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Loss of heterozygosity on chromosome 5 in Iranian esophageal cancer patients

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ABSTRACT. There is a high incidence of esophageal squamous cell carcinoma (ESCC) in Iran. Non-functionality of some tumor suppressor genes has been reported in esophageal cancer. Loss of heterozygosity on chromosome 5 has also been reported in esophageal carcinomas. We assessed loss of heterozygosity along a region of the long arm of chromosome 5 (5q), from 5q23.1 to 5q23.2, by PCR amplifying DNA fragments of tumor tissues from patients with ESCC and their corresponding normal samples. The PCR products were electrophoresed on 6% non-denaturing polyacrylamide gels, and band intensity was shown by silver staining. Of 40 patients with ESCC, 27, 25 and 36% of informative cases showed allelic losses at microsatellite markers D5S1384, D5S1478 and D5S1505, respectively. Two of the 40 patients studied had microsatellite instability at marker D5S1384. Based on the fact that loss of heterozygosity with more than 22% incidence for a specific marker cannot be regarded as a random event, we add support to previous reports concerning the presence of tumor suppressor genes in this chromosome region and that they affect esophageal cancer

development. According to the data in NCBI UniSTS, the PCR product size of human DNA with primers of the D5S1505 marker ranges from 243 to 275 bp, containing about 20 repeats of the TAGA tetranucleotide, while the amplicon size of one allele of one of our cases was 207 bp, with about 10 repeats of the TAGA tetranucleotide, which would be the shortest sequence reported so far.

Key words: Esophagus cancer; Esophageal squamous cell carcinoma; Loss of heterozygosity; Microsatellite instability; Chromosome 5; Iran

INTRODUCTION

Esophagus cancer is the sixth most common cancer worldwide (Parkin et al., 2005). There are two pathological types of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESSCs constitute 98% of esophageal neoplasias (Mathew et al., 2001), but EAC is currently increasing in the world (Wei and Shaheen, 2003; Haghdoost et al., 2008). A high incidence of ESCC has been reported in northern Iran. Esophagus cancer is characterized by a poor prognosis with a post-operative survival of 26 to 54% (Hosch et al., 2003). Seventy-five percent of patients die within one year after diagnosis (Hiyama et al., 2007), while five-year survival is less than 5%. The risk of developing esophageal cancer increases with age, and the highest incidence has been reported to be at the age of 50 to 70 years (Kollarova et al., 2007). Most patients diagnosed with esophageal cancer live in the so-called Asian esophageal cancer belt, including parts of northern Iran (Lam et al., 2000). In general, unlike high-risk populations, in which incidence rates for ESCC among males have been reported to be the same as those of females, in low-risk populations the majority of cases are males (Franco and Rohan, 2002). Epidemiological studies indicate that esophageal cancer is associated with the consumption of alcohol, tobacco, pickled vegetables, and hot drinks. These factors render the esophageal mucosa more susceptible to injuries caused by carcinogenic contaminations such as mycotoxins, nitrosyl compounds and possibly human papilloma and Epstain-Barr viruses (Lam, 2000). In addition to environmental factors, genetic factors have been suggested to play important roles in the development of esophageal cancer (Hiyama et al., 2007).

The activation of oncogenes and inactivation of tumor suppressor genes (TSGs) play roles in the etiology of cancer. Loss of heterozygosity (LOH) studies have proven to be useful for assessing TSG deletions and also for mapping candidate regions harboring potential TSGs (Zheng et al., 2005).

Microsatellite sequences are tandem repeats of short sequences that are scattered throughout the genome. Alterations of these repeats could serve as markers of a mutator phenotype in cancer following the approach of LOH analysis (Loeb, 1994). LOH has been detected at several chromosomal locations, such as chromosome 2q, 3p, 4p, 4q, 5q, 6q, 8p, 9p, 9q, 11p, 13q, 14q, 15q, and 17p in esophageal cancer (Hu et al., 2000)

LOH at loci of the tumor suppressor genes APC and MCC and the mismatch repair gene, MSH3, on chromosome 5q have been reported in esophageal carcinomas (Bodmer et al., 1987; Boynton et al., 1992). Since the observed allelic deletions were not limited to APC/MCC loci (5q21), some other relevant genes have been suggested to be located in this region of chromosome 5 (Boynton et al., 1992).

We used three microsatellite markers that cover chromosome 5 from 5q23.1 to 5q23.2 to analyze both LOH and microsatellite instability (MSI) of this chromosomal region in 40 Iranian ESCC patients.

MATERIAL AND METHODS

Tumor tissues and blood samples

Tumor tissues and peripheral blood samples were collected from 40 Iranian patients with ESCC. The tissue specimens that were examined by a pathologist were stored at -70°C until the time of use. Pathological parameters of tumors are described in Table 1. Blood samples were mixed with EDTA as an anticoagulant and stored at -20°C. DNA derived from blood samples was used as normal DNA.

Table 1. Pathological data of 40 esophageal squamous cell carcinoma from Iran.

Case No.	Gender	Age (years)	Differentiation	LNM	Case No.	Gender	Age (years)	Differentiation	LNM
1	M	55	×	+	21	F	60	Moderately differentiated	+
2	M	73	Poorly to moderately differentiated	-	22	M	65	Well differentiated	+
3	M	55	Well differentiated	+	23	F	55	Moderately differentiated	+
4	M	45	Poorly differentiated	+	24	F	66	Moderately differentiated	+
5	F	40	Moderately differentiated	+	25	M	70	Moderately differentiated	+
6	F	51	Well differentiated	+	26	M	67	Well differentiated	+
7	M	81	Moderately differentiated	+	27	M	68	×	×
8	M	65	Moderately differentiated	-	28	F	56	Moderately to well differentiated	+
9	F	55	Moderately differentiated	-	29	M	58	Well differentiated	-
10	M	53	×	-	30	M	70	Moderately differentiated	-
11	M	60	Well differentiated	+	31	F	52	Well differentiated	-
12	M	67	Moderately differentiated	+	32	M	81	Poorly differentiated	-
13	M	39	Undifferentiated	-	33	F	72	×	×
14	M	60	Moderately differentiated	+	34	M	35	Moderately to poorly differentiated	-
15	F	70	Well differentiated	+	35	F	70	Well differentiated	-
16	F	54	×	×	36	F	55	×	×
17	F	40	Well differentiated	-	37	M	48	Well differentiated	+
18	M	70	Well differentiated	-	38	F	58	Moderately differentiated	+
19	M	55	×	×	39	M	54	Moderately differentiated	+
20	M	60	Well differentiated	+	40	F	42	Moderately differentiated	-

× = data not available; LNM = lymph node metastasis.

DNA extraction

Tissue specimens were dissected, and adipose and connective tissues were removed; the specimens were then suspended in buffer containing 0.15 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.5, and lysed following the addition of 50-100 µg/mL proteinase K and SDS (final concentration of 0.5%). The lysate was incubated at 56°C for 2 h and then mixed with an equal volume of phenol. After gentle mixing and centrifugation at 14,000 rpm for 5 min, the supernatant was mixed with an equal volume of chloroform/isoamyl alcohol (24:1). The mixture was centrifuged at 14,000 rpm for 5 min, and the supernatant was diluted with 2.2 volumes of absolute ethanol. The precipitated DNA was washed with 70% ethanol, dissolved in 50-100 µL TE (10 mM Tris, 1 mM EDTA, pH 7.5), and then stored at -20°C (Lane et al., 1995). DNA was extracted from blood using the procedure of Barlett and Stirling (2003) with

slight modifications. A volume of 3 mL frozen blood was thawed at 37°C and mixed with 12 mL buffer A (containing 320 mM sucrose, 0.01 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1% Triton X-100) and centrifuged at 3000 *g* for 15 min. The pellet was suspended in buffer B (containing 0.4 M Tris-HCl, 0.06 M EDTA, 150 mM NaCl, 1% SDS, pH 8.0) and 250 µL 5 M sodium perchlorate. The mixture was incubated in a 65°C water bath for 20 min; afterwards, 2 mL ice-cold chloroform was added. After centrifugation at 2400 *g* for 2 min, DNA was precipitated with 2-3 mL ice-cold ethanol. The precipitated DNA was washed with 70% ethanol, dissolved in 50-100 µL TE and kept at -20°C.

PCR and microsatellite analysis

The LOH status of 40 ESCC patients was analyzed using polymerase chain reaction (PCR) amplification of three microsatellite repeats on chromosome 5 from 5q23.1 to 5q23.2. Characteristics of the microsatellite markers are shown in Table 2, according to data on the NCBI web-site (NCBI UniSTS, Map Viewer; www.ncbi.nlm.nih.gov/mapview/genome/sts). To amplify the target DNA sequences from tumor tissues and blood samples, we used the PCR in 50 µL PCR mixtures containing 12.5 ng DNA, 5 µL 10X PCR buffer, 3.75 mM $MgCl_2$, 0.31 mM dNTPs, 1.25 µL formamide, 2.5 U Taq DNA polymerase, and 0.62 µM of each primer. The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60-63°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were then mixed with 5 µL loading buffer (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and after denaturing at 95°C for 10 min, electrophoresed on a 6% non-denaturing polyacrylamide gel at 400 V for 1-2 h (Garcia, et al. 2003). Tumor and normal samples from the same patient were loaded side by side and the bands were visualized with a silver-staining method (Blum et al., 1987; Bassam and Caetano-Anollés, 1993). If one of the bands vanished completely or showed a diminution in intensity in the tumor DNA compared with the corresponding normal DNA, the tumor would be considered to have undergone loss of heterozygosity at the aforementioned loci. If some extra band(s) appeared in a tumor DNA in comparison to the corresponding normal sample, it would be interpreted as microsatellite instability.

Table 2. Characteristics of microsatellite markers.

Marker	Position (Mb)	Site	Ta (°C)	Size (bp)	F-primer	R-primer
D5S1384	118,857,860-118,858,038	5q23.1	61	179	CTAAACAGAAAAGAGCTAAGCCTA	TACCTACCTATATGCTCCCAATC
D5S1478	118,938,794-118,939,003	5q23.1	60	194-221	TGGTGTTTCCCAGAATCACT	TTGTGCTACAGGGGAATCAT
D5S1505	119,129,545-119,129,801	5q23.2	63	243-275	TAAGTGCCAGAGTCTCCCAC	TAAGGCATGTCTCGGAGCTA

Mb = mega-base pairs; Ta = annealing temperature.

Statistical analysis

The Fisher exact test was applied to determine the association between some pathological parameters and LOH at a specific marker.

Cloning procedure

PCR fragments of each allele for the D5S1505 marker from patient number 11, whose

one allele showed the shortest length, were ligated into (*Sma*I) linearized pBluescript II KS (+/-) (Sambrook et al., 1989). DH5α competent cells were transformed with plasmids (Perbal, 1988); afterwards, recombinants were identified via blue-white screening. Plasmids were spin-column prepared; the sequences were then determined by Geneservice, Ltd. (UK).

RESULTS

Forty Iranian ESCC patients between the ages of 35 to 81 years were studied. Twenty-four patients (60%) were males and sixteen patients (40%) were females. Representative electrophoresis is exhibited in Figure 1. Six of 22 (27%) heterozygous cases showed LOH at marker D5S1384. Four of 16 (25%) and 8 of 22 (36%) heterozygous cases showed LOH at markers D5S1478 and D5S1505, respectively (Table 3). There is no escaping the fact that the loss of one allele among the homozygous cases is not improbable; however, they are considered to be non-informative cases. The LOH frequencies of markers in tumors with different pathological parameters are shown in Table 1. The Fisher exact test showed no association between LOH and differentiation or metastasis stages of tumors ($P > 0.05$). Two cases showed MSI with marker D5S1384. We also found one patient (number 11) in which the PCR product size of one allele with primers of marker D5S1505 was 207 bp, containing about 10 TAGA repeats, which could be regarded as the shortest sequence reported so far (Figures 2-4).

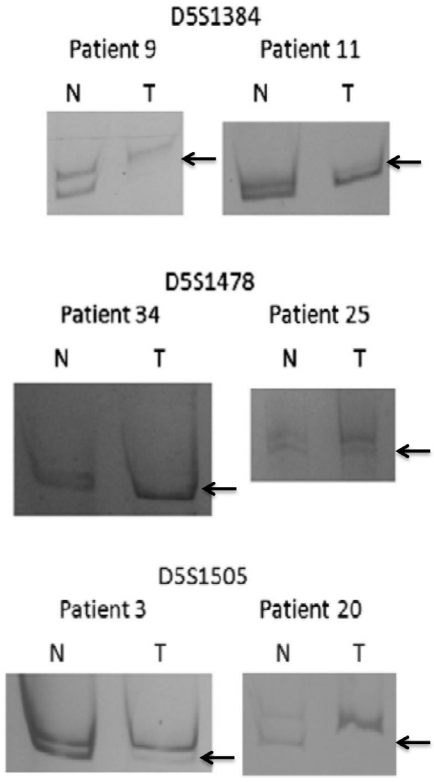

Figure 1. Some samples with loss of heterozygosity. N = normal; T = tumor.

Table 3. Results of loss of heterozygosity analysis.

Sample	D5S1384	D5S1478	D5S1505	Sample	D5S1384	D5S1478	D5S1505
1	○	×	●	21	×	×	●
2	×	×	×	22	○	×	×
3	○	●	●	23	○	×	×
4	×	×	×	24	○	×	×
5	●	×	○	25	●	●	○
6	○	○	×	26	×	●	●
7	●	×	○	27	○	×	○
8	×	○	×	28	×	○	×
9	●	×	●	29	×	○	○
10	×	○	○	30	○	○	○
11	●	×	○	31	MSI	×	×
12	×	○	×	32	×	×	●
13	○	×	○	33	○	×	×
14	×	○	○	34	×	●	×
15	×	×	○	35	×	×	×
16	●	○	●	36	×	×	×
17	○	○	○	37	×	○	×
18	○	×	×	38	○	×	○
19	○	×	×	39	○	○	○
20	MSI	×	●	40	○	×	×

● = loss of heterozygosity; ○ = heterozygous; × = homozygous (non-informative); MSI = microsatellite instability.

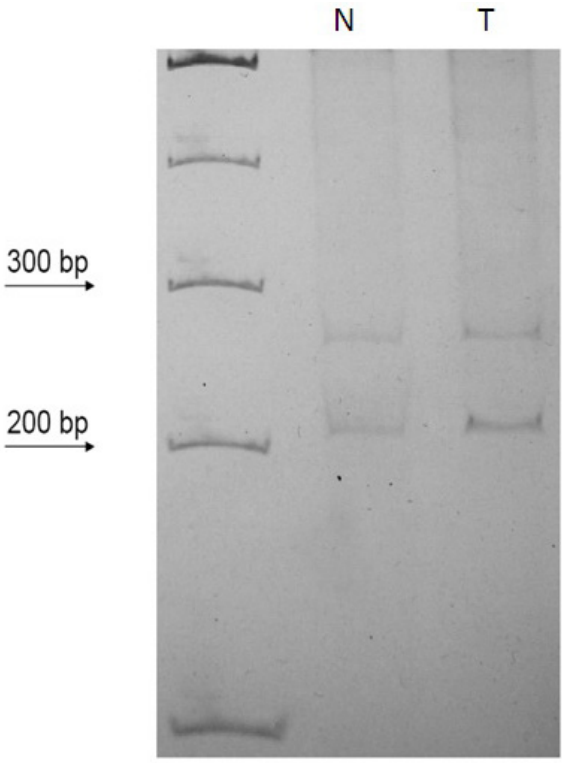

Figure 2. The PCR product size of sample 11 with primers of marker D5S1505. N = normal; T = tumor.

Figure 3. The sequence of one allele amplicon (253 bp) with primers of marker D5S1505 from normal sample of the patient number 11.

Figure 4. The sequence of the other allele amplicon (207 bp) with primers of marker D5S1505 from normal sample of the patient number 11.

DISCUSSION

Generally, it has been accepted that if one of the alleles of a TSG is mutated and the other is deleted, the gene will fail to function (Peralta et al., 1998). Thus, LOH at TSG loci would have an influence on tumorigenesis. Allelic losses on the small arm of chromosome 5 (5p) have been reported in hepatocyte carcinoma, lung cancer and cervix carcinoma (Dolan et al., 2000). Similarly, losses on the 5q arm were observed in many digestive tract malignancies such as of the colon, stomach and esophagus (Kanazawa et al., 2002). The highest incidence of LOH on chromosome 5q (5q35-ter) was detected in well-differentiated gastric adenocarcinoma (Sano et al., 1991). Seventy-seven percent of patients with ESCC, who showed high LOH frequencies, were detected in regions harboring the TSGs APC, MCC or both (Lee et al., 1990). Furthermore, allelic deletion at the tumor suppressor gene irf-1 locus (5q31.1) was observed in 44% of esophageal cancer patients (Peralta et al., 1998). Since deletions often involve several kilo base pairs and are not limited to these genes, some unknown TSG(s) may be present on chromosome 5 whose deletion is important in tumor development.

We carried out a set of experiments using three microsatellite markers to scan for deletions along the regions probably harboring TSG(s) on 5q. In our study 27, 25 and 36% of 40 Iranian patients with esophageal cancer showed LOH at the D5S1384, D5S1478 and D5S1505 markers, respectively. It was suggested that LOH with less than 22% incidence for a marker could be regarded as a non-specific event (Cai et al., 2007). Thus, the deletions we observed were not random occurrences and a TSG(s) is/are probably located near the region studied. Deletions at markers D5S1384 and D5S1505 may involve the APC/MCC and irf-1 genes, respectively. LOH at these TSGs may have an impact on cancer development. It is also possible that some unknown TSGs exist in deleted regions. There are 272 kbp between markers D5S1384 and D5S1505 (Li et al., 2004) containing two genes and three pseudogenes, according to data in NCBI (NCBI Map Viewer; http://www.ncbi.nlm.nih.gov/mapview). One of the genes is HSD17B4, which encodes a bifunctional enzyme that is involved in the peroxisomal beta-oxidation pathway for fatty acids. It also acts as a catalyst for the formation of 3-ketoacyl-CoA intermediates from both straight-chain and 2-methyl-branched-chain fatty acids (Moller et al., 1999). Marker D5S1384 is located in the HSD17B4 gene. The other protein-coding gene is FAM170A. There is only evidence at the transcript level about it (uniprot: A1A519). It is not clear whether alterations of these genes are involved in the process of human carcinogenesis.

Two of the 40 patients studied (5%) showed MSI at marker D5S1384, which is in agreement with previous reports on MSI. It is reported that MSI occurs frequently in Barrett's-associated EAC, but not in ESCC (Meltzer et al., 1994). Additionally, there is evidence implying an inverse relationship between LOH and MSI in other cancers (Cai et al., 2007).

Allelic losses in the region that we studied were previously observed in 50% of the esophageal cancer patients studied in China. This may be due to the nutritional and environmental similarities between Iran and China (Li et al., 2004).

Our statistical analysis showed no association between the differentiation status of tumors and deletions. This result may suggest that LOH in the chromosomal region studied is an initial event leading to cancer development.

The PCR product size of human DNA with primers of marker D5S1505 is expected to be from 243 to 275 bp according to the NCBI UniSTS database. So far, the length of less than

243 bp was not reported for the human genome concerning the marker D5S1505. In one of our samples (number 11), the PCR product sizes of the corresponding normal and tumor DNA with primers of marker D5S1505 were 253 and 207 bp, containing about 20 and 10 repeats of a TAGA tetranucleotide, respectively. This may imply that some of the repeated units were lost, probably due to genomic instability, which makes normal cells susceptible to carcinogenesis.

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