

Interaction of Collagen-related Genes and Susceptibility to Betel Quid-induced Oral Submucous Fibrosis¹

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Abstract

Oral submucous fibrosis (OSF) is a precancerous condition of the oral cavity. It is a collagen-related disorder induced by betel quid chewing, a habit that is common in Taiwan. However, the cumulative exposure to betel quids varies in OSF patients. It seems that there is individual susceptibility to betel quid-induced OSF. This study compared the association of OSF and polymorphisms of six collagen-related genes, collagen 1A1 and 1A2 (COL1A1 and COL1A2), collagenase-1 (COLase), transforming growth factor β 1 (TGF- β 1), lysyl oxidase (LYOXase), and cystatin C (CST3), between patients with low and high exposure to betel quids. A total of 166 patients with OSF from a medical center and 284 betel quid chewers who were free of OSF and oral cancer, from the same hospital and five townships, were recruited. PCR-based restriction fragment length polymorphism assays were used to determine the genotypes of the six collagen-related genes situated on different chromosomes. We found that the genotypes associated with the highest OSF risk for collagen 1A1, collagen 1A2, collagenase-1, transforming growth factor β 1, lysyl oxidase, and cystatin C were CC, AA, TT, CC, AA, and AA, respectively, for the low-exposure group, and TT, BB, AA, CC, GG, and AA, respectively, for the high-exposure group. A trend was noted for an increased risk of OSF with increasing number of high-risk alleles for those with both high and low exposures for betel quid. The cell selection mechanism of oral fibroblasts is proposed to explain the effect of the modification of cumulative betel quid exposure on the risk profiles of collagen-related genes. These results imply that

susceptibility to OSF could involve multigenic mechanisms modified by the betel quid-exposure dose.

Introduction

OSF³ has been identified as a precancerous condition (1). In an epidemiological study on oral cancer and precancerous lesions in rural Indian population, the malignant transformation rate was 7.6% (5 of 66) over a 17-year period (median observation, 10 years; Ref. 2). One of the clinical symptoms of OSF is trismus, a limitation of mouth opening. This may eventually impair the ability to eat and speak, and dental care may become difficult. Chewing betel quid has been recognized as one of the most important risk factors for OSF (3–7), but only a portion of betel quid chewers develops the disease. This disease affects ~0.5% (5 million people) of the population in the Indian subcontinent and is now a public health issue in many parts of the world, including the United Kingdom (8), South Africa (9), and many southeastern Asian countries (10, 11). However, even in countries in which betel quid is widely used, the prevalence of its use is not well surveyed (12). The composition of betel quid showed geographic variations. In Taiwan, it is composed of areca nut (*Areca catechu*), slaked lime, and the inflorescence or leaf of *Piper betle* (13).

The main histopathological characteristic of OSF is the deposition of collagen in the oral submucosa (14). It has been found that alkaloid exposure of buccal mucosal fibroblasts may result in the accumulation of collagen (15). A reduced degradation of the α 1(I) collagen trimer synthesized by OSF fibroblasts may induce the alteration of the ratio of α 1(I): α 2 (I) chains (16). Collagenase activity has been found to be lower in OSF than in normal oral mucosa (17). This evidence implies that OSF may be considered a collagen-metabolic disorder resulting from alkaloid exposure and individual variation in collagen metabolism.

Because collagens are the major structural components of connective tissues, including oral submucosa, the composition of collagens within each tissue needs to be precisely regulated to maintain tissue integrity. Regulation is necessary, not only to control the amount of collagens produced but also to control the fiber architecture. The synthesis of collagens is influenced by a variety of mediators, including growth factors, hormones, cytokines, and lymphokines. A prominent mediator is the TGF- β , which stimulates the production of collagens and other matrix components. TGF- β was also found to modulate the expression of collagenase and metalloproteinase inhibitor (18). This

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³ The abbreviations used are: OSF, oral submucous fibrosis; TGF- β , transforming growth factor β ; CST3, human cystatin C; COL1A1, collagen 1A1; COL1A2, collagen 1A2; COLase, collagenase-1; LYOXase, lysyl oxidase; NTUH, National Taiwan University Hospital; MMO, maximum mouth opening; RFLP, restriction fragment length polymorphism; PCR-RFLP, PCR-based RFLP; OR, odds ratio; OR_{adj}, multivariate-adjusted OR; CI, confidence interval; MMP, matrix metalloproteinase.

growth factor has also been implicated in the development of fibrotic lesions (19). Among the different TGF- β isoforms, TGF- β 1 seems to be the species that plays a major role in wound repair and fibrosis (20). The MMPs are a tightly regulated family of enzymes that degrade extracellular matrix. COLase (MMP-1 or fibroblast-type collagenase) is a member of the MMP family and the principal human enzyme that cleaves native fibrillar collagen (21). In addition to the regulation of collagen biosynthesis and degradation, collagen cross-linking genes also need to be precisely regulated to maintain tissue integrity. The activity of LYOXase, an extracellular enzyme stabilizing the collagen fibrillar array by covalent cross-links, was found to increase in fibroblasts cultured from OSF patients (22). Collagen fibers form three-dimensional scaffolding by combining cross-linked collagen molecules with other extracellular matrix components. The terminal regions of each collagen molecule consist of terminal peptides, which contain the sites of intra- and intermolecular cross-links. These areas are resistant to attack by collagenases but can be attacked by a number of other serine and cysteine proteinases (23). *CST3* encoding a cysteine proteinase inhibitor might contribute to the stabilization of collagen fibrils. In the present study, we tried to study interactive effects of polymorphisms of the above-mentioned collagen-related genes and a complex disease, OSF. Because COLase, *CST3*, and LYOXase have been well documented to be involved in the collagen biosynthesis and degradation, they are hypothesized to contribute to the pathogenesis of OSF.

We have found a dramatic variation in the induction dosage of betel quid exposure for OSF development. This implies the existence of individual susceptibility to betel quid-induced OSF. It was hypothesized that collagen-related genes might play a certain role in OSF pathogenesis. Because of the complexity of OSF pathogenesis, it is important to elucidate independent and interactive effects of polymorphisms of collagen-related genes on OSF risk. We found no studies that examined these issues. This study was carried out to compare associations of OSF and polymorphisms of six collagen-related genes between patients with low and high exposures to betel quids.

Materials and Methods

Study Subjects. A total of 166 men with OSF were consecutively recruited from the Dental Department of NTUH between January and December, 1999. Palpable or strip fibrous bands in the buccal mucosa, labial mucosa, fauces, soft palate including the uvula, and/or floor of the mouth or tongue were regarded as OSF. Scars and oral frenulae were not considered fibrous bands. The diagnoses of OSF were primarily made by oral pathologists. To prevent a keloid-like response after biopsy surgery, further histopathological confirmation was carried out on 90 (54.2%) patients with suspected malignant transformations.

Because there was no confirmed case of OSF among nonchewers of betel quid, only healthy betel quid chewers were recruited as our control subjects. Both hospital and community controls were recruited to avoid possible selection bias. A total of 111 betel quid chewers without OSF or oral cancer were recruited from the dental clinic of NTUH as betel quid-chewing hospital controls. Another 173 betel quid chewers without OSF or cancer were recruited from five townships including Makung, Chutung, Potzu, Kaoshu, and Sanchi as betel quid-chewing community controls. These townships were located in both urban and rural areas and were considered well representative of the catchment area of NTUH. As there were no sig-

nificant differences in OSF risk factors and genetic polymorphisms of collagen-related genes, these two groups were combined in the analysis to increase the statistical power.

Questionnaire Interview and Blood Collection. Experienced nurses conducted a standardized interview based on a structured questionnaire for every study subject to collect information on demographic characteristics and history of exposure to risk factors as well as personal and family history of various cancers. The age at which the betel quid-chewing habit started, average daily consumption, and the age at which the chewing habit stopped were queried in detail. The cumulative exposure to betel quid was derived by multiplying the daily consumption of betel quid (in quids/day) by the duration of betel quid chewing (in years). In other words, the unit for cumulative exposure to betel quid was quid/day \cdot years. The MMO (in mm) between the upper and lower incisor edges was measured and recorded for every patient with standard procedures. The MMO was coded as missing if both incisors of the upper or the lower dentition were lost. Venous blood was also collected from every study subject after informed consent was obtained at recruitment. Serum and buffy coat were separated on the day of blood collection and frozen at -70°C until subsequent analysis.

Genotyping. A 10-ml sample of peripheral blood collected in acetate-citrate dextrose was obtained from each study subject. The buffy coats of these specimens were prepared immediately and stored at -70°C until extraction of the genomic DNA. Genomic DNA was isolated from peripheral WBCs by standard RNase and proteinase K treatment and phenol-chloroform extraction. Genomic DNA was stored at -20°C until genotype analysis. Genetic polymorphisms were determined in peripheral leukocyte DNA using PCR-RFLP methods. All of the photographs of gel electrophoresis were read by two technicians blind to each other's assessment. DNA samples with inconsistent readings were retested. All of the laboratory examinations were carried out by technicians who did not know the disease status of the study subjects.

Table 1 lists the PCR primers and restriction enzymes used for PCR-RFLP analysis of six collagen-related genes including *COL1A1*, *COL1A2*, *COLase*, *LYOXase*, *TGF- β 1*, and *CST3* (24–29). These six collagen-related genes are located on different chromosomes (Table 1).

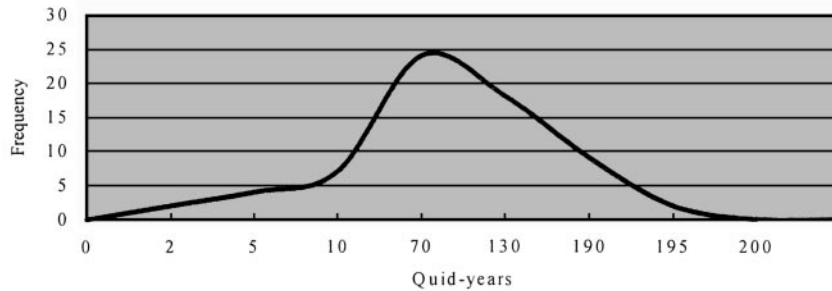
Statistical Analyses. Frequency distribution of betel quid exposure was first analyzed for OSF subjects. After careful examination, an appropriate cutoff point (200 quids/day \cdot years) of cumulative betel quid exposure was chosen to classify OSF patients into low- and high-exposure groups. In addition, the MMO was compared between the two groups. Because hospital and community controls had similar genotype distributions of the six collagen-related genes, they were pooled as one group in the data analysis.

Logistic regression analyses were used to derive $\text{OR}_{95\%}$ s and their 95% CIs. All *P*s were calculated from two-sided statistical tests. The association between different genotypes and OSF risk was evaluated in multivariate logistic regression models with simultaneous consideration of age (years), ethnicity (Fukien Taiwanese, Hakka Taiwanese, Mainland Chinese, and natives), cigarette smoking, alcoholic drinking, and cumulative betel quid consumption (used as a continuous variable; quids/day \cdot year) for low- and high-exposure groups. Biological consideration was the most important criterion for inclusion of variables in the model. Therefore, we included age and ethnicity in the models regardless of statistical significance. The associations with OSF risk for various collagen-related genes were first analyzed by classifying genotypes into three groups, *i.e.*, one

Table 1 Primers and restriction enzymes used in PCR-RFLP assays of collagen-related genes

Gene locus	Chromosomal localization	Sequences (5' to 3') of specific primer set	Restriction enzyme-specific alleles	Ref.
<i>COL1A1</i>	17q	AGA CCA GGA ATT CGG CTT CG TTG GAT CCA AGG TTG AAT GCA CTT TTG G	T: <i>MnII</i> + C: <i>MnII</i> -	Westerhausen <i>et al.</i> , 1990 (24)
<i>COL1A2</i>	7q	GGG ATC CTC GGC CCC GCT GGA AAA GAA CCG AAT TCA CCT TTA TCA CCG TTT TTG CCA	A: <i>PvuII</i> + B: <i>PvuII</i> -	Constantinou <i>et al.</i> , 1990 (25)
<i>COLase</i>	11q	AGG GTA CCA GGC AGC TTA ACA AAG GC TCG AGC TCA GTG CAA GGT AAG TGA TGG C	T: <i>BanI</i> + A: <i>BanI</i> -	Thiry-Blaise <i>et al.</i> , 1995 (26)
<i>TGF-β1</i>	19q	AAG GCA TGG CAC CGC TTC TG GAA GGA GGG TCT GTC AAC AT	C: <i>Bsu36I</i> + T: <i>Bsu36I</i> -	Cambien <i>et al.</i> , 1996 (27)
<i>LYOXase</i>	5q	TCA TCT GGA GTC ACC GCT GG GGT TGT CGT CAG AGT AC	A: <i>PstI</i> + G: <i>PstI</i> -	Csiszar <i>et al.</i> , 1993 (28)
<i>CST3</i>	20p	TGG GAG GGA CGA GGC GTT CC CCG GCC ATG GTC GGC TAG GA	A: (<i>SstII</i>) 172 + 48 bp B: (<i>SstII</i>) 136 + 84 bp	Balbín and Abrahamson, 1991 (29)

Low exposure group



High exposure group

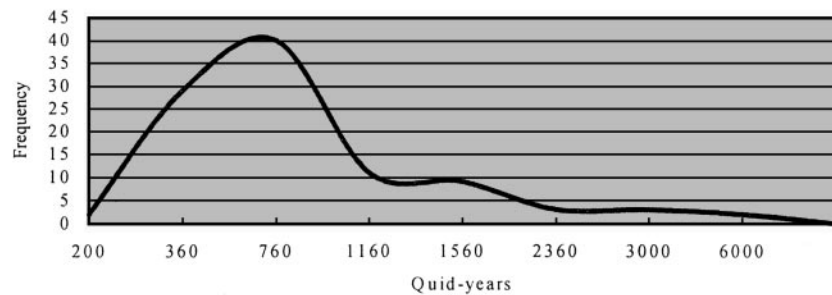


Fig. 1. Distribution of cumulative exposure to betel quid (*Quid-years*) of OSF subjects with low and high exposure.

heterozygous and two homozygous groups. For further analysis, genotypes of the six collagen-related genes were categorized into two groups: the genotype with the highest OR and the genotypes with lower ORs.

In further analysis, genes related to the metabolism and to the cross-linking of collagens were combined to analyze their interactions with collagen genes. We combined the six alleles into two pathways: (a) the collagen-metabolizing pathway including *COL1A1*, *COL1A2*, *COLase*, and *TGF-β1*; and (b) the collagen cross-linking pathway including *COL1A1*, *COL1A2*, *LYOXase*, and *CST3*. The two proposed pathways were evaluated in the low- (cumulative betel quid consumption, <200 quids/day · years) and the high- (cumulative betel quid consumption, ≥200 quids/day · years) exposure groups. The trend relationships between OSF risk and the number of high-risk genotypes were also assessed. The trend statistics for the proposed trend models were based on the Mantel general test of linear trend.

Results

The cumulative exposure to betel quids showed a bimodal distribution with two peaks as shown in Fig. 1. The cumulative exposure was used to classify OSF patients into low- and high-exposure groups. The appropriate cutoff point was set as 200 quids/day · years. The mean ± SE of cumulative betel quid exposure was 68.3 ± 7.1 for 66 low-exposure OSF patients and 764.7 ± 80.9 for 99 high-exposure OSF patients. The low-exposure OSF patients were considered more susceptible to betel quid than those in the high-exposure group. The clinical severity of OSF (30, 31) defined by MMO was quite similar between the low- and high-exposure groups. The mean ± SE of MMO was 30.0 ± 1.3 for 65 low-exposure OSF patients and 27.5 ± 1.1 for 95 high-exposure OSF patients. The discrepancy in the numbers resulted from missing values of MMO for one low-exposure and four high-exposure OSF subjects. The Pearson correlation coefficient between MMO and cumulative betel

Table 2 Frequency distributions of six collagen-related genes in OSF patients and healthy controls with high exposure to betel quids

Gene locus	Genotype	Controls (%)	OSF patients (%)	OR _a ^a (95% CI)
Genetically distinct collagen types				
<i>COL1A1</i>	<i>CC</i>	30 (30.9)	20 (20.2)] 1.0 (referent)
	<i>CT</i>	47 (48.5)	51 (51.5)	
	<i>TT</i>	20 (20.6)	28 (28.3)	
<i>COL1A2</i>	<i>AA</i>	47 (48.5)	43 (43.4)] 1.0 (referent)
	<i>AB</i>	42 (43.3)	37 (37.4)	
	<i>BB</i>	8 (8.3)	19 (19.2)	
Matrix-degrading metalloproteinase				
<i>COLase</i>	<i>AA</i>	39 (40.2)	47 (47.5)	1.3 (0.7–2.4)
	<i>AT</i>	44 (45.4)	42 (42.4)] 1.0 (referent)
	<i>TT</i>	14 (14.4)	10 (10.1)	
Mediator affecting collagen synthesis				
<i>TGF-β1</i>	<i>TT</i>	27 (27.8)	30 (30.3)] 1.0 (referent)
	<i>TC</i>	55 (56.7)	47 (47.5)	
	<i>CC</i>	15 (15.5)	22 (22.2)	
Cross-linking enzyme for collagen molecules				
<i>LYOXase</i>	<i>GG</i>	62 (63.9)	70 (70.7)	1.2 (0.6–2.5)
	<i>GA</i>	31 (32.0)	26 (26.3)] 1.0 (referent)
	<i>AA</i>	4 (4.1)	3 (3.0)	
Cysteine-proteinase inhibitor				
<i>CST3</i>	<i>AA</i>	71 (73.2)	75 (75.8)	1.7 (0.8–3.5)
	<i>AB</i>	23 (23.7)	23 (23.2)] 1.0 (referent)
	<i>BB</i>	3 (3.1)	1 (1.0)	
Total		97 ^b (100.0)	99 ^c (100.0)	

^a OR_a, odds ratio adjusted for age, ethnicity, cigarette smoking, alcoholic drinking, cumulative betel-quid chewing, and other collagen-related genes.

^b The discrepancy in the numbers resulted from missing data of betel-quid chewing exposure in one healthy subject.

^c The discrepancy in the numbers resulted from missing data of betel-quid chewing exposure in one OSF subject.

quid exposure was also calculated ($\rho = -0.2$, $P = 0.12$ for the low-exposure group; $\rho = -0.03$, $P = 0.79$ for the high-exposure group). No significant correlation was found between MMO and cumulative betel quid dose within each exposure group.

The habits of cigarette smoking and alcoholic drinking were found to differ between cases and controls. There were more cigarette smokers among OSF cases (91.0%) than among controls (83.5%). The habit of alcoholic drinking was also more prevalent among OSF cases (68.9%) than among controls (54.2%). The frequency of eating chili or hot pepper was found to be similar between OSF cases and controls.

The frequency distributions of genetic polymorphisms of six distinct collagen-related genes among betel quid-chewing controls and OSF patients are shown in Table 2 for the high-exposure group and Table 3 for the low-exposure group.

Because there is no conclusive evidence in the literature regarding the phenotypic manifestation of various genotypes of these alleles, we defined the genotypes revealing the highest risk among three genotypes of a given allele as the putative high-risk genotype in additional analyses. The high-risk genotypes of *COL1A1*, *COL1A2*, *COLase*, *TGF-β1*, *LYOXase*, and *CST3* were *CC*, *AA*, *TT*, *CC*, *AA*, and *AA*, respectively, for the low-exposure group; and *TT*, *BB*, *AA*, *CC*, *GG*, and *AA*, respectively, for the high-exposure group.

As shown in Tables 2 and 3, there was consistent relationship between genotype distribution and risk for *CC* (*TGF-β1*) and *AA* (*CST3*) genotypes for both low- and high-exposure groups. However, there was no consistency in the other four markers between the two groups. It was hypothesized that the high-risk genotypes might be different in the low- and high-exposure groups. We, therefore, further analyzed the effects of the combination of these six genes to examine the gene-gene interaction.

To assess whether the profiles of genes involved in collagen-metabolizing or collagen cross-linking pathways were associated with OSF risk, we examined the OR_a for different combinations of these genes as shown in Tables 4. A biological gradient of OSF risk with an increase in the number of putative high-risk genotypes of collagen-metabolizing genes ($P < 0.01$) and collagen cross-linking genes ($P < 0.05$) was observed for the high-exposure group. There was also a trend relationship between OSF risk and number of putative high-risk genotypes of collagen-metabolizing genes ($P < 0.01$) and collagen cross-linking genes ($P < 0.01$) for the low-exposure group. The accumulation of more high-risk alleles increases the risk of OSF, stratified by betel quid exposure.

Discussion

In Taiwan, >2,000,000 people have a habit of chewing betel quids and an increasing number are adolescents (32). This habit has resulted in an increase in the incidence of oral carcinoma (33) and OSF (34). Although the etiology of OSF remains unclear, evidence has shown that it is a collagen disorder related to betel quid exposure (14–17, 22). The obvious bimodal distribution in the betel quid exposure among OSF patients, as shown in Fig. 1, suggests individual differences in susceptibility to the development of OSF. It is essential to evaluate the OSF risk associated with polymorphisms of collagen-related genes in low- and high-exposure groups separately to examine whether the high-risk genotypes are consistent or not. Because several genes are involved in the metabolism and cross-linking of collagens, it is, therefore, important to evaluate the combined effects of multiple genes as well as the main effect of single genes. To the best of our knowledge, this is the first study to examine the effects of multiple genes involved in collagen metabolism and collagen cross-linking on OSF risk. We used

Table 3 Frequency distribution of six collagen-related genes in OSF patients and healthy controls with low exposure to betel quids

Gene locus	Genotype	Controls (%)	OSF patients (%)	OR _a ^a (95% CI)
Genetically distinct collagen types				
<i>COL1A1</i>	<i>CC</i>	44 (23.7)	20 (30.3)	1.5 (0.7–3.1)
	<i>CT</i>	92 (49.5)	36 (54.6)	1.0 (referent)
	<i>TT</i>	50 (26.9)	10 (15.2)	
<i>COL1A2</i>	<i>AA</i>	79 (42.5)	40 (60.6)	2.4 (1.2–4.6)
	<i>AB</i>	89 (47.9)	23 (34.9)	1.0 (referent)
	<i>BB</i>	18 (9.7)	3 (4.6)	
Matrix-degrading metalloproteinase				
<i>COLase</i>	<i>AA</i>	95 (51.1)	31 (47.0)	
	<i>AT</i>	75 (40.3)	24 (36.4)	1.0 (referent)
	<i>TT</i>	16 (8.6)	11 (16.6)	2.0 (0.7–5.3)
Mediator affecting collagen synthesis				
<i>TGF-β1</i>	<i>TT</i>	46 (24.7)	18 (27.3)	
	<i>TC</i>	105 (56.5)	31 (47.0)	1.0 (referent)
	<i>CC</i>	35 (18.8)	17 (25.7)	1.8 (0.8–4.0)
Cross-linking enzyme for collagen molecules				
<i>LYOXase</i>	<i>GG</i>	122 (65.6)	42 (63.7)	
	<i>GA</i>	59 (31.7)	22 (33.3)	1.0 (referent)
	<i>AA</i>	5 (2.7)	2 (3.0)	1.4 (0.2–9.1)
Cysteine-proteinase inhibitor				
<i>CST3</i>	<i>AA</i>	150 (80.7)	56 (84.9)	2.0 (0.8–4.9)
	<i>AB</i>	30 (16.1)	10 (15.1)	1.0 (referent)
	<i>BB</i>	6 (3.2)	0 (0.0)	
Total		186 ^b (100.0)	66 ^c (100.0)	

^a OR_a, odds ratio adjusted for age, ethnicity, cigarette smoking, alcoholic drinking, cumulative betel-quid chewing, and other collagen-related genes.

^b The discrepancy in the numbers resulted from missing data of betel-quid chewing exposure in one healthy subject.

^c The discrepancy in the numbers resulted from missing data of betel-quid chewing exposure in one OSF subject.

Table 4 Trend relationship between OSF risk and number of putative high-risk genotypes of collagen-related genes in the high- and low-exposure groups

No. of putative high-risk genotypes ^a	High-exposure group						Low-exposure group					
	Collagen-metabolizing pathway			Collagen cross-linking pathway			Collagen-metabolizing pathway			Collagen cross-linking pathway		
	Controls	OSF patients	OR _a ^b (95% CI)	Controls	OSF patients	OR _a (95% CI)	Controls	OSF patients	OR _a (95% CI)	Controls	OSF patients	OR _a (95% CI)
None	38	22	1	6	1	1	61	15	1	9	3	1
One	40	45	2.1 (0.9–4.4)	33	28	6.8 (0.7–71.8)	83	20	1.2 (0.5–2.8)	94	22	0.9 (0.2–4.6)
Two	15	25	4.0 ^c (1.6–10.1)	47	48	8.1 (0.8–81.9)	35	25	4.0 ^c (1.6–10.3)	65	28	2.2 (0.5–11.2)
Three or more	4	7	3.3 (0.8–13.9)	11	22	18.3 ^d (1.6–204.2)	7	6	4.6 ^d (1.0–20.5)	18	13	3.9 (0.7–22.4)
<i>P</i> for trend	<0.01			<0.05			<0.01			<0.01		

^a The putative high-risk genotypes were *COL1A1-TT*, *COL1A2-BB*, *COLase-AA* and *TGF-β1-CC* for the collagen-metabolizing pathway and *COL1A1-TT*, *COL1A2-BB*, *LYOXase-GG* and *CST3-AA* for the collagen cross-linking pathway in the high-exposure group; *COL1A1-CC*, *COL1A2-AA*, *COLase-TT* and *TGF-β1-CC* for the collagen-metabolizing pathway and *COL1A1-CC*, *COL1A2-AA*, *LYOXase-AA* and *CST3-AA* for the collagen cross-linking pathway in the low-exposure group.

^b OR_a: odds ratio adjusted for age, ethnicity, cigarette smoking, alcoholic drinking, cumulative betel-quid exposure, and the genes not involved in the evaluated pathways.

^c *P* < 0.01

^d *P* < 0.05

six genetic polymorphisms of collagen-related genes that are situated on different chromosomes to evaluate their main and combined effects on low- and high-exposure groups.

Exposure of fibroblasts to the extracts of betel nut, which have been suggested to trigger collagen synthesis and stabilize collagen fibrils (35, 36), underlies the mechanism of collagen accumulation in OSF. However, the individual induction dose of betel quid exposure varied significantly in the present study (Fig. 1). In an attempt to assess the combined effects of collagen-related genes, we defined putative high-risk genotypes of these genes as those associated with the highest risk of OSF. The associations between the number of putative high-risk genotypes and OSF risk support the hypothesis that genetic susceptibility is involved in the development of betel quid-induced OSF. Further study of the combined effect of pheno-

types of these genes on OSF will help to elucidate the mechanisms involved in genetic susceptibility to OSF. The six genetic polymorphisms evaluated in this study (Table 1) are all associated with some biological significance. The polymorphic *MnI* restriction endonuclease site of *COL1A1* (24) was used for null-allele testing in patients affected with osteogenesis imperfecta type I (37). The polymorphic *PvuII* restriction endonuclease site of *COL1A2* is situated in exon 25 of the gene (25). The polymorphic *BanI* restriction endonuclease site of *COLase* is situated in the 5'-upstream region (26). The *COLase* gene contains at least two *cis* regulatory elements in the proximal part of its 5'-upstream sequence. One of them binds with the AP-1 factor, which mediates the down-regulation of the gene by TGF-β (38). *PstI* RFLP results in a nonconservative amino acid substitution encoded within the first exon of human *LYOX-*

ase (28). The polymorphic *Sst*I restriction endonuclease site of human *CST3* is situated in the promoter region (29). The C-509T polymorphism of *TGF-β1* is in a *Bsu*36I restriction endonuclease site (27, 39). Whether this polymorphism is associated with the circulating level of TGF-β1 or with the transcriptional activity of *TGF-β1* remains controversial (39–41). In the present study, we found that the *CC* genotype is associated with a higher risk of OSF than the other two genotypes in both the low- and high-exposure groups. Because TGF-β is a multifunctional cytokine with complex actions, it has been suggested that, under different circumstances, TGF-β may have opposing biological effects (19).

The dose of environmental agents may affect the association between cancer risk and genetic polymorphisms of metabolic enzymes (42). Different collagen-related genotype profiles were found to be associated with an increased OSF risk for the low- and high-exposure groups in the present study. The collagen regulation process is a complex physiological phenomenon including the biosynthesis of collagen molecules and intra- and intermolecular cross-linking, as well as of the degradation and remodeling of collagens. The genes involved in collagen regulation interact with each other. As a causal agent of OSF, betel quid chemicals could interact with *COL1A1*, *COL1A2*, *COLase*, and *LYOXase*, which are mainly expressed in fibroblasts. Such gene-gene and gene-environmental interaction might explain the different putative high-risk genotypes found for the low- and high-exposure groups. It has been shown that fibroblasts are a heterogeneous population of cells that differ in functional properties such as proliferation rates, collagen synthesis, and collagenase production (43–48). This heterogeneity is conserved for at least 12 population doublings (46). Under different selection pressure, cells with the same genetic content may express different phenotypes. Certain subpopulations of fibroblasts may be clonally selected and expanded in diseased tissues (49, 50). The microenvironment around oral fibroblasts may be very different between low- and high-exposure groups during the development of OSF. In other words, the discrepancy in putative high-risk collagen-related genotypes between low- and high-exposure groups may result from fibroblast selection by betel quid ingredients or inflammatory factors (48, 51, 52) during the development of OSF. Although previous experimental studies have proposed the importance of fibroblast selection in the development of OSF (16, 22), the present findings provide preliminary molecular epidemiological evidence for this theory.

The low-exposure group of OSF patients represented those who were more susceptible to betel quid exposure, whereas those in the high-exposure group were less susceptible. In other words, a higher proportion of the OSF risk could be attributed to the genetic component in the low-exposure group than in the high-exposure group. The highest attributable proportion (53) of 25.3% for the *CST3* gene and a summary attributable proportion of 86.9% for the six genes were found in the high-exposure group, and the highest attributable proportion of 42.5% for the *CST3* gene and a summary attributable proportion around 100% for the six genes were found in the low-exposure group.

In the previous study (54), smoking tobacco was not found to increase the risk of developing OSF. Whether alcoholic drinking plays a role in the development of OSF is still uncertain. However, a minor confounding effect was noted after an adjustment for cigarette smoking and alcoholic drinking in the multivariate analysis. There was no obvious increase in OSF risk among alcoholic drinkers with

low betel-quid exposure and cigarette smokers. A significantly increased risk (OR_{adj} 2.2; 95% CI, 1.1–4.3; *P* < 0.05) was noted among alcoholic drinkers with high exposure to betel quids in this study. The role of chili in the pathogenesis of OSF is controversial (55–57). In the present study, we did not find any association between OSF and chili or hot pepper consumption.

Except for the *LYOXase* gene, the allele frequencies of collagen-related genes in the present study showed substantial differences from those in previous studies on Caucasians (24–29). In this study, the genotype distributions of all collagen-related genes other than *CST3* showed no significant departure from Hardy-Weinberg equilibrium among cases and controls, even within different exposure groups. Some deviation from Hardy-Weinberg equilibrium was noted for the genotype distribution of the *CST3* gene among controls with low exposure to betel quid, but not among cases. The deviation was considered a random variation because of the small proportion of *BB* genotype.

In the present study, all of our control subjects were betel quid chewers. It is unusual in epidemiological studies to exclude controls that have not had the exposure of interest. Because there was no confirmed case of OSF among nonchewers of betel quid, betel quid chewers were considered the source population of OSF, rather than the entire nondiseased population, which the controls would usually represent (58).

The assessment of gene-gene and gene-environment interactions requires large sample sizes to attain adequate statistical power, especially when the factors under study are either very rare or very common or when the magnitude of the interaction is modest (59, 60). For the purpose of increasing precision in the present study, genotypes were collapsed into a dichotomous variable, putative high-risk genotype and putative non-high-risk genotypes, for every gene studied. The trend relationships between OSF risk and the number of putative high-risk genotypes were separately evaluated for the collagen-metabolizing pathway and the collagen cross-linking pathway. However, significant trends were found among low- and high-exposure groups for both of these pathways after adjustment for age, ethnicity, cigarette smoking, alcoholic drinking, cumulative betel quid exposure, and the genes not involved in the evaluated pathways. These findings are consistent with those of previous studies, which indicated that a disturbance of collagen synthesis and degradation as well as collagen cross-linking may be involved in OSF pathogenesis. Because these genes are involved in the transcription, translation, and posttranslational processing of collagen, OSF may be considered a complex collagen disorder induced by betel quid exposure. However, susceptibility to OSF could involve multigenic mechanisms modified by the betel quid-exposure dose in OSF induction.

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