

CYP2A6 gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka

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We investigated the relationship between inter-individual difference in CYP2A6 genotype and susceptibility to oral cancer among habitual betel quid chewers in a Sri Lanka population. A total of 286 subjects showing oral malignant or premalignant lesions and 135 control subjects with no lesions were analyzed. The frequency of homozygotes for CYP2A6*4C mutation, a gene deletion type of polymorphism, was significantly lower in the case subjects than the controls. The odds ratio (OR) of the group homozygous for the deletion was significantly lower and calculated to be 0.14 (95% CI; 0.03–0.72). In the allelic base analysis, there was also a significant decrease in the OR of the deletion allele. Our data suggest that deficient CYP2A6 activity due to genetic polymorphism reduces oral cancer risk in betel quid chewers.

Carcinoma of the oral cavity is one of the 10 most frequent malignant tumors worldwide with major predominance in south-east Asia and is a main problem in Sri Lanka (1). Oral squamous cell carcinoma and the most common oral premalignancies such as leukoplakia and oral submucous fibrosis appear to be related to the habit of betel quid (BQ) chewing in these countries, whereas in Western countries cigarette smoking and heavy alcohol consumption are the main causative agents. Areca nut (*Areca catechu*), a major component of BQ, contains certain alkaloids that give rise to nitrosamines, some of which such as *N*-nitrosoguvacoline, 3-(methylnitrosamino)propionitrile, 3-(methylnitrosamino)propionaldehyde and *N*-nitrosoguvacine, are shown to be carcinogenic (2). These BQ-specific nitrosamines may act as an adjunct to tobacco-specific nitrosamines that are strongly implicated as an etiologic factor for leukoplakia and oral

submucous fibrosis. Metabolic activation of several nitrosamines was reported to be catalyzed by cytochrome P450 enzymes (CYPs), large multi-gene family enzymes important in phase I metabolic activation reactions (3). Furthermore, reactive oxygen species are generated in the oral cavity during BQ chewing due to the addition of slaked lime [Ca(OH)₂] into BQ (4).

In Sri Lanka, the predominant habit is chewing BQ with tobacco. Although the influence of other factors such as nutritional conditions is important in the etiology of oral cancer, there is a wide inter-individual difference in the susceptibility to chemical carcinogens. Therefore, the known evidence for relevance of tobacco-specific nitrosamines to the oral cancer and inter-individual difference in the susceptibility to chemical carcinogens due to genetic polymorphism in the xenobiotic metabolizing enzymes may apply to some extent to chewers of BQ.

Among xenobiotic metabolizing enzymes, the CYP2A6 family is characteristic of its catalytic properties to nitrosamines (5). In addition, previous studies in our laboratory reported a novel deletion-type mutant of the *CYP2A6* gene, *CYP2A6*4C*, as one of the major polymorphic genes in Japanese lacking CYP2A6 activity (6–8). The *CYP2A6* gene deletion due to genetic polymorphism reduced lung cancer risk among tobacco smokers in a Japanese population (9). At present, we account for this fact by speculating that the subjects possessing *CYP2A6*4C* might not activate nitrosamines contained in tobacco smoke. Since the presence of the variant gene seems to reduce the activation of xenobiotics, it is possible that the polymorphism of *CYP2A6* could also reduce the metabolic activation of BQ-derived carcinogens. In this study, we intended to clarify whether *CYP2A6* genetic polymorphism, including the gene deletion, could be related to individual susceptibility to oral cancer in habitual BQ chewers in a Sri Lanka population. Because of the known involvement of Phase II enzymes in detoxification of reactive oxygen species, genotyping was extended to cover identification of glutathione-S-transferase μ class isozyme (GSTM1) in the same subjects.

All the subjects in this study were BQ chewers from Sri Lanka with or without smoking habit. Case and control groups were comparable to each other in terms of number of quids chewed per day (~5.5 betel/day). The control group consisted of subjects who visited a hospital for clinical reasons other than oral cancer. The ages shown as means \pm SD of the patients with oral malignancies or premalignancies (246 males and 40 females) and the controls without any lesions (91 males and 44 females) were 51.4 \pm 12.6 years (range, 26–82) and 52.6 \pm 9.0 years (range, 40–85), respectively. Blood samples were obtained from the subjects with informed consent. Genomic DNA was isolated from peripheral blood by using Nucleic Acid Isolation System NA-3000 (Kurabo, Tokyo, Japan). The ethics committee of Hokkaido University, Sapporo, Japan and Peradeniya University ethical committee of Sri Lanka Dental Hospital approved this study.

Abbreviations: BQ, betel quid; CYPs, cytochrome P450 enzymes; GSTM1, glutathione-S-transferase μ class isozyme; PCR, polymerase chain reaction.

Table I. Distribution of *CYP2A6* genotypes in controls and patients with oral lesions

<i>CYP2A6</i>	Control (%)	Case (%)	OR	95% CI
*1A/*1A	49 (36.3)	116 (40.6)	1.00	
*1A/*1B	53 (39.3)	118 (41.3)	0.94	0.59–1.50
*1B/*1B	13 (9.6)	38 (13.3)	1.23	0.61–2.52
*1A/*4C	8 (5.9)	9 (3.1)	0.48	0.17–1.30
*1B/*4C	6 (4.4)	3 (1.0)	0.21	0.05–0.88 ^a
*4C/*4C	6 (4.4)	2 (0.7)	0.14	0.03–0.72 ^a
Total	135	286		

*1A, wild type; *1B, conversion type; *4C, whole deletion type. Significant association between *CYP2A6* genotypes and oral cancer risk is shown as χ^2 value 15.00, $P=0.0104$.

^aSignificantly different from the control group.

The DNA samples were subjected to polymerase chain reaction (PCR) amplification for *CYP2A6* and *GSTM1* analyses. *CYP2A6* genes were analyzed for the wild type (*CYP2A6**1A), the deletion type (*CYP2A6**4C) and the other type of mutant designated as conversion-type (*CYP2A6**1B) by the methods developed in our laboratory (10). Briefly, a region ranging from the exon 8 of the *CYP2A7* or *CYP2A6* gene to a 3'2-untranslated region in the exon 9 of the *CYP2A6* gene was amplified. The amplified DNA samples were treated with two restriction enzymes, *AccII* and *Eco81I*. *GSTM1* amplification was carried out as described by Nair *et al.* (4). The co-amplification of an albumin gene fragment served as an internal positive control for PCR-amplification in determination of the homozygous null polymorphism of *GSTM1*.

To determine an association between *CYP2A6* genetic polymorphism and oral cancer risk, the significance of the difference in the case-control distribution was calculated by χ^2 tests. Odds ratios were calculated by the statistical package StatView (ver. 4.5) (SAS Institute, Cary, NC, USA).

The genotyping method developed in our laboratory allows us to distinguish *CYP2A6**4C (6–8), and *CYP2A6**1B, (10) from *CYP2A6**1A. The prevalence of the *CYP2A6* genotypes in patients with oral lesions of various classes (15 oral squamous cell carcinoma, 62 oral submucous fibrosis and 209 leukoplakia) and control subjects among BQ chewers is summarized in Table I. The individual distribution of six genotypes in Sri Lanka was significantly different from Japanese ($\chi^2=34.90$, $P<0.0001$) (9) and Korean ($\chi^2=17.17$, $P=0.0042$) (11) populations.

The χ^2 test indicated a significant difference in the distribution of six genotypes between the case and control groups ($\chi^2=15.00$, $P=0.0104$) (Table I). Prevalence of *CYP2A6**1A/*1A genotype was not significantly higher in the case group (40.6%) than in the controls (36.3%). The presence of homozygous deletion genotype (*CYP2A6**4C/*4C) was seen in six control subjects (4.4%). A comparison with the patient group (0.7%) showed that this genotype was significantly more frequent in the control group, suggesting that there was a relationship between genetic polymorphism of *CYP2A6* and oral cancer risk. The odds ratio (OR) calculated for the subjects harboring *CYP2A6**4C/*4C was 0.14 (95% confidence intervals, CI, 0.03–0.72), conferring on an individual having *CYP2A6**4C/*4C genotype around seven times lower risk for the development of oral cancer than that of an individual with *CYP2A6**1A/*1A.

The significant association between the *CYP2A6* genetic polymorphism and oral cancer risk was also found in allele-

Table II. Allele frequency of *CYP2A6* in controls and patients with oral lesions

<i>CYP2A6</i>	Control (%)	Case (%)	OR	95% CI
*1A	159 (58.9)	359 (62.8)	1.00	
*1B	85 (31.5)	197 (34.4)	1.03	0.75–1.41
*4C	26 (9.6)	16 (2.8)	0.27	0.14–0.52 ^a
Total	270	572		

*1A, wild type; *1B, conversion type; *4C, whole deletion type. Significant association in distribution of the three alleles between case and control subjects is shown as χ^2 value 18.09, $P=0.0001$.

^aSignificantly different from the control group.

based analysis. Table II shows the frequencies of three allelic variants of the *CYP2A6* gene in the subjects with oral pre-malignant lesions and controls. A significant difference in the distribution ($\chi^2=18.09$, $P=0.0001$) strengthened the role of *CYP2A6* activity in oral carcinogenesis. We also analyzed 25 BQ-chewing subjects diagnosed as lichen planus lesion in our study. Lichen planus is an inflammatory lesion with hyperkeratosis characterized by pearly white papulae (1). This condition has a high prevalence among BQ chewers and is currently considered as a 'probable precancerous' condition. However, there was no significance in the distribution of *CYP2A6* genotypes in the subjects with lichen planus when compared to either the case ($\chi^2=0.98$, $P=0.9639$) or the control groups ($\chi^2=3.30$, $P=0.6545$) (data not shown). A larger sample size for this particular disease is needed for comparison of *CYP2A6* genotypic distribution patterns.

All the subjects in this study were habitual BQ chewers. In addition to BQ chewing, numbers of subjects with the habits of cigarette smoking in case and control groups were 195 (68.2%) and 61 (45.2%), respectively. When we compared the subjects on the basis of their habits, significant association was more relevant in the habitual BQ chewers than in the subjects with both BQ chewing and cigarette smoking habits in either genotypic ($\chi^2=13.38$, $P=0.0201$ vs. $\chi^2=6.24$, $P=0.2839$) or allelic ($\chi^2=8.35$, $P=0.0154$ vs. $\chi^2=7.04$, $P=0.0296$) analyses (data not shown). However, this issue is further complicated because of the way the BQ is practiced in the subjects covered in our study. In Sri Lanka, like some other south-east Asian countries, BQ preparations also contain tobacco to some extent. Therefore, it was not possible to make a clear estimation on the individual contributions of these two habits. No attempt was made to approximate the total amount or lifetime chewing, smoking or alcohol consumption, as these are well-established lifestyle risk factors for oral carcinoma (12).

GSTM1 null genotype is ascribed to a homozygous deletion of the gene *GSTI*. This genotype has been shown to predispose to oral cancer in some (4, 13) but not in all studies (14, 15). A study by Nair *et al.* demonstrated that the homozygous deletion of *GSTM1* null genotype increased the risk for leukoplakia in betel/tobacco chewers in an Indian ethnic population (4), while Tanimoto *et al.* (15) reported that the prevalence of the null genotypes of *GSTM1*, alone, was not statistically significant between the patients who suffered from oral squamous cell carcinoma and the controls. We also analyzed the distribution of *GSTM1* wild type (*GSTM1*_w) and the null genotype among the same Sri Lanka subjects employed for the analysis of *CYP2A6* genotypes. The *GSTM1* assay we employed was based on PCR to determine the presence or

Table III. The distribution of *GSTM1* frequency in controls and patients with oral lesions

<i>GSTM1</i>	Control (%)	Case (%)	OR	95% CI
Wild	99 (73.3)	188 (65.7)	1.00	
Null	36 (26.7)	98 (34.3)	1.43	0.91–2.25
Total	135	286		

χ^2 value 2.44, $P=0.1182$

Table IV. Allele frequency of the *CYP2A6* variants in *GSTM1* wild and null genotypes in controls and patients with oral lesions

<i>GSTM1</i>	<i>CYP2A6</i>	Control (%)	Case (%)	OR	95% CI
Wild	*1A	118 (59.6)	227 (60.4)	1.00	
	*1B	62 (31.3)	136 (36.2)	1.14	0.78–1.66
	*4C	18 (9.1)	13 (3.5)	0.38	0.18–0.79 ^a
	Total	198	376		
Null	*1A	41 (56.9)	132 (67.3)	1.00	
	*1B	23 (31.9)	61 (31.1)	0.82	0.45–1.49
	*4C	8 (11.1)	3 (1.5)	0.12	0.03–0.46 ^a
	Total	72	196		

*1A, wild type; *1B, conversion type; *4C, whole deletion type.

Significant association in distribution of the three alleles between case and control subjects is shown as χ^2 value 8.52, $P=0.0141$ and χ^2 12.67 and $P=0.0018$ for *GSTM1* wild and null genotypes, respectively.

^aSignificantly different from the control group.

absence of the gene without further description of *GSTM1*_w subjects as homozygotes or heterozygotes (4). The frequency of *GSTM1* null genotype in subjects with oral lesions did not differ significantly from the control ($\chi^2=2.44$, $P=0.1182$) (Table III). However, when the genotypes of *CYP2A6* and *GSTM1* were combined, the prevalence of *CYP2A6* deletion allele in *GSTM1*_w and the null genotypes was statistically significant between the case and control subjects ($\chi^2=8.52$, $P=0.0141$ and $\chi^2=12.67$, $P=0.0018$ in the *GSTM1*_w and the null genotypes, respectively) (Table IV). The null genotype of *GSTM1* was more frequently found in cases with the susceptible genotype of *CYP2A6* than among controls. Confirming risk according to the *GSTM1* genotype in the development of oral cancer needs to be analyzed with a larger population.

To our knowledge, two reports (16, 17) have appeared on the relationship between the genetic polymorphism of *CYP2A6* gene and the lung cancer risk after we reported the close relationship (9). One paper showed negative results (16), and the other no conclusive results (17). The two papers showing rather contradictory results seem to be caused by two factors: One is by an unusual low frequency of *CYP2A6**4 employed in that study (16) and the other by an ethnic difference in a sensitivity to cancer (17). We showed that the frequency of *CYP2A6**4C in Caucasians was about one-fifth that seen in a Japanese population (10). Thus, to reproduce our results, the study may require five times bigger size of the population, while a total of 421 subjects in Sri Lanka were employed in this study. Another possibility to explain the apparent discrepancy between our previous results and results reported by other laboratory is ethnic differences. However, the results shown in this paper clearly indicate that the low risk was also seen in a Sri Lankan population, indicating that there are no apparent ethnic differences in the effects of genetic polymorphism of *CYP2A6* gene on the risk of cancer.

In conclusion, this is the first report on the prevalence of

the *CYP2A6* genotypes in habitual BQ-chewers. Our results based on a case-control study suggest that the *CYP2A6* genetic polymorphism has an impact on susceptibility to oral cancer. While there is no current evidence that the incidence of *GSTM1* null alleles predominates in case subjects, the significance of homozygous *CYP2A6**4C prevalence with respect to *GSTM1* genotype is also noteworthy as an additional factor in oral cancer risk. The lesser risk of oral cancer in subjects with the *CYP2A6**4C/*4C genotype may be explained by the fact that individuals with this genotype are incapable of bioactivating BQ-specific procarcinogens to carcinogens. Accumulation of such information will be helpful in identification of high-risk individuals and in developing therapeutic strategies by both reducing the intake of carcinogenic substances and providing novel approaches targeting *CYP2A6* for oral cancer. Inhibition of the activity of *CYP2A6* and/or mimicking the decreased activity variants may produce the same benefits that are imparted by the deletion alleles. Studies on the expression of *CYP2A6* in oral tissues and histopathological examination on biopsy specimens are currently in progress.

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