The novel frameshift mutation, c.3296_97delTT, was identified in a patient who developed colon cancer and belongs to a family that fulfilled the AC (figure 1.a). It was not possible to perform IHC on tumor tissue from this patient, but we were able to perform MSI analysis on tumor DNA, which showed a strong mutator phenotype. The same result was obtained from DNA extracted from colon tumor tissue from a cousin of our index case who was not a carrier of the *MSH6* mutation. Moreover, the sister and brother of our index case were not carriers of the frameshift variant; however, the sister developed a colon adenoma with a high degree of dysplasia at age 31, while, the brother developed polyps at age 49. Therefore, we performed mutation detection analysis of other *MMR* genes (*MLH3*, *MSH3* and *PMS2*) for the index case and for all affected non-*MSH6* carriers belonging to the family. These patients were already known to be negative for mutations in *MLH1* and *MSH2*. No pathogenic mutations were identified. MLPA analysis of *MLH1*, *MSH2*, *MSH6* and *PMS2* in these individuals also showed no rare deletions or duplications [23]. The other frameshift mutation, c.3261dup already described in literature as pathogenic [22], was identified in a patient who developed colon cancer at age 61. This variant was not identified in the sister of the index case who developed a colon adenocarcinoma at age 63. We have not analyzed the other affected members of family because they had died at diagnosis (figure 1.b). For this case, it was possible to perform IHC on paraffin-embedded

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tumor tissue from the index case. This analysis was negative for MSH6 protein expression (figure 2), and MSI analysis showed MSI-L. Analysis of other *MMR* genes did not show pathogenic mutations.

Based on these results, the *MSH6* frameshift variants in these two families could be not pathogenic and could not contribute to the initiation of the disease in the carriers. If this is the case, taking into account the mode of inheritance in the pedigrees, there is most likely another monogenic factor that is responsible for LS. Recently, it has been described that other Mendelian syndromes with autosomal-dominant inheritance patterns, including the phosphatase and tensin homolog (*PTEN*) Hamartoma Tumor Syndrome (PHTS), show an overlapping clinical presentation with LS and sometimes also show a MSI-H phenotype [24]. In line with this, in previous studies by our group, one patient with a Lynch-like phenotype, negative for *MLH1* and *MSH2* mutations underwent germline testing for *PTEN* mutations, which showed a germline mutation in *PTEN* [25], which was associated with disease in the family. As an alternative to the PHTS syndrome, an alteration of inflammatory pathways associated with a dysregulation of cell proliferation pathways (such as WNT/β-catenin) in colon mucosa may be inherited in a Mendelian manner [26, 27], and may have been the underlying cause of disease in these two families.

However, these two variants identified in *MSH6* create a truncated protein and thus it is tempting to assume that there is an effect of these alterations. It remains unclear whether these two truncating mutations truly initiate tumorigenesis. Interestingly, in our study the missense mutations and in-frame insertions were found to segregate with disease in carriers in the families and index-cases, and these mutations showed a typical LS phenotype with MSH-H. The MSH2-MSH6 complex is the major mismatch recognition complex that recognizes both base:base and single base insertion/deletion mismatches, whereas the MSH2-MSH3 complex appears to primarily recognize insertion/deletion mismatches [28, 29]. This difference is also reflected in their relative abundance with MSH2-MSH6 complexes occurring in excess of MSH2-MSH3 complexes in yeast, human, and mouse [30].

Almost 20 years ago, Edelmann et al. showed that mice homozygous for missense mutations in *MSH6* were unable to repair any type of defect, because the mutated protein competes with the wild-type protein in the formation of the MSH2-MSH6 complex [31]. Meanwhile, subsequent studies have shown that truncated proteins caused by frameshift mutations yielded a weak mutator phenotype [32]. This suggested that tumors with missense mutations in *MSH6* show a more severe phenotype because the presence of mutant MSH6 protein in tumor cells interferes with MSH2–MSH3 mediated repair. In contrast, a lack of MSH6 protein does not interfere with the function of the MSH2–MSH3 heterodimer. In cases where MSH6 is not expressed (null mutations), a severe phenotype, if present, is likely to be due to a secondary mutation in a MMR gene, such as MSH3. Taking previous data into consideration, the *MSH6* variants could be responsible for part of the LS phenotype and additional genetic factors could lead to the disease either by themselves or in combination with the *MSH6* mutations; thus operating in a di- or even multi-genic model [7, 33, 34]. The theory of oligogenic disease would also explain the generally variable onset and severity as well as the reduced penetrance between and within *MSH6* families. In summary, we present several variants in *MSH6*. The mutations that do not delete the protein seem to be associated with a typical LS phenotype, while incomplete segregation of two *MSH6* frameshift variants in two independent families may suggest additional genetic factors are involved in the etiology of the disease, possibly acting as an oligogenic model. High-throughput sequencing technologies may help to uncover the genetic basis of LS in the families described in this report [35].

Genetic counseling and specialized monitoring of families with inherited forms of LS is crucial. Identification of a causal gene in the family will have implications for screening and endoscopic surveillance [1, 2, 36, 37]. Therefore, in cases of incomplete segregation of *MSH6* genetic variants, caution should be exercised when family members who do not carry a family-specific *MSH6* variant are counseled, as they cannot be reassured and should still participate in specialized surveillance programs. Furthermore, our study underscores that segregation analysis remains a very important tool in clinical genetics.

PATIENTS AND METHODS

Patients

The patients were recruited from several hospitals in Campania (southern Italy). Seventy-four subjects with Lynch syndrome diagnosed by the AC or BG and negative for mutations in *MLH1* and *MSH2* were selected. As negative controls, 100 samples from healthy patients were collected from the Clinical Department of Laboratory Medicine of the hospital affiliated to Federico II University (Naples, Italy).

Samples from all subjects were collected after being granted authorization from the local ethics committee "Comitato etico per le attività Biomediche Carlo Romano" of the University of Naples, Federico II (protocol no. 120/10). Once the authorization was obtained, the study received ethical approval, and participants' informed and written consent was obtained. The experiments were performed on DNA extracted from peripheral blood lymphocytes and from paraffin-embedded tumor tissues. For the healthy samples, DNA was extracted only from peripheral blood lymphocytes.

Isolation of genomic DNA

Total genomic DNA was extracted from 4 ml peripheral blood lymphocytes using a BACC2 Nucleon kit (Amersham Pharmacia Biotech, Amersham, UK). For each paraffin block, five 20-µm sections were cut and collected in a 1.5-ml micro-tube. DNA was extracted after deparaffinization according to the protocol described by Duraturo et al., 2015 and using a BACC2 Nucleon kit (Amersham Pharmacia Biotech) [38].

DNA amplification and microsatellite analysis

MSI was tested on paired samples of lymphocyte DNA and DNA from paraffin-embedded tumor sections. MSI was evaluated with a fluorescent multiplex system comprising five mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24 and NR-27), three dinucleotide repeats (D2S123, D5S346 and D17S250) and two tetranucleotide repeats using the CC-MSI kit (AB ANALITICA, Padova, Italy) and subsequent capillary electrophoresis analysis using an ABI 3130 Prism (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Tumors were classified as "highly unstable" (MSI-H), if at least 30% of the markers showed instabilities and as "low-level instability" (MSI-L), if at least 10% of the markers showed instabilities; if no allele differences between DNA extracted from normal and tumorous tissues were observed, tumors were classified as microsatellite stable (MSS) [39-41].

Mutation analysis

Amplification, denaturing high-performance liquid chromatography (dHPLC) and sequencing were all performed using standard protocols. All *MSH6* exons were amplified, including intron-exon boundaries, from DNA extracted from blood lymphocytes of 97 patients, using customized primer sets. Prior to dHPLC analysis, the polymerase chain reaction (PCR) products were separated on a 1–2% agarose gel to check for unspecific amplicons. A Transgenomic Wave DNA Fragment Analysis system (3500 HT; Transgenomic, Inc., Omaha, NE, USA) was used to perform dHPLC analysis. For all samples exhibiting abnormal dHPLC profiles, genomic DNA was re-amplified and sequenced in the forward and reverse directions using an ABI 3100 Genetic Analyser (Applied Biosystems).

In silico analysis

Structural analysis of point variants is important to understand the functional activity of the mutated protein. We used three complementary algorithms for functional impact prediction of the novel variants: Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html) [42], Polymorphism Phenotyping (PolyPhen) (http://genetics.bwh.harvard.edu/pph/) [43] and Human Splicing Finder (HSF) (http://www.umd.be/HSF/) [44], as described in our previous studies [7, 38].

Immunohistochemistry (IHC)

IHC was performed on a Benchmark XT automatized immunostainer (Ventana Medical Biosystems, Tucson, AZ, USA). The antibodies used were anti-MSH6, mouse monoclonal clone 44, anti-MSH2, mouse monoclonal clone G219-1129, and anti-MLH1, mouse monoclonal clone M1 (Ventana). The detection system used was an iVIEW DAB Detection Kit (Ventana) which is based on the Streptavidin-Biotin-conjugated system. Nuclear staining was observed with an optical microscope with positivity represented by the presence of brown staining. This positivity was compared with blue nuclear epitopes, in which the specific antigen was not present. The internal positive control was represented by lymphocytes, stroma and functional mucosal crypts, while the negative control was obtained by slides without primary antibody. Nuclear immunoreactivity scores were assigned using range from 0% to 100%.

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REFERENCES

1 Lynch HT, Drescher K, Knezetic J, Lanspa S. Genetics, Biomarkers, Hereditary Cancer Syndrome Diagnosis, Heterogeneity and Treatment: A Review. Curr Treat Options Oncol 2014; 15: 429–442.

2 De Rosa M, Pace U, Rega D, Costabile V, Duraturo F, Izzo P, Delrio P. Genetics, diagnosis and management of colorectal cancer (Review). Oncol Rep 2015; 34: 1087-96.

3 De Rosa M, Galatola M, Borriello S, Duraturo F, Masone S, Izzo P. Implication of adenomatous polyposis coli and MUTYH mutations in familial colorectal polyposis. Dis Colon Rectum 2009; 52: 268-74.

4 Lynch PM. The hMSH2 and hMLH1 genes in hereditary nonpolyposis colorectal cancer. *Surg Oncol Clin N Am* 2009; 18: 611-24.

5 Silva FC, Valentin MD, Ferreira Fde O, Carraro DM, Rossi BM. Mismatch repair genes in Lynch syndrome: a review. Sao Paulo Med J 2009; 127: 46-51.

6 Duraturo F, Liccardo R, Izzo P. Coexistence of MLH3 germline variants in colon cancer patients belonging to families with Lynch syndrome-associated brain tumors. J Neurooncol 2016;129: 577-8.

7 Duraturo F, Liccardo R, Cavallo A, De Rosa M, Grosso M, Izzo P. Association of low-risk MSH3 and MSH2 variant alleles with Lynch syndrome: probability of synergistic effects. Int J Cancer 2011; 129: 1643-50.

8 Sinicrope FA, Sargent DJ. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clin Cancer Res 2012; 18: 1506-12

9 Kim JO, Gyeong Hoon Kang. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. World J Gastroenterol 2014; 20: 4230-4243.

10 Vasen HFA, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999; 116: 1453-6.

11 Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998; 58: 5248-57.

12 Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S. Revised Bethesda Guidelines for hereditary non polyposis colorectal cancer (Lynch Syndrome) and microsatellite instability. J Natl Cancer Inst 2004; 96: 261-8.

13 Hsieh P, Yamane K. DNA mismatch repair: molecular mechanism, cancer, and ageing. Mech Ageing Dev 2008; 129: 391-407.

14 Palombo F, Gallinari P, Iaccarino I, Lettieri T, Hughes M, D'Arrigo A, Truong O, Hsuan JJ, Jiricny J. GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. Science 1995; 268: 1912-4.

15 Drummond JT, Li GM, Longley MJ, Modrich P. Isolation of an hMSH2-p160 heterodimer that restores DNA mismatch repair to tumor cells. Science 1995; 268:1909-12.

16 Edelmann W, Yang K, Umar A, Heyer J, Lau K, Fan K, Liedtke W, Cohen PE, Kane MF, Lipford JR, Yu N, Crouse GF, Pollard JW, Kunkel T, Lipkin M, Kolodner R, Kucherlapati R. Mutation in the mismatch repair gene Msh6 causes cancer susceptibility. Cell 1997; 91:467-77.

17 Miyaki M, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Muraoka M, Yasuno M, Igari T, Koike M, Chiba M, Mori T. Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer. Nat Genet 1997; 17: 271-2.

18 Lucci-Cordisco E, Rovella V, Carrara S, Percesepe A, Pedroni M, Bellacosa A, Caluseriu O, Forasarig M, Anti M, Neri G, Ponz de Leon M, Viel A, Genuardi M. Mutations of the 'minor' mismatch repair gene MSH6 in typical and atypical hereditary nonpolyposis colorectal cancer. Fam Cancer 2001; 1: 93-9.

19 Long Q, Peng Y, Tang Z, Wu C. Role of endometrial cancer abnormal MMR protein in screening Lynch-syndrome families. Int J Clin Exp Pathol 2014; 7: 7297-303.

20 Chu MM, Liu SS, Tam KF, Ip PP, Cheung AN, Ngan HY.The Significance of Mismatch Repair Deficiency in Young Patients With Endometrial Cancer. Int J Gynecol Pathol 2015; 34: 403-10.

21 Ou J, Niessen RC, Vonk J, Westers H, Hofstra RMW, Sijmons RH. A database to support the interpretation of human mismatch repair gene variants. Human Mutation 2008; 29: 1337-1341.

22 Bonk T, Humeny A, Gebert J, Sutter C, von Knebel Doeberitz M, Becker CM. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based detection of microsatellite instabilities in coding DNA sequences: a novel approach to identify DNA-mismatch repair-deficient cancer cells. Clin Chem 2003; 49: 552-61.

23 Duraturo F, Cavallo A, Liccardo R, Cudia B, De Rosa M, Diana G, Izzo P. Contribution of large genomic rearrangements in Italian Lynch syndrome patients: characterization of a novel alu-mediated deletion. Biomed Res Int 2013; 2013: 219897.

24 Bilbao C, Rodríguez G, Ramírez R, Falcón O, León L, Chirino R, Rivero JF, Falcón O Jr, Díaz-Chico BN, Díaz-Chico JC, Perucho M. The relationship between microsatellite instability and PTEN gene mutations in endometrial cancer. Int J Cancer 2006; 119: 563-70.

25 Paparo L, Rossi GB, Delrio P, Rega D, Duraturo F, Liccardo R, Debellis M, Izzo P, De Rosa M. Differential expression of PTEN gene correlates with phenotypic heterogeneity in three cases of patients showing clinical manifestations of PTEN hamartoma tumour syndrome. Hered Cancer Clin Pract 2013; 11: 8.

26 Galatola M, Paparo L, Duraturo F, Turano M, Rossi GB, Izzo P, De Rosa M. Beta catenin and cytokine pathway dysregulation in patients with manifestations of the PTEN hamartoma tumor syndrome. BMC Med Genet 2012; 13: 28.

27 Galatola M, Miele E, Strisciuglio C, Paparo L, Rega D, Delrio P, Duraturo F, Martinelli M, Rossi GB, Staiano A, Izzo P, De Rosa M. Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis. World J Gastroenterol 2013; 19: 8659-70.

28 Lawes DA, SenGupta SB, Boulos PB. Pathogenesis and clinical management of hereditary non-polyposis colorectal cancer. Br J Surg 2002; 89: 1357-69.

29 Jun SH, Kim TG, Ban C. DNA mismatch repair system. Classical and fresh roles. FEBS J 2006; 273: 1609-19.

30 Yang G, Scherer SJ, Shell SS, Yang K, Kim M, Lipkin M, Kucherlapati R, Kolodner RD, Edelmann W. Dominant effects of an Msh6 missense mutation on DNA repair and cancer susceptibility. Cancer Cell 2004; 6: 139-50.

31 Edelmann W, Yang K, Umar A, Heyer J, Lau K, Fan K, Liedtke W, Cohen PE, Kane MF, Lipford JR, Yu N, Crouse GF, Pollard JW, Kunkel T, Lipkin M, Kolodner R, Kucherlapati R. Mutation in the mismatch repair gene Msh6 causes cancer susceptibility. Cell 1997; 91: 467-77.

32 Yang G, Scherer SJ, Shell SS, Yang K, Kim M, Lipkin M, Kucherlapati R, Kolodner RD, Edelmann W. Dominant effects of an Msh6 missense mutation on DNA repair and cancer susceptibility. Cancer Cell 2004; 6: 139-50.

33 Martinez SL, Kolodner RD. Functional analysis of human mismatch repair gene mutations identifies weak alleles and polymorphisms capable of polygenic interactions. Proc Natl Acad Sci U S A 2010; 107: 5070-5.

34 Kumar C, Piacente SC, Sibert J, Bukata AR, O'Connor J, Alani E, Surtees JA. Multiple factors insulate Msh2-Msh6 mismatch repair activity from defects in Msh2 domain I. J Mol Biol 2011; 411: 765-80.

35 Talseth-Palmer BA, Bauer DC, Sjursen W, Evans TJ, McPhillips M, Proietto A, Otton G, Spigelman AD, Scott RJ. Targeted next-generation sequencing of 22 mismatch repair genes identifies Lynch syndrome families. Cancer Med 2016; 5: 929-41.

36 Carethers JM, Stoffel EM. Lynch syndrome and Lynch syndrome mimics: The growing complex landscape of hereditary colon cancer. World J Gastroenterol 2015; 21: 9253-61.

37 Cudia B, Liccardo R, Di Carlo G, Damiano G, Lo Monte AI, Izzo P, Duraturo F. Clinical and anamnestic evaluation role for the diagnosis and treatment of families affected by Lynch syndrome. Case report and review of the literature. Eur J Oncol 2015;19: 265-271.

38 Duraturo F, Liccardo R, Cavallo A, De Rosa M, Rossi GB, Izzo P. Multivariate analysis as a method for evaluating the pathogenicity of novel genetic MLH1 variants in patients with colorectal cancer and microsatellite instability. Int J Mol Med 2015; 36: 511-7.

39 Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, Seruca R, Iacopetta B, Hamelin R. Evaluation of tumor microsatellite instability using five quasimonomorphic nucleotide repeats and pentaplex PCR. Gastroenterology 2002; 123: 1804-11.

40 Xicola RM, Llor X, Pons E, Castells A, Alenda C, Piñol V, Andreu M, Castellví-Bel S, Payá A, Jover R, Bessa X, Girós A, Duque JM, Nicolás-Pérez D, Garcia AM, Rigau J, Gassull MA; Gastrointestinal Oncology Group of the Spanish Gastroenterological Association. Performance of different microsatellite marker panels for detection of mismatch repair-deficient colorectal tumors Gastrointestinal Oncology Group of the Spanish Gastroenterological Association. J Natl Cancer Inst 2007; 99: 244-52.

41 Vilar E, Mork ME, Cuddy A, Borras E, Bannon SA, Taggart MW, Ying J, Broaddus RR, Luthra R, Rodriguez-Bigas MA, Lynch PM, You YQ. Role of microsatellite instability-low as a diagnostic biomarker of Lynch syndrome in colorectal cancer. Cancer Genetics 2014; 207: 495-502.

42 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009; 4: 1073-81.

43 Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013; 07: Unit7.20.

44 Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 2009; 37: e67.