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GERMLINE *hMSH2* AND *hMLH1* GENE MUTATIONS IN INCOMPLETE HNPCC FAMILIES

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Hereditary non-polyposis colon cancer (HNPCC) is a common hereditary disease characterized by a predisposition to an early onset of colorectal cancer. The majority of the HNPCC families carry germline mutations of either *hMSH2* or *hMLH1* genes, whereas germline mutations of *hPMS1* and *hPMS2* genes have rarely been observed. Almost all of the germline mutations reported so far concern typical HNPCC families. However, there are families that display aggregations of colon cancer even though they do not fulfil all HNPCC criteria (incomplete HNPCC families) as well as sporadic cases of early onset colon cancers that could be related to germline mutations of these genes. Therefore, we screened germline mutations of *hMSH2* and *hMLH1* genes in 3 groups of patients from France and Turkey: typical HNPCC (n = 3), incomplete HNPCC (n = 9) and young patients without apparent familial history (n = 7). By *in vitro* synthesis of protein assay, heteroduplex analysis and direct genomic sequencing, we identified 1 family with *hMSH2* mutation and 5 families with *hMLH1* mutations. Two of the 3 HNPCC families (66%) displayed *hMLH1* germline mutations. Interestingly, 4 of 9 families with incomplete HNPCC (44%) also displayed mutations of *hMSH2* or *hMLH1* genes. In contrast, no germline mutation of these genes was found in 7 young patients. Our results show that germline mutations of *hMSH2* and *hMLH1* genes contribute to a significant fraction of familial predisposition to colon cancer cases that do not fulfil all diagnostic criteria of HNPCC. *Int. J Cancer* 73:831–836, 1997.
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Hereditary non-polyposis colon cancer (HNPCC) is an autosomal dominant inherited disease characterized by a predisposition to an early onset of colorectal cancer. HNPCC is estimated to account for 5–10% of all colon cancer cases in Western populations. The families with aggregation of colorectal cancer are identified as HNPCC families if they fulfil the following criteria defined by the International Collaborative Group on HNPCC (Amsterdam Criteria): 1) 3 or more relatives with histologically verified colorectal cancer, one of whom is a first-degree relative of the other 2; 2) colorectal cancer affecting at least 2 generations; and 3) one or more colorectal cancer cases diagnosed before age 50 (Lynch *et al.,* 1993). In addition to colorectal cancer, certain extra-colonic malignancies are characteristic of HNPCC. Carcinomas of the endometrium, ovary, small intestine, biliary tract, ureter, renal pelvis, stomach and pancreas are present at an increased frequency in HNPCC families (Lynch *et al.,* 1993). Germline mutations of 4 human genes involved in DNA mismatch repair (*hMSH2, hMLH1, hPMS1* and *hPMS2*) have been associated with HNPCC. Mutations of these genes have been found in up to 70% of the HNPCC kindreds, with mutations in *hMSH2* and *hMLH1* accounting for the majority of the cases (Liu *et al.,* 1996). Germline mutations of *hMSH2* and *hMLH1* have also been found in sporadic cases developing colon cancer before 35 years of age (Liu *et al.,* 1995). However, little is known about the etiology of familial aggregations of colorectal cancers not fulfilling all HNPCC diagnostic criteria. Such cancers could represent familial predisposition to colon cancer or they could represent aggregation of sporadic cancers independent of germline mutations.

The mutational spectrum of these genes appears to be diverse, in both the localization and the nature of the mutations. Although more than 60% of the described mutations are nonsense or frameshift mutations that generate a premature stop codon, in certain populations the missense mutations are frequent or even predominant (Han *et al.,* 1995; Tannergard *et al.,* 1995). Loss of heterozygosity or somatic mutations of *hMLH1* and *hMSH2* in tumors have been described, suggesting that these genes behave as tumor suppressor genes that require 2 hits to inactivate their function (Hemminki *et al.,* 1994).

Our aim was to investigate germline mutations of *hMLH1* and *hMSH2* genes in 3 categories of colon cancer: typical HNPCC families, familial aggregation of colon cancer not fulfilling HNPCC criteria (incomplete HNPCC) and early onset sporadic colon cancers. We used 3 complementary techniques for mutation screening: *in vitro* synthesis of protein (IVSP) from cDNA, heteroduplex analysis and direct sequencing of all exons as well as intron-exon junction regions. One mutation in the *hMSH2* gene and 5 mutations in the *hMLH1* gene have been identified, with 1 affecting the initiation codon for protein translation.

MATERIAL AND METHODS

Family and patients selection

French families and patients were collected from genetic consultations at the Centre Léon Bérard and Hôpital Edouard Herriot. Three groups of families or patients were selected: 1 family with typical HNPCC syndrome (which fulfils the Amsterdam Criteria), 9 families with incomplete HNPCC syndrome for which one of the Amsterdam Criteria items was missing and 7 patients who developed colon cancer before 50 years of age without apparent familial history of colon cancer (Table I). Two other typical HNPCC families were collected from Turkey. One family (TF1) was from Southern Turkey studied at the Medical School of Akdeniz University. The other family (TF2) was from Istanbul.

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TABLE I – DESCRIPTION OF THE FAMILIES AND THE MUTATIONS

Family	Classification[1]	Tumo spectrum (age)[3] in			Gene	Mutations	
		Individual tested	1st-degree relatives[2]	2nd or more degree relatives		Codon	Nature
FF1[5]	A	C (32)	M: C (51)	C[4] C[4] E[4] E[4] P (57)			
TF1[5]	A	C (40)	B: C (?) F: C (?) S: C (45) B: C (60)	C (13) C (50) C (45) C (?) N (7) C (60)	*hMLH1*	551	AAC → ACC (Asn → Thr)
TF2	A	C (40)	M: C (42) B: C (42)		*hMLH1*	295	AGT → ACT (Ser → Thr)
FF2	B	C (35)	St: CUS (52) F: C? (35)	C (30) CD (60)	*hMLH1*	297	GAA → TAA (Glu → stop)
FF3	B	C (37)/E (38)	M: H (53)	C (46) H (40) E (76)			
FF4	B	C (72)	F: C (57) D: B (48)				
FF5	b	C (51)	S: C (36) B: C (63) B: S (62) B: HN (50) F: L (75)				
FF 6	B	C (31)	F: R (53)	C (70) M (64)			
FF 7	B	C (45)	S: C (32) B: CUS (36) M: CUS (42)	Br (?) CD (?) S (39) H (?)	*hMLH1*	182	AGG → GGG (Arg → Gly)
FF 8	B	R (45)/C (54)/Cae (63)	B: C (44) B: C (54) F: CD (?) S: E? (38) S: Leu (58)	CD (80)	*hMSH2*	Splice donor of exon 5	Deletion of exon 5 (codon 265–314)
FF 9	B	C (39)	S: C (38) F: Leu (82)	P (58) CUS (40)			
FF 10	B	C (45)	S: C (34)	E (43)/C (65)	*hMLH1*	1	ATG → AGG (Met →)
FF 11	C	C (46)	F: L (55)	Lym (53) L (?)			
FF 12	C	C (32)		Leu (?)			
FF 13	C	C (41)	M: CUS (45)				
FF 14	C	C (23)	F: HN (37)				
FF 15	C	C (33)/O (35)	F: L (60)	M (64) Leu (17)			
FF 16	C	R (43)	S: O (34) M: B (69)				
FF 17	C	R (46)	F: L (65)	HN (6)			

[1]A, classical HNPCC families; B, atypical HNPCC families; C, young patients without family history.–[2]For the first-degree relatives: M, mother; F, father; B, brother; S, sister; St, twin sister; So, son; D, daughter.–[3]For cancer spectrum: B, breast cancer; Br, brain tumor; C, colon cancer; Cae, cancer of caecum; CD, cancer of digestive system; CUS, cancer of unknown site; E, endometrum carcinoma; H, hepatocarcinoma; HN, head and neck cancer; L, lung cancer; Leu, leukemia; Lym, lymphoma; M, myeloma; N, neuroblastoma; O, ovarian cancer; P, cancer of pancreas; R, cancer of rectum.–[4]Between 5 and 65.–[5]FF, French families; TF, Turkish families.

In vitro *synthesis of protein assay*

mRNA was obtained from the EBV-transformed lymphoblastoid cell lines of patients using the QuickPrep Micro mRNA purification kit (Pharmacia, Uppsala, Sweden). RT-PCR of *hMLH1* and *hMSH2* was performed using primers and PCR conditions as described (Papadopoulos *et al.,* 1994; Liu *et al.,* 1994). RT-PCR products were then used in coupled *in vitro* transcription-translation reactions using TNT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI) with 40 μCi of ^{35}S-methionine for each reaction. The resulting polypeptides were analyzed on a 7.5–12.5% SDS-PAGE gel and visualized by autoradiography.

Heteroduplex analysis and direct sequencing of genomic DNA

DNA was extracted from peripheral blood, or cell lines or formalin-fixed paraffin-embedded sections of tumor tissues by proteinase K digestion and phenol purification. Each exon and exon-intron junction region of *hMLH1* and *hMSH2* genes was amplified with primers and conditions previously described (Kolodner *et al.,* 1994; 1995). For heteroduplex analysis, each PCR was performed with the addition of 0.05 μl ^{33}P-dATP. Five microliters of PCR reaction was then electrophoresed through a 0.6 × MDE matrix (FMC BioProducts, Rockland, ME) and visualized by autoradiography. For nucleic acid sequencing, the PCR products

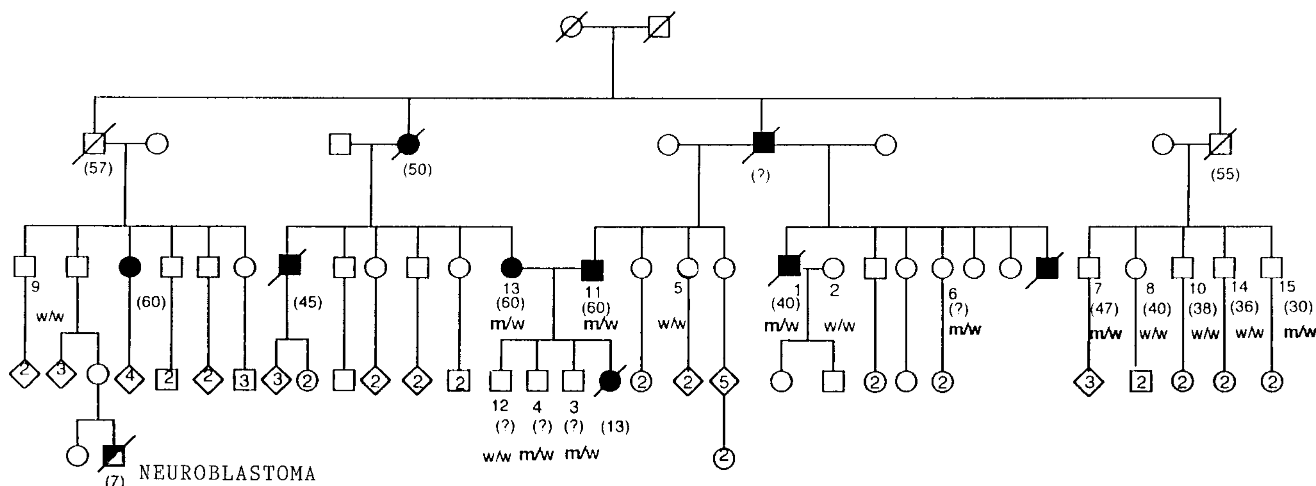

FIGURE 1 – Pedigree of family TF1. □, male (age); ○, female (age); ■ and ●, individuals affected by colorectal cancer (age at diagnosis); m/w, heterozygote for mutation of codon 551: AAC → ACC (Asn → Thr); w/w, wild type on both alleles.

were purified through microSpin columns of Sephacryl S-300 (Pharmacia) and then sequenced on both strands using the PRISM Dye Terminator sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) with an Applied Biosystem model ABI 373A automated sequencer. The same primers as for PCR amplification were used for the sequencing reaction of each exon.

Analysis of in vivo *expression of hMLH1 protein*

Cellular lysate from lymphoblastoid cell lines was prepared as described elsewhere (Laffe *et al.,* 1995). One hundred micrograms of protein were separated on a 7.5–12.5% acrylamide gel. hMLH1 protein was detected by polyclonal antibodies directed against the amino-terminal region (amino acids 2–21, catalogue number sc-58, at a dilution of 1:500) and the carboxy-terminal region (amino acids 737–756, catalogue number sc-582 at 1:2,000 dilution) of hMLH1 (Santa Cruz Biotechnology, Santa Cruz, CA). A chemiluminescence detection system (ECL, Amersham, Aylesbury, UK) was used to visualize the specific products.

RESULTS

We combined 3 technical approaches: IVSP, heteroduplex analysis and genomic DNA sequencing to search for germline mutations in *hMLH1* and *hMSH2* genes, to detect both nonsense or frameshift mutations and missense mutations. Although it is time consuming, direct genomic DNA sequencing remains the most sensitive method for mutation detection. A set of 19 patients was analyzed: 3 from typical HNPCC families, 9 from incomplete HNPCC families and 7 young patients diagnosed before the age of 50 (Table I). Heteroduplex analysis and direct genomic DNA sequencing were performed for all patients, while IVSP was carried out for all but the patient from TF1, whose lymphoblastoid cell line was not available. One germline mutation in the *hMSH2* gene and 5 germline mutations in the *hMLH1* gene were detected (Table I). All mutations were verified by repeated PCR reactions performed on independent DNA or RNA preparations.

Two missense mutations of the *hMLH1* gene were identified in Turkish families out of 3 typical HNPCC families analyzed (Table I). The patient from family TF1 had an A → C transversion at codon 551 of exon 14, leading to a substitution of Asn by Thr. TF1 is a large kindred (Fig. 1), with 11 members through 3 generations affected by colon cancer before 60 years of age. This A → C transversion was found in 2 other affected members. In family TF2, the missense mutation was a G → C transversion at codon 295 of exon 10, which changes amino acid Ser to Thr. As for the previous case, this mutation was present in another affected family member. In addition, a homozygous G → C mutation was revealed in the tumors of these 2 patients, suggesting a somatic loss of wild-type allele. To test whether these 551 A → C, and 295 G → C transversions were true mutations or polymorphisms, we analyzed 50 additional unrelated individuals from the Turkish population. No such transversions were found in any of them.

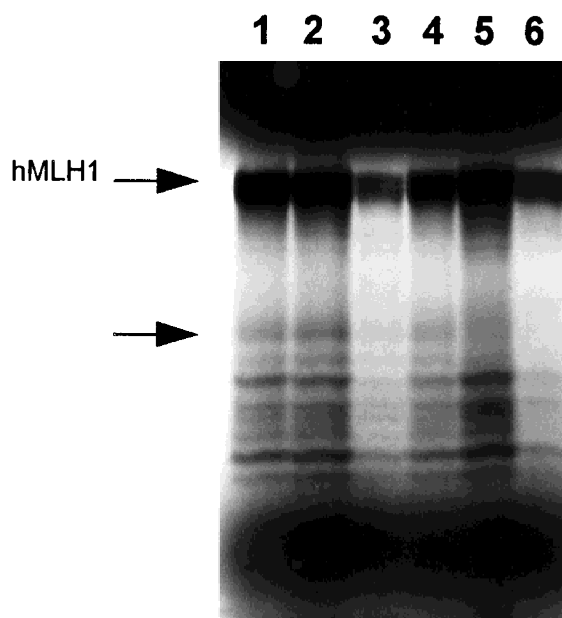

FIGURE 2 – *In vitro* synthesis protein assay. The RT-PCR products from the N-part (fragment A) of *hMLH1* gene (codon 1-394) were transcribed and translated *in vitro* (see Material and Methods) and separated on a 10% SDS-acrylamide gel. The polypeptides in lanes 1–4 were obtained from different cDNA preparations of the patient carrying a nonsense mutation at codon 297. A truncated protein of about 35 kDa is indicated by an arrow. The product in lane 5 was obtained from a healthy individual and the product on lane 6 from a patient with *hMSH2* mutation (supplied by B. Bressac de Paillerets, Institut Gustave Roussy, Paris, France). The normal size of the polypeptide from fragment A is 48 kDa.

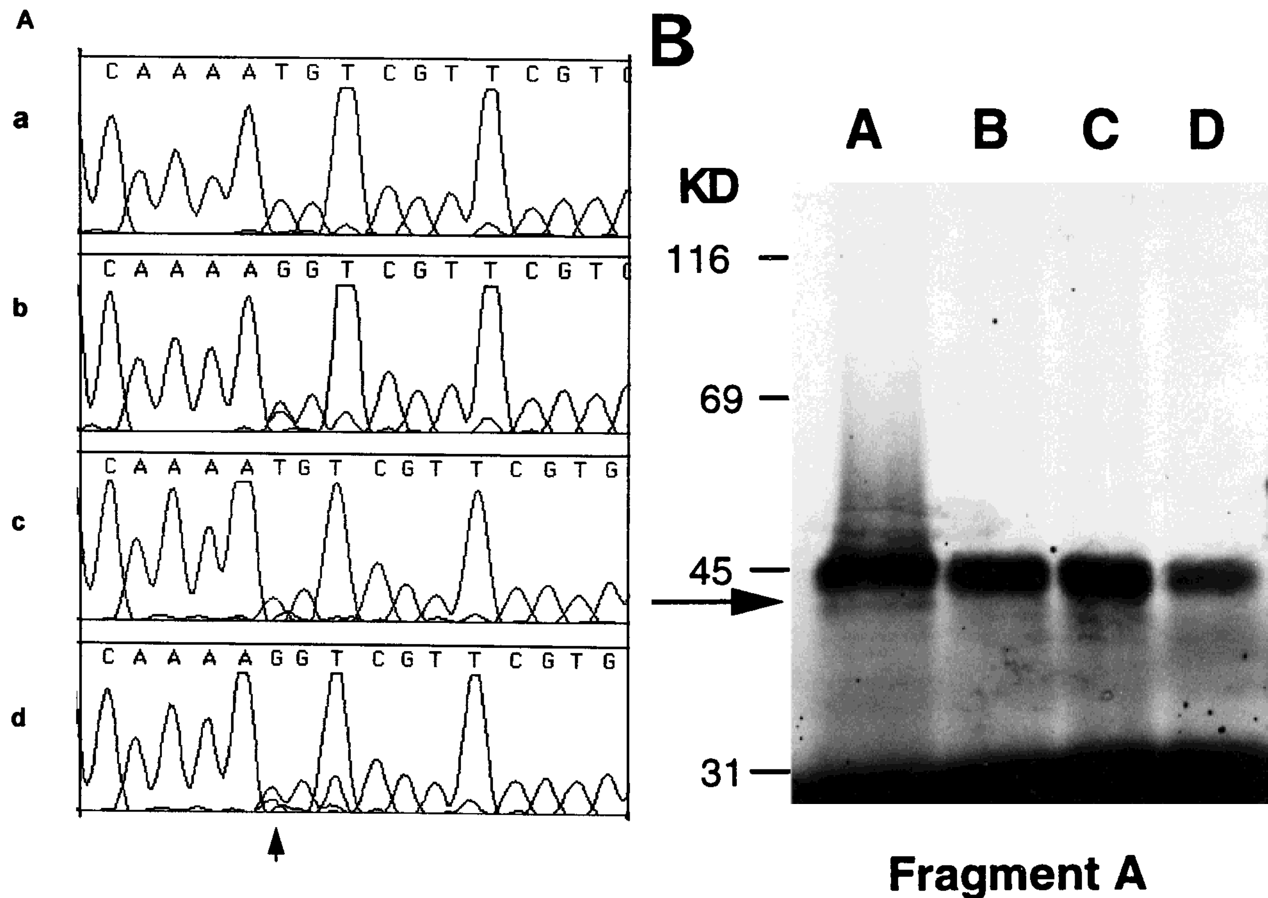

FIGURE 3 – Sequencing and IVSP analysis of mutation at the initiation codon of the *hMLH1* gene. *(A)* A portion of the chromatogram of antisense sequencing of exon 1. *a*, genomic DNA sequence of a healthy individual; *b*, genomic DNA sequence of the patient; *c*, cDNA sequence of a healthy individual; *d*, cDNA sequence of the patient. A heterozygous T → G mutation in both genomic DNA and cDNA is indicated (arrow). *(B)* RT-PCR products of fragment A (codon 1-394) were transcribed, translated *in vitro* (see Material and Methods) and separated on a 7.5% acrylamide gel. A truncated protein was shown in lane A (synthesized from the RT-PCR product of the patient), indicated by an arrow. Products in lanes B–D were obtained from the patients without the *hMLH1* mutation. The normal size of the polypeptide from fragment A is 48 kDa.

Among 9 French families with incomplete HNPCC, we detected 1 mutation of the *hMHS2* gene and 3 mutations of the *hMLH1* gene (Table I). The *hMSH2* gene mutation was located at the splice donor at the 3′ end of exon 5, resulting in an in-frame deletion of this exon. This mutation was found in an incomplete family (FF8) with 3 colon cancer cases and 1 endometrial case among 5 brothers and sisters. The mutation carrier developed a multifocal cancer, a rectal cancer at the age of 45, a primary cancer in the distal colon at 54 and a primary cancer in the caecum at 63.

Among the 3 *hMLH1* mutations detected in French families, 1 was a missense mutation, observed in a family with 2 sisters who developed cancers in the proximal colon ages 45 and 32, respectively (FF7). This mutation was an A → G transition at codon 182 of exon 6, resulting in an Arg → Gly change. The same alteration was identified in the 2 patients of the family but was not observed in 48 additional unrelated individuals from the French population. The second *hMLH1* mutation was a G → T substitution producing a stop codon at codon 297 of exon 11 (FF2). A truncated protein synthesized from a mutated allele was revealed by IVSP (Fig. 2) with different preparations of cDNA. This mutation was identified in a patient with colon cancer (site undefined) at the age of 35. Her father was suspected to be affected by a colon cancer, but this was not confirmed. Her twin sister had died from a generalized cancer at 52. The son of his sister (the nephew of the patient) developed a distal colon cancer at age 30. The third *hMLH1* mutation was identified in a patient with proximal colon cancer at age 45 (FF10). Her sister was affected by colon cancer (site undefined) at age 34. Their maternal aunt developed an endometrial cancer at age 43 and a colon cancer at age 65. The mutation was a T → G transversion affecting the initiation codon (Fig. 3A), abolishing the signal for initiating protein translation. Consequently, 2 fates for the mutant allele were possible: either this transcript was not translated at all, or it used another ATG as a start codon to initiate translation. The second hypothesis would result in a truncated protein missing the amino-terminal part. Sequencing of cDNA demonstrated that the mutant allele was transcribed (Fig. 3*A*). By IVSP, we detected a faint band of about 3 kDa smaller than the wild-type one (Fig. 3*B*). This suggested that the next in-frame codon ATG, located 100 bp downstream, was used as a initiation codon to synthesize the protein in this *in vitro* system. This mutation was also detected in another affected member of the family who developed an endometrial cancer at age 43 and a colon cancer at age 65. To determine whether the mutant alleles were expressed *in vivo,* we studied hMLH1 protein by Western blot. As shown in Figure 4, only full-length proteins were detected in all cell lines from both the controls and the patients carrying nonsense or missense mutations. No predicted abnormal, short polypeptides resulting from nonsense mutations were found (Fig. 4). This indicates that the truncated proteins were not translated or were rapidly degraded after translation in normal cells. Besides the specific hMLH1 protein, other bands of different sizes were revealed, which might be due to

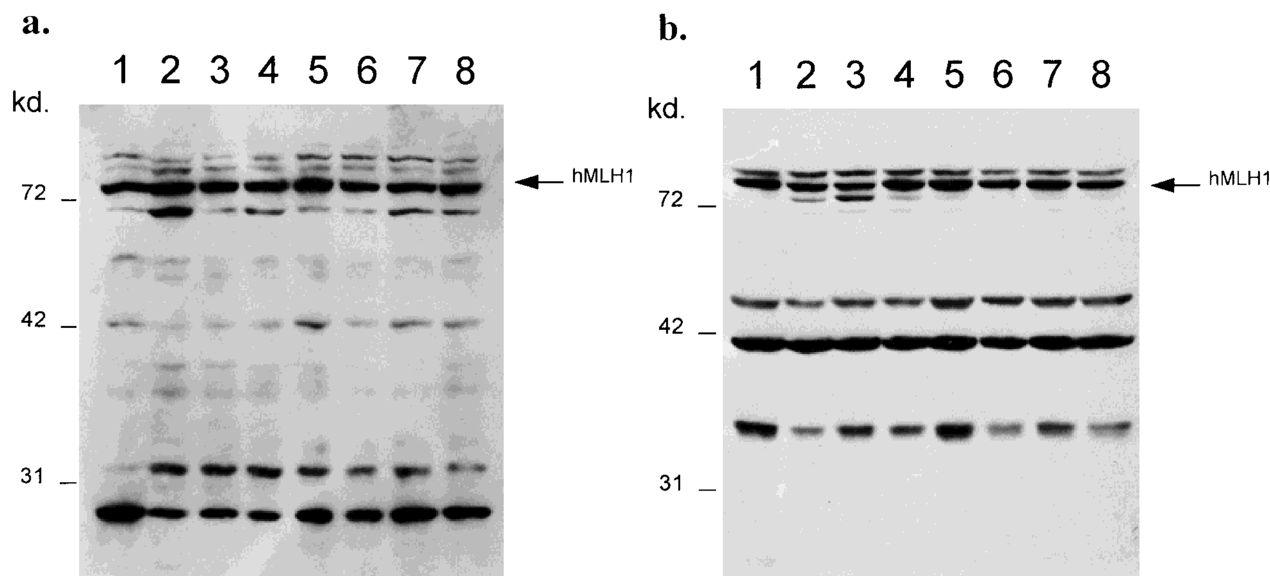

FIGURE 4 – Western blot analysis. Proteins (100 µg) from different individuals were separated on a 12.5% SDS-acrylamide electrophoresis gel. Lanes 1 and 2, unrelated healthy individuals; lanes 3 and 4, HNPCC patients without the *hMLH1* mutation; lane 5, patient with the *hMLH1* mutation at the initiation codon; lane 6, patient with missense mutation at codon 182; lane 7, patient with nonsense mutation at codon 297; lane 8, patient with missense mutation at codon 295. Two blots were hybridized with N-terminus polyclonal antibody *(a)* and C-terminus polyclonal antibodies *(b),* respectively. The size for full-length hMLH1 protein is indicated.

alternative transcriptions (Charbonnier *et al.,* 1995) or non-specific cross-reactions with unknown proteins.

DISCUSSION

Six mutations of *hMLH1* and *hMSH2* genes were found in typical and incomplete HNPCC families. In contrast, no mutation was detected in 7 young patients without a family history, suggesting that neo-mutations of these genes are likely to be infrequent. None of the 5 mutations in the *hMLH1* gene has been previously described, whereas the deletion of exon 5 in *hMSH2* gene resulting from the mutation at the splice donor site was reported in several families of different geographic origins (Liu *et al.,* 1995, 1996). This suggests that exon 5 of *hMSH2* is either a functionally important site or is structurally vulnerable. Among the 5 *hMLH1* germline mutations, 2 were nonsense mutations with one affecting the initiation codon of the gene. A mutation at the initiation codon was reported in the α globin gene, causing α-thalassemia (Pirastu *et al.,* 1984). We have thus detected a mutation affecting the initiation codon in a cancer predisposition gene. The mutant allele of hMLH1 was transcribed and the truncated protein was synthesized *in vitro* probably using the next in-frame ATG as the initiation codon. However, this truncated protein was probably unstable since only small levels of protein were detected by IVSP. *In vivo,* the truncated protein was not detectable. However, the possibility that the abnormal product co-migrated with the product from the wild-type allele cannot be excluded. Similarly, we did not detect the truncated protein in normal cells of the patient carrying the nonsense mutation at codon 297 (family FF2). Consistent with our finding, predicted aberrant proteins resulting from germline mutations of other predisposition genes, such as *hMSH2* and *APC,* are also undetectable by Western blot (Smith *et al.,* 1993; Leach *et al.,* 1996). As shown by immunoblot analysis, levels of wild-type hMLH1 protein were similar in patients carrying nonsense mutations and in control individuals. Although Western blot is a semi-quantitative method, this observation might suggest the existence of functional compensation to the wild-type allele in the normal cells of the patients.

Missense mutations of *hMLH1* reported in our study involve codons 182, 295 and 551 of the gene. Although we cannot definitely exclude the possibility of rare polymorphisms, several observations strongly suggest that these mutations are involved in the familial predisposition to colon cancer: 1) mutations co-segregate with the disease; 2) no polymorphism at codons 182, 295 and 551 have ever been reported, and these mutations were not present in unrelated individuals of the same population; 3) codons 182 and 551 are evolutionally conserved between yeast and human; and 4) the mutation in codon 295 was identified in tumors of affected patients from the same family with the loss of the wild-type allele.

So far, screening for germline mutations in *hMSH2* and *hMLH1* genes has been proposed for members of typical HNPCC families. In confirmation of several other observations (Mauillon *et al.,* 1996; Nyström-Lahti *et al.,* 1996; Maslein *et al.,* 1996), we report a high frequency (44%) of germline mutations of *hMLH1* and *hMSH2* in incomplete HNPCC families. The definition of HNPCC is based on strict clinical criteria, including the identification of several family members with colon cancer. Combined with the observation that disease penetrance is incomplete, this often renders difficult the diagnosis of HNPCC families in small kindreds. Furthermore, the difficulty of fulfilling all criteria may be increased by the lack of necessary clinical information. For instance, in 2 of our 3 incomplete families carrying the *hMLH1* gene mutation (FF2 and FF7), for certain first-degree relatives who died at an early age of a cancer at an unknown site, we were unable to obtain detailed information. Incomplete HNPCC families represent a significant fraction of families encountered in genetic consultations. Consequently, the study of genotype and phenotype correlation in this group of families should be important for better selection of families for whom genetic analysis is required.

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