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ORIGINAL ARTICLE

Four novel germline mutations in the *MLH1* and *PMS2* mismatch repair genes in patients with hereditary nonpolyposis colorectal cancer

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Abstract
Background Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common cause of early onset hereditary colorectal cancer. In the majority of HNPCC families, microsatellite instability (MSI) and germline mutation in one of the DNA mismatch repair (MMR) genes are found.
Materials and methods The entire coding sequence of MMR genes (*MLH1*, *MLH2*, *MLH6*, and *PMS2*) was analyzed using direct sequencing. Also, tumor tests were done as MSI and immunohistochemistry testing.
Results We were able to find three novel *MLH1* and one novel *PMS2* germline mutations in three Iranian HNPCC patients. The first was a transversion mutation c.346A>C (T116P) and happened in the highly conserved HATPase-c region of MLH1 protein. The second was a transversion mutation c.736A>T (I246L), which caused an amino acid change of isoleucine to leucine. The third mutation (c.2145,6 delTG) was frameshift and resulted in an immature stop codon in five codons downstream. All of these three mutations were detected in the *MLH1* gene. The other mutation was a transition mutation, c.676G>A (G207E), which has been found in exon six of the *PMS2* gene and caused an amino acid change of glycine to glutamic acid. MSI assay revealed high instability in microsatellite for two patients and microsatellite stable for one patient.
Conclusion In all patients, an abnormal expression of the MMR proteins in HNPCC was related to the above novel mutations.

Keywords HNPCC · Mismatch repair · MMR · Microsatellite instability · MLH1 · PMS2

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Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) is an autosomal dominantly inherited cancer-susceptibility syndrome and is the most common cause of hereditary colorectal cancer with an early age of onset. It is estimated that HNPCC may account for 5–10% of the total colorectal cancers worldwide [1–3]. In Western countries, patients inheriting this predisposition are at a particularly high risk of developing colorectal cancer [2, 4].

HNPCC is associated with germline mutations in the mismatch repair (MMR) genes, which include *MLH1* (MIM#120436), *MSH2* (MIM#120435), *PMS2* (MIM #600259), and *MSH6* (MIM #600678) [5, 6]. About 90% of the identified germline mutations in the MMR genes are found in two genes, *MLH1* and *MSH2* [7]. Currently, more than 300 different mutations have been described in these genes, which account for approximately 500 HNPCC kindred in the world [8].

Also, microsatellite instability (MSI) is found in some of the HNPCC families and provides an opportunity for

genetic diagnosis. The MMR gene mutations spectrum and MSI variations vary across different populations [9, 10].

Identification of the mutational incidence and spectrum of MMR genes and MSI variations is important to developing successful diagnostics and therapeutic strategies in different populations [10, 11]. Our objective was to detect and study MSI and the MMR gene germline mutation carriers among the Iranian population with colorectal cancer.

Materials and methods

Patients and controls

The study was approved by the local institutional review board. Blood samples (for germline mutation analysis) and paired paraffin-embedded blocks of cancerous tissue (for the MSI analysis) were collected from 592 unrelated patients with HNPCC, and 248 healthy individuals from the Iranian normal population as controls. Cases and controls were recruited through a large multiclinic gastroenterology practice. Paraffin-embedded sections were examined by two experienced pathologists.

Diagnosis of HNPCC was done in a three-stage process including review of family cancer history, tumor testing, and genetic testing.

Review of family cancer history A complete medical history and a physical examination were performed. All patients had a complete physical examination by the same physician. To determine if a patient is at risk for HNPCC, the following criteria are used: (1) At least two relatives with an HNPCC-associated cancer, including colon, rectal, endometrial, small bowel, ureter, or renal pelvis cancer. (2) One of the two relatives is a first-degree relative of the other two, such as a parent, child, or sibling. (3) At least two generations of relatives are affected. (4) At least one relative is diagnosed with cancer before the age of 50. (5) No family history of familial adenomatous polyposis (an inherited disorder unrelated to HNPCC). Individuals who meet the above criteria were considered to have HNPCC in their family.

Tumor testing Tests were done on removed tumors (even those removed years before). Tests included: (1) MSI testing—looks for the presence of genetic instability associated with HNPCC. (2) Immunohistochemistry testing—samples of tumor tissue were stained to look for the presence of proteins associated with this disease.

Genetic testing If the review of family cancer history and tumor testing suggested the likelihood of an HNPCC genetic mutation, the next step was to consider having the blood test for the presence or absence of an HNPCC genetic mutation.

DNA was extracted from 10 mL blood and from three to five sections of each 10-μm-thick paraffin-embedded sample (around 0.01–0.02 g of tissue) using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) and eluted into 150 μL of elution buffer. The eluted DNA was stored at −20°C until further use.

Pathological analysis

The size of each tumor was evaluated by measuring its two greatest axes in order to calculate the surface area in square centimeters. Tumors located from the cecum up to and including the splenic flexure were considered proximal, and those from the descending colon to the rectum distal [12].

The pathological type, grading, and staging were confirmed from the original microscopy slides which were

Table 1 Primer sequences for MSI assay

Name	Gene	GenBank number	Repeat	Primer sequences	Amplicon size (bp)
NR-27	Inhibitor of apoptosis protein-1	AF070674	27 A 5′UTR	F: AACCATGCTTGCAAACCACT R:CGATAATACTAGCAATGACC	87
NR-21	SLC7A8	XM 033393	21 T 5′UTR	F: GAGTCGCTGGCACAGTTCTA R: CTGGTCACTCGCGTTTACAA	109
NR-24	Zinc finger 2	X60152	24 T 3′UTR	F: GCTGAATTTTACCTCCTGAC R: ATTGTGCCATTGCATTCCAA	131
BAT-25	c-kit	X06182	25 T intron 16	F: TACCAGGTGGCAAAGGGCA R: TCTGCATTTTAACTATGGCTC	153
BAT-26	hMSH2	U04045	26 A intron 5	F: CTGCGGTAATCAAGTTTTTAG R: AACCATTCAACATTTTTAACCC	183

Table 2 Primer sequences for germline mutation analysis

Exon and gene	Exon length (bp)	Primer length (mer)	Ta (°C)	Amplicon size (bp)	Primers sequence
Exon4-MLH1	70	22	61.8	479	F: TTCAGATAACCTTTCCCTTTGG
					R: AGCAATACCCCAACTGAAGG
Exon9-MLH1	110	22	65.6	335	F: AATGGATGGATGAATGGACAGG
					R: GTGGGTGTTTCCTGTGAGTGG
Exon19-MLH1	168	21	56.3	397	F: AAAAAATCCTCTTGTGTTCAG
					R: GGAATACAGAGAAAGAAGAACAC
Exon6-PMS2	165	20	62.1	443	F: GCGATGATGTGAGAACCTTG
					R: GAGCCAAGACAACACCACTG

reviewed separately by two pathologists. Grading was evaluated according to the World Health Organization histological classification. Tumors with a regular glandular structure were considered to be "well"-differentiated; those with a structure between well-differentiated and poorly differentiated were defined as "moderately" differentiated. Those with a solid/trabecular architectural structure or mucus-secreting cells which were difficult to recognize were termed as "poorly" differentiated [13].

Immunohistochemical staining

A variety of antigen retrieval methods were evaluated in a pilot study and checker-board titrations were performed to determine the most favorable method of staining. Optimum results were obtained using heat-mediated antigen retrieval and following the Envision method. Immunohistochemical (IHC) analysis for the expression of MLH1, MSH2, MSH6, and PMS2 proteins was performed on 5-μm-thick sections of paraffin-embedded cancerous tissue blocks. The tissue sections were deparaffinized in xylene and rehydrated in graded concentrations of alcohol. Endogenous peroxidase activity was blocked by treating the sections with blocking solution. For antigen retrieval, the sections were treated while boiling in citrate buffer [pH 9.0] in a microwave. Sections were incubated with primary antibodies hMLH1 (BD Biosciences Pharmingen; clone G168-15, 1:100 dilution), hMSH2 (Calbiochem, Oncogene Sciences; clone FE11, 1:100 dilution), hMSH6 (BD Transduction Laboratory; clone 44,1:1,000 dilution), and PMS2 (BD Pharmingen; clone A16-4, 1:500 dilution). After each step, slides were washed with TBS buffer for 3 min. Then, slides were treated with Envision (Dako, REAL Envision) for 20 min. To visualize immunoreaction, 3,2′-diaminobenzidine was used, and samples were counter-stained with hematoxylin. Intramucosal lymphocytes were used as positive controls [14].

Microsatellite instability assay

The MSI analysis was performed on paired tumoral DNA from cancerous tissues and genomic DNA from whole blood, using five loci in the USA National Cancer Institute (NCI) panel (NR-27, NR-21, NR-24, BAT-25, and BAT-26; Table 1). MSI carriers were identified by analysis of tumor tissue using polymerase chain reaction (PCR) according to the NCI recommended panel and classified into microsatellite instability-low (MSI-L), microsatellite instability-high (MSI-H), and microsatellite stable (MSS) [15–17].

Germline mutation analysis

The entire *MLH1*, *MSH2*, *MSH6*, and *PMS2* coding regions and the splice junctions were amplified by PCR, according to previously published methods [18]. The primers which were used for amplification of exons 4, 9, and 19 of the *MLH1* gene and exon 6 of the *PMS2* gene, which included new mutations, are summarized in Table 2. PCR amplicons for each exon were sequenced bidirectionally. Sequencing was performed using the Big Dye

Table 3 Characteristics of the patients with novel MMR gene mutations

Patient's ID	Site of cancer	Age at diagnosis	Stage	Grade	Criteria
832037F	Splenic flexure	52	IIA	Well.diff	B&A
831847A	Ascending colon	52	pT3, pN0, M0	Mod.diff	B&A
882230H	Hepatic flexure	77	IIA	Mod.diff	None

B Bethesda criteria, *A* Amsterdam criteria II

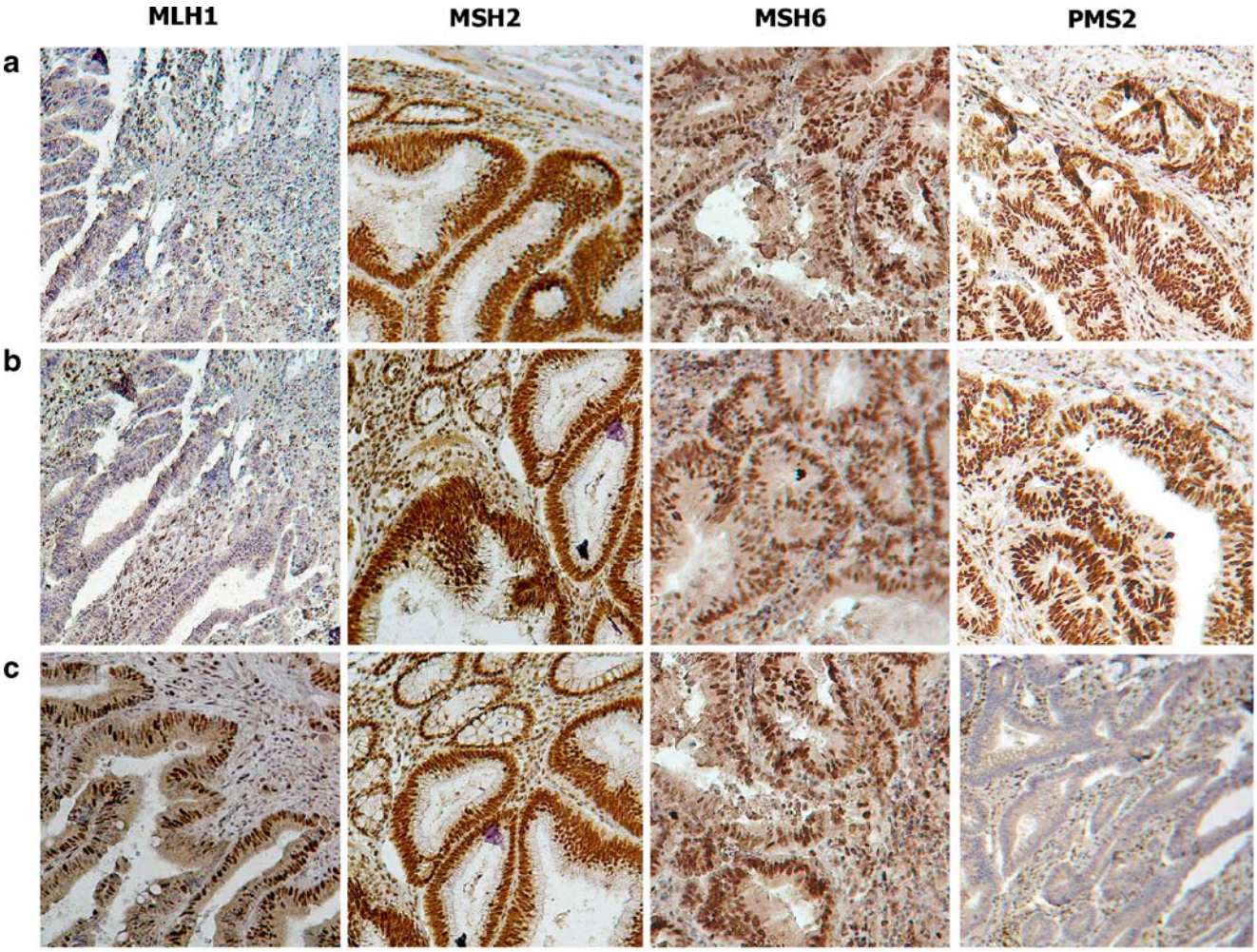


Fig. 1 IHC staining. **a** Patient with two missense mutations in exons four and nine of the *MLH1* gene had abnormal expression of the MLH1 protein (loss of nuclear staining) and normal IHC for the other MMR proteins. **b** Patient with frameshift mutation in exons 19 of the *MLH1* gene had abnormal expression of the MLH1 protein (loss of nuclear staining) and normal IHC for the other MMR proteins. **c** Patient with missense mutations in exon 6 of the *PMS2* gene had abnormal expression of the PMS2 protein (loss of nuclear staining) and normal IHC for the other MMR proteins

terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA), and the products were analyzed on the ABI 3130XL genetic analyzer (Applied Biosystems). Any variation in DNA sequence was confirmed by repeating the bidirectional sequencing on an independent PCR product.

Bioinformatics analysis of frameshift mutation in MLH1 protein

To better understand the pathogenicity of frameshift mutation in exon 19 of the *MLH1* gene which causes the replacement of five amino acids, immature stop codon, and

Table 4 MLH1 and PMS2 germline mutations in patients with HNPCC

Patient's ID	Gene	Exon	Codon	Nucleotide change[a]	Consequence	HIC result	MSI status	GenBank accession no.
832037F	MLH1	4	116	c.346A>C	p.Thr116Pro	Neg.MLH1	MSI-H	EF570786
832037F	MLH1	9	246	c.736A>T	p.Ile246Leu	Neg.MLH1	MSI-H	EF570787
831847A	MLH1	19	715, 716	c.2145,6 delTG	p.Thr715ThrfsX5	Neg.MLH1	MSS	EF125076
882230H	PMS2	6	207	c.676G>A	p.Gly207Glu	Neg.PMS2	MSI-H	EF583852

[a] Numbering based on cDNA sequence, position +1 corresponds to the A of the ATG translation initiation codon in reference sequence; MLH1 [GenBank NM_000249], PMS2 [GenBank: NM_ 000535]

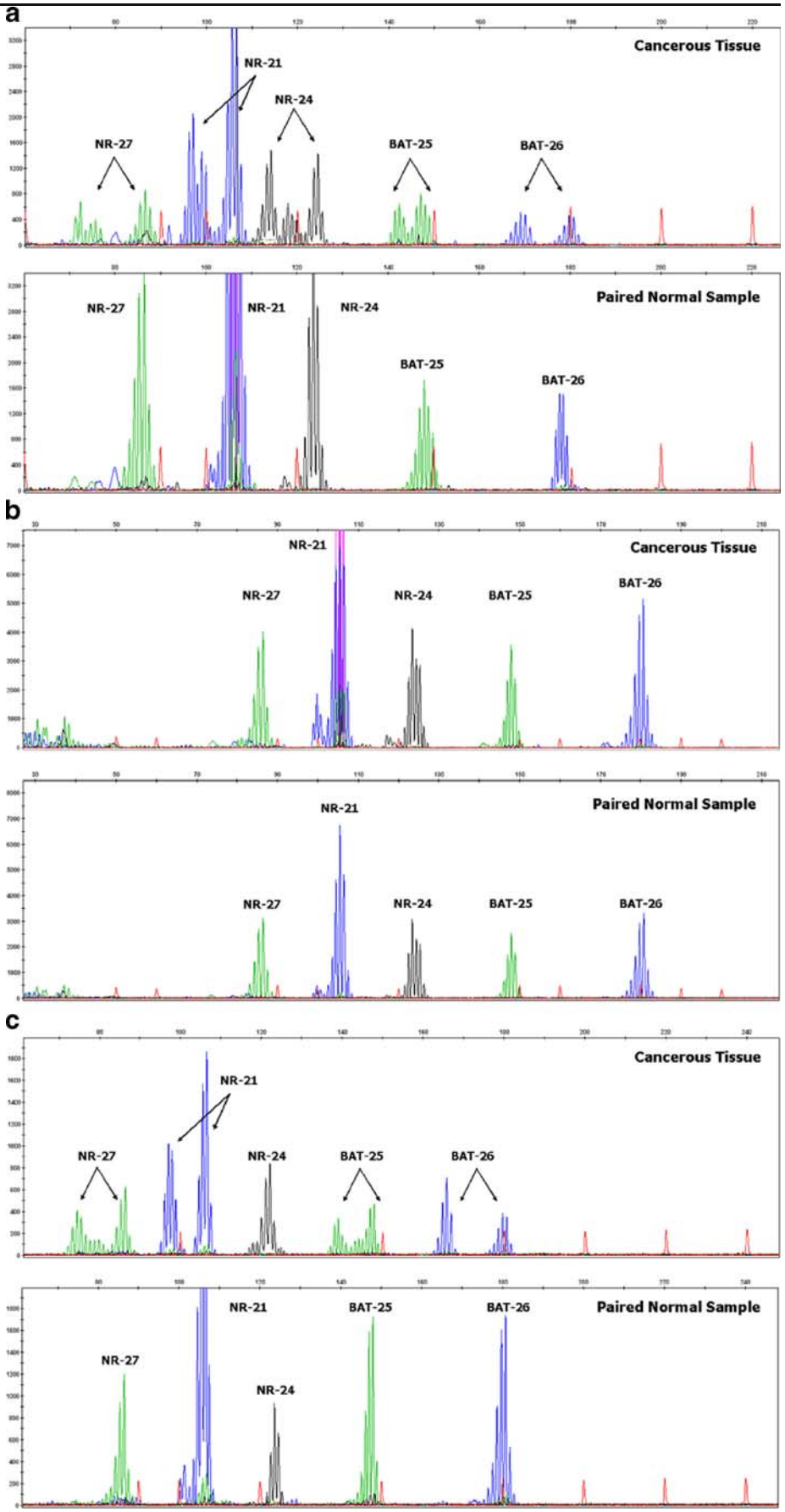


Fig. 2 MSI assay. For each patient, there are two panels, cancerous tissue (*upper*) to check the rate of MSI and paired normal sample (*lower*) as control. **a** Patient with two missense mutations in exons four and nine of the *MLH1* gene had high instability in microsatellite analysis (five out of five instable markers). **b** Patient with frameshift mutation in exon 19 of the *MLH1* gene had completely normal panel (MSS). **c** Patient with missense mutations in exon 6 of the *PMS2* gene had high instability in microsatellite analysis (four out of five instable markers)

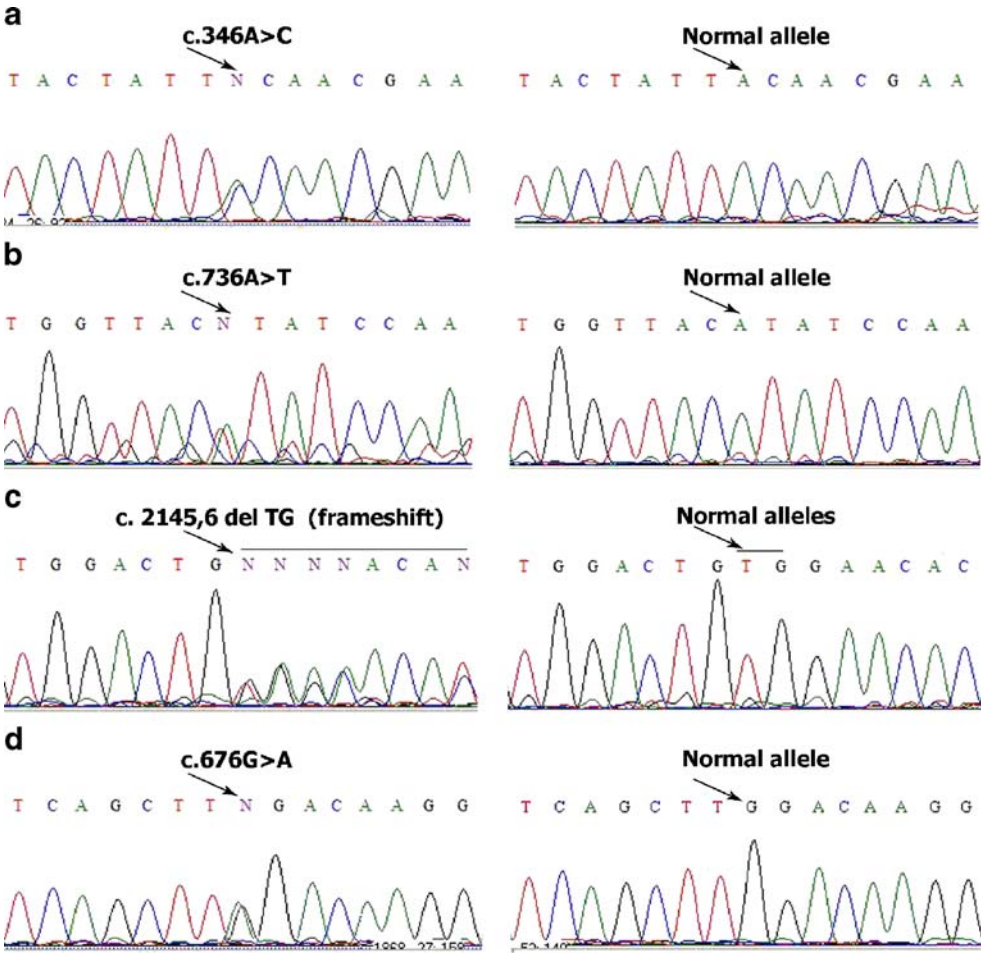


Fig. 3 Sequencing result for novel mutations. **a** c.346A>C (T116P) transversion mutation in exon 4 of the *MLH1* gene. **b** c.736A>T (I246L) transversion mutation in exon 9 of the *MLH1* gene. **c** c.2145,6delTG frameshift mutation in exon 19 of the *MLH1* gene. **d** c.676G>A (G207E) transition mutation in exon 6 of the *PMS2* gene

loss of 41 amino acids in the C-terminal of the protein, we performed the complete bioinformatics analysis of the MLH1 protein including normal sequence and protein sequence after frameshift mutation using CLC Protein Workbench software (CLC bio USA).

Results

After all exons of *MLH1*, *MSH2*, *MSH6*, and *PMS2* were amplified and sequenced in HNPCC patients, we found four novel germline mutations: three missense mutations in exon 4, exon 9, and exon 19 of the *MLH1* gene and one missense mutation in exon 6 of the *PMS2* gene (Table 4).

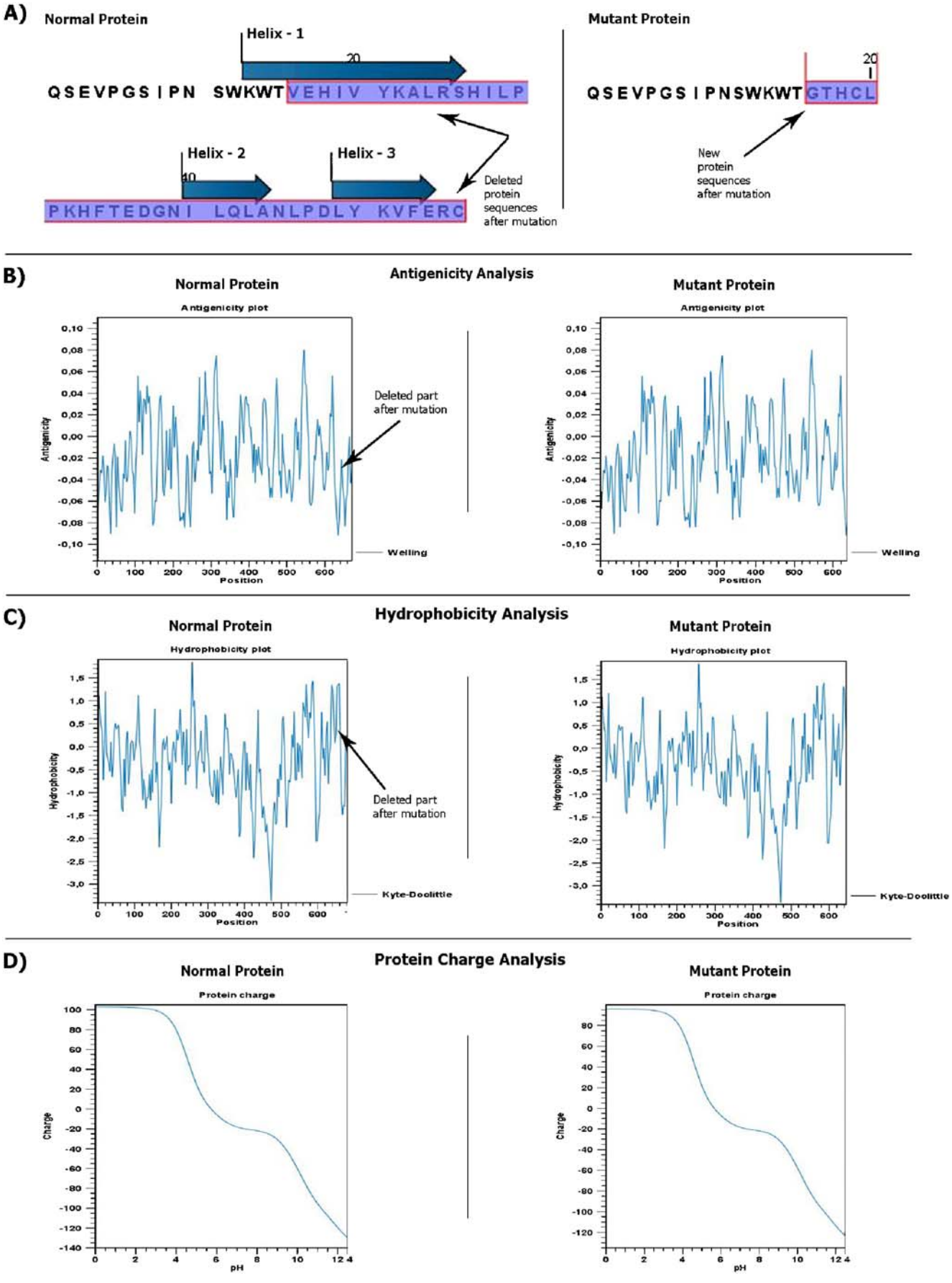
Case report

Patient 1 A 52-year-old woman, 832037F, was diagnosed during a routine colonoscopy with a well-differentiated adenocarcinoma in the splenic flexure of the colon, measuring 5.5 cm^2 in diameter. Lymph node metastases were not present (pt1 (sm1) pN0, M0). The TNM classification of the adenocarcinoma was determined as IIA. The patient fulfilled the Bethesda and the Amsterdam (II) criteria.

Patient 2 Samples were obtained from a 52-year-old man, 831847A, fulfilling the Bethesda and the Amsterdam (II) criteria, with a carcinoma of the ascending colon, measuring 9.9 cm^2 in diameter. The TNM classification was documented as pT3, pN0, M0. The patient was treated by right hemicolectomy. HNPCC screening was initiated, promoted by the findings of colonic cancers with histological features suspicious of HNPCC.

Patient 3 A 77-year-old man, 832230H, who met neither the Bethesda nor the Amsterdam criteria (II), was diagnosed with colorectal carcinoma. Histopathological analysis revealed a

Fig. 4 Bioinformatics analysis of the MLH1protein before and after frameshift mutation. **a** Partial sequence of the MLH1protein contains exon 19 and α-helix domains. **b**–**d** Compared antigenicity, hydrophobicity, and protein charge analysis of normal and mutant MLH1 protein after frameshift mutation in exon 19. *Black arrows* indicate important differences between normal and mutant proteins


A)
Normal Protein
Helix - 1
20
QSEVPGSIPN SWKWT VEHIV YKALRSHILP
Helix - 2
Helix - 3
40
PKHFTEDGNI LQLANLPDLY KVFERC
Deleted protein sequences after mutation
Mutant Protein
20
QSEVPGSIPNSWKWT GTHCL
New protein sequences after mutation
B)
Antigenicity Analysis
Normal Protein
Antigenicity plot
Antigenicity
Position
Deleted part after mutation
Welling
Mutant Protein
Antigenicity plot
Antigenicity
Position
Welling
C)
Hydrophobicity Analysis
Normal Protein
Hydrophobicity plot
Hydrophobicity
Position
Deleted part after mutation
Kyte-Doolittle
Mutant Protein
Hydrophobicity plot
Hydrophobicity
Position
Kyte-Doolittle
D)
Protein Charge Analysis
Normal Protein
Protein charge
Charge
pH
Mutant Protein
Protein charge
Charge
pH

6.8-cm^2 polypoid carcinoma in the hepatic flexure of the colon. TNM classification was documented as IIA. Screening for HNPCC was initiated because of the conspicuous histological findings, although the patient did not fulfill both criteria. Medical histories of the patients carrying these novel mutations are summarized in Table 3.

IHC staining

IHC analysis of MMR protein expression (MLH1, MSH2, MSH6, and PMS2) was performed for all patients. All cases with defects in the expression of one of the MMR proteins were referred for MSI analysis and genotyping. Figure 1 shows the defects of MLH1 protein expression in two patients and PMS2 protein expression in one patient with HNPCC.

MSI assay

After checking the MSI panel for patients with novel mutation, one patient with two missense mutations in the *MLH1* gene (832037F) and the patient with missense mutations in the *PMS2* gene (832230H) had MSI-H. One patient with frameshift mutation in the *MLH1* gene (832047F) had MSS panel (Table 4 and Fig. 2).

Mutation screening

In the *MLH1* gene, the first mutation was a transversion mutation of 346A→C (numbering of nucleotide position followed the GenBank accession number NM_000249), which causes amino acid change of threonine to proline at position 116 of the MLH1 polypeptide chain in exon 4. The second mutation was a transversion mutation of 736A→T in exon 9, which causes amino acid change of isoleucine to leucine at position 736 of the MLH1 polypeptide. The third mutation was a frameshift mutation of 2145, 6, with deletion of two nucleotides (TG) in exon 19, which causes frameshift amino acid change of exon 19 in the MLH1 polypeptide (Fig. 3).

In the *PMS2* gene, the mutation was a transition mutation of 676G→A (numbering of nucleotide position followed the GenBank accession number NM_000535), which causes amino acid change of glycine to glutamic acid at position 207 of the PMS2 polypeptide chain in exon 6 (Fig. 3).

In all mutations, the sequencing from the opposite direction of the product from another PCR was done to confirm these mutations (data not shown).

Mutation nomenclature

Nucleotide numbering was based on the *MLH1* cDNA sequence (GenBank NM_000249; [gi:28559089]), with the A of the ATG translation initiation codon at position 61. Current mutation nomenclature recommendations (http://www.hgvs.org/mutnomen) suggest numbering the A of the ATG translation initiation codon as +1. The numbering of the reported mutations is as follows: c.346A>C or p.Thr116Pro (T116P), c.736A>T or p.Ile246Leu (I246L), and c.2145, 6delTG or p.Thr715ThrfsX5.

Nucleotide numbering for the *PMS2* gene was performed according to the cDNA sequence (GenBank NM_000535; [gi:102470225]), with the A of the ATG translation initiation codon at position 87. The numbering of the reported mutation is as follows: c.676G>A or p.Gly207Glu (G207E). All of these four novel mutations were submitted to the GenBank (GenBank accession numbers are summarized in Table 4).

Bioinformatics analysis of frameshift mutation in MLH1 protein

Bioinformatics analysis showed that, after frameshift mutation in exon 19 of the MLH1 protein, antigenicity and hydrophobicity of MLH1 decreased, but the protein charge did not change. Also, as a result of this mutation, mutant protein lost three important α-helix domains in the C-terminal of the protein. This means that the deleted sequence due to the immature stop codon might have a critical role in protein folding and conformation, and the pathogenicity of mutation is related to these changes (Fig. 4).

Discussion

We report four novel germline mutations (three in *MLH1* and one in *PMS2* MMR genes) in HNPCC patients, which have not been found in the normal Iranian population nor reported by any other member of the research groups. Under these circumstances, polymorphisms could be ruled out.

Patient 832037F showed two missense mutations in exons four and nine of the *MLH1* gene (c.346A>C and c.736A>T, respectively). The c.346A>C mutation was in the highly conserved HATPase-c region of the MLH1 protein and created an amino acid change of threonine with the hydroxyl group to proline with the amide group. Threonine is a completely neutral amino acid, but despite its nonionic nature, the amide groups of praline are markedly polar. As a result of the second mutation (c.736A>T), the amino acid isoleucine was changed to leucine. Both are aliphatic amino acids and do not contain heteroatoms (N, O, or S) in their side chains or a ring system. Their side chains are markedly apolar. This change is considered a tolerated change, and the pathogenicity of HNPCC in this patient mostly related to the first mutation. In this patient, the tumor was immunohistochemically

negative regarding the expression of the MLH1 protein and normal expression of MSH2, MSH6, and PMS2 proteins. This patient had high instability in microsatellite analysis (five out of five instable markers).

Patient 831847A carried pathogenically two nucleotide germline deletions in exon 19 of the *MLH1* gene (c.2145,6 delTG), causing a frameshift and resulting in an immature stop codon in five codons downstream. As a result of this frameshift mutation, five amino acids downstream from the mutation changed (GTHCL vs. VEHIV), and due to the immature stop codon, 41 amino acids in the C-terminal of the MLH1 protein were deleted (VEHIVYKALR-SHILPPKHFTEDGNILQLANLPDLYKVFERC). The tumor was highly microsatellite unstable (five out of five instable markers), and IHC showed no expression of the MLH1 protein, but normal expression of other MMR proteins. Genetic testing in one of his sons was positive for the same mutation.

Patient 882230H demonstrated a missense mutation in exon six of the *PMS2* gene (c.676G>A), causing replacement of glycine with glutamic acid. Glycine is an aliphatic amino acid with an apolar side chain, but glutamic acid is an acidic amino acid which can be uncharged or negatively charged depending on its local environment. Indeed, glutamic acid is often found in the active site of proteins or enzymes where its imidiazole ring can readily switch between these states to catalyze the making and breaking of bonds; so, this mutation can change the conformation of normal protein. IHC revealed a lack of PMS2 protein expression, and the tumor showed high MSI (four out of five instable markers).

Our finding suggests that germline MMR mutations are found in patients with MSS tumors. In all the patients, an abnormal expression of the MMR proteins was related to the above mutations.

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