RESEARCH ARTICLE

Evaluation of MT1XT20 Single Quasi-Monomorphic Mononucleotide Marker for Characterizing Microsatellite Instability in Persian Lynch Syndrome Patients

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Abstract

Background: Colorectal malignancies with high microsatellite instability (MSI-H), either hereditary (Lynch syndrome) or sporadic, demonstrate better prognosis and altered response to 5FU chemotherapy. It is now recommended to perform MSI testing for all new cases of colorectal cancer regardless of being categorized as hereditary or sporadic. For MSI detection, immunohistochemistry or PCR-based protocols using a cohort of various sets of STR markers are recommended. Here we aimed to evaluate a simplified protocol using just a single STR marker, MT1XT20 mononucleotide repeat, for detection of MSI in Lynch syndrome patients. A Promega five-marker MSI testing panel and immunohistochemistry (IHC) were used as the gold standard in conjunction with MT1XT20. Materials and Methods: Colorectal patients with a positive history of familial cancers were selected by evaluating medical records. Based on Amsterdam II criteria for Lynch syndrome 20 families were short listed. DNA was extracted from formalin fixed paraffin embedded tumour and adjacent normal tissues resected from the index case in each family. Extracted DNA was subjected to MT1XT20 mononucleotide marker analysis and assessment with a commercially available five marker MSI testing kit (Promega, USA). IHC also was performed on tissue sections and the results were compared with PCR based data. Results: Eight (40%), seven (35%) and five (25%) cases were MSI positive using with the Promega kit, IHC and MT1XT20, respectively. Among the markers included in Promega kit, BAT26 marker showed instability in all 8 samples. NR24 and NR21 markers showed instability in 7 (87.5%), and BAT25 and MONO 27 in 6 (75%) and 5 (62.5%). Conclusions: Although MT1XT20 was earlier reported as a valid standalone marker for MSI testing in CRC patients, we could not verify this in our Iranian patients. Instead BAT26 among the markers included in Promega MSI testing kit showed instability in all 8 MSI-H CRC samples. Therefore, it seems BAT26 could act well as a single marker for MSI testing in Iranian CRC patients.

Keywords: Colorectal cancer - Lynch syndrome - MSI - IHC - MT1XT20 - quasi-monomorphic repeats - Iran

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Introduction

Lynch syndrome (LS), formerly known as hereditary nonpolyposis colorectal cancer, is the most common form of hereditary colorectal cancers (Lynch et al., 2006). LS is clinically defined as an autosomal dominantly inherited multicancer condition (Zeinalian et al., 2015b) and characterized by germ line mutation in genes responsible for DNA mismatch repair (MMR): MLH1, MSH2, MSH6, MSH3, and PMS2 (Boland, 2013) (Table 1).

In 1993 it was found that loss of MMR function lead to the phenomenon of microsatellite instability (MSI) in CRC tumors (Ionov et al., 1993; Thibodeau et al., 1996; Boland, 2013). Microsatellites are highly polymorphic, short, tandemly repeated nucleotide sequences of 1-6 bases distributed throughout the human genome (Tautz, 1989; Chaksangchaichot et al., 2007). In such tumor cells, typically half or more of all microsatellites demonstrate mutations, contraction or expansion (Shemirani et al., 2011). This phenotype, referred to as high-level microsatellite instability (MSI-H) tumor (Zeinalian et al., 2015a). So microsatellite instability (MSI) serves as

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Table 1. Genes Involved in HNPCC[a]

Gene name	Bacterial homolog	Location	Frequency of mutations	Function	Interactions	Involvement in other cancers
MSH2/HNPCC1	*MutS*	2p22	About 60%	Mismatch recognition	Heterodimerizes with MSH6 to form MutS alpha / Heterodimerizes with MSH3 to form MutS beta	Turcot syndrome Muir-Torre syndrome
MLH1/HNPCC2	*MutL*	3p21	About 30%	Mediates protein-protein interactions during mismatch recognition	Heterodimerizes with PMS2 to form MutL alpha	Turcot syndrome Muir-Torre syndrome
MSH6/HNPCC5	*mutS*	2p16	About 7-10%	Mismatch recognition	Heterodimerizes with PMS2 to form MutS alpha	Turcot syndrome Familial endometrial cancer
PMS2/HNPCC4	*MutL*	7p22	Rare	Endonuclease	Heterodimerizes with MLH1 to form MutL alpha	Turcot syndrome
PMS1/HNPCC3	*MutL*	2q31-q33	Case report	Probably involved in the repair of mis-matches	Heterodimerizes with MLH1	
MLH3/HNPCC7	*MutL*	14q24.3	Under debate	Redundant of PMS2	Heterodimerizes with MLH1 to form MutL alpha	Susceptibility to endometrial cancer
TGFBR2/HNPCC6		3p22	Case report	Tumor suppressor	Bins to another TGFBR2 and 2 TGFBR1 to form receptor complex/ binds TGF-beta/	Somatic esophageal cancer Loeys-Dietz syndrome type 2
EPCAM/TACSTD1/ HNPCC8		2P21	Rare	3' EPCAM deletions lead to MSH2 promoter hyper-methylation and inactivation		

[a] www.omim.org

an excellent, easy to evaluate marker of mismatch repair deficiency in LS patients (Lynch and de la Chapelle, 2003).

LS patients are at risk of developing frequent metachronous colon cancer (Fukutomi et al., 2002) or extra colonic tumors (including endometrium and, to a lesser extent, ovary, stomach, hepatobiliary tract, small bowel, pancreas, brain) and increased risk of cancer in family members (Lynch et al., 2006). In addition, colorectal malignancies demonstrating MSI, have a relatively favorable prognosis (Haghighi et al., 2009), altered response to 5-Fluorouracil (5FU) based adjuvant chemotherapy (Ribic et al., 2003; Deschoolmeester et al., 2008) and altered operative treatment surgery approach (Rodriguez-Bigas and Möeslein, 2013). Several studies indicate that benefit from 5-FU adjuvant chemotherapy may be restricted to non MSI-H colorectal cancer patient (Ribic et al., 2003; Ng and Schrag, 2010; Tejpar et al., 2011).

Accordingly, identification of the MSI phenotype is an independent prognostic tool leading to a better management of LS and is extensively accepted as an standalone method for LS confirmation in suspected colorectal patients (Ward et al., 2002; Deschoolmeester et al., 2008; Shia, 2008; Rodriguez-Bigas and Möeslein, 2013).

In 1997 the National Cancer Institute proposed a panel of five microsatellite markers for MSI detection including two mononucleotide repeats and three dinucleotide repeats known as the Bethesda panel (Boland et al., 1998). In 2004 to improve the accuracy of MSI testing using the Bethesda panel of MSI markers, a panel of five mononucleotide markers (known as the Promega panel) was developed (Bacher et al., 2004).

At present MSI testing offer to the CRC patients demonstrating Amsterdam and/or Bethesda criterion, usually using a recommended panel of five monomorphic markers. However, due to the laborious nature and high cost of multimarker MSI testing, efforts have been employed to find simpler alternatives with less number of markers (Patil et al., 2012). In order to develop an easier, cost effective and rapid method without compromising the sensitivity and specificity issues, various studies have performed to assess the reliability and accuracy of single mononucleotide repetitive markers for MSI testing (Buhard et al., 2006; Xicola et al., 2007; Deschoolmeester et al., 2008).

In this study, we have evaluated the sensitivity and specificity of MT1XT20 mononucleotide marker for MSI testing in colorectal patients showed Amsterdam II criteria. The five markers Promega MSI testing commercial kit used as the gold standard. However, for the sake of comparison between PCR-based methods and IHC we have performed IHC detection of all four important MMR proteins as well.

Table 2. The Promega Panel Mononucleotide Markers in Comparison with MT1XT20 Single Marker

Marker	Gene	GenBank number	Location	Repeats	Length	Fluorescent dye	Number of stable samples	Number of instable samples
Promega markers:								
BAT26	*MSH2*	U41210	Intron 5	26(T)	103-115 bps	FAM/FL	12	8
NR24	*ZNF2*	X60152	3'UTR	24(T)	130-133 bps	NED/TMR	13	7
NR21	*SLC7A8*	XM033393	5'UTR	21(T)	94-101 bps	HEX/JOE	13	7
BAT25	*c-Kit*	L04143	Intron 16	25(T)	114-124 bps	HEX/JOE	14	6
MONO 27	*MAP4K3*	AC007684	5'UTR	27(T)	142-154 bps	HEX/JOE	15	5
Single marker:								
MT1XT20	*MT1X*	KJ891650.1	3' UTR	T20	185-190 bps	Cy5	15	5

Materials and Methods

Population study

The study was approved by Isfahan University of Medical Sciences Ethical Committee. The cancer registry of Poursina Hakim CRC clinical center with 1659 registered cases was screened for selection of CRC patients with LS criteria like age of CRC onset below 50 and occurrence of frequent CRC or other related cancers in his/her family members. All patients requested to fill up a structured questionnaire collecting all important data regarding their own and family history of CRC and other related cancers. Informed written consent was obtained from all individuals participated in the study. Among all 1659 patients, 40 patients were selected with strict criteria of Amsterdam II. Eventually, on the basis of paired tissues availability (CRC resected tissue and its paired normal adjacent tissue) and the patients' cooperation, 20 of them were selected for the present study.

Genomic DNA extraction

Genomic DNA was purified from 20 CRC and 20 adjacent normal formalin-fixed, paraffin-embedded (FFPE) tissues. Briefly, two 10 micrometer tissue slices placed in a clean 1.5 ml Eppendorf tube and incubated at room temperature for 15 minutes in 1 ml xylene for deparaffinization. Then the tubes were centrifuged at 13000 RPM for 5 minutes. Rehydrated by stepwise passing through grades of absolute ethanol to 30% and then distilled water. After rehydration step, centrifugation and drain of supernatant, 600 microliters of 1% SDS, 30 microliters of 20 mg/ml proteinase K and 20 beads of Chelex 20 were added to the samples and incubated overnight at 55 °C in a water. Centrifuged and supernatants were collected in a clean Eppendorf tube and subjected to phenol-chloroform extraction.

Multiplex PCR reactions for Promega MSI testing system

Promega MSI testing kit (MSI Analysis System, Version 1.2) included 5 quasi-monomorphic markers (NR-21, BAT-26, BAT-25, NR-24 and Mono-27) for MSI detection and two pentanucleotide markers (Penta C and Penta D) as sample detector to distinguish probable specimen mix-ups (Table 2). The multiplex PCR assay was carried out according to the manufacturer's recommended protocol. Subsequently, PCR products were analyzed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) as recommended by Promega. The peaks were interpreted employing GeneMapper 3.7 software (Applied Biosystems). Any discrepancy between the number of alleles in tumor and its paired normal tissue was considered as instability in microsatellite repeat sequences. Specimens with more than two of five microsatellite markers alteration were considered as MSI-H, two or one altered microsatellite markers categorized as MSI low (MSI-L) and those without any microsatellites alteration considered as microsatellite stable (MSS).

Mononucleotide marker assay

The amplification of MT1XT20 mononucleotide marker was carried out in a total volume of 25 μl consisted100 ng of tumor or normal tissue extracted DNA, 2.5 μl of 10X PCR buffer, 0.4 mM of each reverse and forward primers (Forward 5'-CAGCTGTGCTCTCAGATGTAAA-3' and Reverse 5'-CCAAGTGCCATATACCCAGTGA-3'), 0.5 Units of Taq polymerase enzyme, 0.2 mM of each dNTPs and 1.5 mM of $MgCl_2$.

Tubes were then placed in an Eppendorf thermocycler (Germany) and subjected to following thermal cycling condition: initial denaturation at 95 C° for 5 minutes, followed by 30 cycles of 94°C, 58°C and 72°C all for 30 seconds and 1 final extension cycle at 72°C for 10 minutes. The 5' end of reverse PCR primer was fluorescently labeled with Cy5 fluorescent dye. PCR products then analyzed using Automated Laser Fluorescence Express (ALF express) sequencer and AlleleLinks software provided by the manufacturer.

IHC analysis

Tumor and paired normal specimens were prepared on at least 4 slides for IHC analysis. The expression assessment of MSH2, MLH1, MSH6 and PMS2 proteins was performed on 1-2 μm thick sections of formalin-fixed and paraffin-embedded tissues. After deparaffinization of sections in xylene and rehydration through graded alcohols, the antigen retrieval step was performed in a pressure chamber at microwave for 20 minutes using Tris-EDTA, pH 9.0 buffer, then sections were washed in dH2O. In order to neutralize the endogenous peroxidase, the slides were incubated in hydrogen peroxide for at least 5 minutes and were washed in PBS twice for 5 minutes each.

For blocking of non-specific background staining, each section were blocked by protein blocking solution for 5 minutes. After washing the sections in PBS two times for 5 minutes each, the primary diluted antibodies, including: NCL-MSH2, NCL-L-MLH1, NCL-L-MSH6 and NCL-

L-PMS2 (Leica Biosystems), were added to each section and incubated overnight at 4°C.

The primary antibody solution was removed after twice washing the slides in PBS and followed by 30 minutes incubation with Post Primary Block reagent. After washing twice in PBS, slides were incubated in Novolink Polymer for 30 minutes, washed and DAB substrate solution added. The process followed by counterstaining with hematoxylin and ultimately dehydration, clearing and mounting of slides. Evaluation of slides was made by an experienced pathologist and categorized as MMR-proficient or MMR-deficient based on the presence or absence of MMR protein nuclear stains.

Statistical analysis

Sensitivity and specificity and positive predictive values of clinical parameters were analyzed using SPSS 19 software package for Windows (SPSS Inc., Chicago, IL, USA).

Results

Cancer type, location, age and gender

In this study 20 CRC patients selected on the basis of Amsterdam II criteria for whom paraffin embedded and formalin fixed tissue blocks from already resected colon tumors were available. In family members of MMR-deficient and MSI-H cases, the following cancers were frequently detected: colorectal, gastric, small bowel, hematopoietic, prostate and breast (Table 3a). In these cases the most frequent tumor locations were ascending and descending colon (Table 4a). However, in family members of IHC-proficient or MSI-L/MSS cases

Table 3. Frequencies of Cancer Locations among Iranian Colorectal Cancer Families (A) MSI-H and MMR-Deficient Groups. (B) MSS/MSI-L and MMR-Proficient Groups

A				
Cancer Type	MSI-H Families		MMR-deficient Families	
	Frequency	Percent	Frequency	Percent
CRC	28	65.1	27	64.2
Small Bowel	3	7	2	4.7
Hematopoietic	3	7	3	7.1
GC	2	4.6	4	9.5
Breast	2	4.6	1	2.3
Prostate	2	4.6	2	4.7
Lung	1	2.3	1	2.3
Other	2	4.6	2	4.7
Total	43	100	42	100
B				
Cancer Type	MSS/MSI-L Families		MMR-proficient Families	
	Frequency	Percent	Frequency	Percent
CRC	22	33.3	23	34.3
Small Bowel	3	4.5	4	5.9
Hematopoietic	1	1.5	1	1.4
GC	10	15.1	8	11.9
Breast	5	7.5	6	8.9
Prostate	3	4.5	3	4.47
Lung	6	9	6	8.9
Other	16	24.2	16	23.8
Total	66	100	67	100

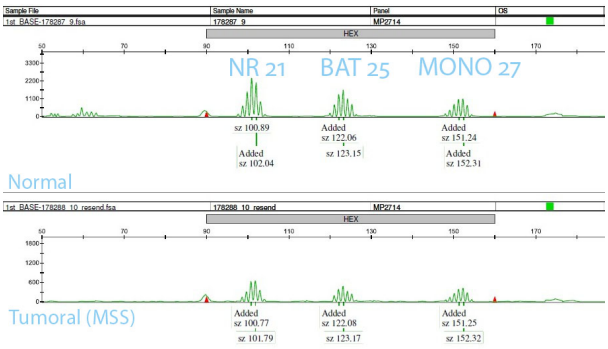

Figure 1. DNA Electropherograms of Promega MSI Analysis System (GeneMapper v3.7 Software), Indicating Stable Status of Both Normal and Tumoral Tissues and Unstable State of Tumoral Tissue Compared with Normal Paired Tissue

gastric, lung and breast cancers was detected as the most frequent cancer types (Table 3b). The most common involved sites in this group of patients were sigmoid and rectum (Table 4b). The average age of MSI-H patients was 38.5 years at tumor diagnosis time (range 31 to 49), and that of MSI-L/MSS patients 46.5 years (range 24 to 64). Most of MSI-H tumors, were detected as poorly or moderately differentiated adenocarcinoma, but there was no mucinous adenocarcinoma among these tumors. The most frequent tumor type among MSI-L/MSS patients was well differentiated adenocarcinoma (Table 5).

Promega five markers MSI testing panel and MT1XT20 single marker MSI analysis

Using Promega commercial MSI testing kit, 8 samples showed high instability in microsatellite markers (MSI-H)

Table 4. Frequency of Tumor Sites in Iranian Colorectal Cancer Patients (A) MSI-H and MMR-Deficient Groups (B) MSS/MSI-L and MMR-Proficient Groups

A				
Cancer Location	MSI-H Cases		MMR-deficient Cases	
	Frequency	Percent	Frequency	Percent
Cecum	1	12.5	1	14.2
Ascending colon	2	25	2	28.5
Transverse colon	1	12.5	1	14.2
Descending colon	2	25	2	28.5
Sigmoid colon	1	12.5	1	14.2
Rectum	1	12.5	0	0
Unknown	0	0	0	0
Total	8	100	7	100
B				
Cancer Location	MSS/MSI-L Cases		MMR-proficient Cases	
	Frequency	Percent	Frequency	Percent
Cecum	1	8.3	1	7.6
Ascending colon	0	0	0	0
Transverse colon	0	0	0	0
Descending colon	0	0	0	0
Sigmoid colon	6	50	6	46.1
Rectum	4	33.3	5	38.4
Unknown	1	8.3	1	7.6
Total	12	100	13	100

Table 5. Frequency of Tumor Phenotypes in Iranian Colorectal Cancer Patients at Risk for LS in both MSI-H and MSS/MSI-L Families

Tumor Grading	MSI-H Cases		MSI-L/MSS Cases	
	Frequency	Percent	Frequency	Percent
Well differentiated adenocarcinoma	2	25	5	41.6
Poorly differentiated adenocarcinoma	3	37.5	3	25
Moderately differentiated adenocarcinoma	3	37.5	3	25
Unknown	0	0	1	8.3
Total	8	100	12	100

Table 6. Comparison between (a) MSI Analysis by Promega kit and IHC Results (b) MSI Analysis by MT1XT20 marker and IHC Results (c) MSI Analysis by Promega kit and by MT1XT20 Marker Results

a	IHC Analysis	
MSI analysis by Promega kit	MMR-proficient	MMR-deficient
8 MSI-H cases	2	6
12 MSS/MSI-L cases	11	1
b	IHC Analysis	
MSI analysis by MT1XT20 marker	MMR-proficient	MMR-deficient
5 microsatellite instable cases	2	3
15 microsatellite stable cases	11	4
c	MSI analysis by MT1XT20 marker	
MSI analysis by Promega kit	Microsatellite instable cases	Microsatellite stable cases
8 MSI-H cases	5	3
12 MSS/MSI-L cases	0	12

(40%) and 12 samples showed low instability or stable status (60%). Female to male proportion in MSI-H cases was 4/4=1 and in MSI-L/MSS was 8/4=2. Among 5 quasimononucleotide markers included in Promega kit, BAT26 marker with instability in all 8 samples was the most instable marker (40%). Both NR24 and NR21 markers showed instability in 7 cases (35%), BAT25 and MONO 27 markers were instable in 6 (30%) and 5 (25%) specimens respectively (Figure 1)

For MT1XT20 quasimonomorphic marker size shift of tumoral sample in comparison to its adjacent normal tissue was considered for categorization of the samples as MSI-H or MSS. Figure 2 shows an example of instable repeats for MT1XT20 marker in tumoral and normal samples. Four alleles were detected for MT1XT20 marker in our study population ranging from 185 to 190 bps. Five samples were detected as MSI-H using MT1XT20 as standalone marker.

IHC staining

Seven cases (35%) showed lack of expression (abnormal staining) of at least one of the MMR proteins in their tumor specimens (designated as IHC-A for absence of one or more MMR proteins). The remaining 13 cases were detected with no defects in any one of the MMR proteins (IHC-P or IHC-present group). Among the 7 IHC-absent cases, 4 samples showed negative staining for either MLH1 and PMS2 proteins (57 %), 2 samples were negative for MSH2 and MSH6 (28.5%) and the remaining one sample showed loss of MSH6 protein expression (14%).

Diagnostic sensitivity and specificity evaluation and positive predictive values

MT1XT20 marker showed 62.5% sensitivity and 100% specificity. Positive predictive values (PPV) for MT1XT20 marker was 100%. The sensitivity and specificity of the IHC staining method was 75% and 91.67% respectively with PPV of 85.71% (Table 6).

Discussion

The annual occurrence of colon and rectum cancers is 6–7.9 per 100,000 in Iran, with approximately 4.7% of them diagnosed as LS (Nemati et al., 2012). About 70-90% of LS patients show instability in microsatellite sequence repeats (Fishel, 1999). In LS one or more MMR genes (MLH1, MSH2, MSH6, and PMS2) undergo inactivation germ line mutation (Vatandoost et al., 2016). Some non-LS colorectal cancer also show MSI which is usually due to the MLH1 silencing because of its promoter hypermethylation. At present, the simultaneous implementation of both microsatellite instability analysis and IHC testing is highly sensitive and reliable screening method for LS diagnosis. IHC alone may not identify LS patients with frame shift mutations, splice site mutations and large genomic rearrangements, because the dysfunctional protein would still be observable with IHC staining (Boland et al., 1998; Shia, 2008). Alternatively MSI testing using PCR based methods is an indispensable method for screening of LS or around 15% of sporadic CRCs exhibiting MSI-H (Boland and Goel, 2010).

Unlike IHC staining which requires several tumor sections, MSI testing being a PCR based method, requires a small amount of tumor and preferably adjacent normal tissues DNA. On the other hand, in comparison with IHC analysis, MSI is an indirect method, hence unable to pinpoint the particular deficient/defective MMR protein(s) involved in the tumor development (Lindor et al., 2002). However, there are many studies conducted on either of these two tests preferences, but still no consensus reached in this regards. According to the preference of every center any one of these two tests hold as the first choice for MSI detection (Beamer et al., 2012).

Apart from the recommended panels for MSI testing (Buhard et al., 2006), several studies performed in order to evaluate the feasibility of the more flexible MSI testing systems with reduced number of markers or even a single marker based detection system (Brennetot et al., 2005; Bouzourene et al., 2006; Deschoolmeester et al., 2008; Bianchi et al., 2009; Haghighi et al., 2010). In some studies CAT25 marker showed high sensitivity and specificity in comparison with standard NCI/ICG-LYNCH SYNDROME marker panel (Findeisen et al.,

2005; Bianchi et al., 2009). Using CAT25 alone or in combination with BAT26 marker is recommended by some authors as a suitable panel for MSI testing (Findeisen et al., 2005; Deschoolmeester et al., 2008).Although BAT-25 usefulness for determination of MSI status was questioned by some authors (Ichikawa et al., 2001), BAT-26 is regarded as superior in terms of sensitivity to identify MSI-H cancers (Xicola et al., 2007; Esemuede et al., 2010). Recently, Morandi et al studied 340 consecutive CRCs using three multiplexed polymerase chain reactions amplifying BAT25, BAT26, TGFBR2, MybT22, BAT40, MT1XT20, NR21, NR24, CAT25, D2S123, D5S346, D17S250, D18S58, CSF1PO, D7S820, and D18S51 markers. MT1XT20 showed very high sensitivity (97.3%) comparable to BAT26 (97.5%) and CAT25 (97.1%). So we decided to conduct a comprehensive comparative study by employing both IHC for the main four MMR proteins and a standard PCR based five marker commercial kit (Promega MSI system covering BAT26, NR24, NR21, BAT25, MONO 27 markers). MT1XT20 standalone marker was also run parallel to two main MSI detection procedures and results were compared.

Eight patients (40%), out of 20 Amsterdam II selected CRC families, showed MSI-H status with Promega MSI testing system while using our in-house developed single marker MT1XT20 MSI testing protocol, 5 samples (25%) detected as MSI-H. However, based on IHC staining 7 samples (35%) were regarded as MSI deficient. Although MT1XT20 showed superior results for MSI detection in Morandi et al study in Spanish population we couldn't replicate the same in Iranian CRC patients. However using Promega MSI testing system, BAT26 was the most instable marker among the five markers included in this kit. So it may be concluded that in Persian population BAT26 could act well as standalone marker for MSI detection. Using single marker would simplify MSI testing considerably hence many more centers can execute this important test. Determination of MSI status in CRC patients with hereditary mode of transmission would be beneficial in confirmation of LS and then start to screen the whole family members in order to categorize them as MMR mutation carriers and non-carriers. In sporadic CRCs MSI detection would help as prognostic factor and essential for making right decision regarding adjuant chemotherapy regimen.

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