Original Article

FREQUENT ALLELIC LOSS/IMBALANCE ON THE SHORT ARM OF CHROMOSOME 3 IN TONGUE CANCER

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Abstract

Frequent allelic imbalances including loss of heterozygosity (LOH) and microsatellite instability (MSI) on the short arm of chromosome 3 (3p) have been found in several types of human cancer. This study was designed to identify the tumor suppressor locus (or loci) on 3p associated with tongue squamous cell carcinoma (SCC). Among 16 patients with tongue SCC tested, 7 (44%) of 16 informative cases showed LOH at one or more loci. Deletion mapping of these 16 tumors revealed two discrete, commonly deleted regions on the chromosome arm. Our data support the notion that tumor suppressor gene(s) contributing to the progression of tongue squamous cell carcinoma reside on 3p24 and 3p25.

Key words: Chromosome 3—Oral squamous cell carcinoma—Tumor suppressor gene—Loss of heterozygosity—Microsatellite instability

INTRODUCTION

Although squamous cell carcinoma (SCC) is the most common malignant tumor of the oral cavity, no useful genetic marker that contributes to determining the prognosis of tongue SCC has been characterized. Despite the clinical relevance, very little is known about the molecular basis of tongue SCC, compared to other human malignancies²¹⁾.

Inactivation of tumor suppressor genes and activation of oncogenes have been considered to play important roles in the multi-step pro-

cess of human tumorigenesis. For example, loss of heterozygosity (LOH) in malignant cells has proven useful for mapping DNA regions potentially harboring candidate tumor suppressor genes¹⁹⁾.

In oral SCCs, we have detected high frequencies of LOH on several chromosome arms, such as 4q, 5q, 7q, 9q, 11q, 13q, 18q, 21q and 22q^{6,7,11,16-18)}, where candidate tumor suppressor genes might be present. Moreover, a previous allelotyping study of head and neck SCC, including tongue SCC, revealed consistent chromosomal deletions involving chro-

Table 1 Summary of clinicopathological features in 16 tongue cancers
T, tumor size; N, regional lymph node; TNM, tumor size, regional lymph node and distance metastasis; pN, post-operative pathological metastasis to lymph node.

		Total	Total(%)
Gender	male	10	62.5%
	female	6	37.5%
Region	tongue	16	100%
T classification	T1	5	31.3%
	T2	6	37.5%
	T3	5	31.3%
	T4	0	0%
N classification	N0	9	56.3%
	N1	2	12.5%
	N2	5	31.3%
TNM stage	I	4	25%
	II	3	18.8%
	III	4	25%
	IV	5	31.3%
Differentiation	well	11	68.8%
	moderately	2	12.5%
	poorly	3	18.8%
pN	(+)	3	37.5%
	(-)	5	62.5%
Mode of invasion	Type 3	10	62.5%
	Type 4C	6	37.5%
Progress	well	8	50.0%
	poor	8	50.0%
	Death	1	6.2%
	Recurrence	2	12.5%
	Lymph node or Distant metastasis	6	37.5%

mosomes 5p, 9q, 11q and 17q¹⁾. Thus, many possible tumor suppressor genes that have not been characterized may be associated with the development of human oral cancer.

Recent allelotype analyses in certain human carcinomas other than oral cancer have identified specific deleted regions on the short arm of chromosome 3 (3p)^{5,8,14)}, suggesting that one or more tumor suppressor gene(s) specific to several types of human cancer may exist on 3p. Therefore, we hypothesized the existence of new tumor suppressor gene(s) specific to tongue SCC on this chromosome arm.

In the present study, as a first step toward the isolation of the putative suppressor gene(s) associated with tongue SCC on 3p, we examined 32 tissue samples (16 sets of primary tumors and corresponding normal tissues) from 16 patients with various grades of tongue SCC. By comparing the results obtained from tumor and normal DNA, a detailed deletion map of the chromosome arm was constructed.

MATERIALS AND METHODS

1. Tissue samples

Sixteen primary tongue SCC tumors and corresponding normal tissues were obtained from 16 patients at the time of surgical resection at Department of Oral and Maxillofacial Surgery, Tokyo Dental College. The study was approved by the Ethics Committee, and each volunteer gave informed consent. The resected tumor samples were trimmed carefully to remove normal fibrous tissue, and one part of each sample was stored at -80°C for DNA extraction. The remaining samples were fixed in 10% formalin to examine them microscopically for the presence of tumor, evaluation of tissue morphology, and grade of differentiation (Table 1). Histopathological classification was performed according to the International Classification of Tumors²⁰⁾. The clinical staging was determined by the UICC TNM staging system¹⁰⁾.

2. DNA isolation

All tumor and most of the normal tissues were processed from fresh frozen specimens preserved in liquid nitrogen immediately after resection. A part of the normal tissues was prepared from corresponding peripheral blood samples at the same time. Genomic DNA was extracted as described previously¹³⁾. The concentrations of extracted DNA were estimated by a spectrophotometric method. From each sample, 50 ng was used as a template for the polymerase chain reaction (PCR) amplification procedure.

Fig. 1 A deletion mapping of chromosome 3p in 16 cases with LOH or MSI at one or more loci. The case number is shown on each column, and the 10 microsatellite markers are indicated on the left.

3. PCR-microsatellite analysis

Ten highly informative microsatellite markers (D3S192, D3S1007, THRB, D3S647, D3S32, D3S966, D3S1228, D3S1079, D3S659 and D3S30) were used for microsatellite motifs at 3p. PCR amplification was performed in a total reaction volume of 50 μ l, as described previously⁴. The forward primers were end-labeled with gamma phosphorus-32-ATP (Amersham, Sylesbury, UK) in the pre-mix step using T₄ polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). Each PCR reaction mixture contained 50 ng of sample DNA, 20 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl₂, 2 mM dNTP and 0.5 unit of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Twenty-two cycles of denaturation at 94°C for 1 min, annealing at 52–58°C for 1 min, and extension at 72°C for 1 min were performed with a DNA Thermal Cycler (Perkin-Elmer). After dilution with

an adequate volume of formamide-dye mixture (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), the PCR products were heat-denatured (98°C, 5 min), chilled on ice, and electrophoresed on 6% urea-formamide-polyacrylamide gel at 3W for 2 to 3 hours, depending on the fragment size. Silver staining of the gels were performed using the DNA Silver Staining Kit (Amersham Pharmacia Biotech AB Sweden). To ensure the reproducibility in each case showing LOH or MSI, second PCR-microsatellite analysis was performed.

4. Assessment of LOH and MSI

LOH for DNA samples was assessed densitometrically, as described previously⁹. Briefly, after correction for differences in the amounts of DNA loaded in each lane, the intensities of the signals in tumor DNA were compared to those of the corresponding normal DNA.

Table 2 Loss of heterozygosity at ten microsatellite regions on 3p in primary tongue cancers

Locus Symbol	Chromosomal Location	Number of Informative Case(%)	Frequency of LOH(%) (LOH/Informative Cases)
D3S192	3p25	8(50.0)	12.5(1/8)
D3S1007	3p25	14(87.5)	21.4(3/14)
THRB	3p24	14(87.5)	21.4(3/14)
D3S647	3p23	8(50.0)	0(0/8)
D3S32	3p21.3–21.2	5(31.3)	0(0/5)
D3S966	3p21.3	7(43.8)	14.3(1/7)
D3S1228	3p14.3–14.1	9(56.3)	0(0/9)
D3S1079	3p13	14(87.5)	14.3(2/14)
D3S659	3p13	7(43.8)	0(0/7)
D3S30	3p12	10(62.5)	0(0/10)

More than 50% reduction in signal intensity was assessed as LOH. Commonly deleted regions were defined by considering the loci most frequently showing LOH, together with multiple interstitial deletions. MSI for DNA samples was also assessed as positive when there were additional bands in the tumor sample that were not observed in the corresponding normal sample or there was a band shift in tumor sample that contrasted to those of the corresponding normal bands.

The Fisher's exact test was performed to evaluate the significant correlation between LOH or MSI and clinical parameters.

RESULTS

A total of 32 tissue samples (16 sets of primary and corresponding normal tissues) from 16 patients with various grades of tongue SCC was studied for LOH using 10 high polymorphic markers mapped on 3p. Of 16 cases that were informative for at least one of the loci, 7 (44%) showed allelic deletions. The results of the LOH analyses in tumor samples showing allelic deletions are summarized in the deletion map in Fig. 1. Frequent allelic deletions were observed at loci on D3S1007 (21.4%) and THRB (21.4%) (Table 2). Among these, one locus, THRB, has already been reported as a candidate tumor suppressor gene locus for oral cancer⁸⁾. The first region of deletion (D3S1007) identified in this study, however,

has not been reported as a putative tumor suppressor locus in any type of human cancer. Microsatellite instability (MSI) was observed in 2 (12.5%) of 16 informative cases in the present study. The most frequent MSI was identified at the marker D3S1228 (22%). No statistically significant correlation was observed between the presence of LOH and TNM stage of tumor differentiation (Table 3). Typical examples of allelic imbalances (LOH or MSI) on 3p are shown in Fig. 2.

DISCUSSION

Carcinogenesis is a multistep process in which several oncogenes and tumor suppressor genes are considered to be involved. Putative tumor suppressor gene(s), which may play an important role in oral squamous cell carcinogenesis, are located on the arms of several chromosomes. Our previous studies of LOH at 21p for primary oral SCCs showed allele loss in 44% of informative cases²¹⁾. Recent studies have indicated that the chromosome 3p arm is frequently altered in patients with several types of malignant tumors, including nasopharyngeal cancer¹⁴⁾, ovarian cancer²⁾ and oral cancer^{7,13)}. Although genetic abnormalities have been reported in many types of tumor, there have been few reports of such changes in tongue cancer.

In the present study, we have confirmed that chromosome 3p-allele imbalance defined

Table 3 Relationship between allelic imbalances and clinicopathological features on 3p

		Total	Total (%)	LOH (+)	LOH (-)	LOH (%)	MSI (+)	MSI (-)	MSI (%)
Gender	male	10	62.5%	5	5	50%	2	8	20%
	female	6	37.5%	2	4	33.3%	0	6	0%
Region	tongue	16	100%	7	9	43.8%	2	14	12.5%
T classification	T1	5	31.3%	1	4	20%	0	5	0%
	T2	6	37.5%	4	2	66.7%	2	4	33.3%
	T3	5	31.3%	2	3	40%	0	5	0%
	T4	0	0%	—	—	—	—	—	—
TNM stage	I	4	25%	1	3	25%	0	5	0%
	II	3	18.8%	2	1	66.7%	1	2	33.3%
	III	4	25%	0	4	0%	0	4	0%
	IV	5	31.3%	4	1	80%	1	4	20%
Differentiation	well	11	68.8%	6	5	54.5%	2	9	18.2%
	moderately	2	12.5%	0	2	0%	0	2	0%
	poorly	3	18.8%	1	2	33.3%	0	3	0%
pN	(+)	3	37.5%	1	2	33.3%	1	2	33.3%
	(-)	5	62.5%	4	1	80%	1	4	20%
Mode of invasion	Type 3	10	62.5%	6	4	60%	2	8	20%
	Type 4C	6	37.5%	1	5	16.7%	0	6	0%

Fig. 2 Microsatellite polymorphism analysis in tongue SCC specimens. Case numbers are shown above and locus symbols are indicated below. Comparison of microsatellite between normal (N) and tumor (T) DNAs. Typical examples of allelic imbalances (LOH and MSI) on 3p are shown.

as LOH and/or MSI is associated with the development of tongue SCC. Seven (44%) of 16 informative tongue SCC cases examined in this study showed LOH at one or more loci (Fig. 1). Based upon our deletion map on the chromosome arm, two discrete candidate regions have been proposed to contain tongue SCC tumor suppressor genes. The most frequently deleted region was identified between markers D3S1007 and THRB on chromosome band 3p24, where frequent LOHs have also been reported in oral cancer⁸⁾. These observations suggest that a putative tumor suppressor gene associated with tongue SCC as well as oral cancer is located in this region. We found a different commonly deleted region at marker D3S1007 mapped on 3p25. Interestingly, this locus includes several genes such as TGF β -RII, FHIT (Fragile Histidine Triad), and VHL (von Hippel Lindau). According to previous studies, those genes seem to be partly associated with the development of human cancer. For example, the TGF β -RII gene is the signal transfer of TGF β that introduces invasive capacity *in vitro*³⁾. FHIT is associated with neoplastic transformation of leukemia¹⁵⁾. The VHL gene is associated with transcription inhibition of colon cancer¹²⁾. Mutational analyses of these genes are needed in further studies, in order to clarify whether they are correlated with tongue SCC. Furthermore, we have identified a novel, commonly deleted region at chromosome band 3p25 (D3S1007), suggesting that other unknown tumor suppressor gene(s) specific to tongue SCC may exist at this region.

In this study, MSI on 3p was also examined in our tongue SCC cases. Overall, the frequency was 12.5% (2 of 16 cases). We found that the incidence of MSI at D3S1228 was the highest among the microsatellite makers tested where no LOH was also detected.

We have statistically analyzed the correlation between the results of 3p-abnormalities with the clinicopathological features of the tumors. A statistically significant correlation was observed only between MSI and TNM tumor staging (Table 3), indicating that such genetic abnormalities may correspond to the

progression of oral SCC, but the sample size was small. At present, it is not known how 3p-MSI is involved in the carcinogenetic process of tongue SCC. Nevertheless, analysis of the MSI in patient with tongue SCC may be useful for determination of prognosis, since it may reflect the tumor staging.

In summary, we have identified two different tumor suppressor gene loci, including a novel deleted region on 3p25, in tongue SCC and have also found frequent MSI on this chromosome arm, which may serve as a useful marker for the evaluation of this disease.

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