Frequent Novel Variations Within *MSH2* and *MLH1* Genes in a Subset of Iranian Families With Hereditary Non-Polyposis Colorectal Cancer

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Abstract- Hereditary non-polyposis colorectal cancer (HNPCC) is the most frequent autosomal dominant predisposition for development of colorectal cancer (CRC) caused by germline defects in mismatch repair (MMR) genes. Current study was aimed to find genetic variations in *MSH2* and *MLH1* genes and their correlation with the serum levels of Carcinoembryonic Antigen (CEA) in seven Iranian HNPCC families. Seven unrelated Iranian families including 11 HNPCC patients and 7 affected family members were selected. They were initially screened for mutations in exons 7 of *MSH2* and exon 15 of *MLH1* gene through polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP). Positive PCR results were further analyzed through exon sequencing. Serum CEA level was determined using the ELISA test. PCR-SSCP was positive in 8 out of 18 patients (44%) for exons 7 of *MSH2* gene, whereas two samples (11%) demonstrated to bear a mutation in exon 15 of the *MLH1* gene. Sequencing analysis of both amplified exons in positive and negative samples have confirmed no mutation in negative samples while revealed 5 and 7 novel mutations in exons 7 and 15, respectively. The mean serum concentration of CEA had a significant difference between HNPCC patients and their healthy family members. Our results demonstrated that the PCR-SSCP method has high specificity and sensitivity in the first step of mutation screening of HNPCC families. High frequency of novel alterations found in the current assay may revise the mutation screening of *MSH2* and *MLH1* genes and abet further assessment of their frequency among individual HNPCC patients.

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Keywords: Hereditary nonpolyposis colorectal cancer; *MSH2*; *MLH1*; CEA; PCR-SSCP

Introduction

Colorectal cancer is the most common gastrointestinal cancer among all populations worldwide. About 5% of colorectal cancers (CRC) are consequences of hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome. HNPCC patients are susceptible to get CRC by 80% before the age of 65-year-old. Emerging of early-onset malignancies in various organs puts the HNPCC in the category of the most important and invasive human cancers. Therefore, the earlier detection of HNPCC would be associated with better and faster control of tumor invasion and increases the survival of these patients. The current clinical diagnosis of HNPCC is based on the international diagnostic criteria (Amsterdam minimum criteria) which has been proposed in 1991 (1). Moreover, carcinoembryonic antigen (CEA) is an antigen expressed in many tissues including the colon. Enhanced CEA expression has been reported in CRC patients, and its serum level could be a good indicator of tumor statue and response to chemotherapy as high CEA serum level has been shown to be associated with poor prognosis.

Although there are some screening methods like a colonoscopy for diagnosis and resection of HNPCC polyps at earlier stages, genotyping of the most important and common genetic mutations of HNPCC patients may be a more robust and precise choice at the microscopic level of changes (2).

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Mutations in any member of mismatch repair (MMR) system genes including *hMSH2*, *hMLH1*, *PMS1*, *PMS2*, *hMSH3*, and *hMSH6* increase the risk of HNPCC development (3). Germline mutations in the first two mentioned genes, *MSH2* and *MLH1*, have determined in more than 90% of HNPCC families with an autosomal dominant pattern of inheritance (4,5). *MSH6* mutations are responsible for 7-10% of the causative genetic alterations of HNPCC and mutations in the remaining aforementioned genes are very rare (6). It was demonstrated that *MSH6* positive HNPCC patients are usually negative for Amsterdam criteria II and they require more sensitive diagnostic guidelines like Bethesda (7). Individuals who inherit these mutations have a high risk of developing colorectal cancer (8).

Herein, given the different and complicated diagnosis of *MSH6* positive patients, only *MSH2* and *MLH1* gene mutations were selected to be screened in HNPCC patients and their family members .In addition, the serum level of CEA was measured to clarify the correlation between *MLH1* and *MSH2* mutations with CEA level and prognosis of these patients.

Materials and Methods

Sample selection

Seven HNPCC families were recruited from the patients referred to a private clinic and Cancer Institute between 2002-2005 years. The diagnosis was based on Amsterdam criteria, and everyone who lacked one or more item of those criteria was excluded from this study to avoid of any other differential diagnosis. All of the enrolled patients and their family members filled the consent form according to the protocol of the local Ethical committee.

DNA extraction

DNA was isolated from 5 ml of whole blood samples obtained from all of the enrolled participants using QIAamp DNA Mini Kit (Qiagen). The quality and quantity of DNA samples were determined using NanoDrop ND-1000 spectrophotometer.

Single strand conformation polymorphism polymerase chain reaction (SSCP-PCR)

Exons 7 of *MSH2* and exon 15 of *MLH1* genes were amplified through PCR to be screened in the following step. Primer sequences were designed using the Primer 3 program and were aligned again using Primer-Blast on the NCBI website. Isolated DNA samples were amplified in a reaction mixture containing 100 ng of template DNA, 10X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of dNTP, 1 unit Taq polymerase and 10 pmol of each primer adjusted with ddH_2O up to final volume of 20 µl. The PCR condition was optimized for exon 7 of the *MSH2* gene as 5 min at 95° C for initial denaturation, 32 cycles of 1 min at 95° C, annealing at 60° C for 1 min, 1 min at 72° C followed by final extension for 10 min at 72° C. PCR reaction and condition was the same for exon 15 of *MLH1* except of annealing temperature which was optimized at 64° C. Each sample was amplified in triplicate and negative control was included in each PCR run. The amplification was confirmed on a 1.5% agarose gel with SYBR Green staining, followed by illumination under UV light.

Screening of mutations in the *MLH1* and *MSH2* genes

Mutations were detected on a non-denaturing polyacrylamide gel by analyzing migration patterns due to the conformational variability of single-stranded DNA. PCR products obtained from the previous step were subjected to polyacrylamide gel electrophoresis (10%), and the differential banding pattern was revealed through silver staining. The positive results were analyzed through full exon sequencing.

Determination of CEA content in serum samples

Enzyme-linked immunosorbent assay (ELISA) test was performed to compare the serum concentration of CEA among HNPCC patients and their family members and healthy controls using CEA specific ELISA Kit (Abcam, UK).

Statistical analysis

Data were statistically analyzed using SPSS software version 17.0 (SPSS Inc, IL, USA). Chi-square, Fisher's exact and *t*-tests were implicated in exploring the correlation between various variants of the patients. In all of the statistical analyses, *P* less than 0<0.05 was considered as significant.

Results

Mutation analysis

SSCP assay revealed abnormal mobility shift of the amplification products of exon 7 of the *MSH2* gene and the exon 15 of the *MLH1* gene in 8 and 4 of all the patients, respectively. Two HNPCC patients with abnormal SSCP assay belonged to four pedigrees (pedigrees 1-4) in which no mutation was found in both genes through exon sequencing (Figure 1). Distribution

of found mutations through SSCP-PCR confirmed by exon sequencing in the two studied exons has been provided within assessed pedigrees (Figure 2-4). The genotyping of *MLH1* and *MSH2* genes were normal in all of the healthy family members confirmed by sequencing.

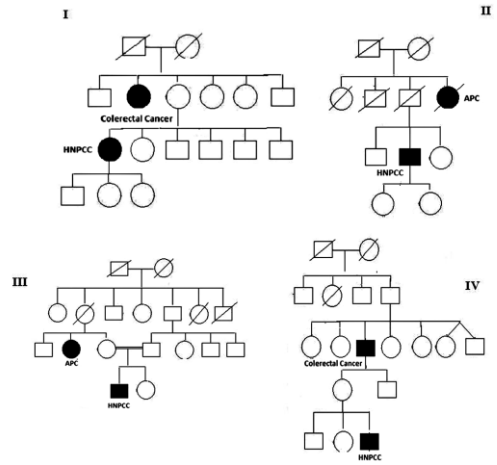

Figure 1. Pedigrees of 4 families in which no mutation found in exon 7 of *MSH2* and exon 15 of *MLH1* genes

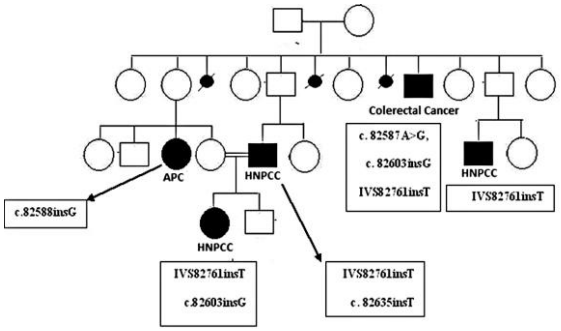

Figure 2. Pedigree of family 5 in which novel mutations have been found in exon 7 of *MSH2* gene

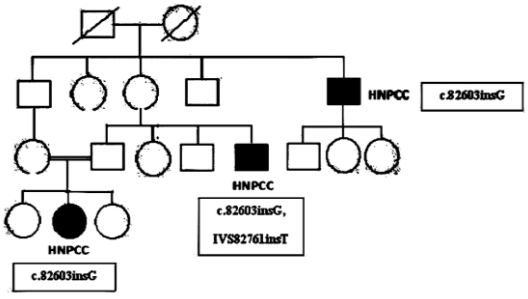

Figure 3. Pedigree of family 6 in which novel mutations have been found in exon 7 of *MSH2* gene

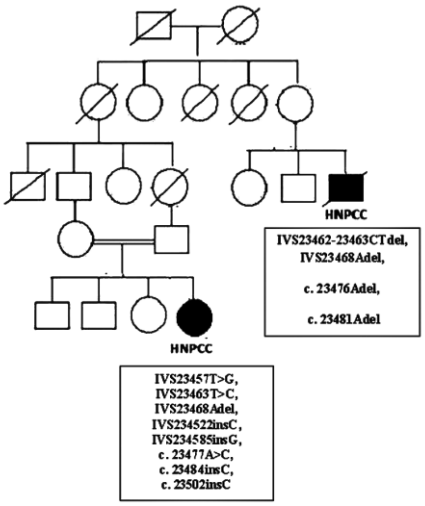

Figure 4. Pedigree of family 7 in which novel mutations have been found in exon 15 of *MLH1* gene

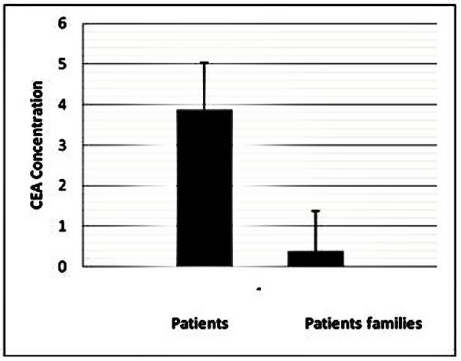

Figure 5. The serum concentration of CEA in patients group compared to their family member and healthy controls

The serum level of CEA

The mean concentration of serum CEA level was 3.87±1.16 µg/ml in HNPCC and cancer patients and 0.383±0.654 µg/ml among unaffected members which was significantly different (P<0.0001) (Figure 5). No meaningful correlation was found between the CEA level and mutation status of *MSH2* and *MLH1* genes in HNPCC and other cancer patients.

Discussion

Combination of tumor-specific biochemical markers and clinical criteria like Amsterdam may enhance the specificity and sensitivity of screening of various malignancies. However, there is not only any unique biomarker for each type of cancer, but also these combination strategies are not usually helpful in the screening of asymptomatic family members of HNPCC patients. Therefore, the implication of molecular

markers can overcome these limits onward prophylactic interventions in high-risk unaffected family members meanwhile can potentially provide an opportunity for earlier diagnosis and treatment of patients. In this regard, identification of the most prevalent genetic alterations in every population can lead the health care provider to move in a straightforward way toward faster and more precise molecular diagnosis and screening of HNPCC patients (9). *MSH2* and *MLH1* gene mutations together comprise more than 90% of all genetic alterations found in HNPCC pathogenesis, so far. Determining the most frequent mutated exon in those genes using the more easy and inexpensive genetic screening methods like SSCP-PCR decrease the cost and time around for focusing on other exons. Herein, it was shown that the mutations within exon 7 and exon 15 of *MSH2* and *MLH1* genes were detected in 44% and 11% of all the patients including HNPCC. There are various divers reports on the frequency of *MSH2* and *MLH1* genes in HNPCC patients. To our knowledge, this is the first study carried out on different types of colon cancer within 7 affected HNPCC families.

In the present study, the c.82603insG frameshift mutation of exon 7 of the *MSH2* gene was found in three HNPCC and the colorectal cancer patients of pedigree 5 (Figure 2). This may be relying on the fact that this novel pathogenic alteration would initiate a common molecular mechanism toward CRC and HNPCC progression. Given that it was not detected in the APC patient of this pedigree, the role of this mutation may be more critical in polyposis variation of CRC. The intronic IVS82761insT variation was common in all of the analyzed patients of this family pedigree. It is consistent with Vasen *et al*., report in which it was described that the risk of CRC was higher in *MSH2* mutation carriers compared to *MLH1* gene mutations carriers (7). Deletion of exon 7 of the *MSH2* gene was identified through sequencing and MLPA assay in two female and male patients that have been suffered from endometrial and colon cancers, respectively (10).

Although some other MMR gene mutations may be responsible for the remaining patient's disease, due to a higher frequency of *MSH2* and *MLH1* mutations, alterations in other exons of these two genes are more likely to have been occurred. Therefore, other exons of those genes were selected to be examined in our ongoing study. Most of the previous studies on HNPCC mutation screening have focused on *MSH2* and *MLH1* genes similar to us. Genotyping of other MMR genes, in particular, *MSH6*, except a founder Ashkenazi duplication (11) almost has been failed to show the frequency of mutation more than the expected range of 7-10% alterations in various populations. This is especially considered when the HNPCC patients don't meet the Amsterdam criteria as mentioned in various studies. Therefore, typing for *MSH6* mutations may not be helpful in both *MSH2* and *MLH1* mutation negative patients (12).

Mutation of exon 15 of the *MLH1* gene was reported in a one member of a Korean HNPCC family affected by gastrointestinal cancer (13). In a separate study, MLH1 mutations including exon 15 were reported to be 24% in all the studied cancer patients of HNPCC families (14).

There was a significant difference between the CEA serum levels in all the patients compared to their healthy family members. However, we have initially demonstrated that serum CEA was not meaningfully different among HNPCC, APC and CRC patients. This is indicating that although CEA would be a good tumor marker for colon cancer, it cannot differentiate between polyposis or non-polyposis types of CRC. Consistent with ours, although the CEA value was lower in HNPCC patients relative to sporadic CRC patients, it was not significant (15). Moreover, no significant correlation was identified between the mutation status of all the patients including HNPCC and other cancer patients and the CEA level. Schiemann *et al*., could not find any significant change in CEA value of HNPCC patients in whom MMR gene mutations have been detected (15). Due to no significant change of CEA value with arising a mutation in MMR genes and progression of HNPCC toward CRC, it may be completely replaced by genetic markers in the near future.

We found *MSH2* gene mutations were more common than *MLH1* gene within studied pedigrees. Moreover, herein, we indicated that SSCP-PCR is a successful mutation screening molecular test in the initial step of genetic analysis of MMR gene alterations in HNPCC families. Determination of the mutation patterns among large numbers of HNPCC patients from different parts of Iran is essential because this will help to decrease the rate of cancer and it also would be helpful for patients' family and abate their stress.

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