Low prevalence of germline hMSH6 mutations in colorectal cancer families from Spain

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INTRODUCTION

The link between defective DNA mismatch repair and the development of tumors has been firmly established. Inherited mutations in DNA mismatch repair genes are associated with the autosomal-dominant cancer susceptibility syndrome, hereditary non-polyposis colorectal cancer (HNPCC [MIM 114400; MIM 114500]). Patients with HNPCC are at high risk for colorectal and endometrial cancer and a variety of other malignancies. Tumors from HNPCC patients frequently show mutations in repetitive sequences, giving rise to a molecular phenotype referred to as microsatellite instability (MSI). Not surprisingly, a significant fraction of sporadic colorectal and endometrial cancers also show MSI.

The hMSH6 (MIM 600678) protein is a member of the MMR system; it interacts with hMSH2 to form the MutSα heterodimer[1-3]. Besides hMSH6, the hMSH2 protein also interacts with the hMSH3 protein to form the MutSβ heterodimer. The MutSα heterodimer is efficient in the repair of both base-base mismatches and insertion-deletion loops, whereas the MutSβ heterodimer is mainly active in the repair of insertion-deletion loops and not in the repair of base-base mismatches[4,5]. These data indicate that the hMSH6 and hMSH3 proteins are partially redundant in their DNA repair function.

Most families with clinically recognized HNPCC and MSI high, have mutations in either hMLH1 or hMSH2. Mutations in hMSH6 gene, appear to be associated with atypical HNPCC and in particular with the development of endometrial carcinoma[6-9]. The age of onset of cancers in HNPCC kindred’s with hMSH6 mutations is higher than in hMSH2 or hMLH1 mutation carriers, and it has been reported that tumors from affected family members are less likely to have high MSI than tumors from hMSH2 or hMLH1 mutation carriers[10].

The estimate frequency of hMSH6 mutations in patients with colorectal cancer ranges between 9% based on a tumor immunohistochemistry study of a consecutive series and 1.5% based on mutation analysis of a combination of sporadic and familiar cases[11,12]. Only a few of these germ-line mutations have been reported in families that fulfill the Amsterdam criteria[12,13]. To date, there have been no studies...
to determine the frequency of germ-line hMSH6 mutations in Spanish HNPCC families. The aim of this study was to verify the contribution of hMSH6 defects to cancer susceptibility in 132 HNPCC Spanish families that have been previously tested for hMSH2 and hMLH1 mutations[14,15].

MATERIALS AND METHODS

Patients
Families were selected through the clinic for familial cancer at the San Carlos University Hospital in Madrid, and they were Spanish by descent. 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, pathology reports, or death certificates.

We classified each pedigree by whether they fulfilled the original Amsterdam I criteria ($n = 56$ families), Amsterdam II ($n = 11$ families), Bethesda guidelines ($n = 37$ families), or none of these criteria ($n = 28$ families)[16-18]. This last category we refer to as “HNPCC-like” where the tumor spectra are reminiscent of HNPCC but not fulfilling Amsterdam I, Amsterdam II, or young age at onset criteria.

All probands, had been previously tested for MSI status and hMLH1/hMSH2 mutations/deletions by either DGGE and direct genomic sequencing and multiplex ligation-dependent probe amplification (MLPA, MRC-Holland).

Control subjects
One hundred unrelated healthy individuals from the Blood Transfusion National Service in Madrid (Spain) were used as controls.

DNA isolation
Genomic DNA was isolated from peripheral blood lymphocytes according to the salting out procedure[19]. Paraffin-embedded tumor samples were used to confirm segregation of identified mutations and to study MSI and LOH. In these cases, DNA was extracted from paraffin blocks as described previously by de la Hoya et al.[20].

Tumor MSI analysis
One hundred and thirteen paired normal and tumor DNA were analyzed for MSI with the five markers of the Bethesda guidelines ($n = 37$ families), Bethesda guidelines ($n = 28$ families)[16-18]. This last category we refer to as “HNPCC-like” where the tumor spectra are reminiscent of HNPCC but not fulfilling Amsterdam I, Amsterdam II, or young age at onset criteria.

All probands, had been previously tested for MSI status and hMLH1/hMSH2 mutations/deletions by either DGGE and direct genomic sequencing and multiplex ligation-dependent probe amplification (MLPA, MRC-Holland).

DNA amplification
The entire MSH6 coding region and the splice-junctions were amplified using the PCR from genomic DNA using 23 primer pairs. All amplicons were subjected to DGGE (below). The sequences of the primers are available upon request.

All DNA amplifications were performed in a 50-μL volume containing 100 ng DNA, 1× PCR buffer (Ecogen SRL, Spain), 0.4 μmol/L of each primer, 200 μmol/L of each deoxynucleotide (Promega, Madison, WI, USA), 1 U Eco Taq DNA polymerase (Ecogen SRL, Spain). Amplification was carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA, USA). After denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 55 °C, or 58 °C for 30 s and 72 °C for 60 s were performed, followed by a final extension step of 10 min at 72 °C. Subsequently, PCR fragments were subjected to one round of complete denaturation and renaturation that is 98 °C for 10 min, 55 °C for 30 min, and 37 °C for 30 min to create heteroduplex molecules.

DGGE
DGGE analysis was performed in a denaturing gradient gel electrophoresis D-Code System (Bio-Rad Hercules, CA, USA). A 6-μL aliquot from the PCR reaction volume was mixed with 2 μL of standard dye-loading buffer and then loaded onto a 10% AA/bis-acrylamide (37.5:1) gel (0-60% or 20-80% urea-formamide chemical gradient according to melting profiles of each PCR fragment) in 1× TAE (40 mmol/L Tris-base, 20 mmol/L NaAC, 1 mmol/L EDTA pH 8) for 16 h at 80 V and 60 °C. The DNA fragments were visualized and photo-documented under ultraviolet transillumination after staining with an ethidium bromide solution.

In addition to mutation analysis, we used DGGE to perform DGGE-LOH analysis where appropriate tissue was available, for tumors belonging to families with identified germ-line mutations in hMSH6. All available tumor tissue was sectioned and subjected to hematoxylin-eosin staining to determine the proportion of tumor cells. Only samples with >95% tumor cells were selected by macrodissection.

DNA sequencing
DNA fragments that displayed an abnormal DGGE pattern were analyzed by cycle sequencing. PCR products were purified with the QIAquick PCR purification Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Cycle sequencing reactions were performed with the ABI Prism dRhodamine Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed in the ABI.
Prism 310 Genetic Analyzer (Applied Biosystems). All mutations were confirmed by two independent sequencing PCR reactions and sequenced in both directions.

**hMSH6 immunohistochemistry**

Immunohistochemical staining of hMSH6 was performed for the 60 MSS cases and for the 12 MSI-H cases negative for mutation in hMLH1 and hMSH2. The tissue sections were cut at 6 μm and mounted on ChemMate Gap microscopy slides (DAKO A/S, Denmark) and dried at 37 °C. After deparaffinization, slides were steam pretreated in EDTA buffer, pH 8-0, in a Magefesa Handy Steamer Plus (Magefesa, Spain) for 30 min, and then cooled and washed in PBS.

The antibody to hMSH6 (clone 44; 1/100; catalog number G70220; Transduction Laboratories, Becton Dickinson, Lexington, UK) is a mouse mAb generated with an NH2-terminal fragment (codons 225-333) of the hMSH6 protein. For the immunohistochemical analysis, avidin-biotin complex immunoperoxidase technique was performed by using a commercial ChemMate detection kit (DAKO A/S) in a Techmate automate machine. Endogenous peroxidase was blocked by incubation in hydrogen peroxide with methanol. Incubation with no immune horse serum was followed by incubation with primary antibody. The sections were then incubated in biotinylated second antibody and peroxidase-labeled avidin-biotin complex. All dilutions were made in PBS (pH 7.2). The stainings were visualized with diaminobenzidine tetrahydrochloride solution. The sections were counterstained in Mayer’s hematoxylin, rinsed with water, and mounted in an aqueous mounting media (Aquamount, BDH, Poole, UK). The percentage of positive nuclei was evaluated by two pathologists. Cases with more than 10% of nuclei staining were considered as positive.

**RESULTS**

MSI testing was carried out in 113 of the 132 families enrolled in our study. Of these 113 families, 60 had tumors that exhibited a non-MSI-high (MSS) phenotype and 53 had tumors that exhibited an MSI-high (MSI-H) phenotype. Nineteen families were not typed for MSI. All families were tested for mutations in hMLH1 and hMSH2, 24 families had a mutation in hMLH1 and 17 had a mutation in hMSH2 and were not studied further (Table 1). The remaining families were studied for mutations in hMSH6. In the 91 index cases tested for mutations in hMSH6, we identified 14 DNA variants. Of these, one was a novel definite deleterious mutation, nine were unclassified variants, six of them not described previously (Table 2) and four were polymorphisms described previously (Table 4). None of the unclassified variants were found in 100 control subjects.

The deleterious mutation Q236X, was found in an Amsterdam II family FC-100; change of cysteine for thymine at position c.706 leads to the formation of a termination codon at amino acid residue 236 that produces an 235 amino acid product instead of the normal 1 360 amino acid product. This mutation was found in a patient with two colorectal cancer cases, one colorectal and one endometrial (Figure 1). MSI testing was carried out in both tumors, colorectal and endometrial, and they exhibited a MSS phenotype; the immunohistochemical analysis of MSH6 showed an absence of staining (Table 3). In this family, we could test at least two affected members, her father affected of colorectal cancer who was a carrier of the mutation and her sister also affected

### Table 1 Classification of colorectal cancer families, frequencies of pathogenic mutations in hMLH1 and hMSH2 genes and MSI phenotype

| Clinical criteria | Number of families | hMLH1 mutations | hMSH2 mutations | MSI-H | MSS | MSI untyped |
|-------------------|--------------------|-----------------|-----------------|-------|-----|-------------|
| Amsterdam I       | 56                 | 17              | 15              | 42    | 13  | 1           |
| Amsterdam II      | 11                 | 2               | 2               | 6     | 2   | 2           |
| Bethesda          | 37                 | 5               | 0               | 6     | 20  | 11          |
| HNPCC-like        | 28                 | 0               | 0               | 3     | 20  | 5           |
| Total             | 132                | 24              | 17              | 53    | 60  | 19          |

### Table 2 hMSH6 mutations in Spanish HNPCC families

| Family | Clinical criteria | Dx aged index patient (y) | No. of tumors in the family | Exon/intron | Nucleotide change | Amino acid change | Predicted consequence | Nfdht data base |
|--------|-------------------|---------------------------|----------------------------|-------------|-------------------|-------------------|----------------------|-----------------|
| 5      | Bethesda          | 42                        | 1E, 1G, 1M, 1H, 1F, 1A      | Ex. 8       | c.3725 C>T        | R1242L            | Inconclusive         | No              |
| 11     | HNPCC-like        | 58                        | 1G, 2C, and 1B              | Ex. 4       | c.3319 C>T        | L775L             | Inconclusive         | No              |
| 68     | Bethesda          | 29                        | 1L, and 1C                  | Ex. 4       | c.1164 C>T        | E388H             | Inconclusive         | No              |
| 81     | HNPCC-like        | 50                        | 4C                          | Int. 5      | c.3439-16 C>T     | NA                | Inconclusive         | No              |
| 94     | Amsterdam II      | 45                        | 3P, 2E, 1G, and 3C          | Ex. 4       | c.2633 T>C        | V878A             | Pathogenic           | Yes             |
| 100    | Amsterdam II      | 54                        | 4C, 1P, and 3G              | Ex. 4       | c.706 C>T         | Q266X             | Truncation           | No              |
| 73     | Amsterdam II      | 29                        | 2M, 1G, 1C, and 1L          | Ex. 4       | c.2633 T>C        | V878A             | Pathogenic           | Yes             |
| 121    | HNPCC-like        | 58                        | 3C, 1G, and 1B              | Int. 5      | c.3439-16 C>T     | NA                | Inconclusive         | No              |
| 138    | Bethesda          | 43                        | 2C and 1 urinary in the same patient Ex. 4 | c.1677 C>T | C599C             | Inconclusive       | No                   | No              |

E, endometrial; G, glioma; M, meningioma; H, hemangioma; F, fibrosarcoma; A, adenoma; G, gastric; C, colon; B, breast; L, larynx; P, prostate; B, brain; SM, small intestine; y, years.
of colorectal cancer that resulted not to be a carrier. Both members had MSS tumors and MSH6 protein staining was absent in the carrier and was present in the non carrier.

The missense mutation V878A was detected in two Amsterdam families, FC-73 and FC-94, this mutation has been reported previously and had been considered as possibly pathogenic. FC-94 (Figure 1) is also a carrier of the unclassified variant K618A in the MLH1 gene (Table 3). Both mutations (V878A in MSH6 and K618A in MLH1) were found in affected members of the family, the proband (affected with colorectal and prostate cancer at ages 51 and 52) and his sister (affected with endometrial cancer at age 45). MSI study in tumors from carriers showed a MSI-H phenotype and the hMSH6 staining was present (Table 3). Of the tumors from the three affected brothers was MSS. The two members who carry the hMSH6 mutation, were also carriers of K618A in the MLH1 gene. The MSI status of the colorectal cancer showed a MSI-H phenotype and the hMSH6 staining was present (Table 3).

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DGGE-LOH analysis was done to further characterize the role of these variants. Figure 3 shows a typical DGGE analysis of different hMSH6 exons amplified from tumor and peripheral blood DNA. Examples of missense mutations R1242L, V878A, and nonsense mutation K263X are shown. The silent mutations H388L and L758L were identified in the same individual. LOH analysis showed loss of the wild-type allele in both informative loci, indicating that these changes were located in the same chromosome. Taking into account our results, no evidence for LOH acting as a second-hit in the hMSH6 locus was found.

**DISCUSSION**

Carrier frequencies of germ-line hMSH6 mutations in selected HNPCC families have been extensively described [6,10,12,23,24]. However, there is no evidence in the literature on this point regarding the Spanish population. We screened 91 HNPCC families (24 Amsterdam I, 7 Amsterdam II, 32 Bethesda guidelines, and 28 HNPCC-like) for the presence of germ-line mutations in the hMSH6 gene and identified 14 DNA changes. Seven of them were previously unreported DNA changes, including the intronic change at -16 base position of intron 5 (intronic mutation) found in two HNPCC-Like families, 1 amino acid substitution, 1 protein truncation mutation, and 4 silent mutations (Tables 2 and 3). The remaining seven DNA changes were reported previously, four of which were attested polymorphisms, one was the silent mutation H388L and the other 2 were V878A substitutions that have been described as a possible disease-causing mutation by other authors [6,9,25]. In our study, the Val878Ala substitution was detected in all affected members tested of both families, FC-73 (Amsterdam I) and FC-94 (Amsterdam II), suggesting that the hMSH6 germ-line mutation segregated with the disease. The MSI study of the tumors revealed an MSI-H phenotype. Moreover, immunohistochemical analysis revealed loss of hMSH6 protein in tumors from these patients. Our data suggest that this alteration may be a disease-causing mutation. However, we cannot discard that another mutation not detected could be linked to V878A in our population. Peterlongo et al. [24], has found the V878A in 4 out of 190

**Figure 1** Pedigrees and DNA nucleotide sequence of the three families with disease-causing mutations of hMSH6 gene. Squares, males; circles, females; diagonal bars, deceased; unblackened symbols, no tumor; semi-blackened symbols, patients with histological-verified carcinomas; arrows, probands; mut, carries the indicated mutation. Abbreviations indicating type of tumor: CRC, colorectal; SI, small intestine; G, gastric; H, Hodgkin; End, endometrium; Pr, prostate; L, liver. Number after abbreviation indicates age at which tumor was diagnosed. Electropherograms of the wild type and mutant nucleotide sequences for V878A and Q263X mutations located in exon 4 of the hMSH6 gene.

**Figure 2** Representative examples of positive (A) and negative (B) immunohistochemical staining for hMSH6 protein (clone 44, Transduction laboratories/Becton Dickinson, Lexington, UK). A: Tumor from family 60, exhibited positive nuclear staining for hMSH6. B: tumor from family 73 carrier of V878A mutation, exhibited loss of hMSH6 expression.
controls (from 18,000 volunteers of varying ethnic backgrounds who live in the New York metropolitan area), suggesting that this variant may not be linked to the disease in their population.

From the study presented here, we propose the following interpretations concerning the role of each previously unreported DNA change in the susceptibility to CRC. We interpret the hMSH6 c.435-16 C>T mutation found in two HNPCC-like families (FC-81 and FC-121) as a non-disease-causing mutation for the following reasons: the 3 435-16 C>T did not segregate in the families, the tumors showed a MSS phenotype and the immunohistochemical analysis showed normal expression of the hMSH6; moreover, the wild type and mutant alleles were both retained in the analysis showed normal expression of the hMSH6; moreover, showed a MSS phenotype and the immunohistochemical study showed expression of hMSH2 proteins from cancer cells. Reduced expression of hMSH2 is low in Spanish HNPCC, they can be found either in tumors of the two carriers were MSS. The frequency of hMSH6 mutations in our series was low, as they were found only in 3 out of 91 families studied (1 of 19 Amsterdam I and 2 of 8 Amsterdam II). The mean age of dx cancer was 55 years. The hMSH6 protein was present in the tumor cells and the LOH study showed retention of mutant allele and perhaps, this fact could produce chromosomal instability, and therefore, mutational events that led to hyperproliferation. At the end, the silent mutation c.1677 C>T (C559C) was found in a patient with two CRCs at age 43 and one urothelial cancer at age 47. This patient was also a carrier of another silent mutation in hMLH1 gene (P350P) and both colorectal tumors showed a MSI-H phenotype and had been tested negative for mutations in hMLH1 and hMSH2. The remaining family exhibiting a MSI-H phenotype and had been tested negative for mutations in hMLH1 and hMSH2. The remaining family was also negative for mutations in hMLH1 and hMSH2 but the tumors of the two carriers were MSS. The frequency of hMSH6 mutations in our series was low, as they were found only in 3 out of 91 families studied (1 of 19 Amsterdam I and 2 of 8 Amsterdam II). The mean age of dx cancer was higher than in hMLH1- and hMSH2-related families. Our data indicate that the frequency of hMSH6 mutations is low in Spanish HNPCC, they can be found either in families with MSI-H or MSS tumors, which on average had an older age at diagnosis of cancer and are associated with other HNPCC tumors-overall gastric and endometrium. These data are consistent with the results of other published studies.

Thus, we can conclude from our study, that the two possible pathogenic mutations of hMSH6 were found in three families, in two of them the patients had tumors exhibiting a MSI-H phenotype and had been tested negative for mutations in hMLH1 and hMSH2. The remaining family was also negative for mutations in hMLH1 and hMSH2 but the tumors of the two carriers were MSS. The frequency of hMSH6 mutations in our series was low, as they were found only in 3 out of 91 families studied (1 of 19 Amsterdam I and 2 of 8 Amsterdam II). The mean age of dx cancer was higher than in hMLH1- and hMSH2-related families. On the other hand, we have not found these variants in site-specific CRC families but in families with a broad
spectrum of cancer types. Therefore, we suggest that hMSH6 testing should be pursued in Amsterdam families that have been negative for mutation in hMLH1 and hMSH2 genes or, as the literature suggests, in families with a history of endometrial, gastric, or other cancers in addition to a history of CRC.

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