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Original Article

Loss of Heterozygosity and Microsatellite Instability on Chromosome 2q in Human Oral Squamous Cell Carcinoma

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Abstract

Allelic imbalance or loss of heterozygosity (LOH) and microsatellite instability (MSI) have been used to identify regions on chromosomes that may contain putative tumor suppressor genes. To obtain a detailed understanding of genetic alterations in oral cancer, 10 highly polymorphic markers mapped on chromosome 2 were used to examine 25 cases of oral squamous cell carcinoma (SCC). With these, we analyzed chromosome 2q for LOH in 25 primary oral SCCs and constructed a deletion map for this arm of the chromosome. LOH was detected in 16 (64%) of the 25 informative samples at one or more of the loci examined. MSI was observed in 5 (20%) of the 25 cases. Among the loci examined, LOHs were restricted to D2S1328 and D2S206 on chromosomes 2q14-21 and 2q36, respectively, with the former locus showing a rate of 5 (20.8%) and the latter a rate of 6 (25%) of the 24 informative cases. These observations taken in conjunction with data from 40 former cases analyzed at our laboratory suggest that the high incidence of LOH at chromosome 2q is associated with carcinogenesis of oral SCC. The regions that comprise the D2S1328 and D2S206 loci may play an important role in the development of oral SCC, perhaps containing sites that harbor a putative tumor suppressor gene.

Key words: Chromosome 2—Oral squamous cell carcinoma—Loss of heterozygosity (LOH)—Microsatellite instability (MSI)—Tumor suppressor gene

Introduction

Oral squamous cell carcinoma (SCC) is the most common malignant disease of the oral and maxillofacial region, and the sixth most frequent cancer worldwide[12]. However, little is known about the molecular basis of oral SCC compared to other human malignancies. Inactivation of the tumor suppressor gene (TSG) is considered to be associated with carcinogenesis, and alterations in TSGs are widely accepted to be critical events in the

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Table 1 Summary of clinicopathologic features in 25 oral SCCs

Case	Gender	Age	Site	T	N	Stage	Differentiation	Mode of invasion
1	M	47	tongue	1	0	1	well	3
2	M	61	tongue	2	2a	4	well	4c
3	M	74	tongue	2	1	3	well	3
4	M	61	tongue	2	0	2	well	3
5	M	39	tongue	1	0	1	well	4c
6	M	46	tongue	1	0	1	poor.	4c
7	M	51	tongue	2	1	3	mod.	3
8	M	59	tongue	2	1	3	well	3
9	M	60	tongue	1	0	1	well	3
10	M	79	tongue	1	1	3	poor.	3
11	M	41	tongue	3	2b	4	mod.	3
12	F	59	tongue	2	1	3	well	3
13	M	46	tongue	3	0	3	mod.	3
14	F	67	tongue	2	0	2	poor.	4c
15	M	36	gingiva of maxilla	2	0	2	mod.	4c
16	F	80	gingiva of maxilla	2	0	2	well	4c
17	F	69	gingiva of mandible	4	2b	4	well	3
18	F	68	gingiva of mandible	2	2b	4	well	3
19	F	55	gingiva of mandible	1	0	1	well	3
20	M	53	gingiva of mandible	4	2b	4	well	3
21	M	77	oral floor	2	1	3	well	4c
22	M	64	oral floor	4	2c	4	well	3
23	M	48	oral floor	4	2c	4	well	3
24	M	66	palate	1	0	1	well	2
25	M	76	palate	1	0	1	well	4c

M: Male, F: Female. well: well differentiated, mod.: moderately differentiated, poor.: poorly differentiated

multi-step process leading toward the development of cancer.

It is generally accepted that the transformation of normal tissue into malignant tissue follows an accumulation of genetic changes in the oncogenes and TSGs[1]. Frequent allelic losses at specific chromosomal loci in several types of human cancer have provided strong evidence to suggest the existence of TSGs on each chromosome where a deletion was detected. Cytogenetic and molecular biologic studies of human cancers have identified chromosomal abnormalities, including partial deletions of specific loci[15]. In particular, loss of heterozygosity (LOH) and microsatellite instability (MSI) on human chromosome 2 is known to be involved in various types of human cancers, such as neuroblastoma[16], thyroid cancer[25], lung cancer[11], stomach cancer[7], head and neck cancer[14], and granulosa cell tumor[24]. Based on these observations, 2 common loss sites have been identified in the 2q14-21 and 2q36 regions, suggesting the presence of possible TSGs at these regions.

In oral cancer, cytogenetic analysis has revealed consistent chromosomal alterations involving chromosomes 3p[13,18], 4q[20], 5q[22], 7q[21], 8p[10], 9p[6], 10q[30], 11q[19], 13q[8], 18q[23], 20q[2], 21q[29] and 22q[5]. Our previous allelotyping study of oral 40 SCCs revealed high frequencies of LOH on the long arm of chromosome 2[27,28]. In the present study, we examined 25 oral tumors with 10 high polymorphic microsatellite markers as a preliminary step toward isolating putative TSGs associated with oral SCC on human chromosome 2, and constructed a detailed deletion map of this chromosome.

Table 2 Allelic imbalances (LOH and MSI) at 10 microsatellite loci on chromosome 2q in 25 oral SCCs

Locus symbol	Chromosomal location	Frequency of LOH: % (LOH/informative cases)	Frequency of MSI: % (MSI/informative cases)
D2S436	2q11.1-14	10.0 (2/20)	5.0 (1/20)
D2S1328	2q14-21	20.8 (5/24)	4.5 (1/24)
D2S111	2q23-33	16.0 (4/25)	0 (0/25)
D2S202	2q32	17.4 (4/23)	0 (0/23)
D2S116	2q32-35	8.3 (2/24)	0 (0/24)
D2S1327	2q33	9.1 (2/22)	4.5 (1/22)
D2S155	2q35	4.1 (1/24)	0 (0/24)
D2S164	2q35	13.6 (3/22)	9.1 (2/22)
D2S133	2q36	4.5 (1/22)	0 (0/22)
D2S206	2q36	25.0 (6/24)	4.2 (1/24)

Materials and Methods

Samples were obtained from 25 Japanese patients with oral SCC who visited the Tokyo Dental College Chiba Hospital over a 7-year period between 1997 and 2003 (Table 1). Informed consent was obtained from all patients and also from their families. This study was also approved by the Institutional Ethics Committee of Tokyo Dental College. The samples consisted of 25 primary tumors and the same number of normal tissues corresponding to those primary tumors taken from the same patients. Three of these cases showed metastatic tumors of the lymph node. In these cases, further samples were taken of the metastasized tumors. They were collected either at the time of surgical resection (no chemotherapy or radiotherapy was given before surgical resection in any of the cases) or at the time of biopsy. Clinicopathologic staging was analyzed using the UICC TNM staging system[17].

1. Tissue samples

The obtained tissues were divided into two segments: one was frozen immediately after careful removal from the surrounding normal tissue and stored at −80°C until extraction of DNA, and the other was fixed in 10% formalin for pathologic diagnosis. Histopathologic diagnosis was performed according to the International Classification of Tumors[26].

2. DNA extraction

All cases had histologically confirmed SCC of the oral cavity, and the tumor samples for DNA extraction were inspected to make sure that they consisted of more than 80% tumor. DNA extraction from all tumor and most of the normal tissue samples was performed from the fresh frozen specimens that had been preserved in liquid nitrogen immediately after resection. First, the tissues were powdered in liquid nitrogen. Next, they were centrifuged to obtain deposition. After that, 1,000 μl of TNES buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.1% SDS) and 30 μl Proteinase K (100 μg/ml) were added to the powder and incubated in a hot water-bath overnight at 50°C. Genomic DNAs were extracted by phenol-chloroform extraction and refined, and then they were washed and precipitated with ethanol[4]. A spectrophotometric method was used to estimate the concentrations of the extracted DNA and the DNA extracts were kept frozen at −80°C.

3. PCR and microsatellite analysis

We used 10 microsatellite markers located on chromosome 2q. All primers were obtained from Research Genetics (Huntsville, AL, Table 2). PCR amplification was performed at

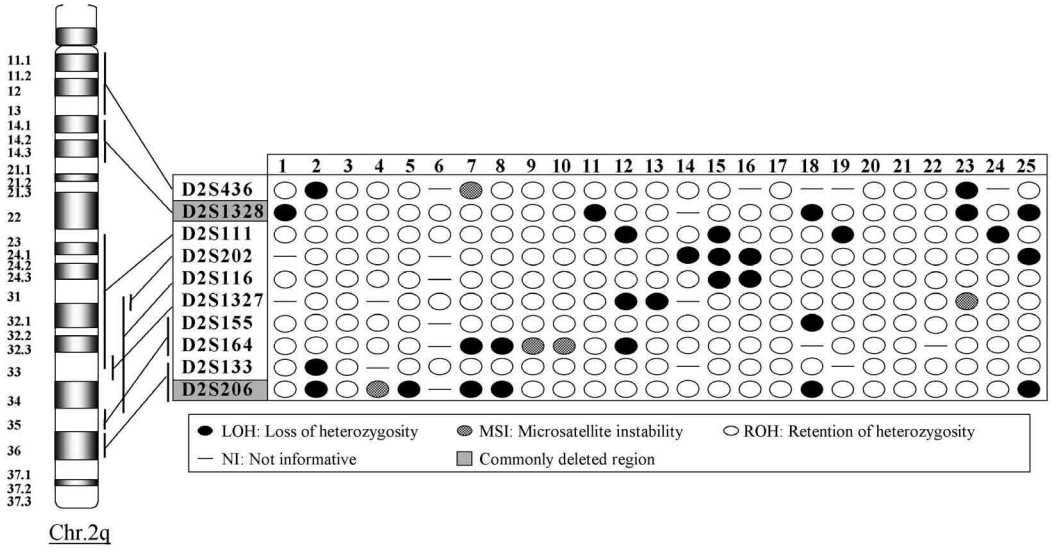

Fig. 1 Deletion mapping of chromosome 2q in 25 primary tumors
The case number is shown above each column, and the 10 microsatellite markers are on the left.

a total reaction volume of 20 μl, as described previously[3]. Each PCR reaction mixture contained 250 ng of sample DNA, 20 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 2 mM dNTP, and 0.5 units of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Thirty-five cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min were performed with a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). 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 was performed using a DNA Silver Staining Kit (Amersham Pharmacia Biotech AB, Sweden). To ensure reproducibility for each instance of LOH or MSI, all tests were performed under the same conditions.

4. Assessment of LOH and microsatellite instability (MSI)

We assessed LOH by scanning a stained gel using a densitometer, followed by analysis with National Institute of Health (NIH) software (Image version 1.62, Dr. W. Rasband, NIH, Bethesda, MD, USA). We compared the intensities of the signals in tumor DNA with those of the corresponding normal DNA. A reduction in signal intensity of more than 50% in a tumor sample compared with that in a normal counterpart was required as the criterion for the presence of LOH. Commonly deleted regions were defined as those loci most frequently showing LOH together with multiple interstitial deletions[27]. MSI in the DNA samples was assessed as positive in those cases which had additional bands in the tumor sample that were not observable in the corresponding normal samples and in those cases which showed a band shift in the tumor sample that contrasted with the pattern of the corresponding normal bands.

5. Statistical analysis

A Fisher's exact test was performed to evaluate the significance of the correlation

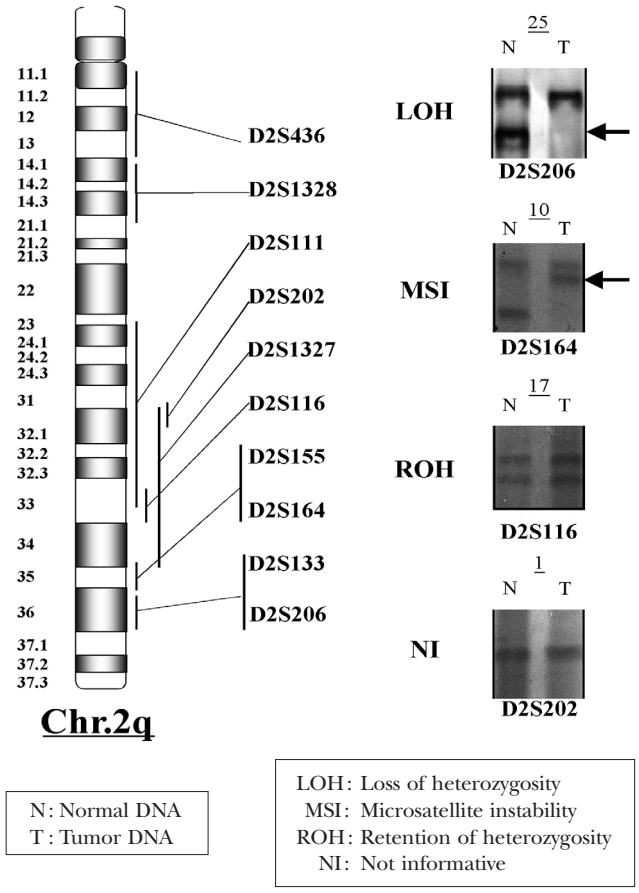

Fig. 2 Typical examples of allelic imbalances (LOH and MSI) on 2q
Microsatellite polymorphism analysis in oral SCC specimens. Case numbers are shown above, and locus symbols are indicated below. Paired normal (N) and tumor (T) samples demonstrating loss of the upper allele (LOH, case no. 25), microsatellite instability (MSI, case no. 10), retain of heterozygosity (ROH, case no. 17), and uninformative (NI, case no. 1).

between LOH and the clinicopathological parameters. The accepted level was $p<0.05$.

Results

Ten microsatellite markers on the long arm of chromosome 2 were analyzed in 25 oral SCCs with various grades of differentiation and clinical stages. At all the loci examined on this chromosome in normal tissues, 20 or more cases were heterozygotes. Sixteen out of twenty-five cases (64%) of the oral SCCs showed a loss of heterozygosity in one or more of the microsatellite markers on chromosome 2. The locations of the markers and frequencies of LOH and MSI are summarized in Table 2 and Fig. 1. A high frequency of LOH was observed at the D2S1328 locus (20.8%) on 2q14-21, and the D2S206 locus (25%) on 2q36. MSI in chromosome 2q was present in 5 out of 25 cases (20%) of tumors (Table 2, Fig. 1). Typical examples of LOH, MSI, retention of heterozygosity case (ROH) and not informative case (NI) on 2q are shown in Fig. 2.

The frequencies of LOH in the lymph node metastases revealed that 2 out of the 3

Table 3 Summary of clinicopathologic features in 25 oral SCCs

Clinicopathologic features		Frequency of LOH: % (LOH/informative cases)	Frequency of MSI: % (MSI/informative cases)
Gender	male	57.9% (11/19)	26.3% (5/19)
	female	83.3% (5/6)	0% (0/6)
Site	tongue	64.3% (9/14)	28.6% (4/14)
	gingiva	66.7% (4/6)	0% (0/6)
	maxilla	100% (2/2)	0% (0/2)
	mandible	50.0% (2/4)	0% (0/4)
	oral floor	33.3% (1/3)	33.3% (1/3)
	palate	100% (2/2)	0% (0/2)
T classification	T1	62.5% (5/8)	25.0% (2/8)
	T2	72.7% (8/11)	18.2% (2/11)
	T3	100% (2/2)	0% (0/2)
	T4	25.0% (1/4)	25.0% (1/4)
pN	+	63.6% (7/11)	0% (0/11)
	−	70.0% (7/10)	20.0% (2/10)
TNM stage	I	71.4% (5/7)	14.3% (1/7)
	II	75.0% (3/4)	25.0% (1/4)
	III	57.1% (4/7)	28.6% (2/7)
	IV	57.1% (4/7)	14.3% (1/7)
Differentiation	well	76.9% (10/13)	23.1% (3/13)
	mod.	75.0% (6/8)	12.5% (1/8)
	poor.	25.0% (1/4)	25.0% (1/4)
Prognosis	well	63.2% (12/19)	21.1% (4/19)
	poor.	66.6% (4/6)	16.7% (1/6)
Mode of invasion	2	100% (1/1)	0% (0/1)
	3	56.3% (9/16)	31.3% (5/16)
	4C	75.0% (6/8)	0% (0/8)

(66.7%) showed LOH at D2S436. In our previous studies on 7 cases of lymph node metastases, LOH was also found at the same locus in 1 out of the 3 informative cases examined. However, among the total 7 cases, LOH was additionally found at D2S1327, D2S155, and D2S164, yielding frequencies of 33.3 (2/6), 100 (3/3), 57.1 (4/7) and 33.3%[28].

We compared our results with the clinicopathological features of each tumor (Table 3). A number of sites displaying LOH at 2q could be detected in early stage lesions, and the frequencies of LOH tended to be higher in the later clinical stages, although no statistical difference was observed. No significant correlation between incidence of LOH and grade differentiation was noted. MSI was present at 4 loci in the chromosome, but again there was no apparent association between the incidence of MSI and histopathological grading or clinical status.

Discussion

In order to isolate putative TSGs via positional cloning, a number of detailed deletion maps have been constructed for each chromosome in relation to a range of cancers in human. In particular, chromosome 2 is well-known to frequently alter in patients with any one of several types of malignant tumor, such as neuroblastoma[16], thyroid cancer[25], lung cancer[11], stomach cancer[7], head and neck cancer[14], or granulosa cell tumor[24]. Two of our recent allelotyping studies on oral SCC

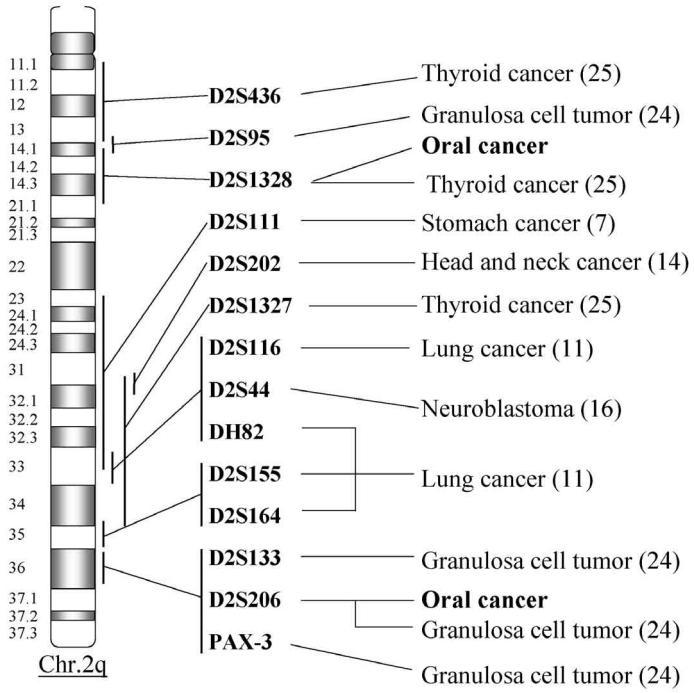

Fig. 3 Commonly deleted regions on 2q in human cancers
Comparisons of reported examples in other cancers on chromosome 2q with the oral cancers in the microsatellite analysis reported herein. Malignant tumors of cancers in thyroid, stomach, lung, head and neck, and granulose cell tumor have been already analyzed on 2q, but no report on oral cancers has been made except by us. The numbers in the parentheses indicate the reference number.

also revealed high frequencies of LOH on the long arm of chromosome 2[27,28]. Therefore, in the present study, in order to confirm the findings of those studies, we further examined the same 10 microsatellite markers on chromosome 2q by using new samples of SCCs.

In our earlier studies on 40 cases of oral SCCs, we found that the incidence of LOH in 10 microsatellite markers on chromosome 2 was 67.5% (27/40) in primary tumors. High frequencies of LOH at these loci (65%) were also found in this study. Among those found in this study, we found 2 distinct putative tumor suppressor loci, D2S206 and D2S1328. High frequencies of LOH at these loci were also found in the results of our earlier studies. Frequent allelic loss of the D2S206 locus located on band 2q36 was also reported in granulosa cell tumor[10]. This finding suggests that a putative suppressor gene located at this region plays a role in both granulosa cell tumor and oral cancer. The second region, including the D2S1328 locus at chromosome 2q14-21, was found to have frequent allelic loss in thyroid cancer[25]. This finding suggests that a putative suppressor gene located at this region plays an important role in both thyroid and oral cancers (Fig. 3). However, among these high-frequency LOHs, oral cancer was found to be the most prevalent. Therefore, this suggests that LOH at this location includes an important TSG in the development of oral cancers. A more detailed deletion map needs to be compiled in order to further clarify this finding.

In 7 metastatic tumors of our previous studies on 40 cases of oral SCC, we found higher frequencies of LOH in at D2S436 (33.3%), D2S1327 (33.3%), D2S155 (100%),

and D2S164 (57.1%) compared with LOH in primary tumors[28]. Deletion of one of these loci D2S436 was also found in 2 of the 3 metastatic cases of this study. These findings suggest that these loci contribute to the development of metastase. However, this may also be attributed either to a sampling bias (types of tumor studied, patient population differences) or to differences in technical and interpretive approaches.

Taking into account the LOHs of primary tumors, it would appear that alterations at several regions on chromosome 2q play an important role in the genesis of oral SCC.

In this study, no statistically significant correlation was found between presence of LOH, TNM stage, and histological grade in the patients. These results indicate that inactivation of the TSG is an early event in oral SCC, because low-stage tumors have a similar frequency of LOH to more advanced cases.

Our findings showed a high incidence of MSI at multiple loci in oral SCC in 20% of the cases examined (5/25). Another important finding of the present study was the higher incidence of MSI in tumors located in the tongue compared with at other anatomic locations in the oral cavity[9]. This suggests that carcinogenesis in tongue tissue may be influenced by different TSGs to that at other sites in the oral cavity, such as the gingiva or the palate. However, more specimens are necessary to confirm this result because our sample size was small.

In conclusion, our present data taken together with the results of our previous studies[27,28], suggest that putative TSGs are located in the 2q14-21 and 2q36 regions and that inactivation may occur at an early stage in the progression of oral tumors.

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