Immunohistochemical Analysis of Mismatch Repair Proteins in Iranian Colorectal Cancer Patients at Risk for Lynch Syndrome

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Abstract

Background: Hereditary non-polyposis colorectal cancer (HNPCC) is a common hereditary cancer predisposing syndrome has molecular and clinicopathological features still have remained ambiguous within Iranian populations. We discuss in this article some molecular and clinicopathological features of the condition.

Methods: The study was a descriptive retrospective and designed on 1659 colorectal cancer (CRC) patients were screened based on early-onset disease and Amsterdam II criteria during 14 years (2000-2013). Immunohistochemistry (IHC) staining was set up to detect expression of mismatch repair (MMR) genes on paraffin-embedded tissue sections of 31 HNPCC-CRC tumors. SPSS 19 software was used to analyze the data.

Results: IHC-MMR staining was absent in 7/31 individuals (22.6%) of which 4 cases showed IHC-Absent (IHC-A) in both MSH2 and MSH6 (57.1%), in 2 cases both MLH1 and PMS2 had negative staining (28.6%), and just in one case, MSH6 was defective (14.3%). The frequency of CRC among IHC-A and IHC-Present (IHC-P) families was 67.5% and 27.9%, respectively. Also the most frequent extracolonic cancers in IHC-A group were: stomach (10%), small bowel (5%), and prostate (5%); and in IHC-P group: stomach (18.4%), lung (10.9%), and breast (7.5%). Average age of IHC-A individuals at diagnosis was 38.0 versus 45.3 years in IHC-P individuals. Overall, 20.8% and 57.1% of our index CRCs were localized proximal to the splenic flexure in IHC-P and IHC-A groups, respectively.

Conclusion: Given the lack of enough information about molecular aspects of hereditary cancer syndromes like HNPCC in Iran, more evaluations are necessary on larger samples using complementary techniques such as MSI-testing and mutation analyses.

Keywords: Immunohistochemistry; mismatch repair; Lynch syndrome; Iran

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Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome (LS) is a chronic disease in which there is familial aggregation of colorectal cancer (CRC) and other associated tumors [1]. This disease now is described as a cancer susceptibility syndrome secondary to a germ-line mutation in at-least one of the DNA mismatch repair genes (*MMRs*) including *MLH1*, *MSH2*, *MSH6*, and *PMS2* or some large deletion mutations in last exon of *EPCAM*, a gene located next to *MSH2*, or *EPCAM-MSH2* locus [2, 3]. It leads to accumulate of mutations in other genes responsible to apoptosis and cell cycle control, accelerating an adenoma-to-carcinoma transition event [3, 4]. Moreover, *MLH1* promoter hypermethylation, an epigenetic phenomenon, can inactivate the gene, mostly in sporadic CRCs [5, 6].

Screening for molecular detection is both time-consuming and expensive due to the heterogeneity

of so variable mutations in *MMRs* [7]. Among two molecular screening tools which are being used commonly to detect LS, Microsatellite Instability (MSI) testing and immunohistochemical (IHC) staining of MMR proteins. MSI is sensitive but not specific for LS, as only 20–25% of all MSI-High (MSI-H) tumors are associated with germline mutations in a *MMR* gene. Although MSI has enough sensitivity to identify LS, it will not be detected in about 5% of all LS tumors [1].

MMR defects can be identified by IHC staining in tumor tissue sections in which expression of *MMR* genes is lost (IHC-Absent), while their expression in healthy adjacent tissue sections is intact (IHC-Present). IHC has about 77-100% sensitivity and 98-100% specificity to detect *MMR* defects compared to MSI-testing [4, 7-9].

Though, IHC has been reproduced as complement of MSI-testing in many studies [1, 10]. It has been suggested as the choice screening method prior to genetic testing by some authors [11]. They believe this technique has some advantages compared to MSI-testing and should be replaced as the first molecular screening tool [8]. IHC is more available than MSI as part of the routine service in the general pathology laboratories. In addition, IHC can be feasible at the time of colectomy efficiently [12]. Also, IHC is regarded more inexpensive than MSI, so earlier analysis indicated that IHC was about threefold less expensive than MSI-testing [13]. IHC, additionally, may consider as genetic testing because it can reveal which particular *MMR* gene may be defective, and as such it enables efficient mutation analysis on the target gene [8, 14]. Meanwhile, there are some limitations in IHC-*MMRs* such as uncertainty in interpretation due to variable tissue fixation and other technical issues which can result in weak or equivocal staining patterns [8]. It has also a low sensitivity to detect mutation of *MLH1* with MLH1 anti body alone, because it is possible that some missense mutations in this gene will not result in the absence of a detectable protein product [15].

Although the incidence of CRC has increased among Iranian population within recent decades [16, 17], it has not been yet established any systematic screening program to identify LS affected families among Iranian population. So, we designed a study to set up a molecular screening program in Central Iran for the first time. We discuss in this article the results of IHC-MMRs concluded from the study.

Materials and Methods

This study was a descriptive retrospective in order to screen CRC patients, in collaboration between two provinces: Isfahan and Charmahal va Bakhtiari, to identify HNPCC families. We screened 1659 CRC patients registered in Poursina Hakim Research Center (PHRC), a famous referral gastroenterology clinic in Isfahan, center of Iran, during about 14 years from 2000 to the end of 2013. At first, we selected all patients with age of fifty years or less, as early-onset patients. Then using Amsterdam II criteria, at risk families for Lynch syndrome were included for next molecular analyses. These HNPCC families were called and invited for genetic counseling. Our study included all individuals participating in counseling sessions with history of bowel resection during the past decade whose paraffin-embedded blocks were available. During genetic counseling, the participants were interviewed about cancer related family history at-least up to three generations. The drawn pedigrees were reconfirmed by at-least two other members of every family. Moreover, the reported malignancies within families were possibly verified by searching for their medical documents, if available. Otherwise, we could trust them. Because according to strong familial relationship among Iranian people, they have usually awareness of serious diseases such as cancer in their relatives.

Immunohistochemistry

We tried to select one paraffin-embedded tissue block for each case from resected bowel specimen containing tumoral and preferably adjacent normal mucosa. About 1-2 µm. thick tissue sections were cut after that deparaffinized in xylene and rehydrated it through graded alcohols.

Then slides were washed in running tap water, immersed in Tris-EDTA buffer at PH 9.0 in a pressure chamber at microwave for antigen retrieval for 20 minutes (5´ in high and 15´ in M-high degree). After washing the slides in deionized water, we used Peroxidase Block reagent to neutralize endogenous peroxidase for at-least 5 minutes. Then we washed the slides twice in TBS (Tris-buffered Saline), each time for 5 minutes.

The slides were incubated with Protein Block reagent for 5 minutes and washed again in TBS twice as mentioned. The next step was incubation the slides overnight with optimally diluted mouse monoclonal primary antibodies as following: MSH2 (Leica Biosystems: Novocastra, UK, Lyophilized,

Product Code (PC): NCL-MSH2) at 1/80 dilution, MLH1 (Leica Biosystems: Novocastra, UK, Liquid, PC: NCL-L-MLH1) at 1/100 dilution, MSH6 (Leica Biosystems: Novocastra, UK, Liquid, PC: NCL-L-MSH6) at 1/100 dilution, and PMS2 (Leica Biosystems: Novocastra, UK, Liquid, PC: NCL-L-PMS2) at 1/100 dilution.

The next morning, after twice washing the slides in TBS, they were incubated with Post Primary Block reagent for 30 minutes. Washing again twice in TBS, we incubated the slides with Novolink Polymer for 30 minutes. Once again, twice washing in TBS with gentle rocking followed with developing peroxidase activity with DAB working solution for 5 minutes. Then we washed the slides in water and counterstained them with Hematoxylin. After rewashing the slides in water for 5 minutes, we finally dehydrated, cleared and mounted sections. Our slides were ready at the time for microscopic observation.

Data Analysis

We used SPSS 19 software package (SPSS Inc., Chicago, IL, USA) to analyze our data.

Results

Overall, of 1659 CRC patients registered in PHRC, 413 patients (24.9%) were ≤50 years at diagnosis. 219/413 successful calls, 45 HNPCC families were screened using Amsterdam II criteria of which 14 affected families were excluded from molecular testing stage. Of excluded families, 10 individuals were omitted because of being unavailable their tumor tissues, and 4 others were excluded due to being unwilling for incorporation.

IHC-*MMR* staining was absent in 7.31 of the individuals (22.6%) (IHC-A (Absent) versus IHC-P (present) families), of which 4 cases determined as IHC-A for both MSH2 and MSH6 antibodies

Table 1. Frequency of cancer locations among Iranian HNPCC families in both IHC-A and IHC-P groups

Cancer Locations	IHC-P families		IHC-A families	
	frequency	percent	frequency	percent
colon / rectum	41	27.9	27	67.5
Lung	16	10.9	1	2.5
Stomach	27	18.4	4	10.0
small bowel	7	4.8	2	5.0
prostate	4	2.7	2	5.0
brain	11	7.5	0	0.0
haematopoietic system	6	4.1	2	5.0
hepatobiliary system	5	3.4	1	2.5
bladder	3	2.0	0	0.0
testis	2	1.4	0	0.0
thyroid	2	1.4	0	0.0
kidney	1	0.7	0	0.0
skin	2	1.4	0	0.0
bone	2	1.4	0	0.0
pancreas	1	0.7	0	0.0
breast	11	7.5	1	2.5
uterus	5	3.4	0	0.0
nasopharynx	1	0.7	0	0.0
Total	147	100.0	40	100.0

HNPCC: Hereditary Non-polyposis Colorectal Cancer; IHC-A: Immunohistochemial Absent; IHC-P: Immunohistochemical Present

Table 2. Frequency of colorectal cancer tumor sites in Iranian HNPCC individuals in both IHC-A and IHC-P patients

Tumor site	IHC-P tumors		IHC-A tumors	
	Frequency	Percent	Frequency	Percent
cecum	3	12.5	1	14.3
ascending colon	2	8.3	2	28.6
transverse colon	0	.0	1	14.3
descending colon	0	.0	2	28.6
sigmoid colon	8	33.3	1	14.3
rectum	10	41.7	0	.0
unknown	1	4.2	0	.0
Total	24	100.0	7	100.0

(57.1%), in 2 cases both MLH1 and PMS2 antibodies showed IHC-A (28.6%), and just in one case MSH6 was defective (14.3%). PMS2 had no deficiency in all studied individuals.

There were 187 cancer patients in all 31 HNPCC families of which 40 affected members (~21%) were related to 7 IHC-A families. The mean of affected members in IHC-A families was 5.7 while it was 6.1 in IHC-P families (p value=0.513).

The most frequent cancers among IHC-A families were: CRC (67.5%), stomach (10%), small bowel (5%), and prostate (5%); while in IHC-P families: CRC (27.9%), stomach (18.4%), lung (10.9%), and breast (7.5%) were the most common cancers (Table 1).

Mean age of IHC-A individuals at diagnosis was 38.0 years (range 31-50), while IHC-P individuals had averagely 45.3 years at diagnosis (range 24-69) (p value=0.146). On the other hand, the mean age of tumor diagnosis in 147 affected members within 24 IHC-P families was nearly the same as 40 cancer patients within 7 IHC-A families (~51 years: range 2-82 years).

The most frequent colorectal cancer tumor sites among IHC-P individuals were: Rectum (41.7%), sigmoid colon (33.3%), cecum (12.5%), and ascending colon (8.3%); while in IHC-A individuals: ascending colon and descending colon (28.6%), and transverse colon, sigmoid colon and cecum (each one 14.3%) were the most common involved sites. Meanwhile, there was no case with rectum involvement among IHC-A individuals (Table 2).

Just 1 of 7 IHC-A individuals (~14%) was diagnosed at I or II colorectal cancer TNM stage, while 8 of 24 IHC-P individuals (~33%) were found at these early stages (p value=0.345) (Table 3).

Although 11/24 of IHC-P individuals (~46%) had been deceased at the screening time, 6.7 of IHC-A individuals (~86%) were alive at this time (p=0.382).

Discussion

In this study we evaluated expression of *MMR* genes containing *MLH1, MSH2, PMS2,* and *MSH6* using IHC staining in 31 index early-onset CRC patients among identified HNPCC families within Isfahan, Iran for the first time.

The gene expression is normal if nuclear staining in CRC cells would be observed intact as in normal adjacent epithelial cells. If nuclear staining is absent in cancer cells versus positive staining in nuclei of normal colon epithelial cells, it would be indicated defective expression of a gene [18].

Prevalence of *MMR* deficiencies

We found absent IHC-*MMR* staining in 22.6% of the early-onset HNPCC individuals (IHC-A) who had met Amsterdam II criteria. Given high sensitivity of four-antibody IHC-*MMR* to identify MSI-CRCs more than 92% [19], it seems a significant portion of Amsterdam positive families in our population has no *MMR* mutations. It suggests

Table 3. Frequency of colorectal cancer TNM stage at diagnosis time among Iranian HNPCC individuals in both IHC-A and IHC-P patients

IHC-MMR State	Stage I		Stage II		Stage III		Stage IV		Total	
	frequency	percent	frequency	percent	frequency	percent	frequency	percent	frequency	percent
Present	7	29.2	1	4.2	11	45.8	5	20.8	24	100
Absent	0	0.0	0	14.3	0	71.4	1	14.3	7	100
Total	7	22.6	2	6.5	16	51.6	6	19.4	31	100

the role of other genes in etiology of the most our samples. Although we cannot find definite data in Iranian population according to other studies, about 35-70% of HNPCC families meeting Amsterdam criteria do not have *MMR* deficiency and are considered "Familial Colorectal Cancer Type X" (FCC-X) or "non-syndromic familial colorectal cancer" [20, 21]. It seems more evaluations on larger samples using complementary techniques such as MSI-testing and mutation analyses are necessary to estimate a more accurate frequency of X syndrome among Iranian population.

The most frequent (~57%) deficiency of gene expression was related to both *MSH2* and *MSH6* genes. MSH6 and PMS2 proteins are accessory to major MMR proteins: MSH2 and MLH1, respectively. So the loss of *MSH2* expression in a tumor tissue leads to loss of *MSH6* expression in that tissue. Germline mutations, however, in *MSH6* or *PMS2*, as minor *MMR* genes, lead to single loss of expression of their associated proteins [3]. Therefore, in 57% of our IHC-A individuals *MSH2* was responsible gene. The prevalence of *MSH2* defect in our study was near to some large early studies [22]. About 29% of the IHC-A tumors didn't show any nuclear IHC-staining for both MLH1 and PMS2 proteins. It predicts existence of germline mutation in *MLH1* of near to 30% of the IHC-A individuals. Although it is similar to some early valid studies [23], developing the IHC results by MSI-testing and mutation analyses, preferably on a larger sample, will be more informative.

We found just one individual with absent IHC-staining in MSH6 protein (14.3%). Interestingly, the genetic pedigree shows the number of affected relatives is fewer on average than MSH2 or MLH1 families. So, there are only two cancer patients among second degree relatives of the individual with more than 65 years old. According to some studies, we expect the patients with *MSH6* mutations would be more likely Amsterdam negative [24], so the Bethesda guidelines are more sensitive than the Amsterdam Criteria to identify it [25]. Consequently, we may identify more patients with MSH6 defect among all our CRC patients using Bethesda guidelines. As some studies have presented, PMS2 loss in IHC-staining is the rarest event [26]. We found also no individual with absent IHC-staining singularly for PMS2.

Clinicopathological features

Although there was no significant difference between average count of cancer patients among the IHC-A and IHC-P HNPCC families, the frequency of CRC among IHC-A families was predominantly more than IHC-P families, nearly 2.5 fold (67.5% versus 27.9%), while we had asked cancer-related family history up to three generations in both groups.

Moreover, the mean age at diagnosis in IHC-A individuals was more than 7 years earlier than IHC-P individuals (38 versus 45.3 years), whereas there was no significant difference between other cancer patients in both groups of families (about 51 years).

Studies on FCC-X families have shown that CRC risk among their kindred's is lower than HNPCC families. Also, CRC diagnosis has occurred averagely 10-15 years later in FCC-X families [20, 27]. Of our index CRCs, 20.8% and 57.1% were localized proximal to the splenic flexure in IHC-P and IHC-A groups, respectively ($p<0.001$).

More studies on families with Amsterdam criteria, with and without MMR deficiency, have shown a higher proportion of CRCs are located proximally of the splenic flexure in patients with MMR deficiency than those with intact MMRs. For example, Mueller-Koch and his coworkers found 68% of CRCs proximal to the splenic flexure in Amsterdam positive families with *MMR* mutations versus 14% in families without MMR mutations [27]. Some other authors have found similar results too [3, 28].

Although the proportion of our alive IHC-A individuals at screening to the alive IHC-P individuals was about 2 fold, the early-stage diagnosis among IHC-P individuals was more than 2 fold of IHC-A individuals, according to their pathologic documents. It may refer to the better survival of MSI-CRCs compared to MSS (microsatellite stable) CRCs, a fact that has been considered in some studies. For example, in one study Malesci and his coworkers showed that MSI was significantly related to a reduced chance of lymph node and distant organ metastases at diagnosis [29].

Some authors have reported a genotype–phenotype correlation in *MMR* mutation carriers. For example, *MLH1* mutations are related to higher risk of early onset CRC cancer and more prevalent CRC cancer than extracolonic cancers, while in *MSH2* mutation carriers there was a higher risk of multiple

extracolonic cancers, and the mean age of diagnosis is more than MLH1 mutation carriers [30, 31].

In our study, the patients with MLH1 defect were identified averagely 8 years earlier than the patients with MSH2 defect (~42 versus ~50 years old). In addition, there were more extracolonic cancer types among families with MSH2 defect in comparison to the families with MLH1 or MSH6 defects (7 types versus 4 and 2 types, respectively).

The phenotype of *MSH6* mutations is somewhat different than *MLH1* and *MSH2* mutations, and this condition has been described as "MSH6 syndrome" [24]. The mean age at cancer diagnosis in *MSH6* mutation carriers is at least one decade more than *MSH2* or *MLH1* mutation carriers [30]. In addition, the risk of CRC affection in families with MSH6 defect is more likely less than HNPCC families with MSH2 or MLH1 defects [32]. In our single family with MSH6 defect, the mean age of three cancer affected members at diagnosis was about 67 years, averagely two decades more than age of the patients in families with MLH1 or MSH2 defects. Moreover, the proportion of CRC patients was significantly lower than HNPCC families with MSH2 or MLH1 defects (33% versus 72 and 66 percent respectively).

Conclusion

Since there is no enough information about molecular aspects of hereditary cancer syndromes like HNPCC in Iran, we have not still a definite known plan for molecular screening of the disease. Given the limitation of our study, we suggest more evaluations on larger samples by complementary techniques such as MSI-testing and mutation analyses to reach more trustworthy results.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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