Expression of the *FAM5C* in tongue squamous cell carcinoma

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Abstract. The purpose of this study was to perform a whole-genome analysis of loss of heterozygosity (LOH) in tongue squamous cell carcinoma (SCC) using the Affymetrix 10K SNP Mapping Array. In the gene which had been identified by whole-genome analysis of LOH, we analyzed allelic imbalance to identify the role of the gene. We applied whole-genome analysis of LOH in the specimens from the 5 cases of tongue SCC using this array. In the chromosomal region which had been identified by whole-genome analysis of LOH, we reconfirmed the existence of LOH in 30 cases using microsatellite markers. The expression levels of the mRNA in the region were examined in 15 cases and in 5 tongue SCC-derived cell lines by real-time quantitative RT-PCR analysis. LOH was observed in all of the 5 cases in the 1q31.1 region. Only 3 microsatellite markers (D1S1189, D1S2151, and D1S2595) existed in the 1q31.1 region. A high frequency of LOH was found at the D1S1189 locus in 18/30 (60%), D1S2151 locus in 16/30 (53%) and D1S2595 locus in 21/30 (70%). Only the Family with sequence similarity 5, member C *(FAM5C)* gene was located in the 1q31.1 region. There was statistically significant difference in the *FAM5C* mRNA expression levels between tongue SCC and normal tissues. All tongue SCC-derived cell lines decreased *FAM5C* mRNA expression compared with normal oral keratinocytes (NOKs). We conclude that *FAM5C* may be a novel tumor suppressor gene (TSG) in tongue SCC.

Introduction

Inactivation of tumor suppressor genes (TSGs) and activation of oncogenes have been considered to play important roles in the multi-step process of human carcinogenesis. Chromosomal loss of heterozygosity (LOH) is a common mechanism for the inactivation of TSGs in human carcinoma. LOH patterns can be generated through allelotyping using polymorphic microsatellite markers (1).

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Several studies demonstrated that multiple genetic alterations were involved in the carcinogenetic process in head and neck squamous cell carcinoma (SCC). Among these alterations, frequent LOH at many chromosome loci was the most striking, indicating that multiple TSGs played important roles in head and neck SCC carcinogenesis (2).

In our laboratory, Yamamoto *et al* analyzed chromosomes 2q, 3p and 21q for LOH in 40 primary oral SCCs using 30 markers and constructed a deletion map for these chromosome arms. LOH were observed at 2 loci in chromosome 2q, 3 loci in chromosome 3p, and 4 loci in chromosome 21q. Our studies suggested that allelic deletions of the 2q, 3p, and 21q loci played a role in oral SCC progression (2). However, because of the limited number of available microsatellite markers, the rather tedious and labor-intensive procedure, and the requirement for large amounts of DNA, only a modest number of microsatellite markers could be screened (3).

High-density whole genome allelotyping cannot be performed; however, single nucleotide polymorphisms (SNPs) offer many advantages for genetic analysis, including their prevalence in the genome and ease of assay. The unique advantage of SNPs for LOH analyses are that, unlike microsatellites, they are not susceptible to the repeat expansion so often observed in cancer and thus provide higher-fidelity markers for tracking the fate of chromosome segments. Analysis using the Affymetrix® 10K SNP Mapping Array is, therefore, a logical approach for genome-wide allelic imbalance profiling and an efficient method for detecting genome-wide cancer LOH (3).

Several studies have successfully utilized this array for identifying consistent LOH regions in breast cancer (4-8), bladder cancer (9,10), prostate cancer (11,12), osteosarcoma (13), lung cancer (1,14) and oral SCC-derived cell lines (3,15). However, only a limited number of studies have utilized this assay for identifying consistent LOH regions in human oral SCCs.

In this study, we analyzed existence of LOH in human tongue SCCs using this array. Then, in the chromosomal region identified by the genome-wide LOH detection, we reconfirmed the existence of LOH using microsatellite markers. The mRNA expression levels of the gene harbored in this region were examined to identify the role of the gene.

Materials and methods

Cells. The five human tongue SCC-derived cell lines used in this study were SCC-4, HSC-3, HSC-4, OSC-19, and OSC-20 (Human Science Research Resources Bank, Osaka, Japan). All

Table I. Summary of clinicopathological features in 30 tongue SCCs.

Case	Gender	Age	T	N	Stage	pN	Differentiation	Prognosis	Progress	Final
1	M	41	2	2b	IV	-	Well	Well	Well	Alive
2	M	58	1	1	III	+	Well	Well	Well	Alive
3	M	63	1	0	I	-	Well	Well	Well	Alive
4	M	33	2	1	III	+	Well	Well	Well	Alive
5	F	60	4	2b	IV	+	Well	Well	Well	Alive
6	F	63	2	0	II	-	Well	Well	Well	Alive
7	M	42	1	0	I	-	Poor	Well	Well	Alive
8	M	68	1	1	III	-	Well	Poor	Death	Died
9	M	56	3	2c	IV	+	Well	Well	Well	Alive
10	M	38	1	0	I	+	Poor	Poor	Meta	Alive
11	M	53	2	2b	IV	+	Well	Well	Well	Alive
12	M	47	2	2b	IV	+	Well	Well	Well	Alive
13	M	52	1	0	I	-	Well	Well	Well	Alive
14	M	44	2	0	II	-	Well	Well	Well	Alive
15	F	54	2	2c	IV	+	Well	Well	Well	Alive
16	F	58	1	0	I	-	Well	Well	Well	Alive
17	M	67	1	0	I	-	Well	Well	Well	Alive
18	F	63	1	0	I	-	Well	Well	Well	Alive
19	M	52	1	0	I	-	Well	Well	Well	Alive
20	M	57	2	2b	IV	-	Well	Well	Well	Alive
21	M	43	1	0	I	-	Well	Well	Well	Alive
22	M	58	2	2b	IV	+	Well	Well	Well	Alive
23	M	66	2	2b	IV	-	Well	Well	Well	Alive
24	M	64	4	2c	IV	+	Well	Well	Well	Alive
25	M	62	2	0	II	+	Well	Poor	Meta	Alive
26	M	61	2	0	II	-	Well	Well	Well	Alive
27	M	75	4	2b	IV	+	Poor	Poor	Meta	Died
28	M	85	2	0	II	-	Well	Well	Well	Alive
29	M	55	2	2c	IV	+	Well	Well	Well	Alive
30	M	48	1	0	I	-	Well	Well	Well	Alive

M, Male; F, Female; pN, pathological Node; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated; Meta, metastasis.

cell lines were maintained at 37˚C (humidified atmosphere 5% CO_2/95% air) on 150x20 mm tissue culture dishes (Nunc, Roskilde, Denmark) and cultured in Dulbecco's modified Eagle's medium F-12 HAM (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (Sigma) plus 50 U/ml penicillin and streptomycin (16).

Two normal oral keratinocyte (NOK) strains from patients who had undergone dental surgery served as the controls, and the two patients provided written informed consent before the start of the study. The normal oral specimens were washed in Dulbecco's phosphate-buffered saline (PBS) (Sigma) and then placed overnight in 0.25% trypsin-EDTA solution (Sigma) at 4˚C. After the epithelial tissue was separated from the connective tissue, it was disaggregated by incubation in 0.25% trypsin-EDTA solution for 15 min with gentle pipetting at 37˚C. Isolated epithelial cells then were seeded into Collagen I Cellware 60-mm dish biocoat cell environments (BectonDickinson Labware, Bedford, MA, USA) and cultured in Keratinocyte Basal Medium-2 (Cambrex, Walkersville, MD, USA) with 0.4% bovine pituitary extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone, 0.1% transferrin, 0.1% epinephrine, and 0.1% GA-1000 (Cambrex) (17).

Tissue samples. Sample tissues were taken from 30 patients with oral tongue SCC who attended the Tokyo Dental College of Chiba Hospital, during the 3 years between 2005 and 2007 (Table I). Informed consent was obtained from all patients, and our protocol was reviewed and approved by the institutional review board of Tokyo Dental College. Thirty primary tumors and 30 normal tissues corresponding to these primary tumors comprised the tissue samples. They were collected either at the time of surgical resection or at the time of biopsy.

The resected tissues were divided into two segments; one was frozen immediately after careful removal from the surrounding normal tissues and stored at -80˚C until DNA

Table II. Microsatellite markers used in our LOH study.

Markers	Locations	Size of PCR Products (bp)	Sequence of primers
D1S1189	1q31.1	341	5'-CTGAACTAACACGGAGAAAC-3' 5'-GTAGACTGTTAAAAGAAGAGC-3'
D1S2151	1q31.1	260	5'-CTGTATAAAGAGCGCTGTGGG-3' 5'-GGATGGGGCAGTGAGAAG-3'
D1S2595	1q31.1	102	5'-GGGCGTTCCAATACTTAGAGG-3' 5'-AGGACACCACAAGTTCCAGG-3'

and RNA extraction, and the other one was fixed in 10% formalin for pathologic diagnosis. Histopathologic diagnosis was performed according to the International Classification of Tumors. Clinicopathologic staging was determined using the UICC TNM staging system.

DNA extraction. All patients had histologically confirmed tongue SCC, and the tumor samples for DNA extraction were checked to ensure that they consisted of >80% tumor. All tumor samples and most normal tissue samples were processed from freshly frozen specimens that had been preserved in liquid nitrogen immediately after resection. First, the tissues were powdered with liquid nitrogen. After they had been collected by single centrifugation, genomic DNAs were then added to 1000 μl of TNES buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.1% SDS) and 30 μl of Proteinase K (100 μg/ml), stirred, digested, and incubated in a water-bath overnight at 50˚C. After the DNAs had been extracted with phenol-chloroform and refined, they were washed and precipitated with ethanol. The concentrations of the extracted DNAs were estimated by a spectrophotometric method, and the DNAs were kept frozen at -80˚C. From each DNA sample, 50 ng/μl was used as the template for the polymerase chain reaction (PCR) amplification procedure.

For the analysis of DNA mapping array, normal cells were prepared from peripheral blood samples as a control. The normal blood samples were analyzed with the Dr.GenTLE® (Takara Bio Inc., Shiga, Japan) gene trapping kit (18).

Analysis of DNA mapping array using Affymetrix 10K SNP Mapping Array. Five cases of tongue SCC were subjected to DNA mapping array analysis of all chromosomes using the Affymetrix 10K SNP Mapping Array to determine the presence of LOH.

DNA labeling, hybridization, washing and staining of the Affymetrix 10K SNP Mapping Array were performed according to the standard Single Primer GeneChip Mapping 10K Assay protocol (Affymetrix). The array was scanned with a GeneChip Scanner 3000, and the scanned array images were processed with GeneChip Operating software (GCOS). The genotype calls and intensities of the SNP probes were generated by GeneChip DNA Analysis software (GDAS). Individual SNP copy numbers and chromosomal regions with gains or losses were evaluated with the Affymetrix GeneChip chromosome copy number tool 2.0 (19).

Analysis of LOH using microsatellite markers. In the region which had been identified by whole-genome analysis of LOH, we reconfirmed the existence of LOH in 30 cases using microsatellite markers. Only 3 microsatellite markers (D1S1189, D1S2151, and D1S2595) existed in the 1q31.1 region. All primers were obtained from Sigma-Aldrich Japan K.K. (Hokkaido, Japan) (Table II). LOH in the tumor DNA samples was assessed by scanning densitometry and analyzed with NIH Image software (version 1.62, http://rsb.info.nih.gov/nih-image/). The intensities of the signals from the tumor DNA were compared with those of normal control DNA. The criterion for the presence of LOH was a reduction in signal intensity of >50%. Commonly deleted regions were defined by considering the loci most frequently showing LOH, together with multiple interstitial deletions (18).

mRNA expression analysis. Only the Family with sequence similarity 5, member C *(FAM5C)* gene was located in the 1q31.1 region (NCBI Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez). Fifteen cases of tongue SCC were examined the expression levels of *FAM5C* mRNA. The expression levels of *FAM5C* mRNA were examined in the tumors, and paired normal oral tissues from the 15 cases with tongue SCC, five tongue SCC-derived cell lines, and two NOKs. We selected 15 cases who could be extracted total RNA. Control reactions were prepared in parallel without reverse transcriptase. Before cDNA synthesis, residual genomic DNA was removed from the total RNA by DNase I treatment (DNA-free; Ambion, Austin, TX, USA). The primer sequences used for analysis of *FAM5C* mRNA expression were forward: 5'-ACCTGAG AAGTCGCATCAAG-3' and reverse: 5'-CCAGGTGGG GAGAGTTTATT-3'. The sequence of specific primers was checked before use to avoid amplification of genomic DNA or pseudogenes using the Primer3 program (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi). The amplified products were analyzed by 3% agarose gel electrophoresis to ascertain the size and purity of the products. To confirm the identity of the PCR products, they were cloned into a pCR 2.1vector (Invitrogen Japan, K.K., Tokyo, Japan) and sequenced as described previously (20).

Real-time quantitative RT-PCR was performed by a single method using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). To prepare the standard curve, 1.5 μg of total RNA from normal oral tissue was reverse transcribed with Superscript

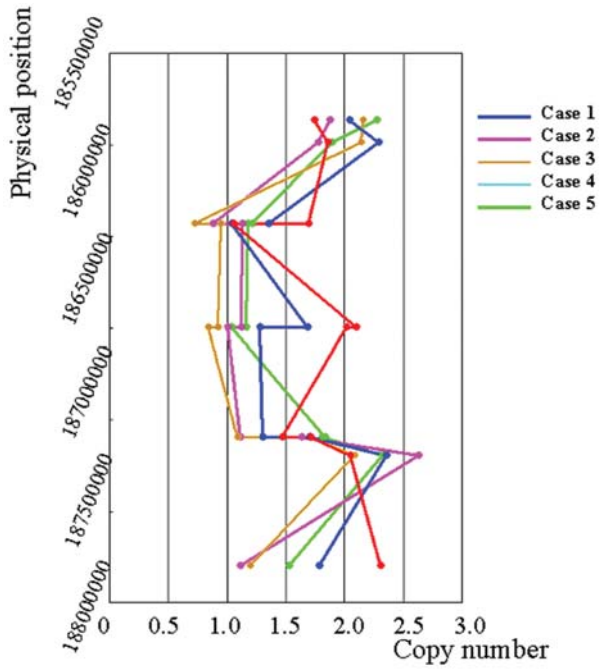

Figure 1. Analysis of copy number abnormality (CNA) on 1q31.1 region. Physical positions of each SNP probe are shown at the vertical axis. The copy number of each SNP probe is shown at the horizontal axis. In the region of 1q31.1, genome copy numbers from 0.8 to 1.2 were confirmed from continual SNP probes in all of the 5 cases.

reverse transcriptase (Life Technologies, Grand Island, NY, USA) and an oligo-d (T) primer, after which serial dilutions were made corresponding to the cDNA transcribed from 300, 30, 3.0, and 0.3 ng of total RNA. The PCR reactions using the LightCycler (Roche Diagnostics) apparatus were carried out in a final volume of 20 μ1 of the reaction mixture consisting of 2 μ1 of FastStart DNA Master SYBR Green I mix (Roche), and 3 mM $MgCl_2$, and 0.2 μ1 of the primers, according to the manufacturer's instructions. The reaction mixture was loaded into glass capillary tubes and submitted to an initial denaturation at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 56˚C (10 sec) for annealing, and 72˚C for extension, with a temperature slope of 20˚C, performed in the LightCycler. The amount of transcript from the *FAM5C* gene was estimated from the respective standard curves and normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript determined in corresponding samples (16).

Clinicopathologic findings and statistical analysis. The patients, 25 males and 5 females, had an average age of 55.5 years for the males (range: 33-80 years) and 59.6 years for the females (range: 54-63 years). The T classifications, which indicate the sizes of the primary clinical tumors, were: 12 patients with T1, 14 with T2, 1 with T3, and 3 with T4. The classifications by TNM stage were: 10 patients with Stage I, 5 with Stage II, 3 with Stage III, and 12 with Stage IV. Thirteen among the 30 patients had histopathologically confirmed cervical lymph node metastasis (LNM) at the time of diagnosis or during the 8-month follow-up period (LNM present). The grades of histological differentiation were: 27 well differentiated tumors and 3 poorly differentiated ones. The prognoses obtained were: 26 favorable prognoses and 4 poor prognoses. The Fisher's exact test was performed to evaluate the significance of correlations between LOH and clinicopathological findings. The accepted level of significance was $P<0.05$.

Results

Analysis of DNA mapping array using Affymetrix 10K SNP Mapping Array. We applied whole-genome analysis of LOH in the specimens from the 5 cases of tongue SCC using

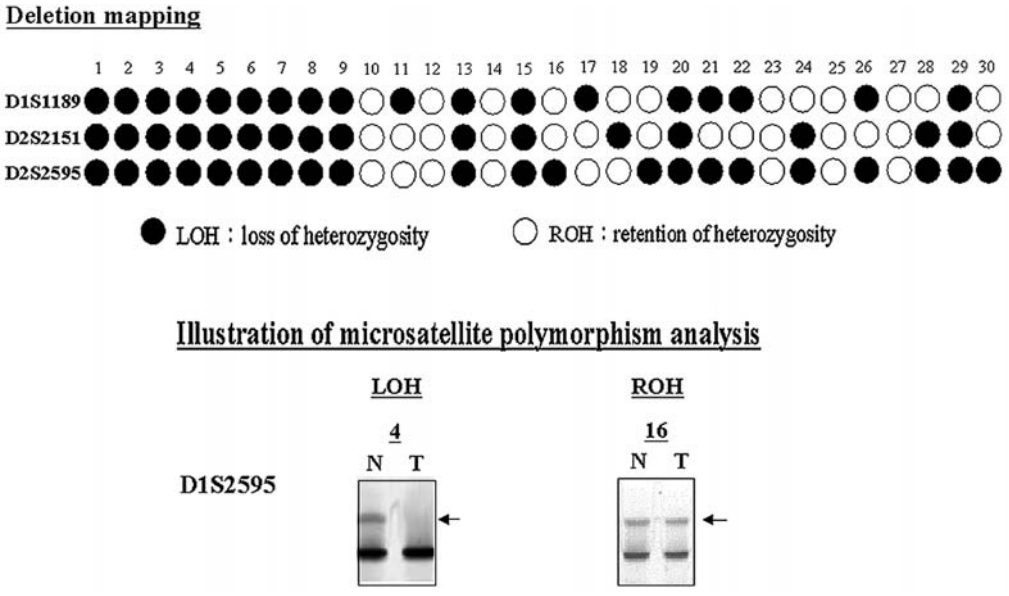

Figure 2. Deletion mapping in the 1q31.1 region in 30 tongue SCCs and illustration of microsatellite polymorphism analysis. Deletion mapping: Case numbers are shown at the top and locus symbols on the left. Illustration of microsatellite polymorphism analysis: Case numbers are shown at the top, and locus symbols on the left. Paired normal (N) and tumor (T) cases for patients 4 and 16 demonstrating loss of heterozygosity (LOH) and retention of heterozygosity (ROH).

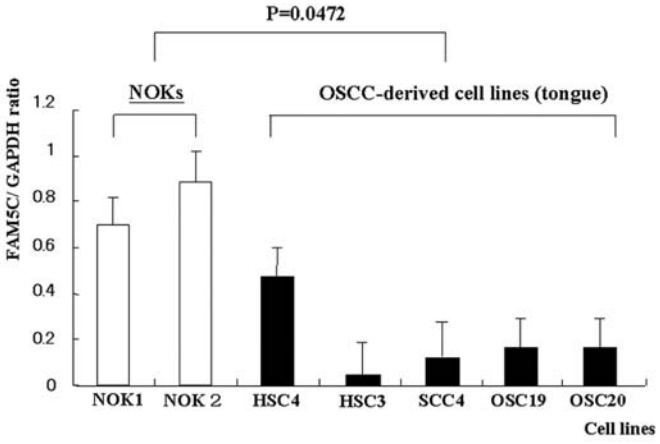

Figure 3. Quantification of mRNA levels in tongue SCC-derived cell lines by real-time RT-PCR analysis. All tongue SCC-derived cell lines decreased *FAM5C* mRNA expression compared with NOKs. There was statistically significant difference in the *FAM5C* mRNA expression levels between tongue SCC-derived cell lines and the NOKs (P=0.0472, Mann-Whitney U test).

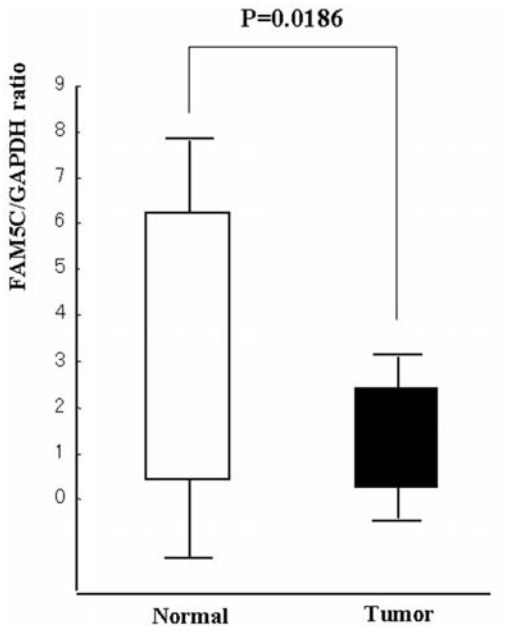

Figure 5. *FAM5C* mRNA expression status in primary tongue SCCs. The mRNA expression level of *FAM5C* was significantly reduced in tumors (n=15) compared with the normal tongue tissues (n=15). There was statistically significant difference in the *FAM5C* mRNA expression levels between tongue SCC and normal tissues (P=0.0186, Mann-Whitney U test).

Affymetrix 10K SNP Mapping Array. In this study, we observed the loss of copy number in copy number abnormality (CNA). At the analysis using this array, a CNA (one copy) suggested LOH (15). A CNA (one copy) was observed in the regions of 1q31.1, 3q13.13, 6p12.3, 9p13.2, 9q33.1, 10p11.21, and 20p11.21. In the region of 1q31.1, genome copy numbers from 0.8 to 1.2 were confirmed using continual SNP probes in all of the 5 cases (21) (Fig. 1).

Analysis of LOH using microsatellite markers. We reconfirmed the existence of LOH in the specimens from the 30 cases using 3 microsatellite markers (D1S1189, D1S2151, and D1S2595) (Table II). A high frequency of LOH was found at the D1S1189 locus in 18/30 (60%), D1S2151 locus in 16/30 (53%) and D1S2595 locus in 21/30 (70%) (Fig. 2).

mRNA expression analysis. The expression levels of *FAM5C* mRNA were examined in 5 tongue SCC-derived cell lines, and two NOKs. All tongue SCC-derived cell lines exhibited decreased *FAM5C* mRNA expression compared with the NOKs. There was statistically significant difference in the *FAM5C* mRNA expression levels between tongue SCC-derived cell lines and the NOKs (P=0.0472, Mann-Whitney U test) (Fig. 3).

The expression levels of *FAM5C* mRNA were examined in the tumors, and paired normal oral tissues from the 15 of the specimens from the cases with tongue SCC. The relative mRNA expression levels in these primary tongue SCCs and the normal tissues ranged from 0.3 to 30.6 (mean: 1.44) and 0.04-37.4 (mean: 2.56), respectively. A significant decrease in *FAM5C* gene expression level was observed in 12 (80%) of the 15 patient specimens (Fig. 4). The expression of *FAM5C* mRNA was significantly reduced in the tumors (n=15) compared with the normal tongue tissues (n=15). There was statistically significant difference in the *FAM5C* mRNA expression levels between tongue SCCs and normal tissues (P=0.0186, Mann-Whitney U test) (Fig. 5). The data are

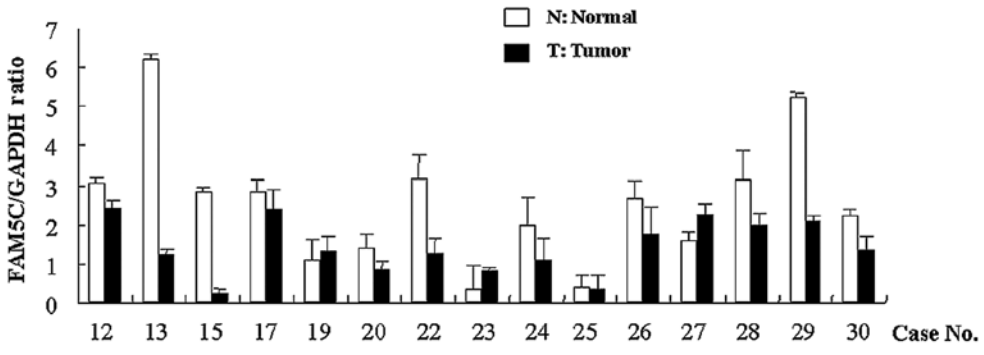

Figure 4. *FAM5C* mRNA expression status in primary tongue SCCs. The relative mRNA expression levels in the primary tongue SCCs and normal tissues ranged from 0.3 to 30.6 (mean: 1.44) and 0.04 to 37.4 (mean: 2.56), respectively. A significant decrease in *FAM5C* gene expression was observed in 12 out of 15 patients (80%).

expressed as the mean ± SD of three independent experiments with samples in triplicate.

Clinicopathological findings and statistical analysis. We compared our results with the clinicopathologic findings for each tumor. A significant statistical correlation between the incidence of LOH in the 1q31.1 region and TNM clinical stage status was observed. A number of the occurrences of LOH in the 1q31.1 region could be detected in early stage lesions; and the frequency of LOH tended to be higher in later clinical stages; however, there was no statistically significant correlation. Moreover, there was no statistically significant correlation between LOH and other clinicopathological findings such as pathological node, grade of histological differentiation, and prognosis associated with the tumor samples.

Discussion

Several studies have successfully utilized the Affymetrix 10K SNP Mapping Array to identify consistent LOH regions (1,3,4-15). However, there are no previous studies identifying CNA and LOH in the 1q31.1 region which we observed in the present study.

Allelic imbalances in whole-genome analyses of tongue SCCs have been reported using the Affymetrix 10K SNP Mapping Array (19). In that study, Zhou *et al* detected genome-wide LOH using the Affymetrix 10K and 100K SNP Mapping Array in tongue SCCs. The results from these two array platforms agreed closely, although more precise allelic imbalance patterns were revealed from the 100K SNP Mapping Array data. This suggests the feasibility of using the Affymetrix 10K SNP Mapping Array for genome-wide LOH detection in tongue SCCs. In addition, Zhou *et al* identified loss of CNA in the 3p11-p12 region. In our study, a rare loss of CNA (one copy) was observed in the region. We clearly established the copy number as one (from 0.8 to 1.2) by analysis of the DNA mapping array. This may be the reason that loss of CNA (one copy) was not observed frequently in the 3p11-p12 region in our study.

We were able to observe a high frequency of LOH in the 1q31.1 region. The only gene known to exist in the 1q31.1 region is *FAM5C*. All tongue SCC-derived cell lines decreased *FAM5C* mRNA expression compared with the NOKs. There was statistically significant difference in the *FAM5C* mRNA expression levels between tongue SCC and normal tissues. These results further support the findings of our previous LOH analysis that the expression of *FAM5C* mRNA is decreased in tongue SCC.

Regarding the role of the *FAM5C* gene, it has been reported that the gene was correlated with the risk of myocardial infraction (MI) (22). To begin to address the functional role of *FAM5C* in MI, Connelly *et al* observed that *FAM5C* was expressed in the human aorta and that its transcript levels decreased with increasing passage of aortic smooth muscle cells in culture, suggesting that the level of gene expression may play a role in proliferation and senescence of this cell type. The correlations between MI and tongue SCC were not clear.

FAM5C was originally identified in the mouse brain as a gene that is induced by the bone morphogenic protein and retinoic acid signaling *(BRINP3)* (23). BRINP3 is a novel protein of unknown function that is normally restricted to the brain. Shorts-Cary *et al* have shown that BRINP3 is a mitochondrially localized protein that is selectively up-regulated in human gonadotropinomas (24). BRINP3 is overexpressed in pituitary gonadotrope cells and promotes proliferation, migration, and invasion, which suggests that it may play an important role in pituitary tumorigenesis. These findings may be useful for the functional analysis of the *FAM5C* gene.

The *FAM5C* gene is also designated deleted in bladder cancer chromosome region candidate 1-like *(DBCCR1-like)*. The *DBCCR1* gene has been reported as the gene functionally affected by frequent loss of 9q32-33 in transitional cell carcinomas of the urinary bladder and identified as a candidate tumor suppressor, which is frequently targeted by promoter hypermethylation in bladder cancer (25). It has been reported that LOH at 9q33 and hypermethylation of the *DBCCR1* promoter are frequent and possibly early events in oral SCC development (26), however, Gao *et al* did not perform DNA mapping array analysis. In our whole-genome analysis of LOH, loss of CNA (one copy) was observed in the 9q33 region. This suggests the *DBCCR1* gene may be a TSG of oral SCC.

Our results suggest that the *FAM5C* gene may be a novel TSG peculiar to tongue SCC, and inactivation of the *FAM5C* gene may play one or more roles in the carcinogenesis of tongue SCCs. In the present study, we established that analysis of DNA mapping array using the Affymetrix 10K SNP Mapping Array was an efficient method to detect genome-wide cancer-associated LOH for tongue SCC. A significant statistical correlation between the incidence of genome-wide cancer-associated LOH and TNM clinical stage status were not clear. We were not able to apply the functional analysis of *FAM5C* gene. Further studies such as immunoblotting, immunofluorescence, and immunohistochemistry are needed to clarify its function in the development and progression of tongue SCC. Furthermore, increasing the number of patient specimens may make it possible to detect genome-wide cancer-associated LOH for tongue SCC in regions other than the 1q31.1 region.

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