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Loss of heterozygosity and microsatellite instability as predictive markers among Iranian esophageal cancer patients

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ABSTRACT

Objective(s): Variation in microsatellite sequences that are dispersed in the genome has been linked to a deficiency in cellular mismatch repair system and defects in several genes of this system are involved in carcinogenesis. Our aim in this study was to illustrate microsatellite DNA alteration in esophageal cancer.

Materials and Methods: DNA was extracted from formalin fixed paraffin embedded (FFPE) tissues from surgical and matched margin-normal samples. Microsatellite instability (MSI) and loss of heterozygosity (LOH) were studied in 50 cases of esophageal squamous cell carcinoma (ESCC) by amplifying six microsatellite markers: D13S260 (13q12.3), D13S267 (13q12.3), D9S171 (9p21), D2S123 (2p), D5S2501 (5q21) and TP53 (17p13.1) analyzed on 6% denaturing polyacrylamide gel electrophoresis.

Results: Statistical analysis indicated a near significant reverse correlation between grade and LOH (P= 0.068, correlation coefficient= -0.272). Specifically, increased LOH in tumor DNA has a significant correlation with increased differentiation from poorly differentiated to well differentiated tumors (P= 0.002 and P= 0.016 respectively). In addition, higher number of chromosomal loci with LOH showed a reverse correlation with lymph node metastasis (P= 0.026, correlation coefficient= -0.485). Furthermore, there was a positive correlation between addiction and MSI (P= 0.026, correlation coefficient= 0.465).

Conclusion: Microsatellite DNA alterations may be a prognostic tool for detection and the evolution of prognosis in patients with SCC of esophagus. It can be concluded that regional lymph node metastasis would be less likely with increased heterozygote loci and addiction with any of opium, cigarette, water pipe or alcohol can be a susceptibility factor(s) for MSI.

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Introduction

Esophageal cancer (EC) is ranked as the eighth common cancer worldwide, with an estimated 456,000 new cases in 2012 (3.2% of the total), and the sixth cause of cancer-related death with an estimated 400,000 deaths (4.9% of the total) (1). Two types of EC are described as squamous cell carcinoma and adenocarcinoma. More than 90% of the malignancy are esophageal squamous cell carcinomas (ESCC) especially in high risk area of "Central Asian Esophageal Cancer Belt", which extended from Northern Iran through the Central Asian republics to North-Central China (2). A wide incidence had been shown for EC by nearly 16-folds. In that context, while Southern, Eastern Africa and Eastern Asia have the highest rate; Western, Middle Africa and Central America are among the lowest (1).

ESCC is the second most common cancer in Iran with incidence rates of 17.6 and 14.4 per 10^5 in males and females, respectively. These rates are

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higher than world standards, but has decreased dramatically compared with the data of 30 years ago (3). This decrease can be attributed to better economic status, improved personal health, nutrition habits, and change in high-risk behavior (4). In Northeastern Iran, there are high incidence areas, including Golestan and Khorasan Provinces with an age-standardized incidence rate (ASR) of 43.4 and 36.3 per 100,000 for men and women, respectively (4). ESCC prognosis is very poor and the patients are diagnosed in advanced stages of the disease. Although surgery and other therapeutic intervenetions such as chemo- and radiotherapy help to suppress tumor growth and development, most of patients show tumor metastasis through the body (due to aggressiveness of EC) leading to increased rate of mortality and decreased rate of patients' 5 year survival (5).

Compared to other common human malignancies, little is known about the molecular basis of ESCC development. Alterations of microsatellite DNA are one of the major factors which may induce immortal and neoplastic transformation of normal cell (6). Microsatellites are repeated DNA sequences widely scattered within the genome, exhibiting length polymorphisms and variations among individuals (7, 8). Due to widespread application and clinical relationship of microsatellite with the malignant phenotypes, their variation seems clinically important (9). Any change in length of microsatellite sequences, as a result of base deletion or insertion, is termed microsatellite instability (MSI). MSI is an indicator of deficient mismatch repair (MMR) system, which is a multi-protein complex responsible for correction of errors arising during DNA replication and cell division (10).

As well as MSI, inactivation of tumor suppressor genes appears as another genetic mechanism involved in ESCC development. This process includes either mutation of one allele, followed by the deletion of the second allele, which called loss of heterozygosity (LOH), or homozygous deletion of both alleles (10).

Analysis of microsatellite using PCR admits elucidation of cancer specific DNA alterations such as LOH and MSI (11). A wide range of MSI frequency (from 2 to 67%) was reported in ESCC. It has been shown that MSI status of long arm of chromosome 17 is associated with ESCC invasion (12). Furthermore, LOH in long arm of chromosome 5 containing tumor suppressor genes can affect ESCC development (13). Interestingly, significant correlations were reported between LOH of MMR system genes and general LOH, as well as MSI status in ESCC patients (14, 15). Therefore, in this study we aimed to analyze a panel of 6 microsatellite markers, based on involved chromosomal locations in ESCC tumorigenesis developing a molecular approach for ESCC detection and elucidating possible indicators for patient's prognosis.

Materials and Methods

Study population

Fifty tumor and margin normal formalin fixed paraffin embedded (FFPE) tissues with histologically confirmed as ESCC were collected from Imam Reza Hospital, Mashhad, Iran. Different criteria were applied to select samples. First, all samples were new ESCC cases without any history of any other malignancy. Second, all recruited samples did not receive any chemo- and radiotherapy treatment before surgery. And finally hematoxylin and eosin (H&E) staining were performed to assure a tumor cell content of more than 70%. The Ethic Committee of Mashhad University of Medical Sciences (MUMS) approved the study (No. 82004).

DNA extraction

DNA was extracted from tumor and related tumor free tissues using proteinase K digestion method. About five to ten sections (5 μm) of FFPEs were deparaffinized by adding 1mL of xylene (Merck, Germany) and washed two times by 500 μl ethanol 96% (Merck, Germany). Digestion buffer contains 50 mM Tris pH 8.5, 1 mM EDTA and 0.5% Tween 20 (Merck, Germany). 20 mg/ml proteinase K (Fermentas, Lithuania) was added to digestion buffer followed by overnight incubation at 37 ˚C. For any 10 mg tissue, 100 μL digestion buffer and 200 μg proteinase K were used. After digestion, the proteinase K was inactivated at 95 ˚C for 10 min. The concentration of extracted DNA was measured using a UV spectrophotometer (UV 1101, Biotech Photometer).

Microsatellite amplification

Microsatellite analysis was performed using 6 microsatellite markers. The selected markers, their chromosome locations and primer sequences are shown in Table 1 (16-18). Various microsatellite markers were amplified using tumor and paired normal DNA. PCR reaction mixture was consisted of 1X CinnaGen PCR buffer, 500 nM of each PCR primer, 1.5 mmol/l $MgCl_2$, 200 μmol/l dNTPs and 1U of Taq DNA polymerase (CinnaGen, Tehran, Iran). 200-300 ng of DNA was used in a reaction volume of 25 μl. Thermal profile of PCR was as follows: 5 min at 95 °C followed by 40 cycles of 50sec at 95 ˚C, 1 min at annealing temperature (49 ˚C -64 ˚C; depending on the primer sequences; Table 1), and 1 min at 72 ˚C followed by 30 min at 72 °C as final extension, with maximum heating and cooling settings in Techne Thermal Cycler (Techgene, Techne, UK). For selecting the appropriate amount of PCR product to load on denaturing gel, 4 microliters of PCR product were electrophoresed through the 2% agarose gel

Table 1. Characteristics of microsatellite markers and the related sequence of used primer sets

Markers	UniSTS	Ta (˚c)	Size (bp)	Location	Sequences (5'→3')
D2S123	888	55	197-227	2p16	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC
D5S2501	70957	54	308-334	5q22.1	TGATTACTCTGAGGAAGAAGGC TTGAAATGGGCACAGAAATT
D13S260	12385	59	158-173	13q12.3	AGATATTGTCTCCGTTCCATGA CCCAGATATAAGGACCTGGCTA
D13S267	53077	49	152-158	13q12.3	GGCCTGAAAGGTATCCTC TCCCACCATAAGCACAAG
D9S171	13653	64	158-177	9p21	AGCTAAGTGAACCTCATCTCTGTCT ACCCTAGCACTGATGGTATAGTCT
TP53	-	65	103-135	17p13.1	ACTGCCACTCCTTGCCCCATTC AGGGATACTATTCAGCCCGAGGTG

and stained with ethidium bromide. PCR products were diluted in formamide loading buffer containing 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA according to the intensity of bands on agarose bands, in 10 to 20 folds; denatured at 95 ˚C for 10 min and chilled on ice for at least 10 min. The volume of 8 µl of diluted PCR products were electrophoresed on 6% denaturing polyacrylamide gel containing 7 M urea, at constant 60 W, 1200 V, 50 ˚C, in TBE 0.5X (44.5 mmol/l Tris-base; 1 mmol/l EDTA; 44.5 mmol/l Boric acid) for 1-2 hr using high voltage power supply (EC600-90, USA), and vertical electrophoresis system (VEU-7703, Iran). The gel was stained by improved silver nitrate staining protocol.

Microsatellite analysis and scoring system

A tumor was classified as having undergone LOH at a particular locus only if the predominant band(s) of one allele showed a decreased intensity in the tumor DNA relative to corresponding normal DNA.

Microsatellite instability was scored when there was appearance of new bands in the tumor as compared to the normal DNA (19-21). It was considered low-level (MSI-L) when 1 of 6 markers was altered and high (MSI-H) when equal or more than 2 markers were changed. If no difference was revealed in electrophoretic banding patterns of tumor DNA in comparison with related margin normal tissues, tumor DNA was considered as microsatellite-stable (MSS) (Figure 1). All positive results were confirmed at least twice. Pathological data and patient demographics were obtained from the patients' medical history to determine any correlation with their microsatellite alterations.

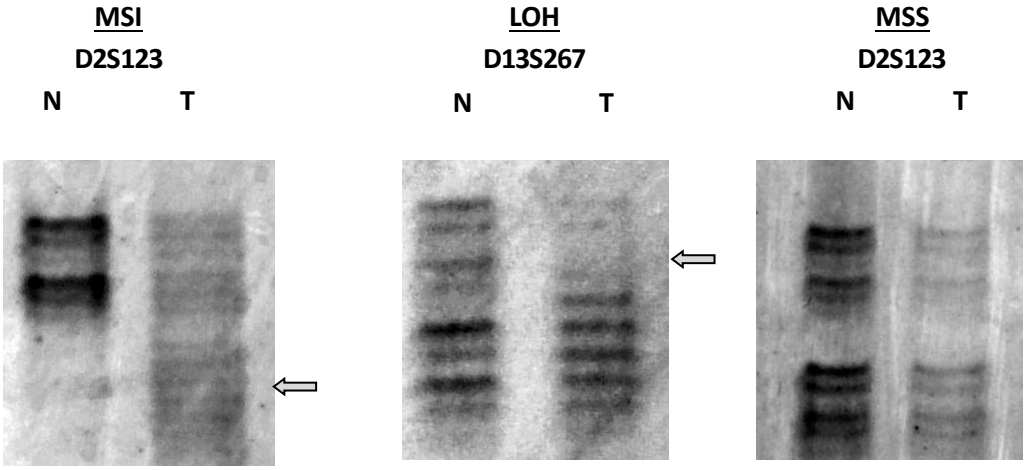

Figure 1. Examples of LOH, MSI and MSS in paired tumor (T) and normal (N) tissues on 6% denaturing polyacrylamide gel. Arrows show lost or reduced alleles of the PCR products for LOH and appearance new bands at tumor cells for MSI. No alteration was seen in MSS samples. [LOH: Loss of heterozygosity; MSI: Microsatellite instability; MSS: Microsatellite stability]

Table 2. Clinicopathological characteristics of 50 patients (the percentages were calculated among the available data)

N (%)	Value	Variable
25 (51)	Male	Sex
24 (49)	Female	
61.9 ± 11.5	Mean ± SD	Age
3.7 ± 2.4	Mean ± SD	Tumor size
6 (24)	Lower	Tumor location
18 (72)	Middle	
1 (4)	Upper	
15 (32.6)	1 (W.D)	Grade
20 (43.5)	2 (M.D)	
11 (23.9)	3 (P.D)	
0	I	Stage (TNM)
7 (36.8)	IIa	
0	IIb	
12 (63.2)	III	
0	IV	
1 (4.8)	T1	T classification
2 (9.5)	T2	(Depth of invasion)
17 (80.9)	T3	
1 (4.8)	T4	
7 (33.3)	Positive	Lymph node metastasis
14 (66.7)	Negative	
9 (39.1)	No addiction	Addiction
14 (60.9)	At least one type of addiction	

Statistical analysis

The data were analyzed by SPSS statistical software version 11.5. The Chi-square, Kruskal Valis, and Spearman tests were used to evaluate the frequency of microsatellite alterations and relationship between alterations of given markers with clinical/pathological parameters. A *P*-value equal or less than 0.05 was considered statistically significant.

Results

Clinicopathological data

Fifty tumors and their corresponded normal tissue samples of patients with ESCC were recruited in this study. Male to female ratio was 1.04 (51/ 49). Mean age (±SD) of patients were 61.9 (±11.5) years. Clinicopathological features of all patients are summarized in Table 2. The mean size (±SD) of tumors was 3.7 (±2.4) cm. For the samples with known pathologic data, 72% (18/25) were located at the middle of esophagus, and 24% (6/25) were located in the lower part of esophagus; An estimate of 43.5% (20/46) of tumors were moderately differentiated; in 81% (17/21) of tumors, the invasion progressed to adventitia (T3); stage III was observed in 63.1% (12/19) of cases and stage IIa for 36.8% (7/19); regional lymph node metastasis was presented in 33.3% (7/21) of tumors; addiction was reported for 60.9% (14/23) of patients which consisted each of cigarette, opium, water pipe, or alcohol.

LOH and MSI analysis

Loss of heterozygosity was shown in 66% (33/50) cases, and a total of 40% (20/50) of cases were microsatellite instable including 30% (15/50) low MSI and 10% (5/50) high MSI. The marker clarified the most loss of heterozygosity was D13S260 26% (13/50), and the most microsatellite instable marker was D5S2501, 20% (10/50). Some markers showed similar instability; D9S171 and TP53 with 18% (9/50), and D13S260 and D13S267 with 10% (5/50) (Table 3). The least frequent LOH and MSI marker were D9S171 (2%, 1/50) and D2S123 (8%, 4/50) respectively.

Association of LOH and MSI with clinical, histological, and pathological parameters. Statistical analysis indicated a near significant reverse correlation between grade and LOH (*P*=0.068, correlation coefficient= -0.272). Specifically, increased LOH in tumor DNA has a significant correlation with increased differentiation from poorly to well differentiated tumors (*P*=0.002 and *P*=0.016 respectively). Higher number of chromosomal loci with LOH showed a reverse correlation with lymph node metastasis (*P*=0.026, correlation coefficient= -0.485). It can be concluded that regional lymph node metastasis would be less likely with increased heterozygote loci. There was a positive correlation between addiction and MSI (*P*=0.026, correlation coefficient= 0.465), therefore any kind of addiction including opium, cigarette, water pipe and alcohol can be a susceptibility factor for microsatellite instability.

Table 3. Frequency and percentage of microsatellite alterations (LOH and MSI) in 50 ESCC tumor

Marker	Related Gene(s)	LOH+ (%)	MSI+ (%)	Total (%)
D2S123	hMSH2	0 (0)	4 (8)	8
D5S2501	APC	3 (6)	10 (20)	26
D13S260	BRCA2	13 (26)	5 (10)	10
D13S267	BRCA2	9 (18)	5 (10)	28
D9S171	p14, p15, p16	1 (2)	9 (18)	20
TP53	P53	6 (12)	9 (18)	30
Total		33 *(66)	20 *(40)	-

*Some samples had more than one LOH+ and/or instable marker, therefore the total number is less than the addition of values for each marker (LOH: loss of heterozygosity, MSI: microsatellite instability)

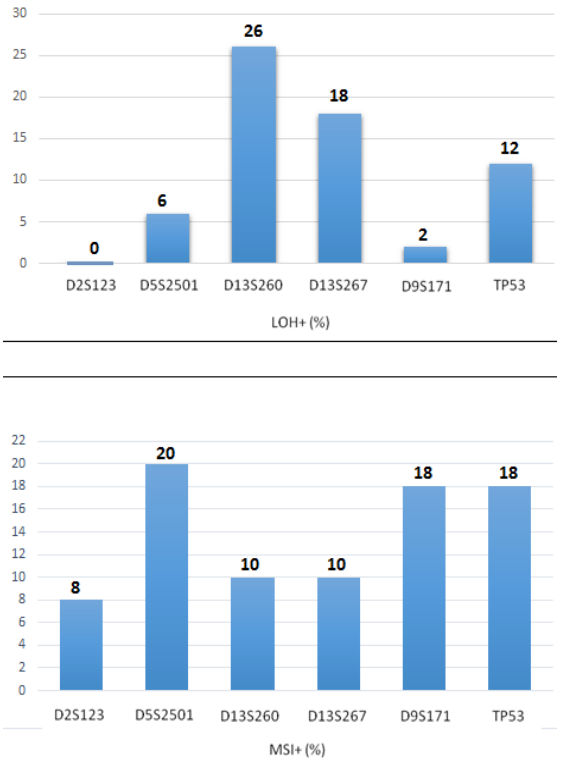

Figure 2. Comparison of the frequency of microsatellite alterations in distinct markers

Discussion

ESCC development is not well understood process as it involve highly complex molecular events. Chromosomal regions which include frequent allelic loss may most likely point to main susceptibility genes which help to understand the involved molecular pathways in esophageal carcinogenesis. These regions may be a probable potential for the development of markers for genetic susceptibility testing and screening for early identification of this type of cancer. While MSI is linked to defects in the DNA mismatch repair system and occurs in hereditary non-polyposis colon cancer (22) and other malig-nancies (21, 23), our knowledge regarding the role of MSI in esophageal carcinogenesis is too limited. In ESCC, MSI has not been considered as a major event in tumorigenesis process. According to the relatively small studies, the frequency of MSI in ESCC is ranged from 2 to 66.7% (14, 23-28). Although criteria to define MSS, MSI-L and MSI-H for colorectal cancer is accepted, for other human solid tumors the standard microsatellite markers, the required number of altered loci, and the degree of shift relative to normal, remained controversial. Considering this fact, we reviewed previous articles about MSI in ESCC and aimed to identify the chromosomal regions which were more informative for ESCC.

The microsatellite DNA in genome is important due to its highly polymorphic nature. It has been shown that these markers are the cause of variations among individuals. It is known that alterations in repeated sequences (microsatellites) are linked to the defects in some critical genes such as mismatch repair genes and tumor suppressor genes (29). According to previous results (30) and frequency percentage of microsatellite alterations, these microsatellite markers may be useful as predictive markers in detection of ESCC (17). In this study, we have investigated two major types of changes including microsatellite instability and loss of heterozygosity in microsatellite sequences. LOH at the specific chromosomal regions was a strong indicator of tumor suppressor genes at the relevant segment (e.g. p16 on chromosome 9, P53 gene on chromosome 17p13.1, BRCA2 gene on chromosome 13q, etc.). The alterations of BRCA2, p16 and P53 genes are common molecular events in esophageal carcinogenesis, and our results showed LOH in D13S260 and TP53 markers can be common events in ESCC patients (31). A high frequency of replication error (RER) and LOH involving 3p loci in the present study suggests that

microsatellite alterations involving 3p loci may be early and frequent events and that multiple tumor suppressor genes harboring these loci may be implicated in esophageal tumorigenesis. MSI is reported as a frequent event in esophageal adenocarcinoma, but not in ESCC (24, 32).

The results suggest that MSI could be an important event in the development of a subset of ESCC. A variation (from 0 to 20%) of MSI frequency was observed among the markers. This result suggests MSI is not accumulated uniformly through the genome, and certain loci are more susceptible to genetic alterations.

One of the most frequent LOH loci observed was D9S171 (9p21), where p15INK4b and p16 reside. With D9S171, LOH was previously reported to be 82% (14/17) in ESCC (32). Furthermore, using the same microsatellite marker D9S171, LOH was observed in 1 out of 10 informative cases (14). In addition, we found frequent genetic alteration events within locus 9p21 (33). Although LOH at D9S171 was found to be associated with frequent homozygous deletion at p15 INK4b while not showing the same association at p16 INK4a, in our study this LOH was only found in 18% of patients and this may confirm that such alterations are random. However, inactivation of p16 gene by hypermethylation of its promoter is a frequent event in ESCC as reported by Taghavi *et al* (34). In a Chinese study of D5S2501 and D2S123 markers, the highest level of MSI was reported with 20-40% frequencies. These results appear to differ from some previous studies in which very low frequency of MSI (2/29) was found in ESCC (35, 36). D13S260 marker showed the highest frequency of LOH among the 6 analyzed microsatellite loci (26%, 13/33) (Figure 2), which is similar to the results of previous studies (6, 31). Our finding regarding frequency of LOH in D9S171 and D13S267 markers was different from previous investigations (6, 37). This difference may in part occurred due to the more advanced stages of the resection specimens, which may have allowed them to accumulate a greater number of genetic alterations.

Lichun *et al* have not detected any micro-satellite instability in 34 patients with esophageal cancer. This group also reported 0% MSI for chromosomes 3, 5, 17, 18 (0/40 patient) and 5% MSI for chromosome 11 (2/38 patients) (38). According to these results, they claimed that MSI does not play an important role in the development of this type of cancer. However, we detected %40 MSI in this cancer by choosing suitable markers. Differences in MSI frequencies in the present and previous studies are possibly due to chance, however, there are alternative explanations that include the knowledge behind choosing microsatellite markers, differences in the type of samples studied (endoscopic biopsies here vs. resection specimens before), microdissection alterations, potential differences in tumor stage, or simply the fact that different population behave differently in a random way. Several studies detected PCR artifacts in using paraffin-embedded tissues as the source of DNA, especially when a small number of cells is analyzed (39). Reported prevalence of MSI in ESCC largely varies between various studies, ranging from 2–65% (24-27). This wide variation may reflect differences in MSI criteria and in markers tested. In most studies, however, the MSI-H frequency in ESCC was low nearly 10%, and the MSI pathway was considered unlikely to be involved in carcinogenesis in ESCC (40). Hayashi *et al* reported a MSI frequency in ESCC of 40% (12 of 30), but 11 of 12 tumors were categorized as MSI-L leading to the conclusion that MSI in esophageal tumors resulted from random replication error and not from mismatch repair defects (41). Our study also showed that MSI-H incidence of ESCC patients was relatively low; 14% (6 of 42), and it may confirm that MSI pathway was not involved in ESCC carcinogenesis. Shimada *et al* found a MSI frequency in 33 ECOPC (esophageal carcinoma with other primary carcinoma) patients of only 3% (1 patient) (42). We defined MSI status as shown at 6 recognized microsatellite markers. In contrast to our microsatellite instability studies in colorectal, and endometrial cancer in Iranian population (43), there was no correlation between the age and MSI status for ESCC patients. This may have occurred due to the differences in the microsatellite markers used in these studies. Some other researchers suggested that there is no relation between MSI status and the age of ESCC patients (26).

Conclusion

In this study we showed significant association of LOH and MSI with different clinicopathological features of ESCC patients, suggesting that development of ESCC is correlated with genetic instability including LOH and MSI. Analysis of these abnormalities can be a useful method for cancer screening and may have potential prognostic value.

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