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ORIGINAL ARTICLE

Colorectal cancer in Iran: immunohistochemical profiles of four mismatch repair proteins

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Abstract
Purpose The aim of the present study was to determine the profile of mismatch repair (MMR) defects in Iranian colorectal cancer patients by using immunohistochemical staining for products of four MMR genes: *MLH1*, *MSH2*, *PMS2*, and *MSH6*.
Methods Tissue samples of 343 patients were immunostained for MLH1, MSH2, PMS2, and MSH6. Clinical and family history and survival data were compared between normal and abnormal staining patterns.
Results Fourteen percent of the patients had abnormal nuclear staining for MMR proteins. *MLH1* was absent in four, *MLH1*/*PMS2* in 15, *PMS2* in five, *MSH2* in 12, and *MSH2*/*MSH6* in 12 patients. These tumors were more proximal, had a nonsignificant better survival, and were more associated with positive family history. Estimation of this study of prevalence of hereditary nonpolyposis colorectal cancer in Iran was 5.5% of the total colorectal cancers.
Conclusions Along with the recommendations of the National Institute of Cancer, we recommend immunohistochemistry staining for *MLH1*, *MSH2*, *PMS2*, and *MSH6* for determining the eligibility of patients for mutation analysis of MMR genes.

Keywords DNA mismatch repair · Hereditary nonpolyposis colorectal neoplasm · MLH1 protein · MSH2 protein · MSH6 protein · PMS2 protein

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Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is an inherited autosomal dominant disorder [1] that accounts for 1–6% of colorectal cancers [2]. It is characterized by early onset colorectal cancer (CRC) [3], more mucinous histology [3], and accelerated transformation from adenoma to carcinoma [4]. It is also associated with synchronous and metachronous colorectal tumors [5], and extra colonic malignancies of brain [6], genitourinary tract, and gastrointestinal system [6, 7].

HNPCC has been shown to be caused by inherited genetic defects in post replication DNA mismatch repair (MMR) system [8] that lead to microsatellite instability (MSI) which is the hallmark of HNPCC [9, 10]. Specific microsatellite markers and PCR can identify MSI [11]. When the instability exceeds 30% of the examined loci, colorectal tumors are classified as high-frequency MSI or MSI-H and otherwise as MSI-L or low-frequency MSI. Absence of alteration in the length of DNA sequence corresponds to be classified as microsatellite stable (MSS). MSI-H is present in about 90% of HNPCC-associated CRCs and four to 15% of sporadic type [10, 12, 13].

The genetic mechanism by which the MSI-H colorectal adenocarcinomas develop is different from that of MSI-L and MSS tumors [14, 15]. These tumors usually involve the

proximal colon and express characteristic histopathologic features including poor differentiation, marked peritumoral and intratumoral lymphoid reaction, and mucinous and medullary histology [16, 17]. Moreover, MSI-H tumors show better clinical outcome irrespective of their stage [7, 17, 18] and are less aggressive than common colorectal tumors [18, 19]. Given these data, the assessment of MSI is exceedingly proposed as a provider of useful prognostic information in CRCs [10, 20]. Moreover, MSI has been shown to be a valuable predictor of double primary malignancies in colorectal patients [21].

In a portion of HNPCC subjects, predisposition to cancer have been associated to germline mutations in four MMR genes: *MSH2*, *MLH1*, *PMS2* [22, 23], and *MSH6* [24]. Inactivation of *MSH2* and *MLH1* is responsible for defects in MMR system in the large majority of MSI-H tumors [10, 22] and can be detected in 50% to 70% of HNPCC families who fulfill the Amsterdam criteria and 30% of atypical HNPCC families [23]. Germline mutations of *MSH6* and *PMS2* have been found in atypical and a minority of typical HNPCC families [24–26] who show a dominant trend towards familial risk of CRC with minimal or no MSI [26].

MMR proteins interact in the form of heterodimers of *MSH2*/*MSH6* and *MLH1*/*PMS2* [27]. Mutations that affect *MLH1* and *MSH2* typically cause concurrent immunohistochemical loss of *PMS1* and *MSH6* through degeneration of heterodimerizing protein partner, respectively [26, 28]. On the contrary, germline mutations affecting the *PMS1* or *MSH6* result, in the majority of cases, in isolated loss of affected protein on immunohistochemistry (IHC) staining, and can be missed by MSI analysis [26].

A growing body of evidence advocates the potential of immunohistochemical analysis of *MLH1* and *MSH2* gene products to identify MSI-H colorectal adenocarcinomas specifically [29–33]. Given the advantage of added value of *MSH6* and *PMS2* and its availability, applicability and lower cost, IHC can be regarded as an effective prescreening tool for identifying HNPCC [30, 34, 35]. The aim of the present study was to determine and study the pattern of MMR defects in a population of CRC patients by using IHC, and to compare the familial, clinical, and survival data of tumors with normal and abnormal MMR status.

Patients and methods

Patients

This study included all colorectal adenocarcinoma patients who had undergone surgical resection from January 2001 to December 2005, at Taleghani Hospital (Tehran, Iran). Data regarding the gender; age at diagnosis; grading, staging, and location of the tumor; histopathology report; and follow up was retrieved from available hospital records for every patient and was completed by telephone interview with the patients or their family members, if necessary. Tumors were originally staged according to TNM system [36] and were graded according to the criteria of the World Health Organization [37]. Tumors were classified as proximal or distal in reference to the splenic flexure of colon.

Paraffin-embedded tissue blocks and histopathology slides were accessed, in advance, from the histopathology archive. Original hematoxylin and eosin slides were reexamined for congruency with previously issued reports or for confirming the diagnosis in case that the original reports were missed. Patients whose pathology specimens were missed and those with history of familial adenomatous polyposis, presurgical radiation therapy, and inflammatory bowel disease were excluded from the study. IHC was employed on tissue specimens of the remaining 343 patients.

Information on family history of CRC was obtained as much as possible from the hospital records of patients in the first place, and measures were taken to verify and complete the familial data by telephone interview with the patients or their close relatives. However, because there was no national registry for cancer in Iran, the CRC patients with a positive family history could not be accurately entered in HNPCC criteria.

Immunohistochemical staining

One tumor specimen from each patient was used for analysis. Four micron-thick sections were obtained from formalin-fixed paraffin-embedded tissue blocks. The tissue sections were deparaffinized in xylene and were rehydrated in graded concentrations of alcohol. Endogenous peroxidase activity was blocked by treating the sections with a blocking solution. For antigen retrieval, the sections were treated while boiling in citrate buffer (pH6.0) in a microwave oven.

Sections were incubated afterwards with primary antibodies against *MLH1* (BD Biosciences Pharmingen, clone: G168-15, dilution of 1:100), *MSH2* (Calbiochem, Oncogene sciences, clone FE11, dilution of 1:100), *MSH6* (BD Trasduction Laboratory, clone: 44, dilution of 1:1000), and *PMS2* (BD Pharmingen, clone:A16-4, dilution of 1:500). After each step, slides were rinsed with TBS buffer for 3 min. Then, slides were treated with Envision (DAKO, REAL Envision) for 60 min. To visualize immunoreactivity, 3,2'-diaminobenzidine was used and samples were counterstained with hematoxylin.

Two different blinded pathologists reviewed all of the slides. Normal epithelial cells, stromal cells, or intramucosal lymphocytes in the same slide were used as internal control for evaluation of IHC staining for *MSH2*, *MLH1*, *PMS2*, and *MSH6* (Fig. 1, a1–d1). Slides were

evaluated for the presence of nuclear staining, and complete absence of nuclear staining for any of the mentioned MMR gene products (MMRP) was considered abnormal MMRP (Fig. 1, a2–d2). The whole procedure was repeated exactly for certain specimens of which the internal control staining was not satisfactory to ensure preservation of maximum sample size and for one pattern of abnormal MMRP (*PMS2* Negative, *MLH1* and *MSH2* positive).

Statistical analysis

Differences of distribution between the categorical variables were examined with chi-square test and Fisher's exact test in case of need. For quantitative variables, Student's *t* test was employed. Multivariate logistic regression analysis was performed with covariates of age at onset, family history, and tumor site included. Multivariate survival analysis was performed with Cox's proportional hazard model while the covariates of gender, age at diagnosis, tumor site, and tumor stage and grade were included. Reported *P* values of less than 0.05 were considered to represent the statistical significance.

Results

Clinical data

Of 343 examined colorectal adenocarcinomas, 295 (86%) showed normal nuclear expression of *MLH1*, *MSH6*, *PMS2*, and *MSH6*, and 48 (14%) showed abnormal staining patterns of MMRP. Tumors with abnormal MMRP staining tended more to be proximal and occur at lower ages; family history of CRC was also higher in these tumors. No significant difference of staging and grading could be found between normal and abnormal MMRP tumors (Table 1). We could find no significant relationship between early onset of CRC (onset before the age of 50) and positive family history.

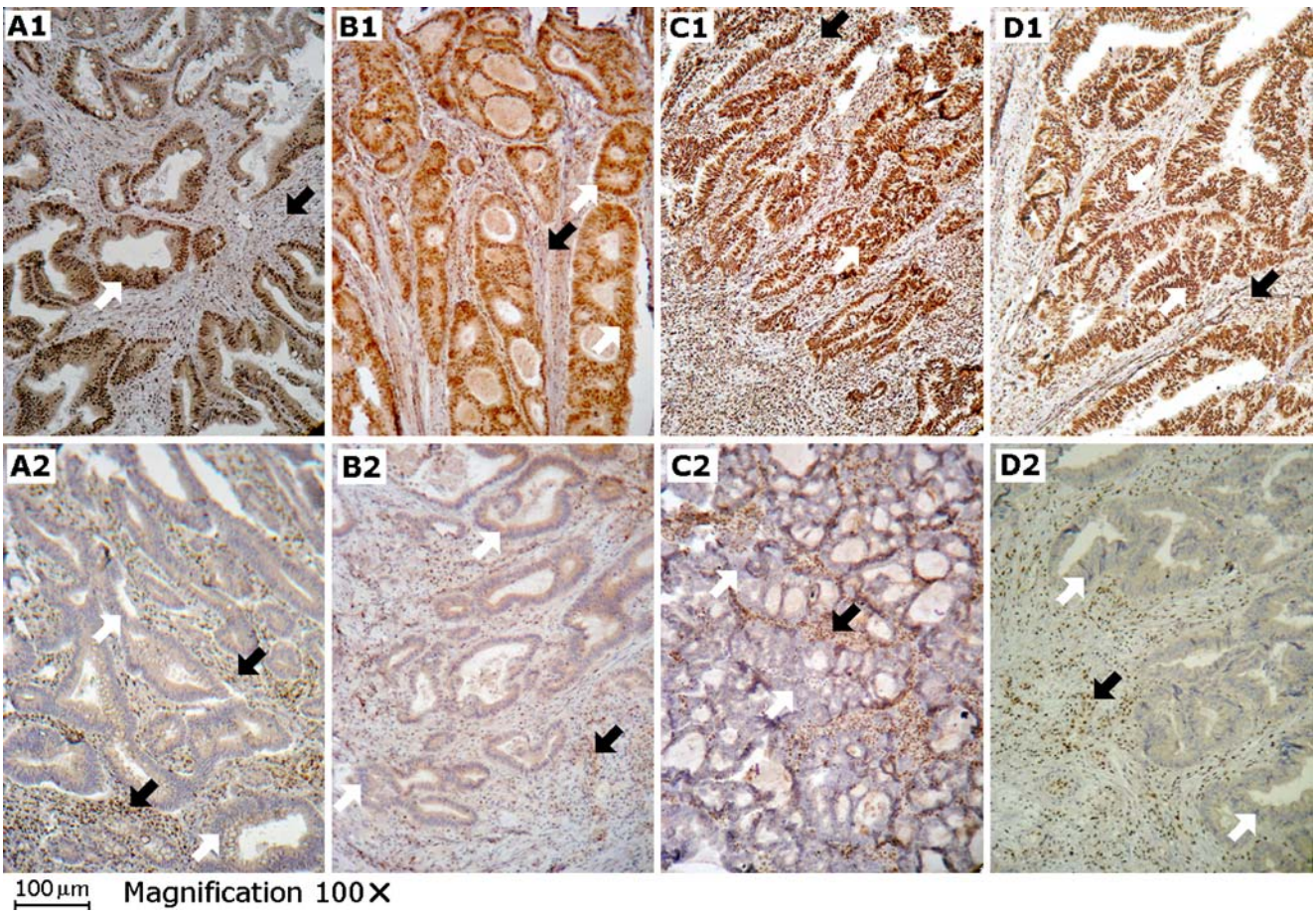

Fig. 1 Normal and abnormal staining patterns of four MMR proteins are presented as eight pictures: **a1**, **2** (MLH1); **b1**, **2** (MSH2); **c1**,**2** (MSH6); **d1, 2** (PMS2). **a1**–**d1** represent normal immunohistochemical staining for MLH1, MSH2, MSH6, and PMS2, respectively. Normal nuclear staining for MMR proteins is notable both in stromal cells (*black arrows*) and epithelial tumor cells (*white arrows*), as brownish accumulation of dye in the nucleus of the mentioned cells. **a2**–**d2** represent abnormal staining for MLH1, MSH2, MSH6, and PMS2, respectively. In these series of pictures, stromal cells (*black arrows*) still show normal staining for MMR proteins, while there is no dye accumulation in nucleus of epithelial tumor cells (*white arrows*)

Table 1 Comparison of clinical features between normal and abnormal MMRP groups

	Normal MMRP (n=295)	Abnormal MMRP (n=48)	Total (n=343)	P value
Mean age at diagnosis (year ± SD)	53.05±14.27	42.81±11.32	51.64±14.33	<0.001
Age at diagnosis				<0.001
<50	130	37	167	
50–70	128	9	137	
>70	36	1	37	
Median age (years)	53	42	51	
Male/female ratio	1.1:1	1.7:1	1.2:1	0.231
Family history of cancer				0.005
Colorectal	60	21	80	
Non-colorectal	68	9	77	
Negative	159	18	177	
Missing	8	–	8	
Location of the tumor				0.001
Proximal of colon	124	33	157	
Distal of colon and rectum	171	15	186	
TNM stage				0.130
Stage I	27	4	31	
Stage II	129	24	153	
Stage III	106	15	121	
Stage IV	24	4	28	
Missing	9	1	10	
Grade of differentiation				0.326
Low	178	27	205	
Intermediate	98	15	113	
High	19	6	25	

MMRP mismatch repair gene products

In order to determine how three variables of age at onset, family history for CRC, and tumor site could have predicted abnormalcy of MMRP patterns, we employed multivariate logistic regression analysis with the mentioned factors included (Table 2). It could be implied from the analysis that there were independent associations between onset age of less than 50 years, positive family history, and proximal tumors with the risk of having abnormal MMRP status.

Staining patterns

Among abnormal MMRP tumors, 19 (39.5%) showed complete loss of *MLH1* with normal *MSH2* immunoreactivity (*MLH1* negative), and 24 (50%) expressed complete loss of *MSH2* with normal *MLH1* immunohistochemical staining (*MSH2* negative). Five tumors (10.4%) showed complete loss of *PMS2* while expressing positive immunoreactivity for both *MLH1* and *MSH2*. Of 19 *MLH1*-negative tumors, 15 had simultaneous loss of *PMS2*, and 12 tumors out of 24 *MSH2*-negative tumors had simultaneous loss of *MSH6* expression.

According to our findings, 80 CRC patients (23%) had a positive family history of CRC, of whom 47 patients (13.7%) had a cancer onset age of less than 50 years old. Out of these 47 patients, 19 (5.5% of total) showed abnormal IHC staining of MMR proteins. The pattern of abnormal IHC staining in this group of CRC patients was

Table 2 Result of multivariate logistic regression analysis

Variable	OR	95% confidence interval		P value
		Lower bound	Upper bound	
Age at diagnosis, ≤50 vs. >50	5.950	2.687	13.175	<0.001
Family history of CRC, positive vs. negative	2.981	1.485	5.984	0.002
Tumor site, proximal vs. distal	3.759	1.835	7.702	<0.001

OR odds ratio

Table 3 Multivariate analysis of colorectal cancer-specific survival

Variable	HR	95% confidence interval		*P* value
		Lower bound	Upper bound	
MMRP status, normal vs. abnormal	2.763	0.613	12.449	0.186
Gender, female vs. male	0.740	0.314	1.748	0.493
Age at diagnosis, ≤50 vs. >50	1.679	0.700	4.331	0.266
Grade, high vs. moderate/low	0.449	0.190	1.062	0.068
Stage, 0/I/II vs. III/IV	0.443	0.184	1.066	0.069
Tumor site, proximal Vs. distal	0.137	0.018	1.036	0.054

HR hazard ratio

as follows: absence of *MLH1* was observed in one, *MLH1*/*PMS2* in five, *PMS2* in two, *MSH2* in six, and *MSH2*/*MSH6* in five patients.

Survival analysis

Twenty-eight (8.2%) patients died because of distant metastasis or local recurrence, four of whom had abnormal MMRP patterns and 24 did not. Multivariate survival analysis was performed on 342 CRC patients with covariates of gender, tumor site, tumor stage and grade, and age at diagnosis included (Table 3). Five-year follow-up was completed in 24 patients including 20 patients with normal MMRP status and four abnormal MMRP cases. A nonsignificant inclination towards better survival was observed among patients with abnormal MMRP (Fig. 2).

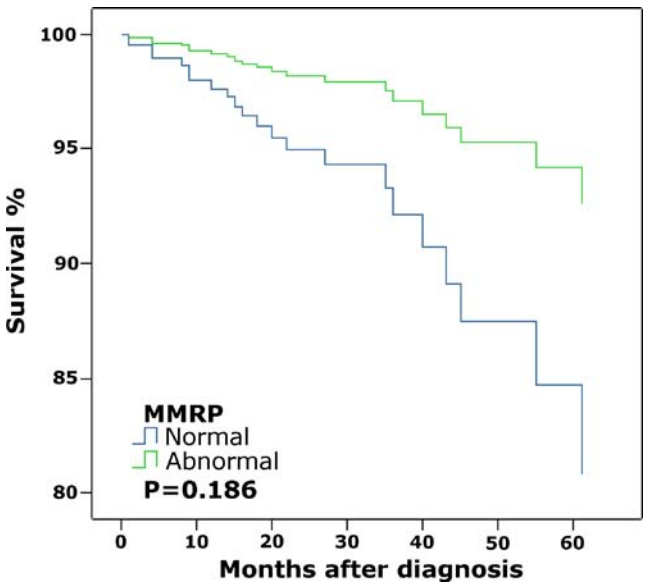

Fig. 2 Cancer specific survival of colorectal cancer patients with normal and abnormal immunohistochemistry for mismatch repair proteins according to Cox's proportional hazard model. The cumulative 5-year survival of patients with abnormal MMRP (*green line*) is better than that of the normal MMRP patients (*blue line*); however, results are not significant

Discussion

While a worldwide familial basis of about 15–20% has been reported for CRC [2, 7], recent studies from Iran have unveiled a familial trend of 29.4–35.1% for these type of cancers [38, 39], which would rise to 53% with the family history of other cancers included [39]. In this study, 23.3% of CRC patient had a positive family history of CRC, and 46% had a positive family history of cancer in general. Our results confirmed the findings of previous studies regarding the high-familial inheritance of CRC in Iran.

The present study was unique because for the first time in Iran, this strived to merge the clinical and familial profile of CRC patients to immunohistochemical evidences of ongoing genetic abnormalities. However, like the previous studies that contended with CRC in Iran, this study faced a major limitation: the bias generated by lack of a national cancer registry system [38–41]. This limitation impeded the inclusion of CRC patients into the accepted HNPCC criteria, just based on positive family history or age of cancer onset confidently, because the recall bias could decrease the accuracy of the data.

Of previous studies about CRC in Iran, only one has tried to depict an overview of HNPCC [39]. The mentioned study, reported a prevalence of 4.7% based on the Amsterdam II criteria. If we hypothetically consider the CRC patients with positive family history, early onset age and an abnormal MMRP on IHC as HNPCC, then the estimation of this study of prevalence of HNPCC will be 5.5% of the total CRC patients. This estimation is the highest among those recorded in Iran and is in accordance with the previously reported prevalence; however, studies from other parts of the world have generally reported lower estimates [2, 3, 6, 11, 42].

In our study, the sensitivity and specificity of IHC in contrast to microsatellite analysis could have been determined only by performing both procedures on every specimen, which was not feasible because of limitation of financial resources. However, high rate of isolated absence of *PMS2*, five (10.4%) of 48 abnormal MMRP, in this study allows us to predict a possible added value of about 10% to

IHC over microsatellite analysis. Absence of *PMS2* staining might be a sign of germline mutations in *MLH1* or *PMS2* [26]; this means that by using immunohistochemical staining for *PMS2*, more carriers of *MLH1* mutations can be identified [43]. Previously, staining for *PMS2* was recommended only in case of high suspicion for HNPCC in the absence of MSI. However, given an extra benefit of 23% in detecting the *MLH1* mutations, a recent study recommended the inclusion of *PMS2* staining in the panel of antibodies to identify families eligible for mutation analysis [43].

No isolated absence of *MSH6* was found in this study. Four tumors had isolated loss of *MLH1*, and 15 had loss of both *MLH1*/*PMS2*. Twelve tumors had isolated abnormal staining for *MSH2*, and 12 had absence of *MSH2*/*MSH6* heterodimer. However, isolated absence of *MLH1* was observed in just one of the 19 assumed HNPCC patients. Germline mutations are usually responsible for the absence of MSH2 proteins [44]; whereas, the absence of MLH1 can be indicative of a germline mutation or of somatic hypermethylation of its promoter that happens in sporadic CRC [45, 46]. Therefore, a likely explanation for the less frequent loss of MLH1 in this study is the higher prevalence of hereditary cancer in Iran.

To conclude, this study demonstrated the importance and feasibility of running large-scale CRC-screening programs in Iran. The guidelines of the National Institute of Cancer for screening CRC is advocated by the present study; moreover, we recommend IHC as a prescreening tool for determining the eligibility of patients for mutation analysis of MMR genes specially in populations with high-familial incidence of CRC like Iran [47]. According to the present study, in addition to *MLH1* and *MSH2*, *PMS2* should be entered into the panel of antibodies used for immunohistochemichal analysis of MMR genes. Although this might exact a substantial financial toll on the prescreening programs, the IHC analysis of *PMS2* seems to be inevitable especially in populations with higher incidence of hereditary colorectal cancer. Moreover, measures should be taken in future studies, to confirm the IHC results by MSI analysis, and to determine the concordance of IHC results with the mutation analysis measures.

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