ONLINE MUTATION REPORT

A founder *MLH1* mutation in families from the districts of Modena and Reggio-Emilia in northern Italy with hereditary non-polyposis colorectal cancer associated with protein elongation and instability

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Hereditary non-polyposis colorectal cancer (HNPCC), or Lynch syndrome, is an autosomal dominant condition predisposing to tumours of the large bowel and other sites. In HNPCC, cancer predisposition is usually inherited as a highly penetrant trait, with a tendency to the development of multiple tumours. Clinical diagnosis of HNPCC is based on the so-called modified ''Amsterdam criteria'',[1] which include: (*a*) the presence of at least three family members—one of whom must be a first degree relative to two other members—affected with carcinoma of the colon, rectum, endometrium, small bowel, or urothelium; (*b*) a direct transmission of the disease from parent to child; (*c*) the occurrence of at least one tumour before the patient reaches 50 years of age; and (*d*) the exclusion of a diagnosis of familial adenomatous polyposis.

The genetic defects underlying most HNPCC cases are represented by constitutional point mutations of one of several genes encoding for proteins of the DNA mismatch repair complex. The vast majority of mutations are located in the major mismatch repair genes *MSH2* and *MLH1* (International Collaborative Group on HNPCC Mutation Database). The constitutional defects most commonly identified are nonsense, splice-site, or frameshift alterations, which all predict the synthesis of shorter, non-functional proteins. Tumours arising in carriers of mismatch repair gene mutations are characterised by a high frequency of insertion or deletion type somatic mutations within microsatellite repeats.[2] These are the expression of mismatch repair deficiency, which arises when a second somatic mutation affecting the wild-type allele fully inactivates the gene locus already altered in the germline.[3] Inactivation of a specific mismatch repair locus in a HNPCC tumour is often revealed by immunohistochemical methods, which show absence of nuclear staining following incubation with antibodies against the mismatch repair protein encoded by the mutant gene.[4]

In addition to genetic heterogeneity, HNPCC is also characterised by a marked degree of allelic heterogeneity. In fact, although some regions of *MLH1/MSH2* appear to be more frequently altered, mutations are spread all over the whole gene sequences, and the mutational mechanisms are heterogeneous[5]. Nevertheless, a few specific mutations are observed at high frequencies in well-defined populations or ethnic groups, because of founder effects. In Finland a major share of HNPCC cases is accounted for by two *MLH1* mutations.[6] Founder HNPCC mutations have also been identified in Denmark, Newfoundland, China, the United States, and among Ashkenazi Jews.[7–11] The presence of common founder mutations can greatly facilitate the molecular diagnosis of HNPCC by targeting mutational analysis to specific gene regions as a first step.

Key points

- Hereditary non-polyposis colorectal cancer (HNPCC) is genetically heterogeneous, with *MSH2* and *MLH1* the most commonly involved genes. The mutational spectrum is highly heterogeneous, with alterations spread over the whole coding sequences of the two genes. Most mutations predict protein shortening by truncation or internal deletion. A few mismatch repair gene founder mutations have also been described.
- We analysed samples from HNPCC and suspected HNPCC families from Modena and Reggio-Emilia. A recurrent 1 bp insertion (2269-2270insT) within the *MLH1* gene was observed in 4/11 (36%) ''Amsterdam'' HNPCC families, but not in 19 ''non-Amsterdam'' familial colorectal cancer pedigrees or 65 unrelated controls from the same region. Tumours developed by mutation carriers did not show expression of the MLH1 protein. To determine the functional consequences of the DNA change, allele specific expression and protein expression were investigated. The mutant and wild-type alleles displayed approximately equal mRNA expression levels. Instability of the MLH1 mutant protein was documented using Western blotting analysis. Haplotype analysis revealed that the mutation could be traced to a common ancestor.
- We investigated the effects of a frameshift elongating mutation occurring in the very last portion of the *MLH1* gene. The pathogenicity of the mutation is largely attributable to protein instability caused by synthesis of a longer polypeptide. The finding of a founder effect is relevant to the molecular diagnosis of HNPCC in this region of Italy. The identification of founder mutations with limited territorial distribution in other regions should be facilitated by analysing HNPCC and suspected HNPCC cases.

We had previously identified an insertion affecting the last codon of *MLH1* in a large HNPCC family originating from northern Italy (2269-2270insT).[12] Analysis of further pedigrees allowed us to detect the same mutation in three additional families from the same geographical area. We then

Abbreviation: HNPCC, hereditary non-polyposis colorectal cancer

performed molecular, immunohistochemical, and in vitro expression studies to ascertain its origin and molecular consequences.

SUBJECTS AND METHODS

Patients

Families enrolled in this study were found either through the colorectal cancer registry of the district of Modena or by interviews of patients attending a familial colorectal cancer clinic. Familial colorectal cancers were subdivided into two groups based on the modified Amsterdam criteria.[1] ''Amsterdam'' or HNPCC families met all of the Amsterdam diagnostic criteria. ''Non-Amsterdam'' or ''suspected'' HNPCC pedigrees did not fulfil all diagnostic criteria, and were characterised by a heterogeneous constellation of family histories, with a minimum of two colorectal cancer occurrences in two first degree relatives diagnosed at any age. A total of 11 Amsterdam and 19 non-Amsterdam families were investigated. All families originated from the districts of Modena and Reggio-Emilia, as ascertained by family names, interviews with family members, and consultation of city registries. A venous blood sample was drawn from each individual who gave informed consent to participate in the study aimed at screening for mismatch repair gene mutations.

Control blood samples (n = 65) were obtained from healthy blood donors without a history of colorectal cancer in first degree relatives, following ascertainment of the origin of their families from the Modena and Reggio-Emilia districts and provision of informed consent.

Molecular analysis

Constitutional *MLH1* and *MSH2* mutations were searched on peripheral leucocyte genomic DNA by a combination of radioactive single-strand conformation polymorphism analysis and direct sequencing, on a Perkin-Elmer Applied Biosystem (Foster City, CA) 373 automated sequencer, and reverse-transcription PCR, as previously described.[13] A restriction fragment length polymorphism PCR assay was designed to detect the presence of the *MLH1* 2269-2270insT mutation in relatives of proband carriers and in control samples. The protocol involves amplifying exon 19 using the forward intronic primer GACACCAGTGTATGTTGG and the reverse primer GAGAAAGAAGAACACATCCC, located in the 3′ untranslated region, followed by digestion of the PCR product with the restriction enzyme *Dra*I (20 000 U/ml; New England BioLabs, Beverly, MA) at 37℃ and direct visualisation onto agarose gels stained with ethidium bromide. The insertion introduces a novel *Dra*I recognition site in the amplified sequence. Therefore, the mutant allele is cut into two fragments, whose sizes are 224 and 44 bp, respectively, while the 268 bp wild-type product remains undigested on incubation with *Dra*I.

Total RNA extraction and cDNA preparation from lymphoblastoid cell lines was performed as previously described.[13] Amplification of a cDNA product spanning the 3′ portion of the *MLH1* transcript was performed in the presence of primers annealing to exon 16 (forward: AAGGCTGAGATGCTTGCAGACT) and to the 3′ untranslated region (reverse: GTTGGTACACTTTGTATATCACAC), respectively. Cycling conditions were: initial denaturation at 94℃ for 5 min, followed by 30 cycles including 30 s at 94℃, 90 s at 60℃, and 60 s at 72℃, with a final extension lasting 5 min at 72℃. 5–10 µl of the reverse transcription PCR product were digested with 0.3 µl of *Dra*I. Samples were then loaded onto agarose gels.

Microsatellite instability was assayed by testing at least five microsatellite markers, always including the five reference markers comprised in the microsatellite instability testing panel advised by the Bethesda guidelines,[14] on matched DNA samples extracted from tumours embedded in paraffin and normal colonic mucosa or fresh peripheral leucocytes, as previously reported.[15] Samples were considered to have high levels of microsatellite instability when the instability was observed at ⩾25% markers.

Segregation of *MLH1* linked polymorphic markers in HNPCC pedigrees was investigated on peripheral blood DNA. The polymorphisms tested included five microsatellite loci (*D3S1609*, *D3S1612*, *D3S1561*, *D3S1611*, and *D3S1298*), and two intragenic single base substitutions located within exon 8 and intron 14[5]. The latter substitutions were typed by direct sequencing. Analysis of microsatellite polymorphisms was performed by PCR amplification in the presence of α^{32}P-dCTP, followed by electrophoresis on 6% denaturing polyacrylamide gels and autoradiographic detection. A total of 42 individuals, including 21 carriers of the 2269-2270insT mutation, were analysed. Allele frequencies in the general population were calculated by analysis of DNA samples of the 65 control blood donors.

Immunohistochemical analysis

Tissues were fixed in formalin, embedded in paraffin, and sectioned at 6 µm. Following deparaffinisation and rehydration with xylene and ethanol, respectively, slides were submitted to microwave antigen retrieval for pretreatment (30 min, 350 W, in 10 mM citrate buffer, pH 6). Immunoperoxidase staining using diaminobenzidine as a chromogen was carried out with a Nexus automated staining system (Ventena, Strasbourg Cedex, France). Mouse monoclonal antibodies against the MLH1 (G168-15; Pharmingen, San Diego, CA) and MSH2 proteins (G129-1129; Pharmingen, San Diego, CA) were used at a dilution of 1:40. Tumours were considered to show inactivation of MSH2 or MLH1 when complete absence of detectable nuclear staining of neoplastic cells was observed. Definite nuclear staining of adjacent non-neoplastic epithelium, stromal cells, or lymphocytes served as an internal positive control.

In vitro expression of *MLH1* 2269-2270insT

The entire open reading frame of a 2484 bp *MLH1* messenger RNA was cloned into a pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). The *MLH1* 2269-2270insT mutation was generated by site directed mutagenesis using the Quick-Change Site-directed mutagenesis kit (Promega, Madison, WI). Human embryonic kidney fibroblast 293T cells lacking expression of intrinsic MLH1 because of *MLH1* promoter methylation were cotransfected with a pSG5 expression vector containing a full length *PMS2* cDNA and with pcDNA3.1 containing either wild-type or 2269-2270insT *MLH1*. Extracts were prepared and 50 µg aliquots were analysed by Western blotting as described.[16]

RESULTS

Mutational screening of the entire *MLH1* and *MSH2* genes was performed in all HNPCC probands and in 19 suspected HNPCC families. Two different mutations were identified in pedigrees complying with the Amsterdam criteria. One of these mutations was present in four apparently unrelated families, two of which originated from the district of Modena and two from the district of Reggio-Emilia. The recurrent mutation was an insertion of a T between nucleotides 2269 and 2270 (c.2269-2270insT) of the *MLH1* cDNA sequence, within exon 19. This mutation is located in the last codon of the gene, and is predicted to displace the termination codon downstream. The new stop codon appears following 33 novel coding triplets which, in the wild-type gene, are contained in the 3′ untranslated region. No other mutation was identified in probands from the four families by direct sequencing of

MLH1 and *MSH2*. Relatives of the 2269-2270insT mutation carriers were assayed by restriction fragment length polymorphism PCR. Seventeen additional carriers were identified (data not shown) and the mutation was shown to cosegregate with the disease phenotype in all families (fig 1). To verify whether the 2269-2270insT could represent a regional polymorphism, 65 control samples obtained by regionally matched blood donors were investigated, and none was found to carry the mutation.

To determine the effects of the mutation on mRNA, we evaluated the expression of the wild-type and mutant alleles by a restriction fragment length polymorphism PCR assay on lymphoblastoid cDNA from two mutation carriers. Direct visualisation of the reverse transcription PCR products on gels stained with ethidium bromide showed that the two alleles are expressed at approximately equal levels in lymphoblastoid cells from both subjects (fig 2).

Microsatellite instability was evaluated in four colorectal cancers and three endometrial carcinomas from seven 2269-2270insT mutation carriers. All were found to have high levels of microsatellite instability. MLH1 protein expression was evaluated by immunohistochemistry in the same specimens. All samples showed absence of the usual pattern of nuclear staining observed in normal colonic mucosa following incubation with anti-MLH1 antibodies (fig 3). Normal MSH2 nuclear expression was present in all specimens.

Western blot analysis performed on 293T cells following transfection of recombinant *MLH1* showed the presence of a larger band in cells containing the 2269-2270insT construct, whose size was compatible with the predicted length of the protein encoded by the mutant allele (fig 4). The intensity of the band corresponding to the mutant product was considerably lower than that of the wild-type *MLH1* sequence (fig 4), whilst expression of β-tubulin, used as an internal

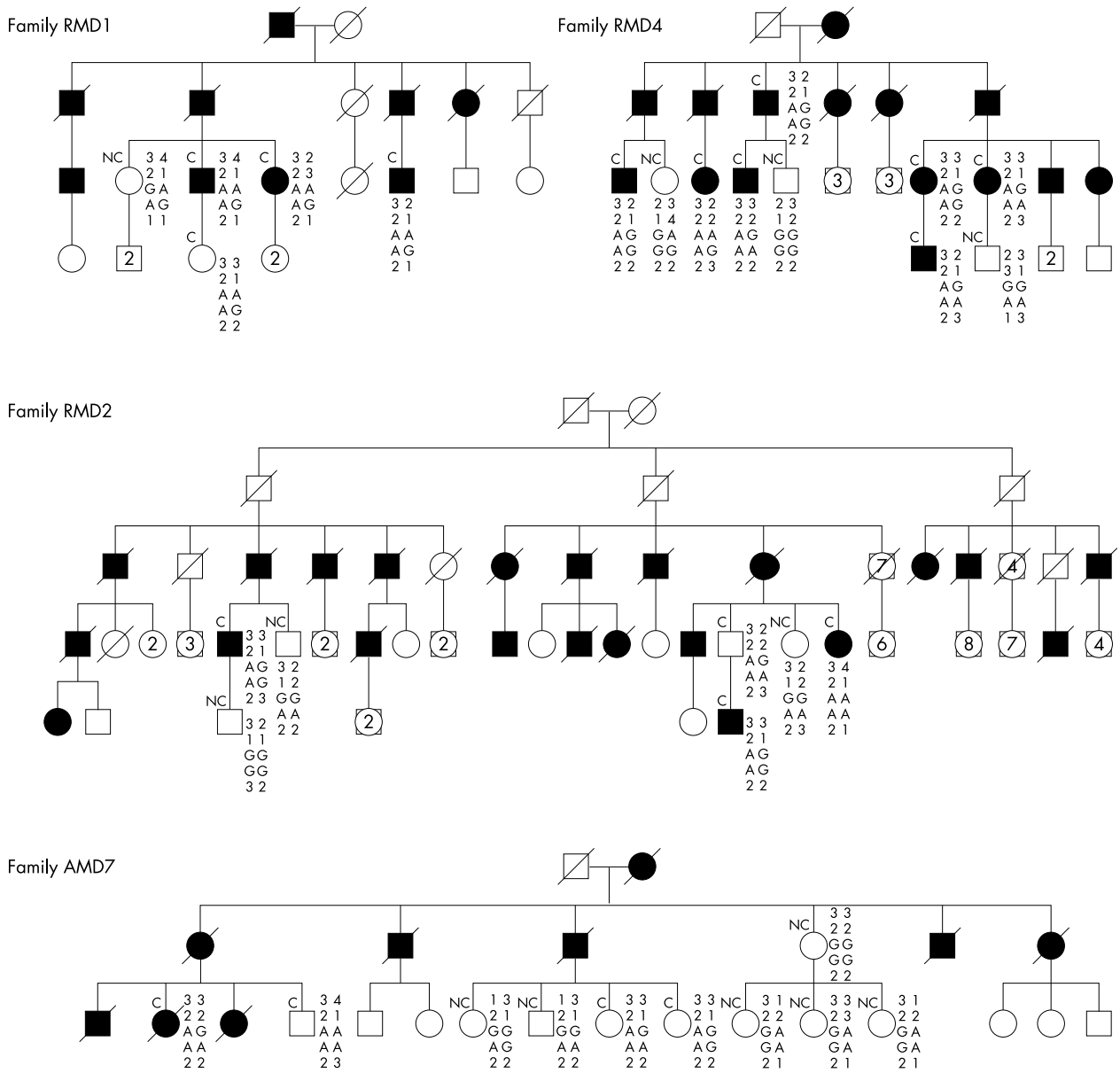

Figure 1 Pedigrees of the families segregating the 2269-2270insT mutation. A detailed description of family AMD7 can be found in Ponz de Leon et al.[12] C, carrier of the *MLH1* 2269-2270insT mutation; NC, non-carrier. Haplotypes in the region surrounding *MLH1* are shown on the left or at the bottom of symbols corresponding to the individuals tested. Alleles are shown for the following markers, from top to bottom: *D3S1561*, *D31612*, exon 8 A/G at nucleotide 655, intron 14 G/A at position -19, and *D3S1611*. For each microsatellite locus, alleles are numbered by decreasing size, (at each locus, number 1 indicates the allele with the highest repeat number).

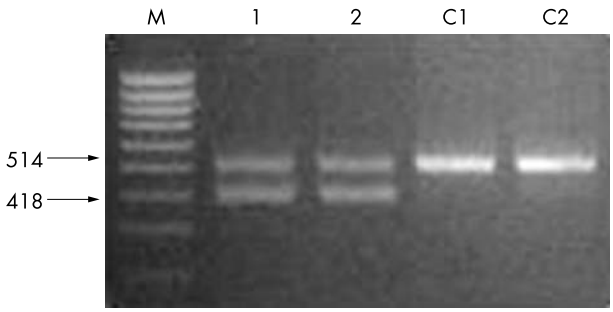

Figure 2 *Dra*I digestion of *MLH1* reverse transcription PCR products extending from exon 16 to the 3′ untranslated region. Analysis was conducted on lymphoblastoid cell lines derived from two c.2269-2270insT mutation carriers (lanes 1 and 2) and from two control individuals (lanes C1 and C2). M, molecular size marker (100 bp ladder). Arrows and numbers on the left indicate the sizes of the wild-type product (514 bp), which is not digested by *Dra*I, and of the larger fragment (418 bp) obtained after digestion of the mutant allele (the smaller 96 bp fragment was barely visible on the gel due to its very low intensity).

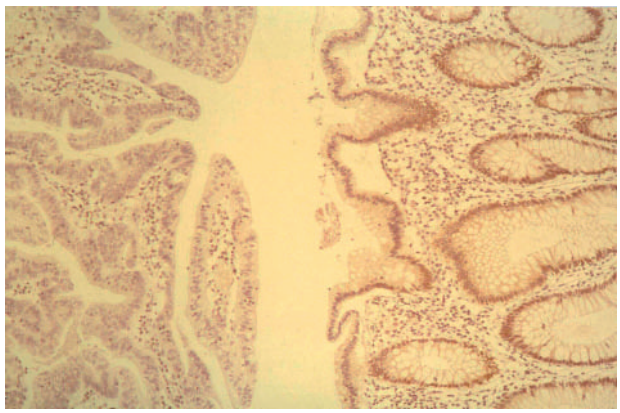

Figure 3 Immunohistochemical analysis with anti-MLH1 antibodies of a colorectal cancer from a carrier of the 2269-2270insT mutation. The tumour (left) shows no staining, whereas a strong positive reaction is visible in normal colonic mucosa (right).

control, was comparable between the two cell lines (data not shown). Expression of wild type recombinant PMS2 was also found to be markedly reduced in the presence of 2269-2270insT construct (fig 4).

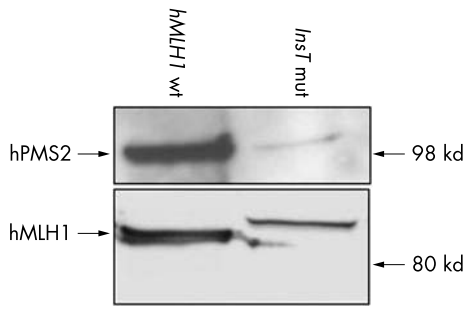

Figure 4 Expression of recombinant *MLH1* and *PMS2* in 293T cells. Extracts were prepared after cotransfection of human embryonic kidney fibroblast 293T cells with the *MLH1* and *PMS2* vectors, and 50 μg aliquots were analysed by Western blotting: *wt*, cotransfection with vector containing wild-type *MHL1*; *mut*, co-transfection with vector containing the *MLH1* 2269-2270insT mutation. Arrows and numbers on the right side indicate the sizes of the expressed proteins.

Finally, to assess whether the mutant 2269-2270insT alleles could be derived from a common founder, we investigated the segregation of alleles at nearby polymorphic markers in the four families (fig 5). A common haplotype was observed in all mutation carriers analysed for this purpose (fig 1). This haplotype spanned three intragenic as well as three very close extragenic markers, and no recombination was observed in this interval. On the other hand, in two families, chromosomes carrying the mutant allele showed recombination between *MLH1* and the more distal locus *D3S1609*.

DISCUSSION

The *MLH1* mutation described in this study is peculiar from both the molecular and the population genetics standpoints. Its unusual molecular characteristic is the location in the last codon of the gene and its predicted chain elongating effect. It is generally assumed that mismatch repair gene mutations contribute to neoplastic development through inactivation of the system, which is rendered unable to repair DNA mismatches, and possibly other functions. Therefore, most proteins encoded by alleles associated with HNPCC lack relevant domains due to either premature truncation or internal deletion. Since the mutation investigated in this study occurs at the very end of the *MLH1* gene, it might well represent a polymorphism restricted to a small geographical region. However, pathogenicity of the 2269-2270insT mutation is demonstrated by several lines of evidence. First, no other significant *MLH1* or *MSH2* sequence change was detected in the families segregating the 1 bp insertion. The mutation segregated with the disease phenotype in all families and was absent in control chromosomes from the same region. It was associated with microsatellite instability in all tumour samples investigated. Furthermore, absence of staining following exposure to anti-MLH1 antibodies in tumour samples confirms that the mismatch repair gene implicated in cancer predisposition in these families is *MLH1*.

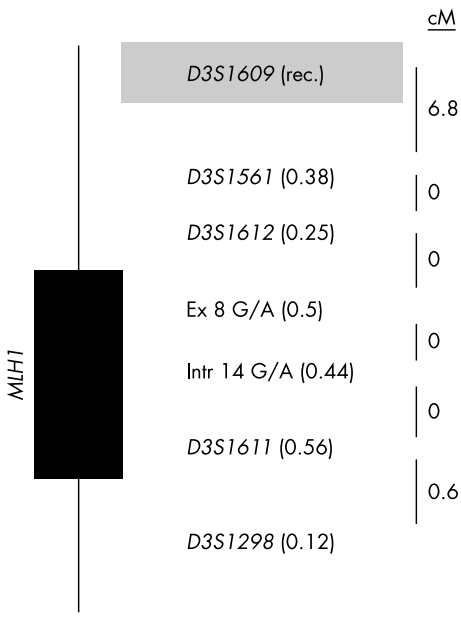

Figure 5 Diagram of the *MLH1* region on chromosome 3p, illustrating the polymorphic markers investigated for haplotype analysis and their approximate distances in cM[17]. The recombinant marker *D3S1609* is shadowed. Alleles at all other markers are conserved in the haplotype carrying the 2269-2270insT mutation. Numbers in brackets indicate the frequency of the mutation-associated alleles in the general population, as determined by analysis of 65 healthy blood donors.

Finally, a 4 bp duplication (TGTT) occurring at the same base position, and therefore causing similar changes of the MLH1 protein, has been previously identified in two HNPCC families.[18 19] This duplication was shown to inhibit interaction with PMS2 in a yeast two hybrid assay.[20]

In principle, the 2269-2270insT could affect mismatch repair function through different mechanisms. Elongation of the reading frame could interfere with mRNA processing and stabilisation, leading to a significant reduction of mRNA levels, as demonstrated for haemoglobin Constant Spring and other mutant globins.[21] Alternatively, the mutant gene could produce normal amounts of mRNA, but protein elongation might have a significant impact on its function or stability. Since the results of expression studies performed in lymphoblastoid cell lines from two *MLH1* 2269-2270insT carriers indicate that mRNA derived from the mutant allele is not significantly under expressed or unstable, it ensues that some properties of the longer protein must be altered by the mutation. Data obtained from in vitro expression studies indicate that the mutation substantially reduces MLH1 protein stability. PMS2 stability was also secondarily affected, since the PMS2 protein is rapidly degraded when it cannot form functional heterodimers with MLH1. MLH1 and PMS2 expression in transfected 293T cells is the preliminary step towards assessment of mismatch repair activity, which requires substantial amounts of these proteins.[16] However, because of their instability, the levels of MLH1 and PMS2 were too low to proceed further with the functional mismatch repair assay. Therefore, an effect of the mutation on the function of residual MLH1 cannot be ruled out.

The 2269-2270insT mutation was identified in HNPCC families originating from a small geographic area, comprising the neighbouring districts of Modena and Reggio-Emilia in Emilia-Romagna. The 2269-2270 insT has not been reported so far in studies of HNPCC families from other areas of Italy[5], including 62 additional kindreds studied by us([22] and unpublished data). These findings are indicative of a founder effect, which is confirmed by the results of haplotype analysis with *MLH1* linked polymorphisms.

In conclusion, we have provided genetic and functional evidence that the 2269-2270insT is an authentic pathogenetic founder mutation, involved in HNPCC causation in a small territory in northern Italy. The identification of founder mutations with limited territorial distribution in other European regions or in other parts of the world should be facilitated by the analytical study of HNPCC and suspected HNPCC cases selected from colorectal cancer registries, as performed for part of the patient population investigated in the present study.

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