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MLH1 and *MSH2* mutations in Colombian families with hereditary nonpolyposis colorectal cancer (Lynch syndrome) – description of four novel mutations

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Abstract

This study searched for mutations in the *MLH1* and *MSH2* genes in 23 unrelated Colombian families with suspected hereditary nonpolyposis colorectal cancer (HNPCC). The families were grouped according to the fulfillment of the Amsterdam II criteria or the Bethesda guidelines. We screened all probands by single-strand conformational polymorphism (SSCP) and direct DNA sequencing. Eleven families fulfilled the Amsterdam criteria II and 12 families the Bethesda guidelines. Germline mutations were detected in 11 families, which corresponds to a mutation detection rate of 48%. When only families fulfilling the Amsterdam II criteria were analyzed, the mutation detection rate rose to 82%. Only 8% of the mutation detection rate was found in families following the Bethesda guidelines. Three mutations were shared by two different families, which corresponds to a total of eight different mutations, seven of them found in the *MLH1* gene and one in the *MSH2* gene. We have identified four mutations that have not been previously reported to the International Collaborative Group of HNPCC. Three of these are pathogenic, a single base substitution (C > T) at codon 640, exon 17, a G deletion at codon 619, exon 16 and in the *MLH1* gene and a two-nucleotide deletion (TG) at codon 184, exon 3 in the *MSH2*. Also, an unclassified variant, a substitution (C > G) at the codon 141, exon 5 of the *MLH1*, was detected.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the developed world, while in developing countries it is the sixth or seventh cause of death, although these last figures are quite heterogeneous and differ racially and by gender [1, 2]. It has been estimated in the US, that 56,730 CRC-specific deaths will occur in the year 2004 [3]. In Colombia, CRC is the fifth cause of cancer mortality and it is estimated that approximately 1500 individuals die annually due to CRC [4, 5]. From 3 to 5% of the total colorectal cancer cases correspond to hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome. This is a cancer predisposition syndrome inherited in an autosomal dominant fashion [6], with high penetrance (85%) and variable expressivity. Besides CRC, other cancers are also common in HNPCC, as endometrial, small-bowel, ureter and renal pelvis carcinoma, and to a lesser extent, stomach, ovary and hepatobiliary carcinoma. It is estimated that up to 75 persons die annually in Colombia due to this autosomal dominant inherited disease.

Colon cancers from patients with HNPCC are characterized by expansion or contraction of short repeat sequences of DNA (microsatellite) at multiple loci. The cause of this phenomenon, known as microsatellite

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instability (MSI), is thought to be responsible for the rapid accumulation of somatic mutations in oncogenes and tumor-suppressor genes that play crucial roles in the initiation and progression of tumors, mainly those with nucleotide repetitions in its coding segments [7]. This instability is due to germline mutations in, at least, 5 DNA mismatch-repair genes (MMR) genes (*MLH1, MSH2, MSH6, PMS1* and *PMS2*). The great majority of germline mutations are found in *MLH1* and *MSH2*, while mutations in the other 3 MMR genes have been reported only in a limited number of cases. Carriers of germline MMR mutations have a >80% risk of cancer by the age of 75. Mutations in MMR genes result in MSI, which is characteristic of >90% of tumors from HNPCC patients and approximately 12% of sporadic colorectal tumors [7, 8].

Like any other cancer, CRC is preventable if detected at an early stage and several strategies have been proposed for this purpose. For the purpose of clinical diagnosis, several criteria were proposed in 1991, and afterwards revised, which are currently known as Amsterdam-II criteria [9]. Additionally, the Bethesda guidelines were proposed, not only for clinical purposes but also for defining the suitability for MSI analysis. These criteria have recently been revised [10]. MSI have been proposed to be tested in CRC tissues and those showing instability, and without promoter methylation, being subsequently sequenced [11, 12].

The aim of this study was to identify mutations in the two major susceptibility genes *MLH1* and *MSH2*, in 23 unrelated Colombian families with suspected HNPCC, by means of single-strand conformational polymorphism (SSCP) and subsequent sequencing of the fragments that displayed an abnormal pattern.

Materials and methods

All aspects of this study were reviewed and approved by the Ethics Committee of the School of Medicine of the Universidad Nacional de Colombia.

Families and patients

Families were searched through proctologic services of different hospitals of Bogotá and Barranquilla, and in the files of clinical and surgical gastroenterologists. All were traditional Colombian families without any recent immigrant ancestors. We classified pedigrees of 23 families by whether they fulfilled the Amsterdam II or followed the Bethesda guidelines. Informed consent was obtained from each participant. Personal and family cancer histories were obtained from the proband and participating relatives, and cancer diagnoses and deaths were confirmed by reviewing the medical records or pathology reports.

DNA analysis

Blood samples were obtained from one or two cancer syndrome affected members from each family. Genomic DNA was isolated from peripheral blood lymphocytes according to the salting out procedure [13]. The entire *MLH1* and *MSH2* coding region and the splice junctions were amplified by PCR from genomic DNA using 19 and 16 primer pairs, respectively, according to reported primers [14], with some modifications for exon 10 of *MLH1* and exons 1 and 11 of *MLH2* genes. Primer sequences modifications are available upon request.

All amplicons were subjected to SSCP [15] and the DNA fragments that displayed an abnormal pattern were analyzed by cycle sequencing with the ABI Prism dRhhodamine Terminator Sequencing Kit and analyzed in the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). All mutations were confirmed by two independent sequencing PCRs and sequenced in both directions.

Bioinformatic analysis procedure

For the mutation analysis of family UN-04 and UN-23, Pfam database and UniProt [16, 17] were used to search for the correspondent domains for the 640 and 141 MLH1 amino acids. Matching sequences were aligned and appointed with ClustalW and Clustal X [18, 19].

Results

Of the 23 families, 11 fulfilled the Amsterdam criteria II, and 12 families fulfilled the Bethesda guidelines. Germline mutations were detected in 11 families, which corresponds to a mutation detection rate of 48%. When only families fulfilling Amsterdam criteria were analyzed, the mutation detection rate rose to 82%. On the other hand, only 8% (1/12) of the mutation detection rate was found in families fulfilling the Bethesda guidelines. Out of the detected mutations in the 11 families, three mutations were shared by two different families, which corresponds to a total of eight different mutations, seven of them found in the *MLH1* gene and one in the *MSH2* gene (Table 1).

We have identified four mutations that have not been previously reported to the International Collaborative Group of HNPCC (www.nfdht.nl). Three of mutations are in the *MLH1* gene. These are a single base substitution (C > T) at codon 640, exon 17 (pathogenic, Family UN-4), a G deletion at codon 619, exon 16 (pathogenic, Families UN-21 and UN-23) and a substitution (C > G) at the codon 141, exon 5 (unclassified variant, Family 23). In the *MSH2* gene, a two-nucleotide deletion (TG) at codon 184, exon 3 was detected (pathogenic, family, UN-2). (Table 1, Figure 1.)

Discussion

The racial admixture among aboriginal populations, Spaniard colonist and African slaves resulted in the Colombian population, mainly during the 17th and 18th

Table 1. Mutations at the *MLHI* and *MSH2* genes in Colombian families.

Family	Criteria	Gene	Exon	Position	Nucleotide change	Consequence	Reported
UN-1	Amsterdam II	*MLH1*	9	790 + 1	G > A	Splicing defect	Yes
UN-6	Amsterdam II	*MLH1*	9	790 + 1	G > A	Splicing defect	Yes
UN-8	Amsterdam II	*MLH1*	13	1558 + 14	G > A	None	Yes
UN-17	Bethesda	*MLH1*	13	1558 + 14	G > A	None	Yes
UN-4	Amsterdam II	*MLH1*	17	1918	C > T	Proline/Serine	New
UN-16	Amsterdam II	*MLH1*	17	1964	A > G	IsoleucinelValine	Yes
UN-3	Amsterdam II	*MLH1*	18	2041	G > A	Alanine/Threonine	Yes
UN-21	Amsterdam II	*MLH1*	16	1856	del G	FrameshiftiStop	New
UN-23	Amsterdam II	*MLH1*	16	1856	del G	Frameshift/Stop	New
UN-23	Amsterdam II	*MLH1*	5	421	C > G	Proline/Alanine	New
UN-2	Amsterdam II	*MSH2*	3	596	del TG	FrameshiftlStop	New

century. Immigrations at the end of the 19th century or during the 20th century, from European or Middle East countries, were remarkably lower than those in other South American countries. For that reason, it is highly unusual to trace back a recent ancestor out of Colombia for the vast majority of the families. None of our studied

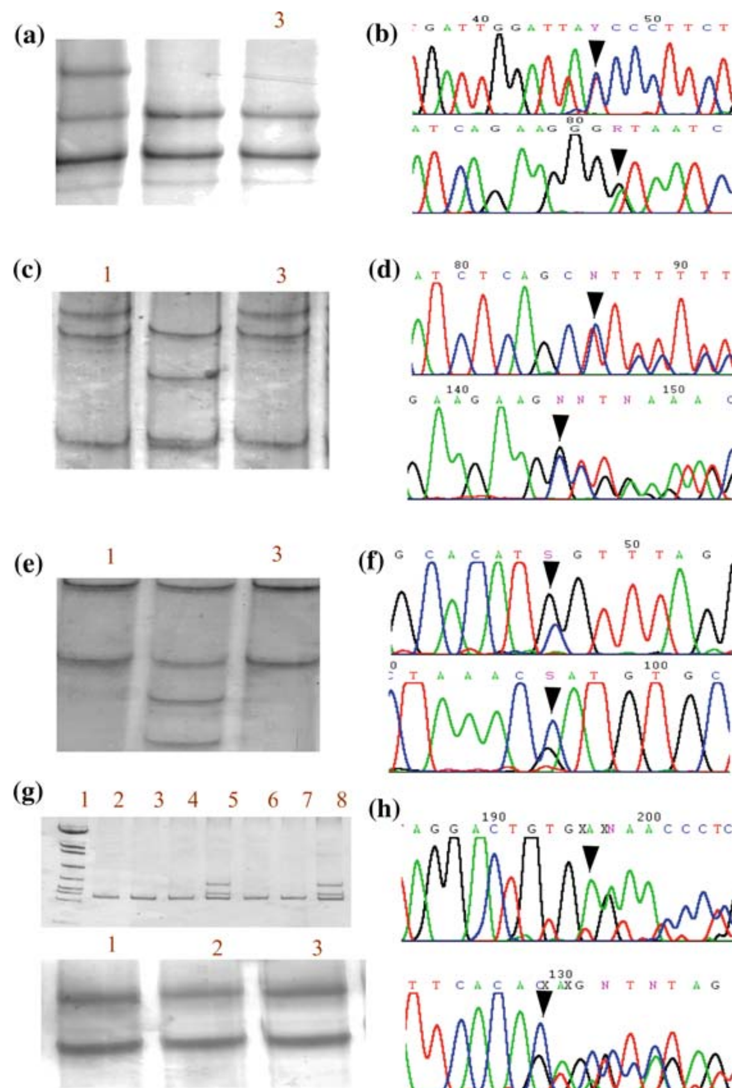

Figure 1. SSCP and nucleotide sequences of four new mutations, a and b, Family UN-04. (a) SSCP analysis of exon 17 of the hMLH1. Lane 1 shows abnormal mobility. (b) Nucleotide sequence of exon 17; the arrowheads show the mutation sites. c and d, families UN-21 and UN-23. (c) SSCP analysis of exon 16 of the hMLH1; Lane 2 shows abnormal mobility. (d) Nucleotide sequence of exon 16; the arrowheads show the G:C deletion in both strands. e and f, Family 23. (e) SSCP analysis of exon 5 of the hMLH1; Lane 2 shows abnormal mobility. (f) Nucleotide sequence of exon 5; the arrowheads show the mutation site. g and h, family UN-2. (g) The upper panel shows a PAGE at the 14%; Lanes 5 and 8, both of exon 3 of the MSH2 gene, show two additional bands in PCR fragments. The lower panel shows a normal SSCP analysis of exon 3. (h) Nucleotide sequence of exon 3 of the hMSH2; the arrowheads show the deletion in both strands.

families has any recent European or non-European ancestors, which is similar to that observed in Brazil [20], but in contrast to NHPCC families from Uruguay [21].

Ethnic variations in both the prevalence of the HNPCC phenotype and carrier frequencies of germline *MLH1* and *MSH2* mutations in selected HNPCC families have been extensively described, mainly in Europe and in the USA [22–26]. There are no previous studies of mutations of *MLH1* or *MSH2* genes in Colombia. This work, therefore, represents the first record of such mutations in Colombian patients with HNPCC and one of the few in Latin America.

Twenty-three unrelated families were studied and germline mutations were detected in 11 families, which corresponds to a detection rate of 48%, which is similar to that observed in several studies, when Amsterdam criteria and Bethesda guidelines are considered together [20, 26]. When only the Amsterdam criteria are considered, the detection rate increases to 82%, which is similar to the rate observed in other studies, when these criteria are exclusively considered [23]. However, these figures vary widely [22–26]. The differences in detection rate could be due to random variation, or population substructure in European older populations, or in Latino American, newer and more heterogeneous or mixed populations.

Of the four new mutations detected, three are predicted to be pathogenic. Observed in Families UN-21 and UN-23, is a nucleotide deletion (delG) in the exon 16, position 1856 of the *MLH1* gene that produces a frame shift and a stop codon. The same consequence produces the two nucleotide deletion (delTG) in the *MSH2* gene observed in family UN-2.

The third predicted pathogenic mutation is observed in the *MLH1* gene of family UN-4. This missense C > T transition in the exon 17, position 1918, causes in the amino acid 640 a proline substitution to serine, an amino acid of different polarity (Figure 2). Proline, a small aliphatic amino acid with a rigid heterocyclic structure, is conserved in this position among eukaryotic species (yeasts, nematodes, plants and mammalians) and also in *E. coli*. In addition, proline is involved in the formation of alpha helices and bends, and the presence of another highly conserved proline eight positions downstream may represent a group of cyclic folding, which could be essential for the protein structure. The high level of conservation of codon for proline 640 of the *MLH1* gene suggests that a mutation that changes this amino acid for a substantially different one might have a remarkable impact on the function and transportation of the protein. Additionally, in this family the mutation co-segregates with the disease in three affected members of the same sibship. Two healthy sibs, 53 and 51 years old were not available for study. The mutation is not present in a healthy 42-year-old woman, but a healthy 35-year-old woman is a mutation carrier. This mutation was not searched in the normal population.

Additionally, in family UN-23, but not in family UN-21 (these two families share a pathogenic mutation in the exon 16 of *MLH1* gene), a missense (C > G) transversion in the exon 5, position 421, was detected in the same *MLH1* gene. This mutation produces a change in amino acid 141, proline to alanine (Figure 3). Both are aliphatic apolar amino acids with chains that can be considered equivalent in relation to their small molecular volumes. The analysis of the three-dimensional structure of MLH1 protein indicates that this position corresponds to a large size linker between the ATPase and the DNA repair domains [27–30]. Notwithstanding, the necessary interaction between these two domains, the change of the amino acid 141 would not produce important modifications because the amino acid in this

MLH1 nucleotide sequence, exon 17, position 1918

Homo sapiens	ATTGGATTACCCCTTCTGATTGACAACTATGTGCCCCCTTT
Mus musculus	ATTGGATTACCTCTTCTGATTGACAGCTATGTGCCACCTTT
S. cerevisiae	AAATCTCTACCACTACTTTTAAAAGGCTACATTCCATCTCT
Family UN-04	ATTGGATTATCCCTTCTGATTGACAACTATGTGCCCCCTTT

MLH1 amino acid sequence (amino acid 640 shadowed)

Homo sapiens	DEEGNLIGLPLLIDNYVPPLEGLPIFILRL
Mus musculus	DEEGNLIGLPLLIDS YVPPLEGLPIFILRL
Family UN-04	DEEGNLIGLSLLIDNYVPPLEGLPIFILRL

Figure 2. Nucleotide sequence of a segment of exón 17 and relative position of amino acid 640 of MLH1 gene.

MLH1 nucleotide sequence, exon 5, position 421

Homo sapiens	CCTCCTAAACCATGTGCTGGCAATCAAGGGACCCAGATCACGGTG
Mus musculus	CCTCCTAAACCCTGTGCAGGCAACCAGGGCACCCTGATCACGGTG
S. cerevisiae	AGCCCCAAACCTGTTGCTGGAAAAGACGGTACCACGATCCTAGTT
Family UN-23	CCTCCTAAAGCATGTGCTGGCAATCAAGGGACCCAGATCACGGTG

MLH1 amino acid sequence (amino acid 141 shadowed)

Homo sapiens	PPKPCAGNQGTLITV
Mus musculus	PPKPCAGNQGTLISV
Family UN-23	PPKACAGNQGTLITV

Figure 3. Nucleotide sequence of a segment of exón 5 and relative position of amino acid 141 of MLH1 gene.

position seems to be involved in a β sheet formation, in which the Van der Waals interactions are still functioning as far as no relevant modification in the molecular volume or in the polarity of the amino acids are given. Compound heterozygosity for two different MLH1 missense mutations has been described, apparently without a highly increased risk of colon cancer [31]. On the other hand, individuals homozygous for pathogenic mutations are expected to display signs of neurofibromatosis type 1 and to develop hematological malignancies early in life [32–34]. Our compound heterozygous patient from family UN-23 is a 45-year-old female with only CRC to date. Notwithstanding the previous considerations, this mutation should be considered an unclassified variant.

The three families sharing the same mutations (UN-1 and UN-6; UN-8 and UN-17, and UN-21 and UN-23), did not know each other, although they came from neighboring regions. A founder effect of these three mutations is to be confirmed, as has been done for the confirmed founder effect for both *MLH1* and *MSH2* genes in several European countries and in USA [35–39].

With regard to the proportion of mutations in *MLH1* and *MSH2* genes, this also varies according to different studies. As in our case, the Brazilian study observed a major frequency of mutations in the *MLH1* gene [16]; a similar proportion has been observed in the Korean population [40]. However, an inverse proportion has been observed in several other studies [41], or similar proportions in both genes [42, 43].

In conclusion, it is clear that identifying HNPCC families and carriers presents a life-saving potential through early cancer detection. We suggest that not only HNPCC families fulfilling the Amsterdam criteria, but also those following the Bethesda guidelines should undergo genetic testing. Identification of the pathogenic mutations would greatly facilitate presymptomatic diagnoses useful in genetic counseling, making therapeutic decisions and developing protocols to implement clinical surveillance of the established carriers prior to disease manifestation.

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