J Oral Pathol Med (2005) 34: 150–6

www.blackwellmunksgaard.com/jopm

Frequent promoter hypermethylation of RASSF1A and p16^{INK4a} and infrequent allelic loss other than 9p21 in betel-associated oral carcinoma in a Vietnamese non-smoking/non-drinking female population

Tu Ngoc Tran[1,2], Yenchun Liu[1], Minoru Takagi[1], Akira Yamaguchi[1], Hiroaki Fujii[2]

[1]*Molecular Pathology, Department of Oral Restitution, Division of Oral health Sciences, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan;* [2]*Department of Pathology (II), Juntendo University School of Medicine, Tokyo, Japan*

BACKGROUND: Betel-chewing, a risk factor for oral carcinoma, is a common habit of elderly Vietnamese females, but concomitant habits of tobacco and alcohol are uncommon.

METHODS: In the present study, 36 paraffin-embedded betel-associated oral carcinoma samples including 27 squamous cell carcinoma (SCC) and nine verrucous carcinomas (VC) were analyzed for the hypermethylation of tumor suppressor genes (TSGs) and loss of heterozygosity (LOH) of important TSG loci. Methylation-specific polymerase chain reaction (MSP) was used to identify promoter hypermethylation of p16^{INK4a} and RASSF1A. For LOH analysis, 39 microsatellite markers at 12 chromosomal arms were examined by p olymerase chain reaction (PCR)-based microsatellite assay.

RESULTS: Hypermethylation of p16^{IKN4a} was detected in 63% of SCC and 67% of VC. In addition, LOH at 9p21 (locus for p16^{INK4a}) was 58% for SCC and 22% for VC, and hypermethylation of RASSF1A was 93% for SCC and 100% for VC. LOH at 3p21.3–3p22.1 (where RASSF1A is located) was detected in only 12% of SCC and 0% of VC. LOH of other chromosomal arms were infrequent.

CONCLUSION: As LOH for chromosomes other than 9p was uncommon, epigenetic silencing of RASSF1A and p16^{INK4a} gene expression by promoter hypermethylation may play a critical role in betel-associated oral carcinogenesis.

J Oral Pathol Med (2005) **34**: 150–6

Keywords: betel; hypermethylation; loss of heterozygosity; oral carcinoma; p16^{INK4a}; RASSF1A; tumor suppressor gene

Correspondence: Hiroaki Fujii, Department of Pathology (II), Juntendo University School of Medicine, 2-1-1 Hongo, Tokyo 113-8421, Japan. Tel.: +81 3 5802 1039. Fax: +81 3 3813 3164. E-mail: hfujii@med.juntendo.ac.jp
Accepted for publication September 29, 2004

Introduction

Oral carcinoma is one of the 10 most common malignancies worldwide and the most common cancer in some Asian countries (1–4). Factors believed to contribute to the etiology of oral cancer include tobacco, betel chewing, and alcohol. Epidemiologically, the habit of betel nut (*Areca catechu* L.) chewing is common in some countries in South and Southeast Asia, especially in India and Taiwan (1, 4). The decline of this habit has been noted in some regions, such as in Cambodia (3) and Vietnam because only elderly women have taken this habit.

The molecular genetic mechanisms responsible for betel-associated oral carcinogenesis are poorly understood. Many previous studies on betel-associated oral cancers focused on alterations of p53 using immunohistochemical staining and p53 mutational analysis (1, 5–12). Their results were variable, but generally the mutational frequency of p53 tended to be lower than conventional squamous cell carcinoma (SCC) among Western countries (5, 11, 12).

In oral carcinogenesis in general, allelic loss of 3p, 9p, and 17p is frequent and is a critical genetic event involved in early stage oral cancer (13, 14). By comparative genomic hybridization, Lin et al. (15) reported on the frequent gains of 8q, 9q, 11q, and the frequent losses of 3p and 4q in Taiwanese betel-associated carcinoma cases.

Most of these studies took place in Taiwan and India, and many of the patients were male and also had tobacco and drinking habits in common. Genetic alterations in betel-associated tumorigenesis may be affected by the synergic effects of tobacco and alcohol as well as a wide variation in ethnic background, in addition to other habits, gender, and the betel preparation. Vietnamese betel-chewing habit is unique among other countries because only elderly females tend to take it up, and betel quid containing the areca nut, lime paste and betel leaf without tobacco and other constituents is introduced into the mouth.

Aberrant promoter hypermethylation of tumor suppressor genes (TSGs) is a major epigenetic mechanism for silencing TSGs. Two TSGs known as $p16^{INK4a}$ (located in 9p21) and RASSF1A (located in 3p21.3) exhibit frequent hypermethylation associated gene silencing in many human cancers including bladder cancer (16), gastrointestinal cancer, hepatocellular carcinoma (17, 18) for $p16^{INK4a}$ and cervical cancer, pancreatic cancer, lung cancer, breast cancer, gastric cancer, and nasopharyngeal cancer for RASSF1A (19–23). Previous studies have also reported on the methylation of $p16^{INK4a}$ and RASSF1A in the development and progression of oral pre-malignant lesions and oral cancer (24–26), but hypermethylation of $p16^{INK4a}$ and RASSF1A in betel chewing associated oral carcinoma have not been studied.

In the present study, we analyzed the hypermethylation of RASSF1A and $p16^{INK4a}$ by the Methylation-specific polymerase chain reaction (MSP) method (27) and allelic loss at the associated 3p21 and 9p21 chromosomal loci in 36 cases of oral carcinoma associated with betel chewing in Vietnamese patients. Furthermore, we investigated allelic losses of other chromosomal arms including 2q, 4q, 5q, 8p, 11p, 11q, 13q, 17p, 18q, and 22q, the loci involved with loss of heterozygosity (LOH) in various human tumors. As all of the betel-associated carcinoma cases in our study were old Vietnamese non-smoking, non-drinking females, we had the rare opportunity to examine purely and predominantly betel-related genetic alterations without interference from other confounding factors.

Materials and methods

Tissue samples

Oral carcinoma cases with a clinical history of betel quid chewing totaled 36 cases (27 cases of SCC and nine cases of verrucous carcinoma, VC). All samples were paraffin-embedded tissues obtained from the archival pathology files at the Department of Pathology, Cancer Center, HoChiMinh City, Vietnam. The study was approved by the Ethics Committee in Vietnam. All patients were Vietnamese females, because only elderly women take up this habit in Vietnam. None of them had concomitant habits of tobacco and/or alcohol consumption. The age of the patients ranged from 65 to 87 with a mean of 75 years. The primary sites of oral carcinoma, along with clinical and pathological finding are shown in Table 1. Histopathological grading was performed according to the World Health Organization International Histological Classification of Tumors, and clinical staging was determined by the UICC TNM staging system. The majority of the SCC cases were histopathological Grade I (22 cases) and five cases were histopathological Grade II. Grade III was not observed in our SCC samples.

DNA extraction

For each case, appropriate tissue blocks were selected and sections 8 μm in thickness were cut, deparaffinized, and stained with hematoxyline and eosin (H&E). For LOH analysis, tumor portions of oral carcinoma samples were microdissected using a 27-gauge needle under an inverted microscope. A laser assisted microdissection system (Leica laser microdissection systems, Leica Microsystems, Wetzlar, Germany) was also used to eliminate heavy inflammatory infiltrates found in most of the tumor sections. Non-malignant stroma and inflammatory cells were microdissected, separately collected, and used as normal control DNA. The microdissected tissue was digested overnight at 50°C in a buffer containing 0.5% NP40, 50 mmol/l Tris-HCl pH8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), and 200 μg/ml proteinase K. The lysates were heated at 95°C for 10 min, and then stored at −20°C until use in polymerase chain reaction (PCR). For the methylation study as described below, the entire tissue (mixture of tumor and inflammatory infiltrates) was extracted without microdissection because a large amount of tissue DNA was needed for bisulfite treatment-based methylation analysis.

Table 1 Clinicopathological summary in 36 cases of betel-associated oral carcinoma

Diagnosis	*SCC (n = 27)*	*VC (n = 9)*
TNM stage		
T1	6	1
T2	10	4
T3	7	4
T4	4	
Grade		
I	22	
II	5	
III	0	
Sites		
Buccal mucosa	11	7
Mandibular gingiva	4	
Maxillar gingiva	2	
Tongue	6	
Palate	1	
Lip	2	2
Floor of the mouth	1	
Age		
60–75	16	5
>75	11	4

SCC, squamous cell carcinoma; VC, verrucous carcinoma.

Microsatellite markers

The PCR amplification was performed using primers for 2q (D2S75, D2S93, D2S95, D2S102), 3p (D3S1234, D3S1263, D3S1286, D3S1293, D3S1568, D3S1597, D3S1581, D3S3624, D3S3678), 4q (D4S175, D4S1544), 5q (D5S346, D5S644, D5S1596), 8p (D8S133, D8S258, D8S264), 9p (D9S1748, D9S1749), 11p (D11S922), 11q (D11S29, Int2), 13q (D13S166, D13S168, D13S171), 17p (CHNRB1, D17S578, D17S786, D17S796, D17S1832), 18q (D18S46, D18S55, D18S474), 22q (D22S282, D22S430). Primers D3S1263, D3S1568, D3S1597, D3S1581, D3S3624, and D3S3678 were designed in our laboratory, and the sequences are available upon request. All other primers were obtained from Research Genetics (Huntsville, AL, USA). The

selected markers covered chromosomal regions containing putative TSG and/or those reported to be frequently lost in head and neck cancer and other human tumors. The locations of the markers on 3p and 9p are 3p14.2 (D3S1234), 3p21.3–3p22.1 (D3S3678, D3S3624, D3S1581, D3S1568), 3p24.3–3p25.3 (D3S1263, D3S1597, D3S1286, D3S1293), and 9p21 (D9S1748, D9S1749).

Analysis for LOH and microsatellite instability

The PCR reactions contained 1 μl of DNA lysate, 0.4 μM [γ-^{32}P] ATP-radiolabeled microsatellite primers, 0.2 mM dNTP, 10 mM tris-HCL pH8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.4 U Taq polymerase in a total reaction volume of 10 μl. Taq was added to the reactions pre-warmed to 94°C (hot-start PCR) and the samples were amplified over 35 cycles. The PCR products were then separated on a 5% denaturing polyacrylamide-urea-formamide gel. The gel was dried, the signals were visualized using an image analyzer (Bas 2500, Bio Imaging System, Fuji Film, Tokyo, Japan), and LOH was determined based on a reduction of over 75% of the relative intensity in one of the two alleles compared with those of the normal controls. Microsatellite instability (MI) was noted when a new allele appeared in the tumor tissues compared with the normal controls. LOH were confirmed by using multiple informative markers and repeating the PCR. When only one informative marker was recognized for a chromosomal region in a tissue sample, LOH was confirmed by repeating PCR amplification of that marker.

Fractional allelic loss

Fractional allelic loss (FAL) was calculated as: the number of chromosomal arms on which LOH was detected divided by the number of chromosomal arms on which allelic markers were informative in the patient's normal cells for each case.

Methylation-specific PCR (MSP)

The methylation status of p16^{INK4a} and RASSF1A in oral carcinoma were examined by MSP using the protocol of Herman et al. (27). To differentiate between methylated and unmethylated sequences, DNA was treated with sodium bisulfite. Briefly, extracted DNA was denatured by 0.2 M NaOH and then incubated in 3 M sodium bisulfite (pH 5.0) and 10 mM hydroquinone for 18–20 h at 52°C. Modified DNA samples were then purified using Wizard purification resin (Promega, Madison, WI, USA). For alkali desulphonation, purified DNA was treated with 0.3 M NaOH, precipitated with ethanol, and resuspended in 50 μl H_2O. After bisulfite treatment, subsequent MSP distinguished methylated alleles of a given gene based on DNA sequence alterations. The MSP primer sequences and PCR conditions for methylated and unmethylated sequences for p16^{INK4a} (27) and RASSF1A (28) were previously described. The PCR products were loaded onto 12% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Statistical analysis

Fisher's exact and chi-squared tests were used to analyze the association of allelic status, RASSF1A and p16^{INK4a} methylation status, and clinicopathological profiles. The Mann–Whiteney *U*-test was used to compare association between FAL and tumor grade or grade. $P < 0.05$ was considered to be statistically significant.

Results

Allelic loss

A summary of LOH for each case is shown in Table 2 and representative gels showing LOH are presented in Fig. 1.

For 21 of 27 SCC cases (78%), LOH was detected at one or more of the chromosomal loci examined. For case OR 77, OR81, and OR 102, LOH were observed in four to six chromosomal arms. In 18 cases, LOH were found in one to three chromosomal arms. There were six cases (Cases OR37, OR73, OR 82, OR 84, OR 85, and OR 100) that showed no LOH at any of the chromosomal loci examined. The highest frequency of LOH for SCC was observed at 9p21 (58%) followed by 3p (23%), 8p (23%), 5q (21%), 2q (20%), 11p (18%), 18q (14%), 17p (13%), 22q (11%), and 11q (4.8%). At chromosomal arms 13q, no case showed LOH. There was no significant correlation between LOH at 3p, 9p and the pathological grading or stage ($P = 0.218/P = 0.711$ for 3p and $P = 0.1/P = 0.893$ for 9p). There was also no significant difference for FAL in grade I vs. II ($P = 0.605$) and for stage T1 and T2 vs. T3 and T4 ($P = 0.820$).

Although only 9 VC cases were included in the present study, we observed LOH at 11p (33%, but for only one case), followed by 5q (29%), 2q (25%) and 9p (22%). No LOH was detected at chromosomal arms 13q, 17p and 18q. The mean FAL was 0.17 for SCC and 0.13 for VC.

Loss of heterozygosity at markers on 3p21.3–3p22.1 (RASSF1A is located at 3p21.3) was 12% for SCC (three of 25 informative cases) and 0% for VC. LOH at markers on 9p21 (p16^{INK4a} is located at 9p21) was 58% for SCC (11 of 19 informative cases) and 22% for VC (two of nine cases).

Mircosatellite instability

There were four cases of SCC showing MI (OR15, OR73, OR82, and OR95). Of these, only Case OR15 showed MI at three loci (D9S1748, D9S1749, D11S29), and the rest showed MI at one locus.

Methylation status of p16^{INK4a}

Hypermethylation of p16^{INK4a} was found among 17 of the 27 SCC cases (63%) and six of the nine VC cases (67%) as shown in Table 2. Unmethylated MSP bands were detected in all of these samples. The unmethylated bands may have been detected because of contaminating normal cells (most cases showed heavy inflammatory cell infiltrates, which were not eliminated by microdissection for the MSP study) and non-methylated alleles of cells in tumor components.

Table 2 Summary of LOH and methylation status in betal-associated oral carcinoma

			LOH analysis													Methylation status	
Cases	**Grade**	**Stage**	**2q**	**3p**	**4q**	**5q**	**8p**	**9p**	**11p**	**11q**	**13q**	**17p**	**18q**	**22q**	**FAL**	*RASSF1A*	*p16*
SCC (27 cases)																	
OR6	I	2	NI/NA		NI/NA	NI/NA	NI/NA	NI/NA	NI/NA		NI/NA	NI/NA		NI/NA	0.33	(+)	(+)
OR9	I	4	NI/NA		NI/NA				NI/NA		NI/NA			NI/NA	0.29	(–)	(–)
OR15	II	2					NI/NA		NI/NA					NI/NA	0.14	(+)	(–)
OR31	I	1								NI/NA	NI/NA				0.2	(+)	(+)
OR37	I	1													0	(+)	(+)
OR47	I	2				NI/NA			NI/NA		NI/NA				0.22	(+)	(+)
OR64	I	1													0.17	(+)	(+)
OR65	I	2						NI/NA	NI/NA				NI/NA		0.11	(+)	(+)
OR66	I	4							NI/NA					NI/NA	0.2	(+)	(–)
OR69	I	3						NI/NA		NI/NA					0.2	(+)	(+)
OR72	I	3							NI/NA						0.18	(+)	(+)
OR73	II	2		NI/NA	NI/NA	NI/NA		NI/NA	NI/NA						0	(+)	(+)
OR74	I	2													0.17	(+)	(+)
OR77	I	1					NI/NA			NI/NA			NI/NA		0.56	(+)	(+)
OR80	I	2							NI/NA			NI/NA	NI/NA		0.33	(+)	(–)
OR81	II	4			NI/NA										0.55	(+)	(+)
OR82	I	2													0	(+)	(+)
OR84	I	1							NI/NA	NI/NA			NI/NA	NI/NA	0	(+)	(–)
OR85	II	3						NI/NA	NI/NA			NI/NA	NI/NA		0	(+)	(–)
OR86	I	3			NI/NA						NI/NA			NI/NA	0.11	(–)	(–)
OR87	I	3							NI/NA						0.09	(+)	(+)
OR95	I	1							NI/NA		NI/NA				0.11	(+)	(+)
OR99	I	2					NI/NA	NI/NA	NI/NA						0.22	(+)	(–)
OR100	II	3					NI/NA	NI/NA	NI/NA	NI/NA		NI/NA			0	(+)	(–)
OR102	I	2												NI/NA	0.36	(+)	(+)
OR104	I	4													0.08	(+)	(+)
OR105	I	3													0.08	(+)	(–)
% LOH and % methylation in SCC			20%	23%	4.50%	21%	23%	58%	18%	4.8%	0%	13%	14%	11%	Mean FAL=0.17	93%	63%
VC (9 cases)																	
OR14		2			NI/NA	NI/NA			NI/NA		NI/NA				0	(+)	(–)
OR18		3							NI/NA		NI/NA				0.3	(+)	(–)
OR27		3													0.08	(+)	(+)
OR29		3	NI/NA						NI/NA						0.3	(+)	(+)
OR33		1			NI/NA		NI/NA		NI/NA						0	(+)	(+)
OR67		2				NI/NA			NI/NA				NI/NA		0	(+)	(+)
OR92		3													0.08	(+)	(–)
OR93		2								NI/NA			NI/NA		0.2	(+)	(+)
OR94		2							NI/NA	NI/NA			NI/NA		0.22	(+)	(+)
% LOH and % methylation in VC			25%	11%	14%	29%	13%	22%	33%	14%	0%	0%	0%	11%	Mean FAL=0.13	100%	67%

Black boxes, LOH; blanks, retention of heterozygosity; NI/NA, chromosomal arms which were non-informative or not amplified; gray boxes, microsatellite instability; (+), methylated; (-), not methylated.

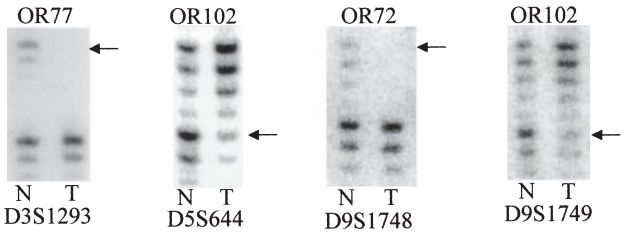

Figure 1 Representative gels showing loss of heterozygosity (LOH) in betel-associated oral carcinoma. N, normal control DNA; T, microdissected tumor DNA; arrows, LOH.

Representative MSP results of $p16^{INK4a}$ are presented in Fig. 2a. Concomitant $p16^{IKN4a}$ hypermethylation and 9p21 LOH were detected for nine of 19 informative SCC cases (47%) and for two of nine VC cases (22%) (Table 3). No correlation was found between 9p21 LOH and methylation of $p16^{INK4a}$ ($P = 0.319$ for SCC and $P = 0.5$ for VC).

Methylation status of RASSF1A

The methylation status of RASSF1A is summarized in Table 2. Bands for RASSF1A methylation were found in 93% of the SCC samples (25 of 27) and all of the VC samples (nine of nine). Representative MSP results of RASSF1A are presented in Fig. 2b. As shown in Table 4, the concomitant occurrence of RASSF1A hypermethylation and 3p21.3–3p22.1 LOH was detected for three of 27 informative SCC cases (11%). All of the VC cases showed hypermethylation of RASSF1A but retention of 3p21.3–3p22.1. No correlation was found between 3p21.3 and 3p22.1 LOH and RASSF1A hypermethylation ($P = 1$ for SCC and VC).

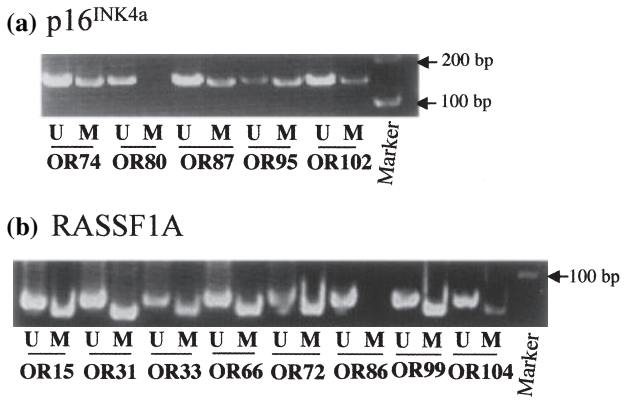

Figure 2 Methylation analysis of $p16^{INK4a}$ (a) and RASSF1A (b) by MSP. U, PCR with primers for unmethylated DNA; M, PCR with methylation-specific primers. PCR product sizes of unmethylated allele and methylated allele for $p16^{INK4a}$ are 151 and 150 bp, respectively. PCR product sizes of unmethylated allele and methylated allele for RASSF1A are 81 and 76 bp, respectively.

Table 3 Methylation status and allelic status of the gene loci for p16

	Methylation of p16	
Histology	*Methylated*	*Unmethylated*
SCC		
LOH of 9p21 (58%)	9	2
Retention 9p21	4	4
VC		
LOH of 9p21 (22%)	2	0
Retention 9p21	4	3

SCC, squamous cell carcinoma; VC, verrucous carcinomas; LOH, loss of heterozygosity.

Table 4 Methylation status and allelic status of the gene loci for RASSFIA

	Methylation of RASSF1A	
Histology	*Methylated*	*Unmethylated*
SCC		
LOH of 3p21.3–3p22.1 (12%)	3	0
Retention 3p21.3–3p22.1	22	2
VC		
LOH of 3p21.3–3p22.1 (0%)	0	0
Retention 3p21.3–3p22.1	9	0

SCC, squamous cell carcinoma; VC, verrucous carcinomas; LOH, loss of heterozygosity.

Discussion

In previous studies, allelic loss of 3p and/or 9p was reported to be an essential step in the early development of head and neck cancer (14, 29). 9p21 is one of the most frequent regions for allelic loss in head and neck SCC (24) and oral precancerous lesions (13, 29, 30). In our study, we found LOH at 9p21 in 58% of the SCC cases, and 22% of the VC cases. The frequency of LOH at 9p21 for SCC is similar to previously published data on oral SCC (13, 14, 29, 30).

In the present study, we found that $p16^{INK4a}$ hypermethylation occurred at 63%. Our result is different from a study by Waber et al., (31) who examined 21 paired set tumors and blood samples in head and neck SCC and found no $p16^{INK4a}$ hypermethylation. Contrary to the report by Waber et al., hypermethylation of $p16^{INK4a}$ in oral cancer has been reported in up to 50% of cases in other studies (25, 32, 33). Kresty also reported on $p16^{INK4}$ methylation in severe oral dysplasia, (24) indicating that alteration of this gene may be a critical early event in oral carcinogeneis. The high frequency of $p16^{INK4a}$ in the present result indicates that betel chewing habit preferentially affects the methylation of $p16^{INK4a}$.

The concomitant occurrence of 9p21 LOH and hypermethylation of $p16^{INK4a}$ was frequently found in our SCC samples, satisfying the two-hit mechanism of TSG suppression. Cases with hypermethylation of $p16^{INK4a}$ but retention of 9p21 LOH may show biallelic methylation, partial methylation or haploinsufficiency.

Frequent 3p allelic loss is one of the most common genetic alterations in a wide range of tumor types such as cervical cancer, esophageal SCC, lung cancer, renal cancer, and head and neck SCC (23, 26, 34). Three discrete regions of deletions at 3p including 3p24, 3p21.3 (locus for RASSF1A), and 3p14 were identified in head

and neck cancer (35). In the present study, RASSF1A hypermethylation frequently occurred in our SCC and VC samples at rates of 93% for SCC and 100% for VC. Unexpectedly, the percentage of LOH at 3p21.3–3p22.1 in our study was low (12% for SCC and 0% for VC), and LOH at one or more 3p markers was only 23% for SCC and 11% for VC. Our results differ considerably from other reports. For example, Hogg et al., (26) found a high frequency of allelic loss at 3p (81%) and at 3p21 (66%), but RASSF1A hypermethylation was detected for only 17% (four of 24 cases) of sporadic HNSCC. The lower frequency of 3p21.3 allelic loss compared with frequent RASSF1A methylation in our samples may be explained by the following mechanisms: (i) our cases were non-smokers and non-drinkers, and genetic pathways for betel-associated cancer and betel/smokers and/or drinkers may be different, (ii) ethnic, and gender differences, (iii) the majority of our samples were grade I – well differentiated SCC (81%; 22 of 27 cases), showing that allelic loss occurred at a lower rate than that of poorly differentiated carcinoma (36), (iv) betel chewing may preferentially cause aberrant hypermethylation of RASSF1A gene, and both alleles of RASSF1A may be methylated, and (v) RASSF1A may behave as a haploinsufficiency.

Loss of heterozygosity of 10 other chromosomal arms were all infrequently detected in the present study. Previous studies described the 17p region as a common loss and early event in HNSCC, and the frequency of 17p LOH to be 31–69% (30, 37, 38). We found allelic loss of 17p for only 13% of the SCC cases. Similarly, LOH at 5q and 8p was low compared with other studies on HNSCC (13, 29, 37, 39). As mentioned above, the genetic pathway responsible for betel-associated SCC without concomitant effects of ethanol and tobacco may be different from conventional oral SCC (tobacco and alcohol as major risk factors) and may belong to the low FAL pathway. These results also suggest that 9p21 is an early and critical genetic event in betel associated oral SCC carcinogenesis. The existence of a distinct genetic population in the development of oral SCC with low FAL has also been described for the English population (30).

Verrucous carcinomas is a lesion of the oral cavity that is distinct from SCC in morphology and behavior. Tobacco and human papilloma virus (HPV) are important risk factors for VC. Genetic studies on verrucous carcinoma are still scarce. Poh et al. (40) reported a high frequency of LOH in oral VC. Many of the allelic losses including 3p, 4q, 8p, and 11q were present in verrucous hyperplasia, the precursor of VC, and 17p LOH (p53 locus) occurred at a lower frequency than SCC. Contrary to their report, positive p53 immunohistochemical staining was detected among 15 of 29 of the VC cases (41). These studies were conducted in the western hemisphere, were not associated with betel-chewing and had tobacco and HPV as major risk factors. Although the number of VC cases examined (9) was small in the present study, and the HPV status of each was unknown, the high frequency of hypermethylation for $p16^{INK4a}$, and RASSF1A and low FAL with infrequent LOH for all chromosomal arms examined suggests that betel-chewing related VC may develop via a genetic pathway distinct from Western VC.

In conclusion, Vietnamese betel-associated oral SCC/VC cancer without a history of concomitant tobacco and alcohol habits shows infrequent LOH except at 9p21 for SCC. However, hypermethylation of RASSF1A and the $p16^{INK4a}$ promoter is a frequent event in SCC and VC. Thus, epigenic silencing of TSG gene expression may play a critical role in betel-associated oral carcinogenesis.

References

1. Kaur J, Srivastava A, Ralhan R. Overexpression of p53 protein in betel- and tobacco-related human oral dysplasia and squamous-cell carcinoma in India. *Int J Cancer* 1994; **58**: 340–5.
2. Reichart PA. Oral cancer and precancer related to betel and miang chewing in Thailand: a review. *J Oral Pathol Med* 1995; **24**: 241–3.
3. Reichart PA, Schmidtberg W, Scheifele C. Betel chewer's mucosa in elderly Cambodian women. *J Oral Pathol Med* 1996; **25**: 367–70.
4. Ho PS, Ko YC, Yang YH, Shieh TY, Tsai CC. The incidence of oropharyngeal cancer in Taiwan: an endemic betel quid chewing area. *J Oral Pathol Med* 2002; **31**: 213–9.
5. Thomas S, Brennan J, Martel G, et al. Mutations in the conserved regions of p53 are infrequent in betel-associated oral cancers from Papua New Guinea. *Cancer Res* 1994; **54**: 3588–93.
6. Kuttan NA, Rosin MP, Ambika K, Priddy RW, Bhakthan NM, Zhang L. High prevalence of expression of p53 oncoprotein in oral carcinomas from India associated with betel and tobacco chewing. *Eur J Cancer B Oral Oncol* 1995; **31B**: 169–73.
7. Ranasinghe AW, Warnakulasuriya KA, Johnson NW. Low prevalence of expression of p53 oncoprotein in oral carcinomas from Sri Lanka associated with betel and tobacco chewing. *Eur J Cancer B Oral Oncol* 1993; **29B**: 147–50.
8. Ranasinghe A, MacGeoch C, Dyer S, Spurr N, Johnson NW. Some oral carcinomas from Sri Lankan betel/tobacco chewers overexpress p53 oncoprotein but lack mutations in exons 5–9. *Anticancer Res* 1993; **13**: 2065–8.
9. Chiba I, Muthumala M, Yamazaki Y, et al. Characteristics of mutations in the p53 gene of oral squamous-cell carcinomas associated with betel-quid chewing in Sri Lanka. *Int J Cancer* 1998; **77**: 839–42.
10. Kaur J, Srivastava A, Ralhan R. Prognostic significance of p53 protein overexpression in betel- and tobacco-related oral oncogenesis. *Int J Cancer* 1998; **79**: 370–5.
11. Kannan K, Munirajan AK, Krishnamurthy J, et al. Low incidence of p53 mutations in betel quid and tobacco chewing-associated oral squamous carcinoma from India. *Int J Oncol* 1999; **15**: 1133–6.
12. Thongsuksai P, Boonyaphiphat P, Sriplung H, Sudhikaran W. p53 mutations in betel-associated oral cancer from Thailand. *Cancer Lett* 2003; **201**: 1–7.
13. Partridge M, Emilion G, Pateromichelakis S, Phillips E, Langdon J. Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas. *Int J Cancer* 1999; **83**: 318–25.
14. Mao L, Lee JS, Fan YH, et al. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral

premalignant lesions and their value in cancer risk assessment. *Nat Med* 1996; **2**: 682–5.
15. Lin SC, Chen YJ, Kao SY, et al. Chromosomal changes in betel-associated oral squamous cell carcinomas and their relationship to clinical parameters. *Oral Oncol* 2002; **38**: 266–73.
16. Valenzuela MT, Galisteo R, Zuluaga A, et al. Assessing the use of p16(INK4a) promoter gene methylation in serum for detection of bladder cancer. *Eur Urol* 2002; **42**: 622–8.
17. Rocco JW, Sidransky D. p16(MTS-1/CDKN2/INK4a) in cancer progression. *Exp Cell Res* 2001; **264**: 42–55.
18. Schagdarsurengin U, Wilkens L, Steinemann D, et al. Frequent epigenetic inactivation of the RASSF1A gene in hepatocellular carcinoma. *Oncogene* 2003; **22**: 1866–71.
19. Lo KW, Kwong J, Hui AB, et al. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. *Cancer Res* 2001; **61**: 3877–81.
20. Byun DS, Lee MG, Chae KS, Ryu BG, Chi SG. Frequent epigenetic inactivation of RASSF1A by aberrant promoter hypermethylation in human gastric adenocarcinoma. *Cancer Res* 2001; **61**: 7034–8.
21. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000; **25**: 315–9.
22. Dammann R, Yang G, Pfeifer GP. Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. *Cancer Res* 2001; **61**: 3105–9.
23. Yu MY, Tong JH, Chan PK, et al. Hypermethylation of the tumor suppressor gene RASSFIA and frequent concomitant loss of heterozygosity at 3p21 in cervical cancers. *Int J Cancer* 2003; **105**: 204–9.
24. Kresty LA, Mallery SR, Knobloch TJ, et al. Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. *Cancer Res* 2002; **62**: 5295–300.
25. Viswanathan M, Tsuchida N, Shanmugam G. Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. *Int J Cancer* 2003; **105**: 41–6.
26. Hogg RP, Honorio S, Martinez A, et al. Frequent 3p allele loss and epigenetic inactivation of the RASSF1A tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. *Eur J Cancer* 2002; **38**: 1585–92.
27. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996; **93**: 9821–6.
28. van Engeland M, Roemen GM, Brink M, et al. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002; **21**: 3792–5.
29. Jiang WW, Fujii H, Shirai T, Mega H, Takagi M. Accumulative increase of loss of heterozygosity from leukoplakia to foci of early cancerization in leukoplakia of the oral cavity. *Cancer* 2001; **92**: 2349–56.
30. Nunn J, Scholes AG, Liloglou T, et al. Fractional allele loss indicates distinct genetic populations in the development of squamous cell carcinoma of the head and neck (SCCHN). *Carcinogenesis* 1999; **20**: 2219–28.
31. Waber P, Dlugosz S, Cheng QC, Truelson J, Nisen PD. Genetic alterations of chromosome band 9p21-22 in head and neck cancer are not restricted to p16INK4a. *Oncogene* 1997; **15**: 1699–704.
32. Huang MJ, Yeh KT, Shih HC, et al. The correlation between CpG methylation and protein expression of P16 in oral squamous cell carcinomas. *Int J Mol Med* 2002; **10**: 551–4.
33. Shintani S, Nakahara Y, Mihara M, Ueyama Y, Matsumura T. Inactivation of the p14(ARF), p15(INK4B) and p16(INK4A) genes is a frequent event in human oral squamous cell carcinomas. *Oral Oncol* 2001; **37**: 498–504.
34. Kuroki T, Trapasso F, Yendamuri S, et al. Allele loss and promoter hypermethylation of VHL, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. *Cancer Res* 2003; **63**: 3724–8.
35. Maestro R, Gasparotto D, Vukosavljevic T, Barzan L, Sulfaro S, Boiocchi M. Three discrete regions of deletion at 3p in head and neck cancers. *Cancer Res* 1993; **53**: 5775–9.
36. Kanazawa T, Watanabe T, Kazama S, Tada T, Koketsu S, Nagawa H. Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. *Int J Cancer* 2002; **102**: 225–9.
37. Ah-See KW, Cooke TG, Pickford IR, Soutar D, Balmain A. An allelotype of squamous carcinoma of the head and neck using microsatellite markers. *Cancer Res* 1994; **54**: 1617–21.
38. Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. *Cancer Res* 1994; **54**: 1152–5.
39. Huang JS, Chiang CP, Kok SH, Kuo YS, Kuo MY. Loss of heterozygosity of APC and MCC genes in oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* 1997; **26**: 322–6.
40. Poh CF, Zhang L, Lam WL, et al. A high frequency of allelic loss in oral verrucous lesions may explain malignant risk. *Lab Invest* 2001; **81**: 629–34.
41. Gimenez-Conti IB, Collet AM, Lanfranchi H, et al. p53, Rb, and cyclin D1 expression in human oral verrucous carcinomas. *Cancer* 1996; **78**: 17–23.

Acknowledgments

We sincerely acknowledge the Department of Pathology, Cancer Center, HoChiMinh City, Vietnam for the archival pathology materials. We sincerely acknowledge Dr Joseph Califano MD, for critical review of the manuscript and appreciate D. Mrozek for English proof reading of the manuscript. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, Japan.