Iranian Journal of Pathology (2009)4 (1), 26 - 31

Original Article

Molecular Analysis of Microsatellite Instability in Hereditary Non Polyposis Colon Carcinoma Patients from North-East Iran

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ABSTRACT

Background and Objectives: **Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant cancer predisposition syndrome caused by germ-line mutations in DNA mismatch repair genes. Tumors arising as a result of these mutations display instability in a sequence area known as microsatellites. Studies have shown that some Bethesda markers (BAT25, BAT26) are more efficient than others in identifying Microsatellite Instability (MSI) in tumors of HNPCC patients. The aim of this study was to evaluate the possible benefits of two MSI markers BAT25 and BAT26 to identifying microsatellite instability in tumor tissues from HNPCC patients.**

Material & Methods: **We used 49 cases gathered from north-east Iran. Microsatellite Instability analysis was performed using fluorescent-labeled primers. Statistical analysis was achieved using SPSS software.**

Results: **24/5% (12/49) and 34.7% (17/49) of the cases showed MSI in BAT25 and BAT26, respectively. None of tumor sample was MSI positive for both markers.**

Conclusion: **MSI frequency is considerably lower, compared to other findings. This might be due to the fact that environment and Race has great influence on MSI frequency.**

Key words: Hereditary nonpolyposis colorectal cancer, Mismatch repair, Microsatellite instability, Mononucleotide markers, Iran

Introduction

Hereditary colorectal cancer (CRC) is a major public health problem worldwide (1) and the third cause of mortality in western countries, which is classified into hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (2).

FAP is associated with germline mutations in the adenomatous polyposis coli (APC) gene, whereas germline mutations in the MMR genes (hMSH2 or hMLH1) are the common cause of HNPCC (2). The mean age of the HNPCC patients at the time of diagnosis is 42 to 65 years for sporadic CRC (1).

With the discovery of the mismatch repair (MMR) genes, it has become clear that two major molecular

Received: 2 November 2008
Accepted: 13 June 2008
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genetics pathways could be discerned in colorectal carcinogenesis. The first pathway, the chromosomal instability pathway, is characterized by allelic losses and gains, and by aneuploidy. The second pathway, the microsatellite instability pathway, is characterized by an abundance of subtle DNA mutations and diploidy (3). Microsatellite instability is caused by inactivation of a MMR system, in particular *hMLH1*, *hMSH2,* and *hMSH6*. Dysfunction of MMR genes leads to loss of MMR protein expression, and often to microsatellite instability (MSI) (4, 5). Microsatellite instability seems to be an early event in the carcinogenesis of HNPCC, as up to 57% of colorectal adenomas from patients with HNPCC show MSI (6). Microsatellite instability is also detected in 10–20% of sporadic colorectal cancers. Areas of human genomes, especially those with repeated sequence, known as microsatellites, appear to be error prone, because of slippage of the DNA polymerase during DNA replication. Some genes, including those of MMR system, contain such repeats that are making them prone to be expanded. Genome wide scan have also indicated that distinct markers can be used to detect such expansion as early events in cancer pathogenesis. The five suitable markers including BAT25, BAT26, D_2S_{123}, $D_{17}S_{250}$ and D_5S_{346} are often used in previous studies to identify microsatellite instability (7). Nevertheless, the markers BAT25 and BAT26 are considered to be more sensitive in detecting microsatellite instability in tumors due to the negligible size variation (8-10). Some classifications tend also to distinguish between tumors with instability at many loci (more as two markers) that is referred to MSI-High, and those with single unstable locus that are defined as MSI-low phenotype (11).

However, the aim of this study was to establish the frequency of MSI by the markers BAT25 and BAT26 in individuals that have been diagnosed for HNPCC. To our best knowledge, the presented study is the first, conducted in Iranian population.

Material and Methods

Subject

The postmortem study was approved by the Ferdosi Medical University (Mashhad) Ethics Board. Paraffin embedded tumor tissues from 49 patients with gender distribution of 40% (20/49) male and 60% (29/49) female and with the age average of 53.7 years collected from Mashhad's hospitals were diagnosed with colon cancer according to the Bethesda Guidelines. For each case, tumor and respective normal tissue from individuals were obtained separately. Regarding to the tumor differentiation, the samples were classified to 22% (11/49) poorly, 60% (29/49) moderately and 18% (9/49) well differentiated.

DNA Extraction

Formalin-fixed, paraffin-embedded tissue blocks were sectioned with 5 µm thickness, stained by Hematoxylin - Eosin and viewed to confirm histopathological finding. Using the stained dissected slides as templates, two 20-µm section of paraffin embedded tissue were placed in two sterile tubes as source of tumoral and normal samples, separately. Deparaffinization was performed with Xylene, followed by DNA extraction as described previously (12).

Polymerase chain reaction (PCR)

One hundred nanogram extracted DNA from each tumor and normal tissue was amplified by PCR using 2 mononucleotide microsatellite markers BAT25 and BAT26, respectively. Two primer pairs with following sequences:

BAT25-Forward
(5-TCGCCTCCAAGAATGTAAGT-3),
BAT25-Reverse
(5-TCTGCATTTTAACTATGGCTC-3),
BAT26-Forward
(5-TGACTACTTTTGACTTCAGCC-3) and
BAT26-Reverse
(5-AACCATTCAACATTTTTAACCC-3)
were fluorescent-labeled (Genefanavaran), which sequences were adopted as described previously (12). The 25 µL reaction mixture contained 2.5 µL PCR buffer, 0.75 µL $MgCl_2$ (1.5 m*M*), 0.5 µL of all four deoxynucleoside triphosphates (each at 0.2 m*M*), 0.5 µL of each forward and reverse primers (each at 25 pmol/µL), and 2.5 U of *Taq* DNA polymerase. After an incubation time at 94°C for 3 min, 30 cycles including 94° C for 30 s, 56° C for 30 s and 72° C for 30 s, were performed, followed by a final 5-min extension at 72° C in a thermal cycler (BIO-RAD thermo cycler, Germany). To eliminate false positive MSI, all PCR reactions from tumor and normal samples were replicated and a PCR reaction without genome was achieved to control for cross-contamination.

Gel electrophoresis and Microsatellite-instability testing and analysis

The amplified products were visualized by staining with ethidium bromide after electrophoresis on 1.5 % agarose gel to control for accurate size and specificity. Five µL of the labeled PCR products was then loaded onto a 60 g/L polyacrylamide gel containing 7 mol/L urea, 450 mmol/L Tris-borate (pH 7.5) and 1 mmol/L EDTA (pH 7.0) running buffer. Electrophoresis carried out for 2-3 h (depending on PCR product size bands) on the gel-Scan 2000 (Corbert Research) according to the manufacturer's instructions. The data were collected automatically and analyzed by GeneScan software (Corbert Research).

Statistical Analysis

The chi-square test was performed to calculate the significance of MSI frequency in patients related to the gender, age and tumor stage.

Results

The PCR products generated by tumor and normal tissues were verified on a 1.5 % agarose gel with appropriate size of 125 and 130 base pairs for BAT25 and BAT26, respectively (Fig. 1).

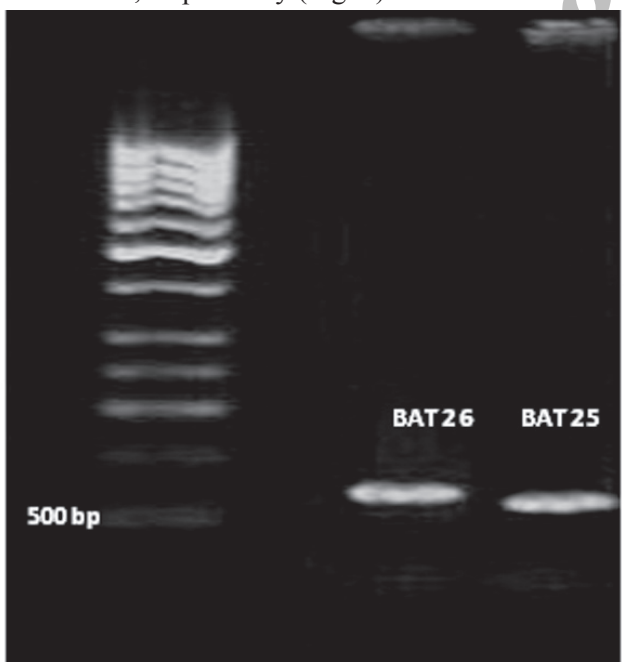

Fig. 1: PCR products were electrophoresed on a 1.5% agarose gel to examine the excepted size; 125 base pair for BAT25 and 130 base pair for BAT26. The 50 base pair DNA size marker (Fermentase) was used to control for fragment length.

Only fragments with excepted length were selected for subsequent analysis. Afterwards, the fragments were analyzed on an 8% polyacrylamide gel and monitored on the automated gel scan. Any shifted bands in tumor samples relative to the normal tissue was scored as MSI positive (Fig. 2). Of 49 HNPCC suspected patients, 34.7% have shown MSI in BAT26 and 24.5% in BAT25. Forty percent of MSI+ for BAT26 was in right colon, 46.7% in rectum and 13.3% in sigmoid horizontal colon but the ratios for BAT25 were 40%, 50% and 10% respectively. None of patients was MSI positive for both markers. Table 1 and 2 represent some characteristic of patients, such as age, sex and stage of tumors, which did not show significance in any category. However, samples without enough data in pathological survey assumed to be non significant in SPSS analysis.

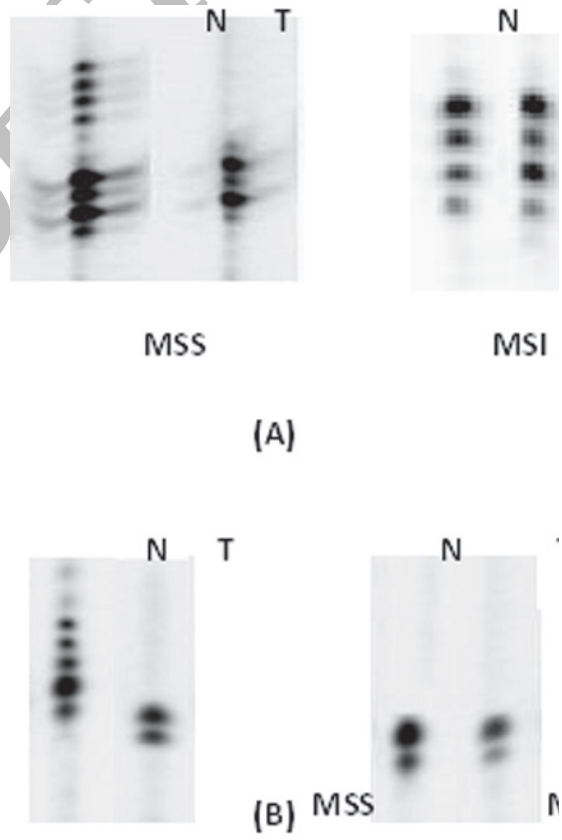

Fig. 2: Microsatellite instability (MSI) positive tumor samples (T) compared to normal tissue (N) defined as microsatellite stability (MMS) for BAT25 (A) and BAT26 (B). The characteristic pattern of allele extension can be shown in both markers in the case of MSI. Indeed, MSI negative tumor samples did not expose size variation relative to the normal tissue.

Table 1: General properties and histopathology of HNPCC patients and MSI results for BAT25.

Patients' characteristics	Classification	MSI in %	Total MSI, n=12	*P* value
Age	< 40 years	58.33	7	
	40-60 years	41.66	5	0.015
	> 60 years	0.0	0	
Sex	Female	66.66	8	
	Male	33.33	4	0.464
Tumor Stage	B1	16.66	2	
	B2	41.66	5	
	C1	8.33	1	0.175
	C2	33.33	4	
	D	0.0	0	
Tumor Differentiation	Well	8.33	1	
	Moderate	83.33	10	0.881
	Poorly	8.33	1	

Table 2: General properties and histopathology of HNPCC patients and MSI results for BAT26.

Patients' characteristics	Classification	MSI (%)	Total MSI	*P* value
Age	< 40 years	47.05	8	0.325
	40-60 years	15.34	6	
	> 60 years	17.64	3	
Sex	Female	35.34	6	
	Male	64.7	11	0.184
Tumor Stage	B1	0.0	0	
	B2	82.35	14	
	C1	5.88	1	
	C2	5.88	1	0.224
	D	5.88	1	
Tumor Differentiation	Well	23.53	4	0.320
	Moderate	58.82	10	
	Poorly	17.64	3	

Discussion

In the present study, 49 patients diagnosed with HNPCC were analyzed using two markers BAT25 and BAT26, which showed 24.5% and 34.7% MSI, respectively. As are presented in Table 1 and 2, there was no significance related to the stage and location of tumor in comparison with both markers that has been used in this study. Remarkably, none of tumor sample did show MSI for both markers. As mentioned above, the classification of tumors are based on MSI+ tumors if instability is observable in a proportion of microsatellite loci, but some molecular biologists define the microsatellite instability only at single

locus relevant for further tumor progression, i.e. just one marker, such as BAT 25 or BAT26, may be sufficient to detect nearly all MSI positive tumors (12). Furthermore, some researchers distinguish between tumors with instability at many loci, referred to MSI-High, from those with few new alleles, known as the MSI-Low phenotype (12). Therefore, the individuals in this investigation count to MSI-Low phenotype.

Additionally, the average of MSI frequency including both markers in this study was relative lower, compared to the previous reports (13). Low MSI frequency in Iranian HNPCC individuals may be resulted from at least two reasons: The frequency rate of MSI is not the same in different populations. As well as race and environment may be not irrelevant to the MSI in the population study (14). Furthermore, different ethnic groups may have different predictive MSI markers (15). In addition, some patients in this study might harbor point mutation in the MMR genes that did not examine by us. Recently, Pistorius *et al*. have postulated that microsatellite instability is rare in HNPCC patients carrying point mutation in MSH2, MLH1 or MSH6. Furthermore, they did not find compound heterozygous mutations comprising point mutations and genomic instability in HNPCC patients, those biallelic inactivations were not infrequent (16). Further mutation screening of MMR genes in Iranian HNPCC patients may also clarify that suggestion.

Although extensive data indicate that tumors with low-frequency of microsatellite instability are not biologically distinct from those that exhibit microsatellite stability and these two molecular phenotypes were also grouped together in previous studies (17, 18).

Nevertheless, the genetic background of a substantial number of HNPCC patients remains unexplained, indicating that other genes may participate in MMR system and play important role in cancer susceptibility. Despite increasing knowledge in genetics and biochemistry of the MMR, little is known about factors that contribute to the etiology and tissue specificity of MSI-associated carcinogenesis. The identification of the tumor-suppressor genes containing hyper-mutable repeated sequences those make them potential targets for MSI-driven mutations should provide valuable insights into this issue.

However, increasing evidence indicates that the prognosis and the chemo-sensitivity of the MSI+ colorectal tumors differ from those without instability (19), while the detection of MSI is simple and economical and has high correlation with the clinicopathologic feature of HNPCC (20).

Conclusion

Consequently, we consider the MSI phenotype could be routinely assessed in order to improve the clinical management of Iranian individuals with HNPCC.

Acknowledgments

We thank Research Deputy of Shahid Chamran University of Ahwaz supporting this work and all the participants have been making this research possible. The authors declare that they have no conflict of interests.

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