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Investigating the prevalence of point mutations in the human mutL homolog 1 gene in colorectal cancer patients in the Northwest of Iran

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Abstract

Background/aims: *Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common form of colorectal cancer (CRC) syndrome, in which the mutations in mismatch repair genes have been implicated in the disease etiopathogenesis. The aim of this study was to investigate the prevalence of human mutL homolog 1 (hMLH1) gene point mutations in patients with HNPCC in Northwest of Iran.* **Methodology:** *In this study, 30 patients with HNPCC were selected who fulfilled the Amsterdam II criteria. Moreover, 30 subjects were selected as the control group. Total RNA was extracted from peripheral blood sample, and complementary DNA (cDNA) was synthesized using specific primers. Screening of germline mutations within the hMLH1 gene was performed by direct cDNA sequencing. Functional evaluation was conducted through real-time polymerase chain reaction mRNA expression using the TaqMan Gene Expression Assay.* **Results:** *In this study, c.655A >G polymorphism was found in 13.3% of patients and 10% of the control group, while c.1959G > T polymorphism was found in only one of the patients. Two novel variants, including c.973C > A and c.1086C > A, were found in 13.3% and 20% of patients, respectively, that were identified for the 1st time in this study. Moreover, the mRNA expression of hMLH1 gene in patients carrying the c.973C > A and c.1086C > A mutations showed no statistically significant difference in comparison to patients with no mutation and healthy controls.* **Conclusions:** *This study establishes that the frequency and type of mutations do not follow a similar pattern in other populations due to genetic and geographical differences.*

Key words: *Hereditary nonpolyposis colorectal cancer. Human mutL homolog 1. Human mutL homolog 2. Mismatch repair genes. Point mutation.*

Investigando la prevalencia de mutaciones puntuales en el gen humano mutL homólogo1 en pacientes con cáncer colorrectal en el Noroeste de Irán

Resumen

Antecedentes/objetivos: *El cáncer colorrectal hereditario sin poliposis (HNPCC) es la forma más común del síndrome de cáncer colorrectal (CCR) en el que las mutaciones en los genes reparadores de desajustes (MMR) se han implicado en la etiopatogenia de la enfermedad. El objetivo de este estudio fue investigar la prevalencia de mutaciones puntuales del gen homólogo humano MUTL1 (hMLH1) en pacientes con HNPCC en el noroeste de Irán.* **Metodología:** *En este estudio, se*

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seleccionaron 30 pacientes con HNPCC que cumplían los criterios de Amsterdam II. Además, se seleccionaron 30 sujetos como grupo control. Se extrajo el RNA total de la muestra de sangre periférica y se sintetizó el cDNA usando iniciadores específicos. La detección de mutaciones en la línea germinal dentro del gen hMLH1 se realizó mediante secuenciación directa del cDNA. La evaluación funcional se realizó mediante expresión de mRNA de PCR en tiempo real utilizando el ensayo de expresión génica TaqMan. **Resultados:** *En este estudio, se encontró polimorfismo c.655A > G en el 13.3% de los pacientes y el 10% del grupo control, mientras que se encontró polimorfismo c.1959 G > T en solo uno de los pacientes. Dos nuevas variantes, incluidas c.9730 > A y c. 1086C > A, se encontraron en el 13.3 y el 20% de pacientes, respectivamente, que fueron identificados por primera vez en este estudio. Además, la expresión de mRNA del gen hMLH1 en pacientes portadores de las mutaciones C.973C > A y c.1086C > A no mostró diferencias estadísticamente significativas en comparación con pacientes sin mutación y controles sanos.* **Conclusiones:** *Este estudio establece que la frecuencia y el tipo de mutaciones no siguen un patrón similar en otras poblaciones debido a diferencias genéticas y geográficas.*

Palabras clave: *Cáncer colorrectal hereditario sin poliposis. hMLH1. hMLH2. Genes reparadores de desajustes (MMR). Mutación puntual.*

Introduction

Colorectal cancer (CRC) is one of the most prevalent gastrointestinal cancers worldwide, especially in Western and European countries and has been introduced as the most common cancer type in Asia[1,2]. Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome, the most frequent inherited syndrome, is a highly penetrant disease (about 85%) with the autosomal dominant inheritance patterns that account for approximately 5-10% of total CRC cases[3]. Patients with HNPCC are at a high risk to develop a range of cancers, including colorectal and endometrial cancers as well as extracolonic gastrointestinal, genitourinary, and ovarian and brain cancers[4,5]. Mutations in MLH1 and MSH2 are considered the major cause of HNPCC, since germline alterations of these genes have been found to be responsible for more than 90% of mutation carrier HNPCC families. According to the database of the International Society for Gastrointestinal Hereditary Tumors (InSiGHT), currently more than 450 different pathogenic mutations have been described in these genes, accounting for approximately 750 HNPCC kindreds worldwide[6].

Lynch syndrome is caused by mutations in one of the mismatch repair (MMR) genes, including *human mutL homolog 1* (*hMLH1*), *human mutS homolog 2 (hMSH2)*, *hMLH3*, *hMSH3*, *hMSH6*, *human PMS1 homolog 1, MMR system component* (*hPMS1*), *hPMS2,* and *human epithelial cell adhesion molecule*[7]. Mutations in one of the alleles and additional somatic mutations result in defects of MMR system and accumulation of mutations in the genome, leading to carcinogenesis[8]. Pathogenicity of mutations associated with HNPCC is due to loss of main interaction domains or alterations in conformational structure of proteins encoded by MMR genes that disrupt their ability to interact other components of MMR pathway[8].

Germline mutations of *hMLH1* and *hMSH2* genes account for greater than 90% of overall mutations[9]. *hMLH1*, the human homolog of bacterial *MutL*, is the main gene[1]. *hMLH1* gene is located on 3p21 chromosome and contains 19 coding exons[6]. To date, more than 1600 variants of this highly polymorphic gene have been reported (http://genecards.org/cgi-bin/carddisp.pl?gene=MLH1&search=mlh1%23snp)[10]. The prevalence and type of mutations in these genes have not been similarly distributed in various populations that stem from genetic and geographical differences. Hence, identifying the common mutations in each region is potentially useful in the diagnosis of high-risk individuals, better management of disease, and designing effective prevention methods. The objective of this study was to investigate the germline mutations identified in *hMLH1* gene in a population of HNPCC suspected families in Northwest of Iran. Moreover, the functional impact of the mutations was evaluated.

Materials and methods

Study population

In this paper, 30 patients with HNPCC of Azeri descent that met the Amsterdam II criteria (revised International Collaborative Group on HNPCC criteria 1998) were selected from the individuals recruited to the Ebne Sina Medical Genetic Center (Tabriz, Iran). According to Amsterdam II guideline, participants should have the following criteria; first, at least three relatives need to have cancer associated with HNPCC (colorectal, endometrial, stomach, ovary, ureter or renal-pelvis, brain, small bowel, hepatobiliary tract or skin [sebaceous

Table 1. List of primer sequences used for amplification of cDNA of *hMLH1* gene and their respective sizes of PCR products

Primer	Primer sequence (5'→3')	T_m (°C)	T_a (C C)	Size (bp)
Forward 1	CTTGGCTCTTCTGGCGCC	63	63	675
Reverse 1	GGAGCGAATATTGTCCACGG	60.5		
Forward 2	ACAACATAGCCACGAGGAGAAAAG	63.5	64	769
Reverse 2	ATTTTTGGCAGCCACTTCAGC	59.4		
Forward 3	GGAACAGAAGCTTGATGCATTTC	61.1	64	638
Reverse 3	CATCTTCCTCTGTCCAGCCA	60.5		
Forward 4	CTGTGTGAATCCTCAGT	50	68-58	822
Reverse 4	AAGGAATACTATCAGAAGGC	54.3		

Bp: base pair; T_a: annealing temperature; T_m: melting temperature.
cDNA: complementary DNA; PCR: polymerase chain reaction.

tumors]). Second, one needs to be a first-degree relative of the other two. Third, at least two successive generations need to be affected. Fourth, at least, one of the relatives with CRC needs to have received the diagnosis before age 50[11]. Cancer diagnosis was confirmed by pathology reports. As a control group, 30 healthy volunteers were selected who had no cancer disorder either in themselves or their relatives. Personal and family cancer histories and written informed consent were obtained from all participants. The local ethical committee of Tabriz University of Medical Sciences approved this study.

Real-time polymerase chain reaction (RT-PCR) quantification

After taking 3 µl of peripheral blood sample of all subjects, RNA extraction was performed immediately. Total RNA of the peripheral blood was extracted using QIAamp RNA Blood Mini Kit (Cat No: 52304) according to the manufacturer's instructions (Qiagen, Germany). About 500 ng of total RNA was used to synthesize the complementary DNA (cDNA), which was performed using Prime ScriptTM RT Reagent Kit (Cat No: RR037Q) according to manufacturer's instructions (Takara, Japan).

Specific primers were designed using Primer 3 software[12] to amplify the cDNA of *hMLH1* gene (2484bp) in four overlapping fragments. Sequences of these particular primers are described in table 1. The primers were synthesized by Macrogen Company (Korea). RT-PCR was performed in a final volume of 25 µl reaction mixture, containing Red Mix: Taq DNA Pol 2X masters mix Red, 1.5 mM $MgCl_2$ (No: 5200300-12121, Ampliqon, Denmark) which was diluted at a ratio of 1-1, 0.7 µl of each forward and reverse primers, and 1 µl of cDNA.

The reaction conditions were as follows: activation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 40 s, specific annealing temperature for each fragment (as listed in Table 1) for 40 s, and 72°C for 45 s and a final extension at 72°C for 5 min. For the fourth fragment, the reaction conditions were as follows: activation step at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 25 s, 68°C for 25 s, 72°C for 25 s, 25 cycles of denaturation at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min.

Germline mutation analysis

The PCR products were visualized by 2% agarose gel electrophoresis. The purified PCR fragments were sequenced by Macrogen Company (Korea). The reference sequence of *hMLH1* gene was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/NG.007109.1?form=4863&report=genbank), and INSIGHT-group database was used for mutation analysis (http://www.insight-group.org/mutations).

Functional analysis

Quantitative analysis was conducted using RT-PCR through the TaqMan Gene Expression Assays containing the FAM dye-labeled probes (TaqMan

Table 2. *hMLH1* mutations and polymorphism detected by cDNA sequencing

Gene	Exon region	Nucleotide change	Altered codon	Consequence	Number of affected patients	Type of variation
hMLH1	Exon 8	c. 655A > G	219	p.Ile219Val	4	Polymorphism
hMLH1	Exon 11	c. 973C > A	325	p.Arg325Arg	4	U.V.
hMLH1	Exon 12	c. 1086C > A	362	p.Ser362Ser	6	U.V.
hMLH1	Exon 17	c. 1959G > T	653	p.Leu653Leu	1	Polymorphism

cDNA: complementary DNA; *hMLH1*: human mutL homolog 1.

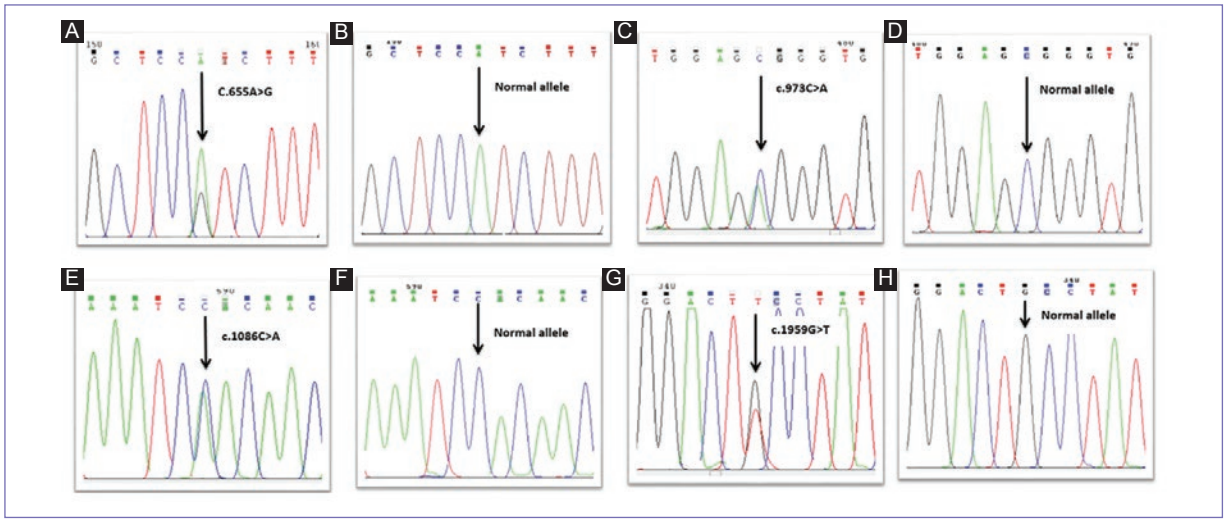


Figure 1. Sequence chromatograms of *hMLH1* gene showing the germline mutation identified in the study group. **A:** c.655A > G polymorphism in exon 8; **C:** novel mutation, transversion mutation c.973C > A in exon 11; **E:** novel mutation, transversion mutation c.1086C > A in exon 12; **G:** c.1959G > T polymorphism in exon 17 (**B, D, F, and H** represent wild-type sequences).

Pre-designed Gene Expression Products, Applied Biosystems, Foster City, CA, USA) and StepOne RT-PCR System (Applied Biosystems, Foster City, CA, USA). mRNA expression was conducted using the cDNA obtained through previous experiments. Moreover, the cDNA of 30 healthy controls was prepared and applied in the gene expression analysis. Each reaction mixture contained a total volume of 25 µl (master mix 12.5 µl, cDNA 4.5 µl, assay mix 3 µl, and H_2O 5 µl). The quantitative RT-PCR conditions were as follow: 50°C for 1 min, 95°C for 5 min, then 40 cycles of 95°C for 30 s, and 60°C for 30 s, and finally 72°C for 30 s. The comparative C_T method ($2^{\Delta\Delta Ct}$ method) was exerted to evaluate the expression as previously described by Livak and Schmittgen[13]. As the housekeeping gene, β-Actin mRNA transcript level was used for normalization of relative amounts of target mRNAs.

Statistical analysis

SPSS software version 21 (SPSS, Chicago, IL, USA) for data analysis was also used. The GraphPad Prism version 6.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA, www.graphpad.com) was exerted to represent data by plots. Evaluation of the comparisons for mRNA expression level between groups was conducted through Kruskal–Wallis nonparametric test. All data were shown as mean ± standard deviation with statistical significance set at 5%.

Results

In this study, 56.7% of patients were men with an average age of 47 ± 5.6 and 43.3% were women with an average age of 43.8 ± 6.8.

DNA sequencing results revealed four *hMLH1* gene mutations in 50% of patients. The first variant

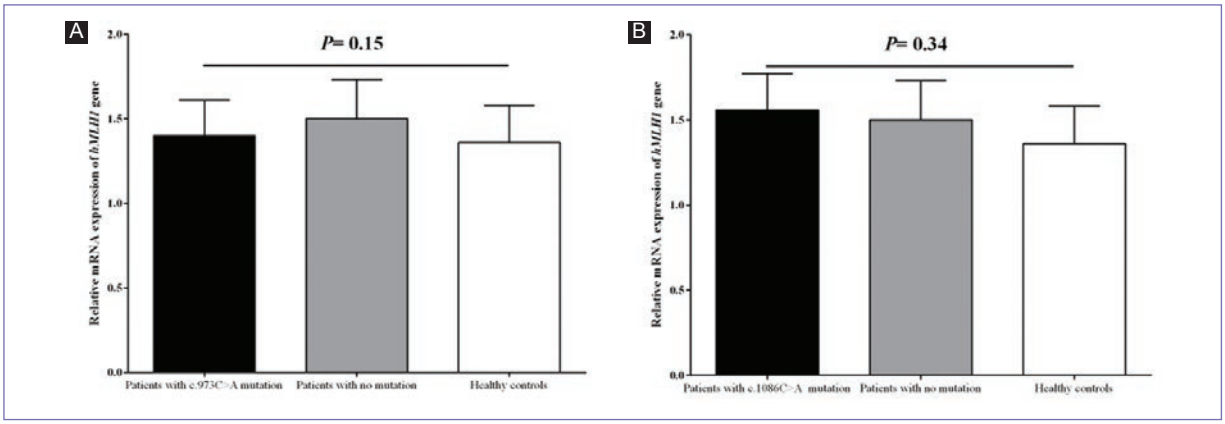


Figure 2. Bar graphs illustrate the mRNA expression of human mutL homolog 1 gene in patients carrying the c.973C > A **(A)** and c.1086C > A **(B)** mutations in comparison to patients without any mutations and healthy subjects.

(c.655A > G) was a polymorphism that occurred in exon 8 of *hMLH1* gene that resulted in an A > G change at nucleotide position. This single nucleotide polymorphism (SNP) led to the substitution of the isoleucine amino acid by the valine at codon 219. The second variant (c.1959G > T) was a polymorphism which was caused by a change of G to T base in exon 17, which was found in only one of the patients.

In addition, two novel variants were found in the studied group that had not been reported before in any of the HNPCC mutation databases. The first variant was a transversion mutation (c.973C > A) which was detected in 4 out of 30 patients (13.33%). The second variant was a transversion mutation (c.1086C > A) which was found in exon 12 of six patients (20%). All of the detected variants were summarized in table 2 and figure 1.

With respect to functional analysis (Fig. 2), it was found that mRNA expression level of *hMLH1* had no statistically significant difference (p = 0.15) between patients who carried the novel c.973C > A mutation (n = 4) in comparison to the patients without any mutation (n = 4) as well as healthy controls (n = 30). Furthermore, no statistically significant difference (p = 0.34) was detected in the mRNA expression of *hMLH1* gene in patients carrying the c.1086C > A novel mutation (n = 6) in comparison to the patients without any mutation (n = 4) as well as healthy controls (n = 30).

Discussion

CRC is one of the most common cancers in Iran. According to the recent epidemiological reports, the overall age-standardized incidence rates of CRC are higher in men (14.8/100,000 person-years) and the urban populations (35.4), relative to women (11.5) and the rural populations (17.1), respectively. The overall incidence rate of CRC has been reported to significantly increase from 2004 to 2013 in both men and women[14]. CRC accounts for 9.7% of all cancers incidences[15], which is increasing continuously[4]. Furthermore, it shows a younger age distribution in Iranian population rather than western countries[16]. According to different molecular mechanisms, CRC is divided into two groups: sporadic (70% of all CRC cases) and inherited (nearly 20% of all CRC cases). HNPCC or Lynch syndrome is the most common form of hereditary CRC, which constitutes 5-8% of all CRC cases[17].

The defects of different genes of MMR system result in HNPCC syndrome. Among these genes, *hMLH1* gene, which is the most frequently mutated gene, plays the main role in the etiopathogenesis of this syndrome[18]. Here, in this study, the *hMLH1* gene germline mutations in 30 HNPCC patients were investigated using RT-PCR and cDNA sequencing methods. cDNA sequencing is a useful technique in recognizing the mutations and decreases the cost of sequencing mainly[19]. We found four variants in the studied population. The first variant was c.655A > G in a conserved region of exon 8 of 4 patients. This SNP leads to the substitution of the isoleucine by the valine at position p.219 in the *hMLH1* protein, but the influence of this common missense polymorphism on colon cancer risk is poorly understood[4]. There are conflicts on the importance of this variant in the hMLH1 protein performance. On the one hand, both mutant and wild alleles result in non-polar and pH-neutral amino acid residues[20]. On the other hand, functional analyses suggest that this variant may impress the efficiency of DNA repair system[21]. In

another study, it was found that in male patients, G-allele carriers (AG or GG genotype) had higher risk to develop CRC relative to homozygous AA wild-type individuals. Therefore, sexuality should be considered in analyzing the results. Furthermore, patients with G-allele show a better pattern of the disease, angiogenesis, and metastasis, and disease recurrence are less observed among these people[22]. A few reports have shown that the hMLH1 p.Ile219Val polymorphism is not entirely benign and is associated with nearly 5-fold increased risk of ulcerative colitis, which is a major risk factor for colon or rectum cancer[23,24] and is related to early onset of CRC in patients[25]. Decreasing in hMLH1 protein expression is associated with this polymorphism among sporadic CRC cases from Korea[26]. Thus, further extensive studies are necessary to clarify the real impact and importance of this polymorphism toward the susceptibility to CRC.

The second variant detected in our investigations was c.1959G > T (p.Leu653Leu) in exon 17 of hMLH1 gene in only one of the patients. This variation has been reported as a neutral variant that does not have a consequential effect on MMR system. According to available information from the insight group database, it is suggested that this mutation forms alternative splicing site, which causes exon skipping, and since there is no phenotypic consequence, it cannot be considered as a rare polymorphism[27].

On the other hand, two novel variants were recognized in this study. The novelties of these variants are based on the insight database (www.INSiGHT-group.org). The c.973C > A variant is located in the middle part of exon 11 of *hMLH1* gene. Since exon 11 encodes the terminal part of MutS interaction domain, hence this variant can affect the hMLH1 interaction with hMSH2 or hMSH6, which are necessary for accurate performance of MMR system. The second novel variant was c.1086C > A in the primary part of exon 12 of *hMLH1* gene. Since this region has no interaction with other MMR proteins, thereupon, it seems that this variant has no important effect on *hMLH1* gene performance. However, the high frequencies of these variations indicate the predominance of these variants among Iranian population. On the other side, functional analysis indicated that these novel mutations did not impress the mRNA level of *hMLH1* gene. It can be speculated that other regulatory feedback mechanisms might correct the mRNA expression and final functional impression of these mutations.

Considering all the results, this study identified two already reported polymorphisms (including c.655A > G and c.1959G > T) as well as two novel variants (c.973C > A and c.1086C > A) in *hMLH1* gene for the 1st time in HNPCC patients. The novel mutations had no impression of the mRNA expression level of *hMLH1* gene. The most reliable way to diagnose Lynch syndrome is to detect a mutation in the MMR genes in the suspected patients. By investigating and determining the common mutations in each region, researchers will be able to use genetic testing for early detection of CRC in the relatives of the patients. This will result in decreasing the mortality and morbidity of CRC and better management of the disease.

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Conflicts of interest

None.

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