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Frequent loss of heterozygosity at 3p25-p26 is associated with invasive oral squamous cell carcinoma

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Abstract Recent molecular evidence suggests that allelic deletions of chromosomes are involved in the carcinogenesis of various neoplasms, including oral squamous cell carcinoma (OSCC). To determine the role of 3p deletions in Japanese OSCC and to define the localization of putative tumor suppressor genes, we initially examined loss of heterozygosity (LOH), using nine microsatellite markers in 36 OSCCs and 28 oral epithelial dysplastic lesions (OEDLs). LOH on chromosome 3p was observed at one or more loci in 72% of OSCCs and 18% of OEDLs. Fourteen (61%) of 23 OSCC patients informative at D3S2450 (3pter-p24.2) showed LOH most frequently, in contrast to OEDL, where LOH was never seen at this locus. Interestingly, we found a significant association between an allelic deletion at this locus and the histologic grade of mode of tumor invasion. Therefore, we also examined allelic deletion on chromosome 3p telomeric to where D3S2450 was located. A common deletion region was identified between D3S2450 and D3S3591. Our results provide evidence for the presence of a tumor suppressor gene in a 0.8-cM region bordered by D3S2450 and D3S3591 at 3p25-p26, which may play a role in carcinogenesis and invasion of OSCC.

Key words Loss of heterozygosity · Microsatellite instability · Oral squamous cell carcinoma · Oral epithelial dysplastic lesion · Chromosome 3p

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Introduction

An estimated 5500 new cases of oral cancer are expected to be diagnosed each year in Japan, with 80% of these histologically designated as squamous cell carcinoma; approximately 2500 people will die of oral cancer. More than 80% of oral cancers occur in patients over the age of 50 years. The incidence increases steadily with age until age 70 years, when the rate levels off. The primary risk factors for oral cancer in Japanese men and women are tobacco and alcohol use. Although easily detected and often cured in its early stage, the prognosis of the advanced stage is poor. Most patients, after multidisciplinary treatment, suffer from severe cosmetic and/or functional difficulties.

Among genetic changes, loss of heterozygosity (LOH) is a frequent mechanism of inactivation of tumor suppressor genes where one allele is already altered. Frequent allelic deletion at specific loci in both hereditary and sporadic tumors may indicate the location of a putative tumor suppressor gene (Ponder 1988). For determination of the location of some putative tumor suppressor genes, various studies have been carried out, using polymorphic markers. To date, in head and neck cancer, including oral squamous cell carcinoma (OSCC), LOH has frequently been identified on chromosomes 2q, 4q, 7q, 8p, 9p, 10q, 11q, 13q, 14q, 17p, 18q, and 22q (Uzawa et al. 1996; Jares et al. 1997; Matsuura et al. 1998; Pearlstein et al. 1998; El-Naggar et al. 1998; Ransom et al. 1998; Ogawara et al. 1998; Mutirangura et al. 1998; Miyakawa et al. 1998; Wang et al. 1999; Gasparotto et al. 1999; Califano et al. 1999). Other studies have also reported a high frequency of deletion at chromosome 3p in diverse neoplasms, as well as in oral cancer (Fullwood et al. 1999; Chino et al. 1999). Investigation of the 3p locus in head and neck cancer, including oral cancer, has shown a relatively high incidence of LOH, ranging from 58% to 81% (Ishwad et al. 1996; Partridge et al. 1994). These findings suggest that the chromosome 3p locus may contain a tumor suppressor gene that plays a role in OSCC carcinogenesis. However, it remains unclear whether LOH in this region is of biological significance, or whether it only

reflects a consequence of the mechanism generating the deletion of 3p. The existence of a putative tumor suppressor gene masked by allelic deletions is suggested by the correspondence of LOH with clinicopathological factors. LOH on 3p was reported to correlate with poor survival in lung and oral cancer (Mitsudomi et al. 1996; Partridge et al. 1996). These findings suggest a tumor suppressor gene on 3p involved in the development of several types of tumor.

Microsatellite instability (MI) is a form of genomic instability demonstrated in neoplasms in which the lengths of microsatellite DNA sequences in patients' tumors diverge from those in their normal tissue counterparts. Tumors diagnosed as hereditary nonpolyposis colorectal cancer show MI at many tested microsatellite loci. However, sporadic carcinomas, in general, display quantitatively less MI (Schmitt et al. 1999). Investigation of MI in OSCC and oral epithelial dysplastic lesions (OEDLs) has provided evidence that mismatch-repair genes are implicated in the genesis of oral cancer.

The aim of this study was to perform a preliminary assessment of genomic instability of 3p in Japanese patients with OSCC, and to determine the common deletion region that may include the tumor suppressor gene. We also examined the differences in LOH between OSCC and OEDL to determine the timing of genetic changes.

Table 1. Clinicopathological features of OSCC and OEDL

Characteristics	No.
OSCC	(36)
Tumor site	
Tongue	15
Lower gingiva	9
Upper gingiva	4
Buccal mucosa	4
Maxillary sinus	3
Floor of mouth	1
Tumor size[a]	
1, 2	27
3, 4	9
Lymph node metastasis	
(+)	12
(−)	24
Differentiation	
Well	19
Moderate	10
Poor	7
Mode of tumor invasion[b]	
Grades 1, 2	17
Grades 3, 4C, 4D	19
TNM stage[a]	
I, II	17
III, IV	19
OEDL	(28)
Leukoplakia	26
Carcinoma in situ	2

OSCC, Oral squamous cell carcinoma; OEDL, oral epithelial dysplastic lesion
[a] Classified according to the criteria of the International Union Against Cancer
[b] Classified according to the criteria of Yamanoto et al. (1983)

Patients and methods

Patient materials

A total of 36 OSCC and 28 OEDL tissue samples were obtained by incisional or excision biopsy from Japanese patients at Ehime University Hospital from 1996 to 1998. None of the patients had received any prior radiation or chemotherapy. Heparinized peripheral blood was collected as corresponding normal tissue. OSCC was classified by tumor site, tumor size, stage, differentiation, mode of tumor invasion, and presence or absence of local lymph node metastasis (Table 1). The mode of tumor invasion was classified as low (grade 1, 2) or high invasive type (grade 3, 4C, 4D) according to previously reported criteria (Yamamoto et al. 1983). Tumor size and stage were classified into two types according to the criteria of the International Union Against Cancer (UICC). The OEDLs consisted of 26 cases of mild dysplasia and 2 of severe dysplasia. All patients gave informed consent before enrollment in this study.

DNA preparation

Ten 5-μm sections from formalin-fixed, paraffin-embedded specimens were used for DNA extraction from OSCC and OEDL tissues. One section was stained with hematoxylin and eosin to facilitate precise identification of neoplastic and nonneoplastic cells. After the stained sections were examined, unstained sections of the specimens were microdissected under a light microscope to separate the tumor areas from adjacent nonneoplastic areas. After deparaffinization, the microdissected samples were incubated in sodium dodecyl sulfate (SDS)-proteinase K at 50°C for 72 h, with additional proteinase K being added every 24 h. Digested products were purified by treatment with phenol-chloroform. DNA was precipitated by the ethanol precipitation method. Normal control DNA from peripheral blood was isolated by the standard method using phenol-chloroform extraction.

Polymerase chain reaction (PCR)-LOH assay

In this study, we used trinucleotide or tetranucleotide microsatellite markers mapped on the short arm of chromosome 3 for the mapping of putative tumor suppressor genes on 3p. These were D3S2450 (3pter-p24.2), D3S1516 (3p25-p24.2), D3S1537 (3p24.2-p22), D3S2447 (3p24.2-p22), D3S2414 (3p24.2-p22), D3S2411 (3p24.2-p22), D3S2420 (3p21.3-p21.2), D3S1514 (3p21.1-p14.2), and D3S1540 (3p21.1-p14.2). Primers for PCR amplification were obtained from Research Genetics (Huntsville, AL, USA). The PCR mixture was prepared as follows: 100 ng of sample DNA, 10 pmol of each primer, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.5 unit Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) in a final volume of 20 μl. PCR was performed with 30 cycles of dena-

turation at 94°C for 1 min, annealing at 55–57°C for 1 min, and extension at 72°C for "!min. Samples were then resolved by electrophoresis on 6% polyacrylamide gel and ethidium bromide staining. Allelic reductions in the intensity of alleles between tumor and normal DNA were calculated by quantitative densitometry, utilizing National Institutes of Health (NIH) Image software, and a value greater than 0.5 was considered indicative of LOH. MI was identified as a novel band in tumor DNA not seen in the paired normal DNA. Each PCR was performed at least twice, and was defined visually for LOH and MI by three independent observers.

Assessment of LOH and MI at 3pter-p24.2

As LOH at locus D3S2450 (3pter-p24.2) was associated with the mode of tumor invasion and was found most frequently in the previous preliminary assessment, we examined the 36 OSCCs to determine the common deletion region. We used dinucleotide microsatellite markers for examination by fluorescent microsatellite assay. These were: D3S1560 (3p26-p25), D3S1304 (3p26-p25), D3S3591 (3p26-p25), D3S1038 (3p26.1-p25.2), D3S1597 (3pter-p24.2), D3S1293 (3pter-p24.2), and D3S2335 (3pter-p24.2). One primer of each pair was fluorescence-labeled at the 5′ end with 6-FAM. Fluorescent PCR was performed as well as the nonlabeled PCR described above. Fluorescent DNA products were mixed with internal standard-size markers (Tamra 500; Applied Biosystems, Tokyo, Japan) and fractionated by capillary-electrophoresis, using an ABI PRISM 310 Genetic Analyzer. The sizes of two alleles for heterozygous cases were assigned according to the two peaks of greatest height in the normal sample. The allele ratio was calculated for each paired normal and tumor sample and then the tumor ratio was divided by the normal ratio. A ratio of less than 0.5 was taken to be indicative of LOH to allow for up to 50% contaminating normal cells in the tumor cells (Cawkwell et al. 1994). All assays were performed at least twice to ensure that consistent results were obtained. Two point linkage analysis with these markers was performed in Centre d'Etude du Polymorphisme Humain (CEPH) pedigrees; 102, 884, 1331, 1332, 1347, 1362, 1413, and 1416.

Statistical analysis

Fisher's exact test was used for statistical analysis of the results. One-tailed P values less than 0.05 were considered significant.

Results

Allelic deletion of chromosome 3p in OSCC and OEDL

We analyzed matched pairs of normal/tumor DNA from 36 OSCCs and 28 OEDLs by PCR-LOH assay with nine microsatellite markers spanning from 3p14.2 to 3pter. Of the 36 OSCCs that were informative for at least two of the loci, 26 (72%) showed LOH, while in OEDLs, LOH was detected in 18% (5/28). Figure 1 shows representative cases with typical LOH and MI results. The results of the PCR-LOH assay are summarized in Table 2. Frequencies for individual marker loci in OSCC ranged from 23% to 61%, with a maximum at D3S2450 (61%), at which no allelic deletion was detected in OEDL. Comparing the frequency of LOH in OSCC and OEDL, LOH frequency in OEDL was apparently lower at each locus than that in OSCC. The loci D3S2450, D3S1514, D3S2420, and D3S1540 had no LOH in OEDL.

Frequency of microsatellite instability on chromosome 3p

Except for one OSCC sample that had MI at two loci, most OSCCs had no band shift on gel electrophoresis (Fig. 1). Three OEDLs showed evidence of MI. Of these, one showed MI at two loci, and two showed MI at one locus. In total, only four (6%) of the 64 samples of OSCC and OEDL showed MI (Table 2). It was suggested that mismatch-repair genes are not usually involved in carcinogenesis in OSCC.

Table 2. Cumulative results of LOH and MI on chromosome 3p in 36 OSCCs and 28 OEDLs

	OSCC			OEDL		
Locus symbol (chromosome location)	Informative cases (%)	Frequency of LOH (%)	MI cases	Informative cases (%)	Frequency of LOH (%)	MI cases
D3S2450 (3pter-p24.2)	21 (58%)	14 (61%)	0	10 (36%)	0 (0%)	0
D3S151 (3p25-p24.2)	23 (64%)	12 (52%)	0	13 (46%)	2 (15%)	0
D3S1537 (3p24.2-p22)	28 (78%)	9 (32%)	0	16 (57%)	2 (13%)	2
D3S2447 (3p24.2-p22)	25 (69%)	9 (36%)	1	19 (68%)	1 (5%)	0
D3S2414 (3p24.2-p22)	21 (58%)	5 (23%)	0	12 (43%)	1 (8%)	0
D3S2411 (3p24.2-p22)	12 (33%)	6 (50%)	0	6 (21%)	1 (17%)	1
D3S2420 (3p21.3-p21.2)	26 (72%)	8 (30%)	0	18 (64%)	0 (0%)	1
D3S1514 (3p21.1-p14.2)	27 (75%)	10 (37%)	0	21 (75%)	0 (0%)	0
D3S1540 (3p21.1-p14.2)	24 (67%)	10 (41%)	1	19 (68%)	0 (0%)	0

LOH, Loss of heterozygosity; MI, microsatellite instability

Relationship between LOH on chromosome 3p and clinicopathological factors

We investigated the correlation between clinicopathological factors and LOH on chromosome 3p. An interesting result was obtained at D3S2450 (3pter-p.24.2), in which LOH was most frequently observed (Table 3). Ten of the 14 OSCCs with LOH were the highly invasive type (grade 3, 4C, 4D), while 6 of the 7 without LOH were the low invasive type (grade 1, 2). There was a significant association between LOH of D3S2450 and mode of tumor invasion ($P < 0.05$). Furthermore, lymph node metastasis was not observed in OSCC without LOH at this locus. Although the relation between LOH of D3S2450 and lymph node metastasis did not reach statistical significance, because of the small sample size, detection of LOH at the D3S2450 locus may be indicative of progressive disease with lymph node metastasis. For other markers, we failed to demonstrate any relationship between LOH and clinicopathological factors, in terms of differentiation grade, lymph node metastasis, and mode of tumor invasion.

Common deletion region at 3p25-p26

We observed the maximum LOH at locus D3S2450, which is located at 3pter-p24.2. To find a common deletion region at 3pter-p24.2, we examined normal and tumor DNA from the 36 OSCCs for allelic deletions at eight microsatellite markers located in 3pter-p24.2, including D3SD2450, by fluorescent microsatellite assay. Figure 2 shows the pattern of LOH and MI. Frequencies for individual marker loci in OSCC ranged from 24% to 61%, with a maximum at D3S2450 (Fig. 3). At D3S1304, sited telomeric to D3S2450,

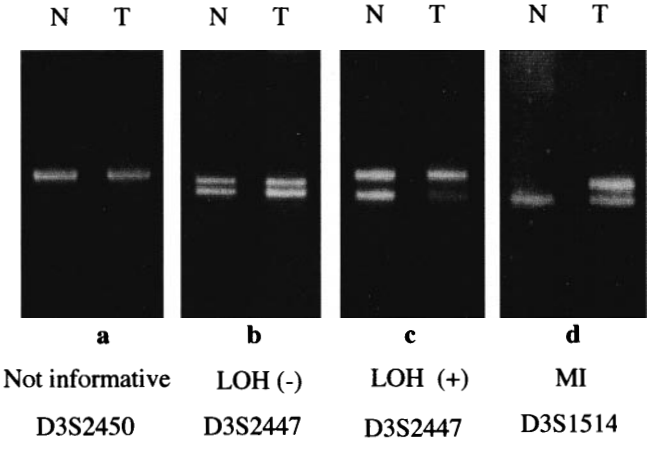

Fig. 1a–d. Representative patterns of **a** not informative, **b** retention of both alleles (loss of heterozygosity; *LOH* (−)), **c** allelic deletion (*LOH* (+)), and **d** microsatellite instability (*MI*) on chromosome 3p. *N*, Normal DNA; *T*, tumor DNA

Table 3. Relationship between LOH of D3S2450 (3pter-p24.2) and clinicopathological features in OSCC

Characteristics	LOH of D3S2450 (+)	(−)	Percentage	*P* value
Differentiation				
Well	5	5	50	
Moderate, poor	9	2	82	0.27
Mode of tumor invasion				
Grades 1, 2	4	6	40	
Grades 3, 4C, 4D	10	1	91	<0.05
Lymph node metastasis				
(+)	5	0	100	
(−)	9	7	56	0.20

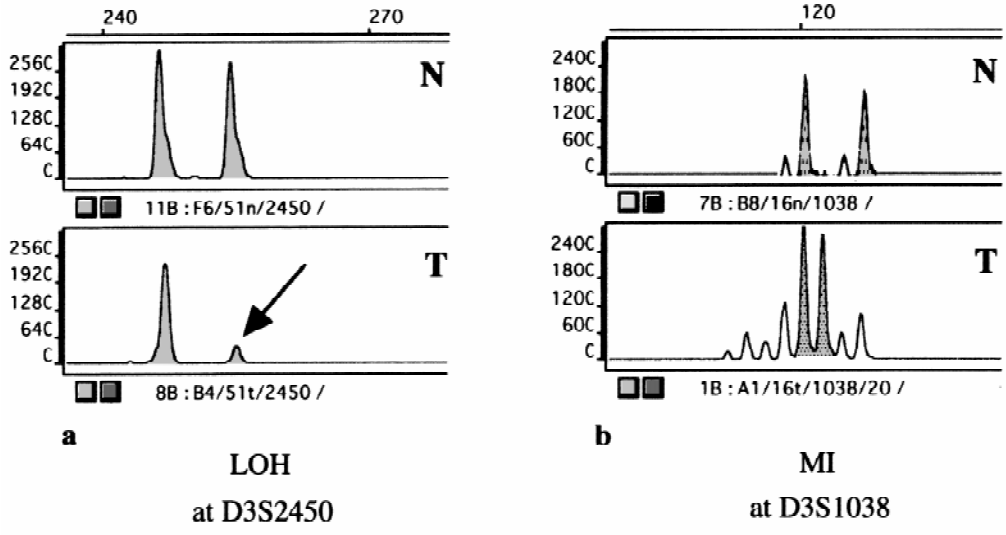

Fig. 2. Loss of heterozygosity (LOH) of D3S2450 and microsatellite instability (MI) of D3S1038 in oral squamous cell carcinoma (OSCC) detected by fluorescent polymerase chain reaction (PCR). Data were obtained with the ABI PRISM 310 Genetic Analyzer. The X axis shows fragment size in bp. *T*, DNA from tumor; *N*, DNA from peripheral blood. **a** A heterozygous individual (246 bp, 254 bp) is shown for each normal/tumor DNA. *Arrow* shows deletion of a longer allele in tumor DNA. An LOH value of 0.20 clearly indicates LOH in the tumor sample. **b** In the normal sample, the shorter allele is 120 bp and the longer allele is 126 bp. In the tumor sample, the longer allele is shifted to 122 bp, indicating MI

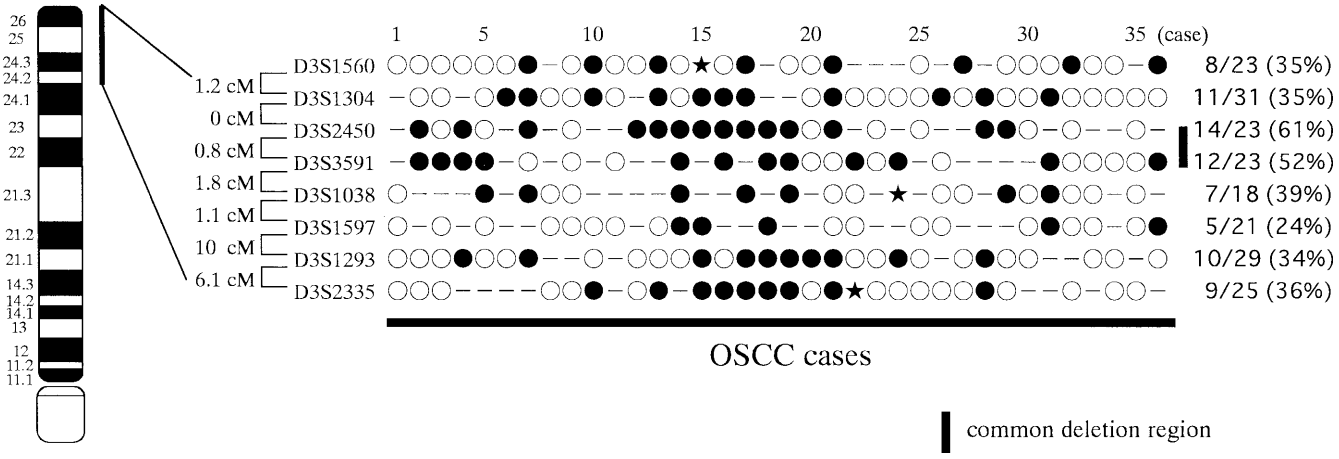

Fig. 3. Deletion mapping of chromosome 3p terminal in oral squamous cell carcinomas. *Closed circles*, Loss of heterozygosity; *open circles*, retention of both alleles; *dashes*, not informative; *stars*, microsatellite instability. Although the region bordered by D3S1304 and D3S2450 was 0-cM by our linkage analysis, the distance was 50 kbp according to the database of sequence tagged sites of the National Center for Biotechnology Information. We found a common deletion region within a 0.8-cM region bordered by D3S2450 and D3S3591 at 3p25-p26

11 of 31 informative cases (35%) showed LOH, whereas 12 of 23 informative cases (52%) showed LOH at D3S3591, sited centromeric to D3S2450. In case 22, LOH at D3S3591 and retention of heterozygosity at flanking markers D3S1304 and D3S1038 were clearly seen. In addition, cases 3 and 5, which have a typical interstitial deletion, showed LOH at D3S3591 and retention at D3S2450, indicating that a telomeric breakpoint of the common deletion region is between D3D3591 and D3S2450. Conversely, LOH at D3S2450 and retention at D3S3591 were seen in cases 7 and 21, indicating that a centromeric breakpoint is between these two markers. An interstitial deletion between D3S2450 and D3S3591 was strongly suggested in OSCC, and, accordingly, we found a common deletion region within a 0.8-cM region containing D3S2450 and D3S3591 in 3p25-p26. Next, using LOH analysis of D3S2450 and D3S3591, we tested whether the common deletion region was associated with any clinical features of OSCC. Twenty (65%) of 31 informative cases showed LOH at D3S2450 and D3S3591. No strong correlation was found among allelic deletions with respect to differentiation grade, tumor size, lymph node metastasis, and stage. However, a significant correlation between LOH and mode of tumor invasion was observed (Table 4). These results provide evidence for the presence of a tumor suppressor gene in a 0.8-cM region bordered by D3S2450 and D3S3591 at 3p25-p26, which may play a role in carcinogenesis and invasion of OSCC. On comparing groups with and without lymph node metastasis we found that 8 (40%) of 20 patients in the LOH-positive group had lymph node metastasis, versus only 2 (18%) of 11 in the LOH-negative group. Although there was no significant association, LOH detection in this common deletion region may be a useful tool for the prediction of lymph node metastasis prior to treatment. In the analysis of the 3p terminal by fluorescent microsatellite assay, only three (cases 15, 22, and 24) showed MI, and a low frequency of MI was revealed in OSCC, as had been shown in a previous preliminary assessment.

Table 4. Relationship between LOH of common deletion region (3p25-p26) and clinicopathological features in OSCC

Characteristics	LOH of common deleted region		*P* value
	LOH (+)	LOH (−)	
Differentiation			
Well	8	7	
Moderate, poor	12	4	0.19
Mode of tumor invasion			
Grades 1, 2	5	10	
Grades 3, 4C, 4D	15	1	<0.001
Tumor size			
1, 2	12	10	
3, 4	8	1	0.08
Lymph node metastasis			
(+)	8	2	
(−)	12	9	0.20
	40%	18%	
TNM stage			
I, II	8	8	
III, IV	12	3	0.08

Discussion

To evaluate the role of 3p deletions in the pathogenesis of OSCC, we studied 36 OSCCs for LOH, using microsatellite markers at 3pter-p14.2. LOH was observed in 72% of the OSCCs, and a common deletion region, at 3p25-p26, was identified. The common deletion region is consistent with that reported for renal cell carcinoma by Alimov et al. (2000), who evaluated deletions on 3p by LOH and comparative genome hybridization (CGH) analysis; the common deletion region is also consistent with that reported for OSCC by Uzawa et al. (1998), who analyzed DNA of the fragments derived from transferred 3p in microcell hybrid clones. Our present data provide evidence for the presence of a tumor suppressor gene at 3p25-p26 in OSCC. The gene

responsible for von Hippel-Lindau (VHL) disease was isolated from 3p25. VHL disease is a dominantly inherited familial cancer syndrome that predisposes affected individuals to a variety of tumors, in particular, hemangioblastoma of the central nervous system and retina, renal cell carcinoma, and pheochromocytoma. The *VHL* gene lies within 10 cM between D3S1597 and D3S1293, which is centromeric above 2.9-cM from the common deletion region that we found. LOH frequencies at D3S1597 and D3S1293 were 24% and 34%, respectively, with lower frequencies than those of the common deletion region. It was also reported that mutation of the *VHL* gene was not found in OSCC by PCR-single strand conformation polymorphism (SSCP) analysis (Uzawa et al. 1995). Our results may also provide evidence for the presence of a tumor suppressor gene, other than the *VHL* gene, that may play a role in carcinogenesis and invasion of OSCC. Moreover, LOH at D3S2411 in OSCC showed a relatively high incidence (50%) in the preliminary assessment, although the frequency of informative cases was low (33%). The transforming growth factor (TGF)-beta type 2 receptor has been mapped approximately 5-cM centromeric to D3S2411 at 3p22. It has been suggested that alterations in the nucleic acid sequence and mRNA expression of TGF-beta type 2 receptor are prevalent events in the development of head and neck cancer, which may deregulate cell cycle control (Wang et al. 1997). It is possible that TGF-beta type 2 receptor contributes to the suppression of tumorigenesis in OSCC.

To determine whether LOH on chromosome 3p occurs as an early event or as a late event in OSCC tumorigenesis, we investigated LOH on 3p in 28 OEDLs, as well as in OSCCs. Regarding this, there is still controversy in the literature: El-Naggar et al. (1995) suggested that LOH at 3p was a late event, because they found LOH in only 5% (1/20) of oral dysplastic lesions, while Roz et al. (1996) provided evidence that LOH may be an early event in OSCC tumorigenesis. Most recently, Rosin et al. (2000) examined LOH to compare progressive and nonprogressive low-grade oral epithelial dysplasias. They reported that 3p and 9p losses were increased in lesions that later progressed to cancer, and they found a significant difference between lesions with and without progression. Hence, as all of our OEDL samples showed no progression during follow-up, we considered that the LOH frequency in OEDL was low. We found no LOH at 3p21.3-p14.2 and 3p terminal in OEDL samples. Taking all these results together, we assume that there may be a common deletion region between D3S2450 and D3S3591 at 3p25-26, at most 0.8-cM long, telomeric to the *VHL* gene locus.

As mentioned in the Introduction, the findings with relation to clinicopathological factors are provocative, because a tumor suppressor gene can be expected at that locus. To our knowledge, in head and neck cancer: (a) LOH of 9p21-p23 is related to advanced local invasion, lymph node metastasis, and stage IV tumor (Jares et al. 1997); (b) LOH of 9p21 and 7q31 is related to a high incidence of recurrent tumor (Matsuura et al. 1998); (c) LOH of 18q is related to survival (Pearlstein et al. 1998); (d) LOH of 2q is related to poor prognosis (Ransom et al. 1998); (e) LOH of 13q14.3 is related to lymph node metastasis (Ogawara et al. 1998); and (f) LOH of 11q23 is related to recurrence (Lazar et al. 1998). In this study, we found a locus exhibiting a high rate of LOH at 3p25-p26, which was associated with the mode of tumor invasion. Elucidation of allelic deletions in human cancers is regarded as important for the understanding and treatment of tumors. Therefore, in OSCC, assessment of LOH at the aforementioned 2q, 3p, 7q, 9p, 11q, 13q, and 18q loci promises to be valuable in improving the survival rate.

MI comprises length mutations in tandem oligonucleotide repeats. This type of mutation is believed to be caused by altered DNA mismatch-repair genes. Recent literature suggests that *hMLH1* promoter hypermethylation is associated with *hMLH1* transcriptional inactivation and mismatch-repair deficiency in a large proportion of MI-positive gastric carcinomas (Fleisher et al. 1999). However, in oral cancer, our findings suggest that mismatch-repair genes are not usually involved in carcinogenesis, because, in our 64 samples, MI-positive samples were relatively few (6%).

In conclusion, we used microsatellite polymorphism analysis to screen LOH frequency of 3p in OSCC and OEDL, and identified a common deletion region (3p25-p26), where a tumor suppressor gene associated with invasive OSCC may be located. We are currently investigating the target genes at 3p25-26, using bacterial artificial chromosome (BAC) contig and subsequent complete genome sequencing.

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