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High frequency of microsatellite instability in sporadic colorectal cancer patients in Iran

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ABSTRACT. Microsatellite instability in sporadic colorectal cancer patients was assessed, and the clinicopathological associations were evaluated in northeastern Iran, which is a high-risk region for gastrointestinal malignancies. Microsatellite instability (MSI) status of tumoral tissue, compared to normal tissue, was assessed with

a standard panel of MSI markers on paraffin-embedded surgically resected tissues from 67 consecutive sporadic colorectal cancer patients. Eleven of the patients were under 40 years old. Female patients were significantly younger than male patients (mean age 54.2 *vs* 62.1 years, P = 0.020). MSI analysis revealed 18 cases of MSI-H (26.9%), 11 MSI-L (16.4%) and 38 MSS (microsatellite stable tumors; 56.7%). While a greater proportion of patients consisted of males, 56.7 *vs* 43.3% females, MSI-H was more frequent in females (34.5 *vs* 21.5%). MSI was associated with proximal location of tumor (P = 0.003) and lower stages of tumor (P = 0.002), while MSS tumors were associated with node metastasis. MSI has a higher frequency in sporadic colorectal cancer patients, suggesting that molecular epidemiology of the genetic alterations involved in colorectal cancer carcinogenesis has a different pattern in the Iranian population, which deserves further epidemiological attention. The high frequency of MSI-H in this population suggests that we should look at microsatellite instability prior to chemotherapy to determine the most appropriate chemotherapeutic strategy in our population.

Key words: Microsatellite instability; Sporadic colorectal cancer; Iran; Mismatch repair

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent and the third leading cause of cancer-related deaths in Iran (Mousavi et al., 2009). The origin of CRC isdue to accumulation of genetic and epigenetic abnormalities in the intestinal epithelium, which may lead to the development of colorectal adenocarcinoma. The majority of sporadic CRCs (approximately 85%) originate from chromosomal instability involving the adenomatous polyposis coli and wingless type signaling pathway genes. Other tumors (10-15%) originate from a microsatellite instability (MSI) pathway which is characterized by the mismatch repair (MMR) genes leading to failure for DNA mismatches repair during replication and generation of inserted or deleted bases (Fearon and Vogelstein, 1990; Thibodeau et al., 1993; Liu et al., 1996; Soreide et al., 2006). This phenomenon caused novel alleles detectable as a change in allele size between tumor and normal DNA. Microsatellites are simple DNA sequences which consist of a repeating unit of 1-5 bp with 25-60 bp in length and are commonly in the form of CA_n (Beckman and Weber, 1992). Expansion or contraction of these sequences due to increase or decrease in the repeat units gives rise to what is referred to as MSI. Although different frequencies have been reported, MSI is detected on an average of 15% of CRCs (Jenkins et al., 2007), 20-25% of them occur in Lynch syndrome (or hereditary non polyposis colorectal cancer (HNPCC), while the other 75-80% are due to the acquired loss of DNA MMR activity caused by promoter hypermethylation of the hMLH1 gene (Kane et al., 1997). In contrast to sporadic CRC in which MSI is supposed to be associated to 10-15% of malignancies (Aaltonen et al., 1993; Boland et al., 1998), MSI is the important underlying event in in 85-90% of HNPCCs (Aaltonen et al., 1993; Moslein et al., 1996).

To overcome the discrepancies in MSI results of the different studies, the standard testing procedure recommended by the National Cancer Institute/International Collaborative Group/HNPCC (NCI/ICG-HNPCC) has been proposed, introducing five microsatellite markers including two mononucleotide repeats (BAT26 and BAT25) and three dinucleotide repeats (D2S123, D5S346, and D17S250) (Boland et al., 1998). Using this reference panel, three various MSI phenotypes have been described; microsatellite instability-High (MSI-H) is characterized by presence of MSI in more than 40% (two of five markers) examined, microsatellite instability-Low (MSI-L) by presence of fewer than 40% of markers and microsatellite stable tumors (MSS) by absence of any MSI, in markers examined. Regarding the clinicopathological relevance of MSI various reports have been published with some contradictory results in different populations, suggesting that MSI may have contrasting features in different populations based on the distinct and various set of risk factors responsible for genetic alterations and tumor development. In the current study, standard NCI/ICG panel of biomarkers was assessed in the sporadic colorectal patients and the clinicopathological associations were evaluated in the northeastern Iran, which is a high risk region for gastrointestinal malignancies.

MATERIAL AND METHODS

Sample collection and DNA preparation

Sixty-seven consecutive patients, histologically confirmed as colorectal cancer, were enrolled in this study. Paraffin-embedded archival surgical tumoral and their adjacent normal, tumor-free tissues were obtained from 3 general hospitals in Mashhad, the capital city of the Khorasan province in northeastern Iran. Demographic and clinicopathological data of each patient including age of cancer onset, familial history of CRC and related cancers, location, grade, and stage of tumor were collected from their medical records and directly by contacting the patients and their family. The patients with positive family history of cancer were excluded from the study. A written consent form was obtained from the patients. The study was approved by the research Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

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DNA extraction

Paraffin-embedded tissues were retrieved by using xylene and alcohol, digested by proteinase K (Fermentas, Latvia). DNA was extracted with phenol/chloroform/isoamyl alcohol, and precipitated in ethanol.

MSI testing

DNA extracted from tumor and normal tissues used as template in separate PCRs by using a Bethesda panel of microsatellite markers for MSI testing (Boland et al., 1998). PCR was carried out in 20 µL reactions containing 1 µL DNA, 0.2 µL Taq polymerase (5 U/µL, GENET BIO, Korea), 0.4 µL dNTP (10 mM,GENET BIO), 0.5 µL of each primer, 2 µL 10X reaction buffer (GENET BIO) and 1.6 µL $MgCl_2$ solution (25 mM,GENET BIO). Amplification was performed in a thermocycler (Techgene, Korea) which consisted of one cycle of

95°C for 5 min, 40 cycles of 95°C for 45 s, 54°C for 45 s and 72°C for 1 min, followed by a final extension of 72°C for 30 min. PCR products were loaded on 2% agarose gels and stained with ethidium bromide for visualize bands. The products were then subjected on denatured polyacrylamide gel (8%) electrophoresis (BIO RAD, USA) and stained with silver nitrate (MERCK, Germany) solution.

Statistical analysis

Statistical analysis was performed using the SPSS software (ver. 16). The correlation between two variables was evaluated using Pearson's χ^2 and Fisher's exact test when required. Regression analysis was performed to rank the factors associated with MSI and identify the predictors of MSI. Statistical significance was defined as $P < 0.05$.

RESULTS

A total of 67 sporadic colorectal cancer patients including 38 (56.7%) men and 29 (43.3%) women were enrolled in the study. The mean age of patients was 62.1 years in males and 54.2 years in females (ages ranged between 25 and 86) with a statistically significant difference ($P = 0.020$).

MSI analysis

A representative of MSI analysis is shown in Figure 1. Microsatellite instability was detected in 29 of 67 cases (43.3%) with colorectal cancer. MSI analysis revealed 18 cases of MSI-H (26.9%), 11 MSI-L (16.4%) and 38 MSS (56.7%). Figure one represents MSI analysis of the studied markers in representative patient's samples. Instability is observed as the bands shift in the tumoral DNA compared to the DNA from the adjacent normal tissue. The most instable markers were BAT25, D17S250 in which instability was detected in 16 patients (55.2%). Among 18 MSI-H patients, instability in BAT-25 occurred in15 (83.3%) cases, BAT-26 in 13 (72.2%) cases, D2S123 in 11 (61.1%) cases, D17S250 in 11 (61.1%) cases, and D5S346 in 8 (44.4%) cases (Figure 2). Among 18 MSI-H patients, instability of two markers was detected in 7 patients, 3 patients had instability in three markers, 5 patients had instability in four markers and instability of all the markers was observed in 3 patients.

Clinicopathological data

Clinicopathological features of all patients and associations with MSI status are presented in Table 1. Although the overall percentage of males was greater in the studied population (56 *vs* 44%) MSI-H was more frequent in females, compared to males (34.5 *vs* 21.5%), with no statistical significance. In the MSI-H group, the mean age was 62.3 years whereas in MSH-L and MSS groups, it was 55 and 58 years respectively. Tumors were in the right side of the colon, proximal to the splenic flexure, in 50.7% of patients; among them 44.1% were MSI-H. Significant correlation between MSI and location of the tumor was observed with the majority of instable tumors (MSI-H) located in the proximal colon (83%) while a greater proportion of MSI-L tumors were observed in the distal colon (72%) ($P = 0.003$). Among the MSI markers, instabilities in BAT25 ($P = 0.002$), BAT26 ($P = 0.022$), D2S123 ($P = 0.010$)

and D5S346 (P = 0.015) were significantly more frequent in proximal colon tumors and only instability of D17S250 was not associated with the location of tumor (table 2).

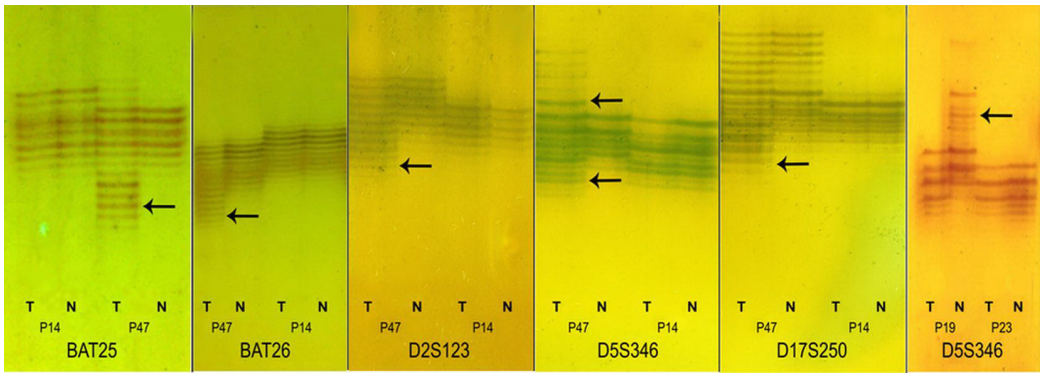

Figure 1. Illustration of microsatellite instability (Arrows) in five MSI markers. All the five markers showed instability (MSI) in patient number 47; in contrast all of the markers were stable in patient 14 (MSS). LOH is represented in D5S346 in patient number 19 (LOH +) compared to normal heterozygosity in patient 23 (LOH -). (N = normal; T = tumor; LOH = loss of heterozygosity).

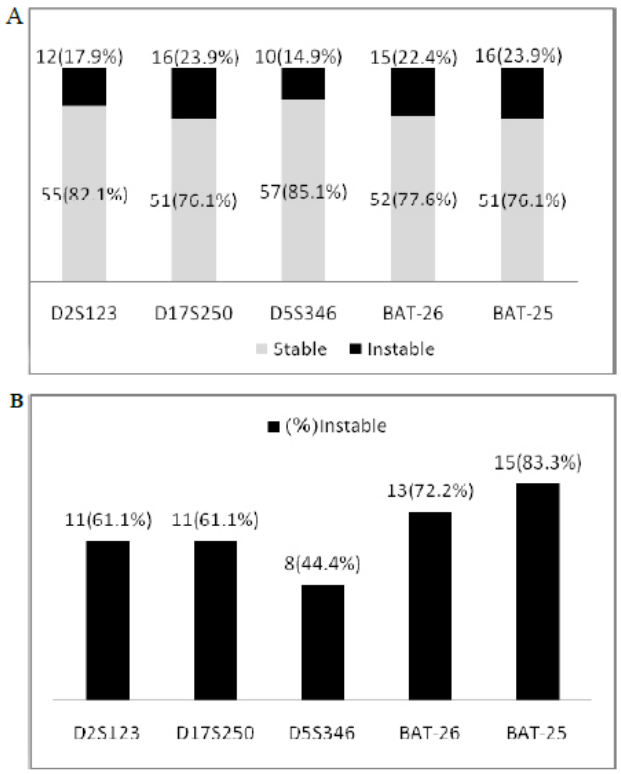

Figure 2. Microsatellite instability in the panel of 5 markers. **A.** Histograms show the stability status of each marker in the studied population. **B.** Rate of Instability of each MSI marker in the tumors with MSI-H.

Table 1. MSI status in sporadic colorectal cancer patients and correlations with clinicopathological features

	Total	MSI-H (%)	MSI-L (%)	MSS (%)	P value	LOH -	LOH+	P value
Patients	67	18 (26.9)	11 (16.4)	38 (56.7)		57 (85.1)	10 (14.9)	
Mean age (years)	58.7 ± 15.7	62.4 ± 17.3	55 ± 14.6	57.9 ± 15.3		58.4 ± 15.9	59.9 ± 15.3	
Gender					0.171			0.551
Male	38	8 (44.4)	10 (90.9)	20 (52.6)		32 (47.8)	6 (60)	
Female	29	10 (55.6)	1 (9.1)	18 (47.4)		25 (52.2)	4 (40)	
Location					0.003a			0.707
Proximal	34	15 (83.3)	3 (27.3)	16 (42.1)		29 (43.3)	5 (50)	
Distal	30	3 (16.7)	7 (63.6)	20 (52.6)		26 (38.8)	4 (40)	
Proximal/distal	3	-	1 (9.1)	2 (5.3)		2 (17.9)	1 (10)	
Grade					0.547			0.599
W.D	40	9 (50)	6 (54.5)	25 (65.8)		33 (57.9)	7 (70)	
M.D	25	8 (44.4)	5 (45.5)	12 (31.6)		22 (38.6)	3 (30)	
P.D	2	1 (5.6)	-	1 (2.6)		2 (3.5)	-	
Lymph node					0.286			0.026c
Yes	28	6 (33.3)	2 (18.2)	20 (52.6)		27 (47.4)	1 (10)	
No	39	12 (66.7)	9 (81.8)	18 (47.4)		30 (52.6)	9 (90)	
Stage					0.002b			0.242
1 (B1)	4	1 (5.6)	3 (27.3)	-		3 (5.4)	1 (10)	
2 (B2)	34	12 (66.7)	6 (54.5)	16 (42.1)		27 (47.3)	7 (70)	
3 (C1,C2)	29	5 (27.7)	2 (18.2)	22 (57.9)		27 (47.3)	2 (20)	
Age (years)					0.382			0.480
<40	11	2 (11.1)	2 (18.2)	7 (18.4)		10 (17.5)	1 (10)	
>40	56	16 (88.9)	9 (81.8)	31 (81.6)		47 (82.5)	9 (90)	
Depth of tumor					0.973			0.436
T2	11	3 (16.7)	3 (27.3)	5 (13.2)		8 (14)	3 (30)	
T3	35	9 (50)	5 (45.4)	21 (55.3)		30 (78.9)	5 (50)	
T4	21	6 (33.3)	3 (27.3)	12 (35.5)		19 (7.1)	2 (20)	

[a]MSI-H *vs* MSS/MSI-L; [b]MSI-H/L *vs* MSS; [c]LOH + *vs* LOH-.

Table 2. Clinicopathological significance of instability in MSI markers independently.

	Location		P value	Stage			P value	Node	Metastasis	P value
	Proximal	Distal		1	2	3		Yes	No	
BAT25	14	2	0.002	1	10	5	0.521	7	9	0.540
BAT26	12	3	0.022	1	11	3	0.097	5	10	0.327
D5S346	9	1	0.015	1	6	3	0.609	2	8	0.120
D17S250	9	7	0.414	2	11	3	0.049	4	12	0.101
D2S123	10	1	0.010	1	9	2	0.099	1	11	0.008

MSI was associated with the lower stages of tumor (P = 0.002). Moreover, among the markers, instability in D17S250 were independently associated with the lower stages of tumor (P = 0.049).

Lymph node metastasis was significantly less frequent in the tumors with MSI (P = 0.039). This significant difference was observed for D2S123 independently, as well; in 12 tumors with instability in D2S123, 11 cases had no metastasis to lymph nodes (P = 0.008).

MSI-L was a rare event in the females, i.e. 1/12, compared to 11 out of 12 males (P = 0.022). MSI was not associated with the tumor size, depth of tumor (T) or tumor grade (although the majority of tumors were well or moderately differentiated and only 1 (3%) of samples was poorly differentiated).

Regression analysis was performed to rank the factors associated with MSI-H. Among the associated factor location of tumor was the most important predictor (OR: 10.1, 95%CI = 2.07-49.27; P = 0.004). Stage of the tumor was the second important factor (OR: 6.36, 95%CI

= 1.16-34.74; P = 0.033). Gender of patient was the other factor which was associated with a slight increase in the probability of MSI-H (OR: 2.48, 95%CI = 0.60-10.26; P = 0.210).

Loss of heterozygosity (LOH)

LOH of the studied markers was observed in 10 patients (males = 6, females = 4). Among the total of 11 markers with LOH, 45.5% were in D17S250 and LOH was not observed in BAT25. Among the patients with LOH, 90% (9/10) had no metastasis to lymph node (P = 0.026). The clinicopathological features of LOH patients are shown in Table 1. Figure 1 shows LOH in patient 19 as compared to patient 23.

DISCUSSION

Northeastern Iran is a cancer prone region, in which the incidence gastrointestinal cancers, including CRC, is above the average in Iran (Sadjadi et al., 2005; Mousavi et al., 2009). In order to better clarify the genetic features of CRC and its clinicopathological relevance in this high risk area, we studied MSI, as an important genetic pathway of CRC carcinogenesis, for the first time in this region. The frequency of microsatellite instability in sporadic colorectal cancer is estimated to be 10-15% and in patients with HNPCC up to 90% (Aaltonen et al., 1993). Previously Bishehsari et al., analyzed 170 sporadic CRCs in Tehran, capital city of Iran, with two MSI markers, BAT25 and BAT26, and reported 19.4% MSI-H (Bishehsari et al., 2006). In the present study, MSI was detected in 43.3% of our patients with 26.9% of them having MSI-H phenotype which is higher than the previous reports, suggesting that the molecular epidemiology of genetic alterations, involved in the CRC carcinogenesis, has different patterns in the Iranian population. It is believed that different genetic pathways involved in CRC carcinogenesis are associated with different environmental exposures and thus, introduces a divergent accumulation of risk factors and related genetic alterations in a different subset of patients. A well-known example is dietary risk factors, including western diet and red meat which is suggested to be associated with the MSS CRC (Diergaarde et al., 2003). While the dietary patterns are different in our population in contrast to western diet, such variety of risk factors may be responsible for different epidemiological trends and molecular patterns in our population and requires further in-depth evaluation in future large-scale studies.

Among all markers, BAT25 had the highest sensitivity with 83.3% and D5S346 was least detected (44.4%). However, instability in each of the MSI markers was detected both independently and concomitant with other markers, confirming the fact that the complete panel of five MSI makers are required in order to have a better assessment of MSI status in sporadic colon cancer. Various studies demonstrated that using mononucleotide markers without applying dinucleotide markers were highly effective for determining the MSI-H status of CRCs (Dietmaier et al., 1997; Hoang et al., 1997; Cravo et al., 1999). While in this study 2 samples with MSI-H had instability in dinucleotide markers (D17S250, D2S123 and D5S346), in addition we observed 3 samples with instability in one mononucleotide and one dinucleotide marker and by solely assessing mononucleotide markers, we could have missed a considerable proportion of MSI-H patients. The overall pattern of instability in the studied marker did not reveal any specific manner, indicating that analysis of both groups of dinucleotides and mononucleotides are required for this purpose.

In line with previous reports, majority of tumors with MSI were located in proximal colon (Liu et al., 1996; Ashktorab et al., 2003, 2005, 2008). However, Brim et al. (2008) have reported higher frequency of MSI-H in the distal colon in Omanian population. We observed that the rate of MSI-H in females is higher than males, although the proportion of males was greater than females among the patients, inconsistent with the trend in Iran, previously reported by Mousavi et al. (2009). Issa et al. (1994) have shown that colon tumors generally arise from cells that have lost estrogen receptor (ER) expression and through the process of aging, the colon harbors hypermethylation, and consequently reduced expression of the ER. Notarnicola et al. (2001) reported a significant association between MSI and ER status in colorectal tumors and showed that MSI tumors harbored low levels of ER expression. Furthermore, they described an increasing risk of MSI CRC tumors with the withdrawal of estrogens. Breivik et al. (1997) hypothesized that gender difference in CRC is linked to estrogens through a mechanism involving MSI. On the other hand, Slattery et al. (2001) suggested that estrogen is associated with preventing ER methylation; at least one of the major MMR genes is estrogen responsive and that loss of estrogen results in loss of DNA mismatch repair capacity. They concluded that estrogen prevents MSI^+ tumor and abstinence from estrogen will lead to development of tumors with MSI. The gender preference observed in this study could be described with this model.

Recent studies suggest that MSI-H tumors, compared to MSS tumors, have shown different sensitivity to chemotherapeutic agents (Warusavitarne and Schnitzler, 2007), resistant to cisplatin, carboplatin (Aebi et al., 1996) and 5 Fluorouracil (5FU) (Ribic et al., 2003) but sensitive to topoisomerase inhibitors such as irinotecan (Bras-Gonçalves et al., 2000) and it is anticipated that the clinical demand for MSI analysis in sporadic CRC patients to likely increase in the near future (Sinicrope et al., 2010).The rate of resistance to the current chemotherapeutic strategies is reasonably assessed along with the MSI status, in this population with a high frequency of MSI^+ tumors. The high frequency of MSI-H in the studied population suggests the consideration of MSI analysis prior to chemotherapy to determine the sensitive regimen in our population.

Several epidemiologic studies from Iran revealed that the proportion of young CRC patients is significantly higher than western countries. About 20% of patients are under 40 years old in Iran in contrast to 2-8% in western countries (Sadjadi et al., 2003; Hosseini et al., 2004; Yazdizadeh et al., 2005; Ansari et al., 2006; Fazeli et al., 2007; Azadeh et al., 2008; Mousavi et al., 2009). Although the early onset of CRC is thought to be a consequence of HNPCC in western countries, the differences in the age trend in this study, in the northeastern Iran (16.4% under 40), and similarly in the previous reports from the capital, Tehran, may be influenced by the fact that our country is among the young populations in the world, therefore a significant proportion of CRC patients are expected to be young (Malekzadeh et al., 2009). This small cohort, in addition to other reports from Iran, suggests different epidemiological and molecular trends in the CRC and warrants further large-scale studies for the assessment of genetic and epidemiological features in this region. Lower age of onset offers the requisite of reconsideration of screening guidelines based on the epidemiological features of this region and increases the possibility that the age threshold for screening should be lower. Developing non-invasive screening tools deserves further attention to screen a greater proportion of younger individuals (Abbaszadegan et al., 2007).

In conclusion, This is the first report, to our knowledge, of MSI status of CRC patients

in this high risk region, demonstrating a higher frequency of MSI^+ sporadic CRC. Both CRC and MSI were more frequent in younger patients, compared to the common trend in the world, which deserves further assessment of involved risk factors in association with the genetic alterations and probably reconsidering the screening guidelines for sporadic CRC in Iran. Despite some previous studies which indicated that applying mononucleotide markers alone would be sufficient to determine the MSI status, it seems that analysis of all the biomarkers are essential for a more sensitive detection. MSI was more frequent in female gender, right-sided tumors and lower stages of tumor while MSS was correlated with node metastasis. High frequency of MSI suggests consideration of MSI testing to determine the more sensitive and effective regimen prior to chemotherapy in our population.

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