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Original Article

Loss of Heterozygosity (LOH) on Chromosomes 2q, 3p and 21q in Indian Oral Squamous Cell Carcinoma

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Abstract

Around the world, 200,000 people a year are affected by oral cancer, and the incidence of this disease is 10 times higher in India than Japan, mainly due to the custom of chewing tobacco. Loss of heterozygosity (LOH) on the long arm of chromosome 2 (2q), the short arm of chromosome 3 (3p) and the long arm of chromosome 21 (21q) are observed in several human cancers. We identified novel tumor suppressor loci on these regions in primary oral squamous cell carcinomas (OSCCs) in Japanese. However, there has been no detailed analysis of LOH on these chromosomes in Indians. In the present study, we investigated LOH at 2q, 3p and 21q using 9 microsatellite markers in 26 Indian OSCCs. LOH was detected in 25 (96.2%) out of 26 informative samples at one or more of the loci examined. On the basis of the results, two commonly deleted regions were identified and a detailed deletion map was constructed. In the first region, a high frequency of LOH was observed at the D3S1007 locus (53.8%) on 3p25, which is located in the region neighboring the *VHL* (von Hippel-Lindau) gene. In the second region, LOH was concentrated at the D3S966 locus (50.0%) on 3p21.3, suggesting the presence of a putative tumor suppressor gene (TSG) associated with OSCCs. These results strongly suggest that there are at least two candidate TSGs located on chromosome 3p, and that alteration in them is associated with the tumorigenesis of OSCCs.

Key words: Oral squamous cell carcinoma—Loss of heterozygosity (LOH)—Chromosome 2q—Chromosome 3p—Chromosome 21q

Introduction

Approximately 350,000 new cases of oral and oropharyngeal SCCs are diagnosed annually, worldwide[21]. Two major etiological factors are recognized: tobacco and alcohol. The type and usage of these agents is dependent on social and cultural factors, which to a variable extent underlie the geographic variation in the incidence of OSCCs. In Indians, in

particular chewing tobacco is considered to be a personal habit. This chewing tobacco, locally known as 'Khaini', which is a raw tobacco pasted/powered in lime (calcium hydroxide) is very dangerous in the development of oral cancer.

Studies on the molecular mechanisms of cancer have revealed that carcinogenesis proceeds from the accumulation of genetic alterations in a multistep process[5,12]. For example, inactivation of tumor suppressor genes (TSGs) and activation of oncogenes play important roles in the development and progression of carcinomas[5,12]. One strategy of locating putative TSGs is to survey tumors for high rates of loss of heterozygosity (LOH). Oral squamous cell carcinoma (OSCC) is one of the most frequently occurring human malignancies[21]. In OSCC, several LOH studies have revealed consistent chromosomal alterations involving chromosomes 2q, 3p, 4q, 5q, 7q, 8p, 9p, 10q, 11q, 13q, 18q, 20q, 21q and 22q[2,3,9,14,16,18,19,21,31–35,38,39,41]. Additionally, we have previously demonstrated LOHs on 2q, 3p and 21q in 67.5%, 60% and 65% of OSCCs, respectively in Japanese[40]. However, to the best of our knowledge, chromosomes 2q, 3p, and 21q have not yet been studied for LOH in Indian OSCCs. Given the high frequency of LOH in these chromosomes, we hypothesized that LOH in these regions was a target loci for the development of Indian OSCC.

In the present study, as a preliminary step in isolating putative TSGs associated with Indian OSCC on human chromosomes 2q, 3p and 21q, we examined 26 oral tumors by using 9 high polymorphic microsatellite markers and constructed a detailed deletion map of these chromosomes. In addition, we compared the data with various clinicopathological features, including personal habits.

Materials and Methods

1. Tissue samples and clinicopathologic findings (Table 1)

Sample tissues were taken from 26 Indian patients with OSCC. Tissue samples consisted of 26 primary tumors and normal blood corresponding to those primary tumors.

The subjects consisted of 12 men and 14 women. Average age was 49.5 years for the men (between 34 and 76 years old), 49.4 years for the women (between 39 and 69 years old), and 49.4 years for both sexes (between 34 and 76 years old). T classifications to indicate the sizes of the primary clinical tumors were: 5 T1, 8 T2, 10 T3 and 3 T4. The TNM stage classifications of was as follows: 3 Stage I, 2 Stage II, 15 Stage III and 6 Stage IV. Grades of histological differentiation were as follows: 18 well differentiated tumors, 5 moderately differentiated ones and 3 poorly differentiated ones.

2. DNA extraction from paraffin sections

All patients had histologically confirmed OSCCs, and the tumor samples for DNA extraction were checked to ensure that they consisted of more than 80% tumor. Deoxyribonucleic acid was extracted from the paraffin blocks after microdissection of tumor tissue. Multiple 10-μm sections were cut and mounted on silanated slides and left overnight at 37°C. They were then dewaxed in xylene and taken to water through descending grades of alcohol. The sections were lightly stained in Harris hematoxylin and compared with a hematoxylin and eosin-stained section on the same block. Tumor cells were identified and selected by cutting them out of the section using a scalpel blade and fine forceps. Normal cells were prepared from peripheral blood samples at the same time. Genomic DNA was extracted from several paraffin-embedded sections from each tumor sample using Takara DEXPAT (Takara, Tokyo, Japan).

After the DNA samples had been extracted by phenol-chloroform extraction and refined, they were washed and precipitated with ethanol. Concentrations of extracted DNA were determined by spectrophotometric method and kept frozen at −80°C. From each DNA sample, 1 μl (2.5 ng/μl) was used as a template for the polymerase chain reaction (PCR) amplification procedure.

Table 1 Summary of clinicopathologic features in 26 oral SCCs

	Sex	Age	Personal habits	T	N	Stage	Differentiation
1	F	39	Chewing tobacco	3	0	III	well
2	M	45	Chewing tobacco	4	2	IV	poor
3	M	40	Chewing tobacco	2	0	II	well
4	F	62	Chewing tobacco	1	0	I	well
5	M	67	Chewing tobacco	3	1	III	mod
6	F	43	Chewing tobacco	1	1	III	poor
7	M	50	Chewing tobacco	2	1	III	well
8	F	52	Chewing tobacco	1	2	IV	well
9	F	45	Chewing tobacco	4	2	IV	poor
10	F	40	Chewing tobacco	3	1	III	well
11	M	60	Chewing tobacco	3	1	III	well
12	M	34	Chewing tobacco	2	1	III	well
13	F	45	Chewing tobacco	2	1	III	well
14	M	45	Chewing tobacco	3	2	IV	well
15	F	40	Chewing tobacco	3	1	III	well
16	M	38	Chewing tobacco	1	0	I	well
17	F	41	Chewing tobacco	2	1	III	well
18	F	69	Chewing tobacco	4	1	IV	mod
19	M	51	Chewing tobacco	1	0	I	well
20	M	76	Chewing tobacco	3	0	III	well
21	F	64	Chewing tobacco	3	1	III	mod
22	F	45	Chewing tobacco	3	0	III	well
23	F	50	Chewing tobacco	2	0	II	well
24	M	43	Chewing tobacco	2	1	III	mod
25	F	56	Chewing tobacco	3	2	IV	mod
26	M	45	Chewing tobacco	2	1	III	well

M: man, F: woman.
well: well differentiated, mod: moderately differentiated, poor: poorly differentiated.

3. PCR and microsatellite analysis

We investigated whether these tumor suppressor genes were involved in the generation and development of oral SCCs. We used 9 microsatellite markers from among those on 2q, 3p and 21q. All primers were obtained from Research Genetics (Huntsville, AL) (Table 2). PCR amplification was performed in a total reaction volume of 20μl as described previously[15]. Each PCR reaction mixture contained 2.5 ng sample DNA, 20 pmol each primer, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM dNTP and 0.5 unit Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Thirty-five cycles of denaturation at 9°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min were performed using the DNA Thermal Cycler 9700 (Perkin-Elmer Cetus). 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 fragment size. Silver staining of the gels was performed using the DNA Silver Staining Kit (Amersham Pharmacia Biotech, Piscaway, NJ). To ensure reproducibility in each case with LOH or MSI, all tests were performed under the same conditions.

Table 2 Sequence of primers used for PCR-LOH analysis

Locus symbol	Sequence of primers
D2S1327	5′-TGACCAGGGGAAGATACTGA-3′ 5′-TGAATTGAATAATAACACTCTGTGC-3′
D2S206	5′-TTAAAAATTAAGTAGGCTTTTGGTT-3′ 5′-GTCCTCATGTGTTTATGCTGT-3′
D3S1007	5′-GAAGGGTCACTTGAGTCTAGGAG-3′ 5′-ATTTGCCACCATGCCTGGCTAG-3′
D3S966	5′-TACCTCCTCACTGTTTCATATTAG-3′ 5′-CACATAGTATGTCTCGGCTAACAG-3′
D3S1079	5′-GGGAGATAGGTAGTATCATCT-3′ 5′-ATCTACCATTAAGGCAACCTG-3′
D21S369	5′-ATGGCCTTGGCTAAATGCTG-3′ 5′-CTAAGCTGATATGGTAAGTACA-3′
D21S236	5′-CCCAAATAAAAAAGAGAACAG-3′ 5′-CTAAAGAGGACTTCAGAGTAAGG-3′
D21S11	5′-GTGAGTCAATTCCCCAAG-3′ 5′-GTTGTATTAGTCAATGTTCTCC-3′
D21S1254	5′-AAATACTGATGATCCTTAATTTTGGG-3′ 5′-GGTGGCTGAGCGAGAC-3′

4. Assessment of loss of heterozygosity (LOH)

LOH in the tumor DNA samples was assessed by scanning densitometry and analyzed with National Institute of Health (NIH) software (Image version 1.62, Dr. W. Rasband, NIH, Bethesda, MD, USA). The intensities of the signals in tumor DNA were compared with those of the corresponding normal DNA. A reduction in signal intensity of more than 50% was required for LOH. Commonly deleted regions were defined by considering the loci most frequently showing LOH, together with multiple interstitial deletions.

5. Statistical analysis

The Fisher's exact test was used to determine significance of correlation between LOH and the clinicopathological parameters. Level of significance was set at $p<0.05$.

Results

We analyzed tumors from 26 unrelated Indian patients with OSCCs by PCR-LOH assay with 9 microsatellite markers. LOH was observed on at least one of the loci in 25 out of the 26 informative cases (96.2%). LOH on chromosomes 2q, 3p and 21q was observed at one or more loci in 26.9% (7/26), 84.6% (22/26) and 61.5% (16/26), respectively, in the tumors examined. We detected a high concentration of LOH at D3S1007 and D3S966 on 3p25 (53.8%) and 3p21.3 (50.0%), respectively, suggesting the presence of putative TSGs associated with Indian OSCC. Other loci on the other chromosomes showed lower frequencies (0 to 42.3%) of LOHs. The results of PCR-LOH assay are summarized in the deletion map in Fig. 1. Location of the 9 markers and frequencies of LOH are summarized in Table 3. Examples

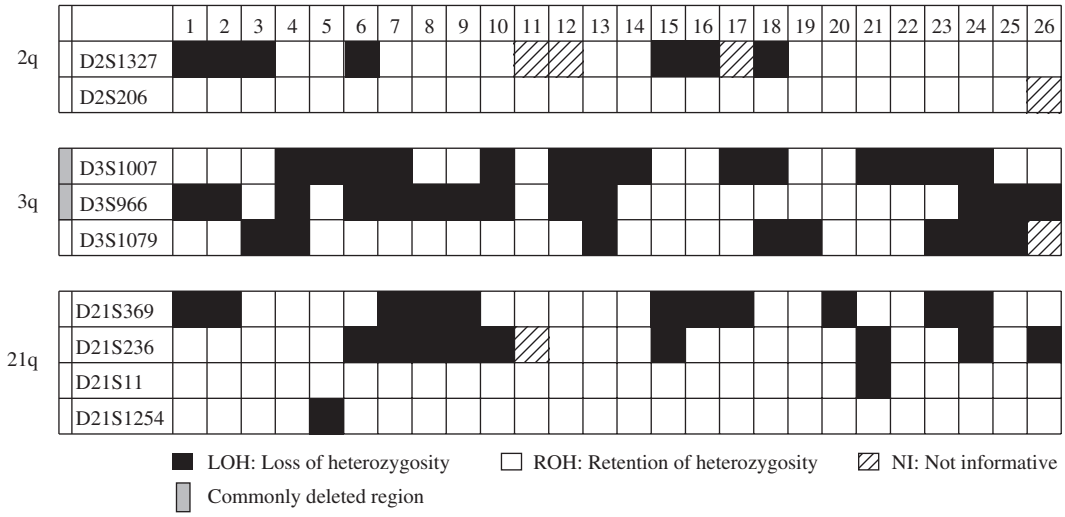

Fig. 1 Deletion mapping of chromosomes 2q, 3p and 21q in 26 Indian oral SCCs
Case numbers are shown at top and locus symbols on left.

Table 3 LOH at 9 microsatellite loci on chromosomes 2q, 3p and 21q in 26 Indian oral SCCs

Chromosomal location	Locus symbol	Frequency of LOH (%) (LOH/informative cases)
D2S1327	2q32-35	30.4% (7/23)
D2S206	2q36	0% (0/25)
D3S1007	3p25	53.8% (14/26)
D3S966	3p21.3	50.0% (13/26)
D3S1079	3p13	32.0% (8/25)
D21S369	21q11.1	42.3% (11/26)
D21S236	21q11.1	36.0% (9/25)
D21S11	21q21	3.8% (1/26)
D21S1254	21q22.1	3.8% (1/26)

of a typical LOH, an informative case (INF), and a non-informative case (NI) on 2q, 3p and 21q are shown in Fig. 2. One tumor sample exhibited no LOH at chromosomes 2q, 3p or 21q loci (Case No. 11).

We found commonly deleted regions at 3p25 and 3p21.3, the most frequently detected LOHs by Arai *et al.*[3] in Japanese OSCC. Among these, we detected a high frequency of LOH on D3S1007 at 3p25, near which the *VHL* (von Hippel-Lindau) gene was mapped in the present study.

We compared our results using Fisher's exact test to analyze the correlation between LOH and clinicopathological features. The results revealed no statistically significant correlation. No significant statistical correlation between incidence of LOH and TNM staging or grade of differentiation was noted. As all the patients investigated in this study had the habit of chewing tobacco, no connection with LOH was revealed.

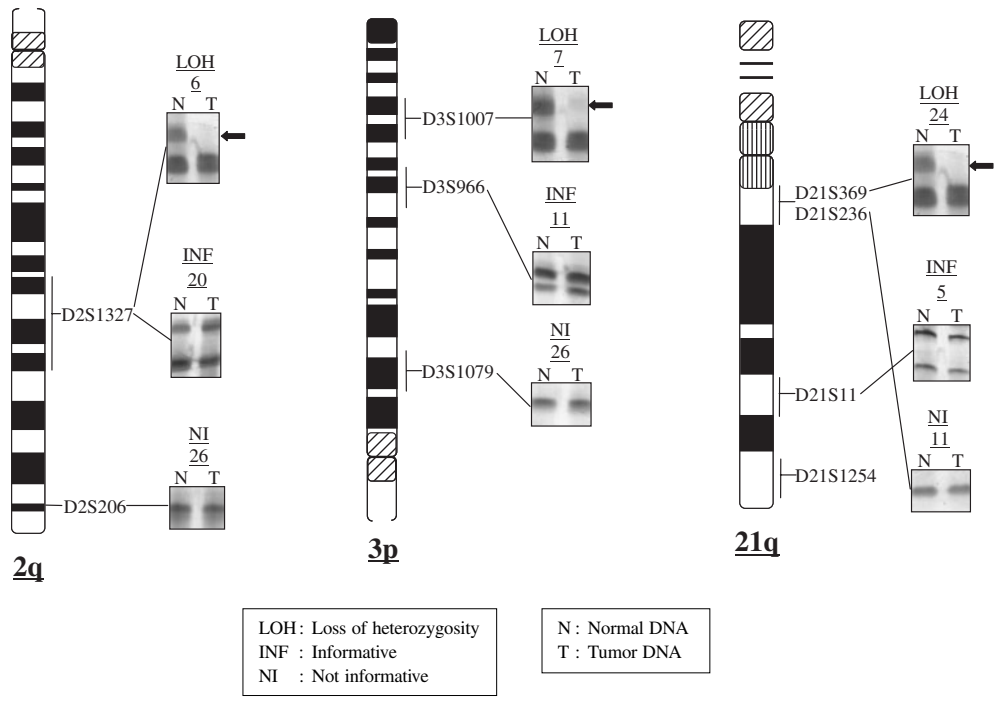

Fig. 2 Illustration of microsatellite polymorphism analysis in oral SCC specimens

Case numbers are shown at top and locus symbols on left. Paired normal (N) and tumor (T) samples from patients 5, 6, 7, 11, 20, 24 and 26 demonstrating loss of upper allele (LOH), retained heterozygosity (INF) and non-informative (NI), respectively.

Discussion

A number of detailed maps of each chromosome have been constructed for many types of human cancer for the isolation of putative TSG(s). LOH on chromosomes 2q, 3p, and 21q has frequently been reported in several types of malignant tumor such as gastric cancer[17,23], lung cancer[20,24,29], head and neck cancer[22], neuroblastoma[30], granulose cell tumor[36], thyroid cancer[37], renal cancer[28], breast cancer[11], uterine cervical cancer[6], ovarian cancer[4,27] and esophageal cancer[1,13]. However, studies on chromosomes 2q, 3p and 21q with respect to the oral region have been few in number and inconclusive in the Indian population. In our previous study, 2, 3 and 4 potential TSGs loci were identified on 2q, 3p and 21q in Japanese OSCCs[40]. In the present study, our findings showed LOH at multiple loci on D3S1007 (53.8%) in 3p25 and D3S966 (50.0%) in 3p21.3 in Indian OSCCs. This was comparable to our previous results on OSCCs[3]. Interestingly, the 3p25 locus includes the *VHL* gene, and alterations have been found in patients with von Hippel-Lindau disease or sporadic clear cell renal carcinoma[25]. In particular, it has been suggested that LOH at the *VHL* locus occurs most frequently in SCC of the upper aerodigestive tract including the oral cavity[10]. As our sample size was small, we were unable to demonstrate a statistically significant correlation between incidence of LOH and clinicopathological features. However, we believed that it was necessary to do a follow-up survey on the connection between chewing tobacco and LOH, a personal habit common in Indians. Therefore, we hypothesized that loss of function in the *VHL* gene might contribute

to tumorigenesis in Indian and Japanese OSCCs. We have identified a novel commonly deleted region on 3p21.3 (D3S966), suggesting that another unknown TSG specific to Indian and Japanese OSCCs exists at this region.

The death rate of oral cancer in Japan is 3 to 4 patients in 100,000 peoples. It has been reported that geographical location, family, influence of luxury goods, chemical stimulation, chronic physical stimulation, drinking and tobacco may play a role in the development of this disease, although further clarification is required. Every year, 200,000 people are affected by oral cancer, worldwide. Moreover, the incidence in India where there is a tradition of tobacco chewing is 10 times higher than that in Japan[7,8,26,42].

This suggests that a comparison of data on lifestyle habits and cancer-associated genes in areas with a high rate of oral cancers with data on the Japanese population offers a way to obtain a better understanding of the underlying mechanisms of this disease. In further collaborative work with Poole hospital, we intend to carry out multivariate studies, increasing the number of cases investigated, clinical indices and lifestyle habits taken into consideration.

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