Molecular Analysis of Iranian Colorectal Cancer Patients at Risk for Lynch Syndrome: a New Molecular, Clinicopathological Feature

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ORIGINAL RESEARCH

Molecular Analysis of Iranian Colorectal Cancer Patients at Risk for Lynch Syndrome: a New Molecular, Clinicopathological Feature

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Abstract
Purpose Microsatellite instability (MSI) and mismatch repair (MMR) gene expression present a hallmark mutational signature of Lynch syndrome as a common hereditary cancer predisposing condition. Since there is not enough data of molecular and clinicopathological aspects of the disease in Iranian populations, this article is a new description in Central Iran.
Methods It is a descriptive analytical study in which we screened 1659 colorectal cancer (CRC) patients based on early-onset disease and Amsterdam II criteria during 14 years (2000–2013). MSI testing was applied through a commercial kit evaluating five mononucleotide markers (BAT-25, BAT-26, MON0-27, NR-21, and NR-24) using a fluorescent multiplex PCR method. Immunohistochemistry (IHC) staining was set up to detect expression of four mismatch repair (MMR) genes including MLH1, MSH2, MSH6, and PMS2. SPSS 16 software was used to analyze the data.
Results Overall, 31 of 45 screened at-risk families were eventually included to MSI testing of which 9/31 patients (~29 %) showed MSI in their tumor tissues including 6 (19.4 %) MSI-H (high). BAT-26 was the most instable marker with instability in 7/31 MSI tumors (22.6 %). IHC-MMR staining was absent in 7/31 probands (22.6 %) of which in 4 cases, both MSH2/MSH6 (57.1 %) and, in 2 cases, both MLH1/PMS2 showed deficiency (28.6 %), and just in one case, MSH6 was defective (14.3 %). IHC-MMR was absent in all 6 MSI-H tumors while none of 3 MSI-L tumors were MMR-deficient. Just single MSH6-defective tumor showed MSS state. The frequency of CRC among MMR-deficient and MMR-proficient families was 67.5 and 27.9 %, respectively. The most common extracolonic cancer among both MMR-deficient and MMR-proficient groups was stomach, respectively, with 26.7 and 16.5 %.
Conclusions A different molecular and clinicopathological phenotype of tumors in CRC Iranian patients at risk for Lynch syndrome could suggest some new molecular mechanisms about which more evaluations are necessary.

Keywords Molecular analysis · Mismatch repair genes · Clinicopathological · Lynch syndrome · Iran

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome (LS) is a cancer susceptibility syndrome secondary to a germline mutation in at least one of the DNA mismatch repair genes (*MMRs*) including *MLH1*, *MSH2*, *MSH6*, and *PMS2* [1]. It leads to accumulate of mutations in other genes responsible to apoptosis and cell cycle control which accelerate some tumorigenesis events [2, 3]. Mutation analysis of *MMRs* is both time-consuming and expensive because of so heterogeneous mutations in these genes [4]. Two molecular screening tools are commonly being used to detect LS, including microsatellite instability (MSI) testing and immunohistochemical (IHC) staining of MMR proteins.

MSI refers to genomic instability of short tandem repeats (STRs), the stretched sequences of 1–5 base pairs repeating

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units with 25–60 bp in length distributed throughout the genome [5]. Actually, MSI is a mutational signature or a molecular fingerprinting [6], the hallmark of CRC concluded from DNA mismatch repair system deficiency [7]. MSI is identified as a change in allele size between tumor and normal DNA. It is found in almost 15–24 % of all CRCs [8] while in LS, including nearly 3 % of all CRCs, about all cases show MSI in tumor cells [7]. This contains 20–25 % of all MSI-CRCs. Other MSI-CRCs (75–80 %) are concluded from an epigenetic DNA-MMR deficiency due to the promoter hypermethylation of the *MLH1* gene [9]. Although MSI has enough sensitivity to identify LS, it will not be detected in about 5 % of the all LS tumors [10]. A fluorescent multiplex PCR-based method is currently applied for MSI testing. A pentaplex panel including mononucleotide repeats has had a sensitivity more than 95 % and specificity more than 98 % to identify CRC tumors with MMR deficiency [11].

MMR deficiency can be also detected using IHC staining in tumor sections; so, proficient nuclear staining implies the normal expression of *MMR* genes (IHC-present), and absent nuclear staining indicates the loss of *MMR* gene expression (MMR-deficiency). IHC has been reproduced as the complement of MSI-testing [10, 12], although it has been advised by some authors as the first screening method before genetic testing because of some advantages compared to MSI-testing [13]. IHC is more accessible than MSI as a routine service in the most pathology laboratories and a proficient, viable procedure at the time of colectomy [14]. Moreover, IHC is more inexpensive than MSI [15] being considered as a genetic testing by which a defective *MMR* gene would be recognized leading to efficient mutation analysis of the target gene [16, 17]. IHC-MMRs, however, have some limitations such as uncertainty in interpretation and low sensitivity to discover mutation of *MLH1* with MLH1 antibody alone [16].

CRC is the third most prevalent leading cause of cancer-related deaths in Iran [18], and its incidence has been increased over the last three decades in the country [19]. However, no systematic screening program has been set up so far in Iran to detect and evaluate hereditary colorectal cancer. Therefore, our study is a new trial to set up a screening system in center of Iran (Isfahan).

Materials and Methods

Study Design

A descriptive retrospective study was designed to select CRC patients at risk for Lynch syndrome in Central Iran. Among 1659 CRC patients registered in Poursina Hakim Research Center (PHRC), an important referral gastroenterology clinic in Central Iran, within 2000–2013, we at first selected all early-onset patients (patients with age ≤50). Then, we used Amsterdam II criteria for primary clinical screening including the following: having at least 3 affected members with one of the HNPCC-associated cancers (CRC, other GI cancers, endometrial, renal, breast (according to some resources), brain, skin, and pelvic cancers) in at least two successive generations, and one of these three members being a first degree relative of the other two and at least one diagnosed before the age of 50 years.

MSI Testing

Both tumor and normal tissue DNA are necessary for MSI testing. Although a pentaplex panel including two mononucleotide markers (BAT25 and BAT26) and three dinucleotide markers (D2S123, D5S346, and D17S250) is currently recommended by NCI, the National Cancer Institute, to analyze MSI [11, 20], the pentamerous mononucleotide markers have shown a higher specificity and similar or better sensitivity than dinucelotide markers to detect an MSI-H phenotype [21, 22].

We used a commercial kit from Promega (MSI Analysis System, Version 1.2) by which five mononucleotide markers (BAT-25, BAT-26, MON0-27, NR-21, and NR-24) are evaluated. It also contains two pentanucleotide markers (Penta C and Penta D) as specimen detector markers to identify specimen mix-ups. The markers are amplified through a fluorescent multiplex PCR-based method. Then, the amplified alleles are evaluated in matching pairs of test samples, which may be MMR-deficient and normal tissue samples. If new alleles are observed in the tumor sample without their presence in the corresponding normal sample, MSI is confirmed. Tumors would be considered as MSI-High (MSI-H) if at least two of five quasimononucleotide markers show instability and MSI-low (MSI-L) if only one marker is unstable.

Immunohistochemistry

A formalin-fixed paraffin-embedded (FFPE) tissue block is necessary for IHC on each case preferably from resected bowel specimen included tumoral and adjacent normal mucosa. After cutting the block and preparing at least four slides per case to evaluate four MMR proteins, the slides are incubated with Protein Block reagent and primary antibodies according to IHC guideline specific for each immunologic product. Then, they are incubated with Post Primary Block reagent for several minutes. In each step, the slides must be washed in TBS with gentle rocking for a short moment. The next step is developing peroxidase activity with DAB working solution for some minutes. Then, the slides are counterstained with hematoxylin and finally dehydrated, cleared, and mounted. Our slides were ready at the time for microscopic observation. If the MMR protein has been expressed, the nuclear staining will be present. MMR deficiency leads to absent nuclear staining in tumor section compared to normal adjacent tissue.

Data Analysis

We analyzed the obtained data by SPSS 16 software package (SPSS Inc., Chicago, IL, USA).

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Results

Altogether, 413 patients (24.9 %) were identified as early onset (age ≤50 years at diagnosis) after screening of 1659 CRC patients registered in PHRC within a 14-year period (2000–2013). Among 219/413 successful calls, 45 HNPCC families met finally Amsterdam II criteria and were candidate for molecular analyses of which 14 probands were excluded due to lack of their tumor tissues or being unwilling for incorporation.

Altogether, 9 of 31 studied HNPCC probands (~29 %) showed MSI in their tumor tissues (6 patients (19.4 %) with MSI-H). Male to female proportion in MSS probands was 11/11=1, and in MSI probands, it was 6/3=2. BAT-26 was the most instable quasimononucleotide marker with instability in 7/31 MSI tumors (22.6 %). In 6/31 cases (19.3 %), both BAT-25 and NR-24 markers showed instability, and both MONO-27 and NR-21 markers were instable in 5/31 (16.1 %) MSI-CRC tumors. All markers were instable (66.6 %) in 4/6 MSI-H patients, and among two rest patients, one showed instability in four markers (except MONO-27) and the other showed instability in two of them (BAT-26 and NR-24) (Figs. 1, 2, and 3).

IHC-MMR staining was absent in 7/31 probands (22.6 %) of which 4 cases were MMR-deficient (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 %). IHC-MMR staining was absent in all 6 MSI-H tumors while none of 3 MSI-L tumors were MMR-deficient. Among all IHC-MMR absent tumors, just single MSH6-defective tumor showed MSS state (4.5 %).

The age of MMR-deficient probands at diagnosis was averagely 38.0 years (range 31–50), while MMR-proficient probands had averagely 45.3 years at diagnosis (range 24–50) (*P* value <0.05).

There was a positive history of cancer in 186 members within 31 HNPCC families of which 140 affected members were in 24 MMR-proficient families and 46 cancer patients in 7 MMR-deficient families. The mean age of cancer patients among MMR-proficient and MMR-deficient families was 51.7 and 51.0, respectively (*P* value=0.817).

The most frequent tumor sites among MMR-deficient probands were as follows: rectum (41.7 %), sigmoid colon (33.3 %), cecum (12.5 %), and ascending colon (8.3 %); while among MMR-proficient probands were as follows: 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 MMR-deficient probands (Table 1).

The most frequent cancers among MMR-deficient families were as follows: CRC (67.4 %), stomach (8.7 %), hematopoietic system (6.5 %), and prostate, hepatobiliary tract, and lung (4.3 %), while in MMR-proficient families, CRC (39.3 %), stomach (10 %), lung (8.6 %), breast (7.9 %), and brain (6.4 %) were the most common cancers (Table 2).

Although 1 of 7 MMR-deficient probands (~14 %) was diagnosed at I or II pathological TNM stage, a third of MMR-proficient probands (8/24~33 %) were found at these early stages (Table 3). On the other hand, 11/24 of MMR-proficient probands (~46 %) had been deceased at the screening time while 6/7 of MMR-deficient probands (~86 %) were

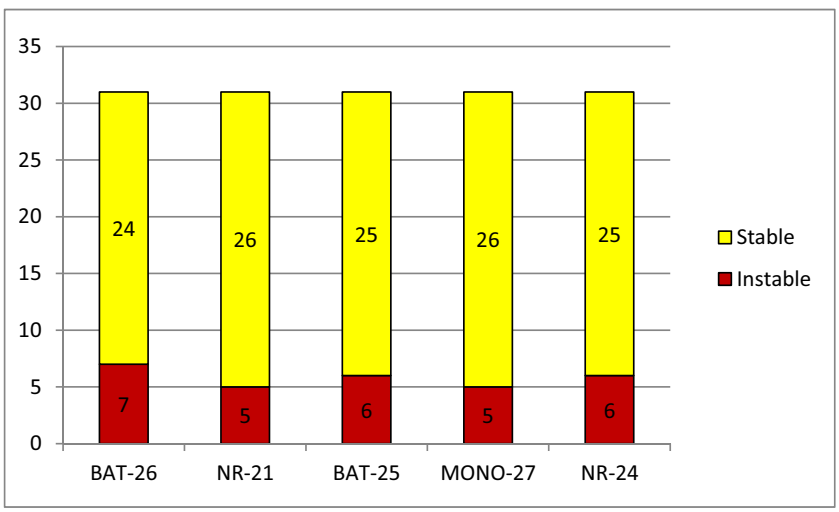

Fig. 1 Frequency of five quasimononucleotide markers in 31 Amsterdam positive tumors according to instability state

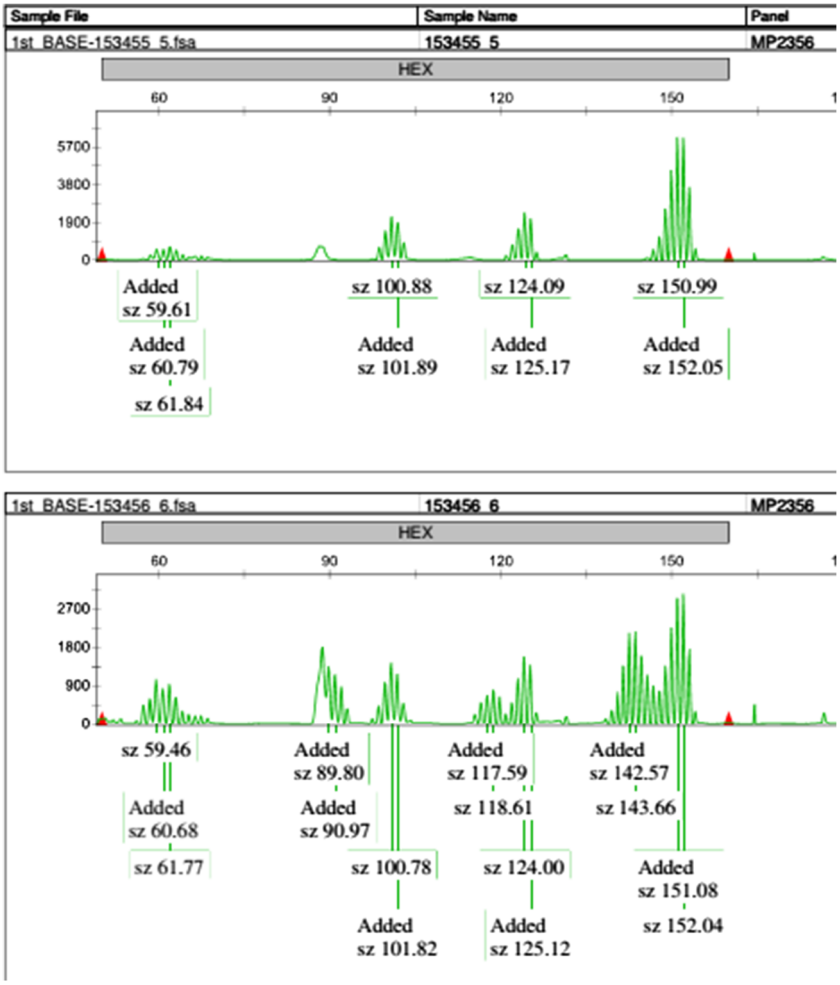

Fig. 2 An example of microsatellite instability in one of our DNA electropherogram trace: It is related to three quasimononucleotide markers including NR-21, BAT-25, and MONO-27, indicating normal stability state in upper sample (153455 5) as normal healthy tissue, and instability state in every three markers in lower sample (153456 6) as tumor tissue

alive at this time. The survival period of the probands, the interval between diagnosis time, and date of death or date of analysis were averagely calculated for MSS, MSI-L, and MSI-H group of the probands as 6.1, 2.0, and 5.8 years, respectively (P value=0.341).

Discussion

We used being early-onset disease (age at diagnosis ≤50 years) and Amsterdam II criteria, simultaneously, to primary selection of our samples. Somewhat, high prevalence of familial

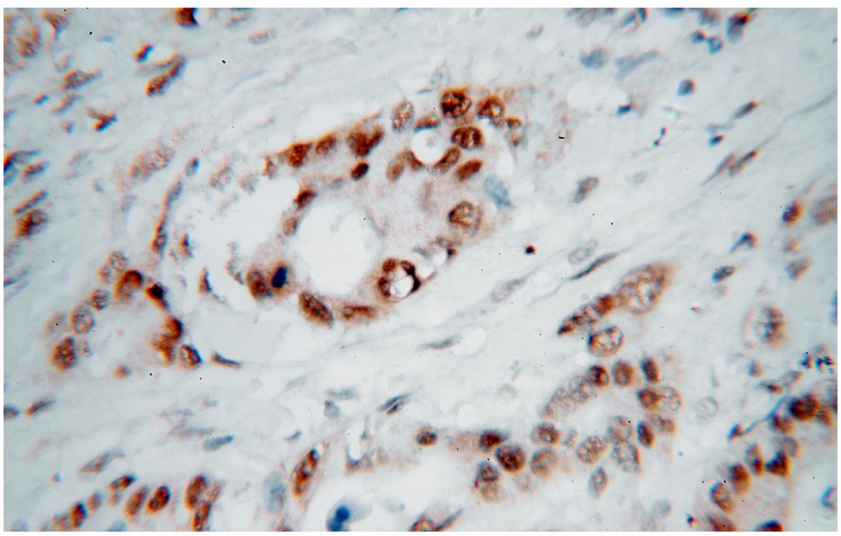

Fig. 3 A positive immunohistochemical staining of paraffin-embedded colorectal cancer tissue using MSH2 antibody at dilution of 1:100 (under ×40 lens)

Table 1 Frequency of tumor sites in Iranian colorectal cancer patients at risk for LS in both MMR deficient and MMR proficient groups

Tumor site	MMR-proficient families		MMR-deficient families	
	Frequency	Percent	Frequency	Percent
Cecum	3	12.5	1	14.3
Ascending colon	2	8.3	2	28.6
Transverse colon	0	0.0	1	14.3
Descending colon	0	0.0	2	28.6
Sigmoid colon	8	33.3	1	14.3
Rectum	10	41.7	0	0.0
Unknown	1	4.2	0	0.0
Total	24	100.0	7	100.0

LS Lynch syndrome, *MMR* mismatch repair genes

CRC among our population and the limitation of our financial resources made us using these more stringent specific criteria.

Microsatellite Instability Analysis

According to instability rate among five quasimononucleotide markers of pentaplex Promega panel in our MSI-CRC tumors, 16.1 to 22.6 %, apparently all of these five markers were determinant to detect MSI status of our probands. In different studies, however, variable molecular phenotypes for these markers have been described. For example, in a recent Iranian study on 80 sporadic CRC patients, the most instable markers were NR-21, NR-24 with 45 %. In this study, NR-27 with zero and BAT-26 with 6.7 % instability were the most stable markers [23]. Also, in another Iranian study on 80 sporadic CRC and 80 HNPCC patients, NR-21 was determined as the most frequent instable marker among five quasimonomorphic markers with 53 and 25.6 % in sporadic CRC and HNPCC tumors, respectively. NR-27 was also determined as the most stable marker with 0 and 19.2 % in sporadic CRC and HNPCC tumors, respectively [24]. In the only performed study on MSI testing in HNPCC patients in Isfahan, just BAT-26 marker was analyzed in blood samples of 40 HNPCC patients of which 12 cases (30 %) showed instability [25]. Since no specific clinicopathological criteria have been mentioned to screen the patients in the study and just blood DNA has been tested, apparently, we cannot compare our results to their findings. Other similar studies among different ethnic populations around the world have shown various results [26–28]. It seems that instability feature of mononucleotide markers varies among different populations due to ethnical variation in frequency of these markers. It may be also variable in sporadic CRCs compared to LS-CRCs, an issue about which more evaluations on larger samples are necessary.

Table 2 Frequency of cancer locations among Iranian colorectal cancer patients at risk for LS in both MMR-deficient and MMR-proficient groups

Cancer type	MMR proficient		MMR deficient		Sum
	Frequency	Percent	Frequency	Percent	
CRC	55	39.3	31	67.4	86
GC	14	10.0	4	8.7	18
Lung	12	8.6	2	4.3	14
Breast	11	7.9	1	2.2	12
Brain	9	6.4	0	0.0	9
HBC	7	5.0	2	4.3	9
Intestine	6	4.3	0	0.0	6
Prostate	4	2.9	2	4.3	6
Uterus	4	2.9	1	2.2	5
Skin	3	2.1	0	0.0	3
HP	3	2.1	3	6.5	6
Bladder	3	2.1	0	0.0	3
Thyroid	2	1.4	0	0.0	2
Testis	2	1.4	0	0.0	2
Bone	2	1.4	0	0.0	2
Kidney	1	0.7	0	0.0	1
Pancreas	1	0.7	0	0.0	1
Nasopharynx	1	0.7	0	0.0	1
Total	140	100	46	100	186

LS Lynch syndrome, *MMR* mismatch repair genes

Immunohistochemistry of MMR Proteins

Given the high sensitivity of simultaneous usage of both IHC and MSI testing to identify MMR deficiency [29], apparently, a significant portion of Amsterdam positive families in our population has no *MMR* mutations. It highlights the role of other genes in etiology of the most our samples. Although there is no explicit data of 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" [30, 31]. More studies on larger samples using complementary techniques such as mutation analyses could estimate a more accurate prevalence of FCC-X among Iranian population.

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 [2]. Consequently, *MSH2* was responsible gene in 57 % of the MMR-deficient cases. The frequency of *MSH2* defect in our study was similar to some large early studies [32]. MMR-deficiency for both MLH1 and PMS2 proteins in about 29 % of the tumors

Table 3 Frequency of TNM pathological stage at diagnosis time among Iranian colorectal cancer patients at risk for LS in both MMR-deficient and MMR-proficient groups

MMR genes function	Stage I		Stage II		Stage III		Stage IV		Total	
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent
Deficient	7	29.2	1	4.2	11	45.8	5	20.8	24	100
Proficient	0	0.0	1	14.3	5	71.4	1	14.3	7	100
Total	7	22.6	2	6.5	16	51.6	6	19.4	31	100

LS Lynch syndrome, *MMR* mismatch repair genes

predicts existence of germline mutation in *MLH1* of near to 30 % of the MMR-deficient probands. It is comparable to some early valid studies [33]; however, developing the results by mutation analyses, preferably on a larger sample, will be more informative. Since individual PMS2 loss in IHC-staining is the rarest event [34], we identified also no proband with absent IHC-staining for PMS2.

Although all our MMR-deficient cases showed MSI-H in MSI testing for main MLH1 and MSH2 antibodies, the single IHC-MSH6 absent case presented MSS (14.3 %). Some mutations in MSH6 gene may not be identified by MSI testing. It is due to the secondary nature of MSH6 protein in MutS complexes in which functional redundancy of this protein with MSH3, another accessory MMR protein, could explain MSS or MSI-L phenotype of the tumor, at least in some cases [18, 35].

Phenotype–Genotype Correlation

Genetic pedigree related to MSH6-defective proband shows fewer affected relatives than MSH2/MLH1-defective probands (3 versus 6.5 cases, averagely). According to some studies, we expect that the patients with *MSH6* mutations would be more likely Amsterdam negative [36]; so, the Bethesda guidelines are more sensitive than the Amsterdam Criteria to identify it [20]. Consequently, we may find more MSH6-defective patients among all our CRC patients using Bethesda guidelines.

Age at diagnosis in MMR-deficient probands was averagely more than 7 years earlier than probands without MMR-deficiency (38 versus 45.3 years) (P value <0.05). Meanwhile, there was no meaningful difference between other cancer patients in both groups of families (51 versus 51.7 years) (P value=0.817).

Mean age at diagnosis in the patients with MLH1 deficiency was 8 years earlier than the MSH2-deficient patients (~42 versus ~50 years old, P value <0.01). Moreover, there were more extracolonic cancer types among the MSH2-deficient families compared to the families with MLH1 or MSH6 defects (7 types versus 4 and 2 types, respectively). A genotype–phenotype correlation has been reported 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 is a higher risk of multiple extracolonic cancers, and the mean age of diagnosis is more than MLH1 mutation carriers [37, 38] .

Mean age of three cancer patients at diagnosis in the single MSH6-deficient family was about 67 years, averagely two decades later than the patients in families with MLH1 or MSH2 defects. The proportion of CRC patients in this family was also considerably lower than LS families with MSH2 or MLH1 defects (33 % versus 72 and 66 %, respectively). *MSH6* mutations present a phenotype somewhat different than *MLH1* and *MSH2* mutations; so, this condition has been described as "MSH6 syndrome" [36]. Mean age at cancer diagnosis in *MSH6* mutation carriers is at least one decade more than *MSH2* or *MLH1* mutation carriers [37]. In addition, the risk of CRC affection in families with MSH6 defect is more likely less than LS families with MSH2 or MLH1 defects [39]. Some studies have shown that CRC risk among FCC-X families is lower than HNPCC families. Also, CRC diagnosis has occurred averagely 10–15 years later in FCC-X families [26, 40].

According to our findings, 57.1 and 20.8 % of our index CRCs with and without MMR deficiency were localized proximal to the splenic flexure, respectively (P<0.01). More studies on families with Amsterdam criteria that have shown a higher proportion of CRCs are located proximal to the splenic flexure in patients with MMR deficiency than those with proficient MMRs [40–42].

Although there was no meaningful difference between average count of cancer patients among families in both MMR-deficient and proficient groups (6.6 versus 5.8, P value= 0.513), the relative frequency of CRC to all cancer patients among MMR-deficient families was mainly more than families without MMR deficiency, nearly 2.5-fold (67.5 versus 27.9 %). Meanwhile, we had asked cancer-related family history up to three generations in both groups.

The proportion of the survivors at screening in MMR-deficient group to proficient one was about 2-fold. Meanwhile, the early-stage diagnosis among MMR-

proficient group was more than 2-fold of MMR-deficient probands, according to their pathological documents. It may refer to better survival of MSI-CRCs compared to MSS CRCs, a fact that has been considered in some studies [43].

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Conflict of Interest The authors declare that they have no conflict of interest.

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