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Polymorphism in *CYP1A1* and *CYP2E1* genes and susceptibility to leukoplakia in Indian tobacco users

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Abstract

Inter-individual genetic differences may contribute to differences in susceptibility to human diseases triggered by environmental exposures. In this study, we investigated polymorphisms at two sites in the *CYP1A1* and three sites in the *CYP2E1* genes in 99 leukoplakia patients and 227 controls from one Indian population. The frequencies of genotypes at these polymorphic sites (*MspI* and *Ileu/Val*) in the *CYP1A1* and (*PstI*, *RsaI* and *DraI*) in the *CYP2E1* genes, were similar in patient and control groups. But the combined rare and heterozygous genotypes (*CC* + *CD*) at the *DraI* site in the *CYP2E1* gene were over-represented among patients compared with controls (age-adjusted odds ratio (OR) = 2.02, 95% confidence interval (CI) = 1.21–3.35). Light tobacco smokers (i.e. <21 pack-year) and light tobacco chewers (i.e. <104 chewing-year) with a "rare" *C* allele at the *DraI* site had high risk of leukoplakia (OR = 2.88, 95% CI = 1.16–7.22; OR = 2.94, 95% CI = 1.15–7.65, respectively). The "mixed tobacco" users with "rare" *C* allele are more susceptible to the disease than "exclusive" tobacco smokers and chewers. The results indicate that the "rare" *C* allele at the *DraI* polymorphic site in *CYP2E1* gene may enhance susceptibility to leukoplakia among tobacco users in this population. But the low sample size limited the power to precisely estimate the tobacco–genotype interactions.

Keywords: Indian tobacco user; Leukoplakia; *CYP1A1*; *CYP2E1*; Susceptibility

1. Introduction

Oral leukoplakia, a common pre-malignant lesion among smokers, is defined as "a chronic white mucosal macule which cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except the use of tobacco" [1]. The different types of leukoplakia are classified as homogeneous, ulcerated, nodular and verrucous in order of their increasing severity. The annual incidence of oral leukoplakia among subjects over 15 years of age was reported as 0.2–11.7% in different populations of India [2]. Tobacco chewing and smoking has been reported as major risk factors for oral leukoplakia [2–4]. A study in India found that keeping chewed tobacco in the cheek overnight increases the risk of oral leukoplakia [5]. Tobacco smoking and alcohol consumption have been ident-

ified as main risk factors for oral cancer in Western population [6]. Polycyclic aromatic hydrocarbons (PAHs), aldehydes and nitrosamines are thought to be carcinogenic components present in tobacco smoking. But chewing of tobacco with betel quid increases the concentrations of carcinogenic tobacco-specific nitrosamines and reactive oxygen species in mouth [7]. As an early sign of damage to oral mucosa, tobacco smokers and chewers often develop precancerous lesions such as leukoplakia and submucosal fibrosis. These lesions are easily accessible to diagnosis and can be considered as indicators of oral cancer risk. About 2–12% of these lesions becomes malignant within several years [4]. Molecular epidemiological studies have now provided evidence that an individual's susceptibility to cancer is mediated by both genetic and environmental factors. Inherited differences in the effectiveness of the activation/detoxification of carcinogens play a crucial role in host susceptibility. Thus, there is an urgent need to know host genetic markers, which could predispose an individual to leukoplakia and ultimately to cancer.

Most procarcinogens require metabolic activation by Phase I enzymes (e.g. cytochrome P450 oxidases like *CYP1A1* and *CYP2E1*) to act as carcinogens. But detoxification of it by Phase II metabolic enzymes (e.g. glutathione *S*-transferase (*GST*) M1, T1) is also maintained by body to protect itself against the ill effects of carcinogens. *CYP1A1* and *CYP2E1* genes are considered to play important roles in the activation of PAHs and nitrosamines, respectively [8]. The polymorphic *MspI* restriction enzyme site in *CYP1A1* gene, at the 264th base downstream from additional poly (A) signal in the 3′-flanking region, could modulate expression of gene and has been shown to be associated with susceptibility to lung and oral squamous cell carcinoma (SCC) [9–11]. Another polymorphic site (*Ileu*/*Val*) in exon 7 of same gene has been reported to increase risk of oral cancer among tobacco users in different ethnic populations [12–14].

Another *CYP* enzyme, *CYP2E1* is responsible for the metabolism of various xenobiotics [15–17]. *CYP2E1* is expressed in cultured human oral epithelial cells [18,19]. In addition to metabolizing potentially important carcinogens such as benzene, butadiene, carbon tetrachloride, vinyl chloride and low molecular weight nitrosamines [20], *CYP2E1* is also involved in the activation of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [21]. Two polymorphisms in this gene, at nucleotides −1259 and −1019 bp upstream of the *CYP2E1* transcriptional start site, are detectable by *PstI* and *RsaI* restriction enzyme digestions, respectively [22]. Based upon the presence [+] or absence [−] of *RsaI* and *PstI* recognition sequences at these polymorphic sites, the "wild-type" haplotype (*RsaI*[+].*PstI*[−]) and one of the variant haplotypes (*RsaI*[−].*PstI*[+]) were designated as the "c1" and "c2" alleles, respectively. The *PstI*/*RsaI* polymorphic sites are present in a putative HNF-1 binding site and may play important roles in the regulation of *CYP2E1* transcription and subsequent protein expression [23]. The *DraI* polymorphism in intron 6 of the *CYP2E1* gene leads to a wild-type D allele (presence of *DraI* restriction site) and a variant *C* allele (absence of *DraI* restriction site) [24]. Several studies have reported that the variant "c2" and *C* alleles (of the *RsaI*/*PstI* and *DraI* sites, respectively) are associated with enhanced enzyme activity [25]. Results from other studies also suggested that there was no difference in *CYP2E1* activity in human liver with different *CYP2E1* genotypes [26,27]. But the reasons for this discrepancy are not clear yet. Other *CYP2E1* variant alleles identified so far are very rare in many populations and hence may lack functional significance in an association study [28,29]. The hypothesis that polymorphisms in the *CYP2E1* gene influence susceptibility to tobacco-related oral cancer has attracted attention in the recent past [30–32].

It is reported that homozygous deletions in *GSTM1* and *GSTT1* genes increase risk of leukoplakia in Indian tobacco user [33]. But the association study relating polymorphisms in *CYP1A1* and *CYP2E1* genes and risk of leukoplakia in tobacco users has not been reported yet. In this study, we examined whether the polymorphisms in these two genes could modify the risk of oral leukoplakia in individuals with tobacco smoking and/or chewing habits.

2. Materials and methods

2.1. Subjects

All the patients, who were clinically diagnosed

leukoplakia in oral cavity, were identified between 2000 and 2001 in the Department of Oral Pathology and Maxillofacial Surgery, R. Ahmed Dental College and Hospital, Kolkata, India. The same department histologically confirmed all patients as leukoplakia cases. The controls were both in- and out-patients that were treated at the same hospital for dental ailments. Individuals with any prior or present diagnosis of lung, colon, gastric, bladder cancers or respiratory ailments were excluded from the controls. All participants gave written consent for participation in the study and the study was approved by the Institute's Review Committee for Protection of Research Risks to Humans.

All subjects answered a questionnaire on demography, ethnicity, economic status, occupation, lifelong smoking, chewing and smokeless (i.e. keeping in mouth) tobacco use, alcohol consumption and the duration of their habits. All patients and controls were ethnically similar, called *Bengalee*, and living in and around the city, Kolkata. Most of the patients and controls belonged to low-income group (family income <100 US$ per month) and this is one of the reasons for which they visited Government hospital for treatment. Both patients and controls had occupations in diverse areas such as agriculture, industry, office, driving, private security, etc. Most of the females were housewives and doing only household jobs.

The patients and controls had three different ways of tobacco habits: smoking, chewing and keeping in mouth. Tobacco was smoked as cigarettes or in the form of *bidi*, a native cigarette-like stick made of coarse tobacco hand rolled in a dry *tembuhurni* leaf. In India, tobacco chewers use betel quid or *pan*, i.e. a combination of betel leaf, areca nut, lime and tobacco. Smokeless tobacco users keep a mixture of tobacco flakes and lime between the lower lip and gum for 2–3 h, and as a result, this part of the lip is mostly affected. Tobacco use was classified into three groups: (a) "exclusive" smoking, (b) smoking as well as chewing and/or keeping in mouth simultaneously (i.e. mixed habit) and (c) "exclusive" chewing/keeping. Doses of tobacco smoking were converted into pack-year (PY) of cigarettes/*bidi* (10 cigarettes equivalent to 20 *bidi* = 1 pack; 1 pack/day for 1 year = 1 PY) consumed [34]. In the subsequent sections, the "smokers" included individuals who had "exclusive" smoking as well as smoking and chewing habits (i.e. mixed habits) simultaneously. The patients and controls had lifetime 2–90 and 1–90 PY smoking habit, respectively. Using these PY estimates, smokers were classified as light (<21 PY) and heavy (>21 PY) smokers as 21 PY is median dose of all smokers in patients and controls. The smokeless tobacco dose was estimated as "chewing-year" (i.e. CY = frequency of tobacco chewed–kept/day × duration in year). The "chewers" also included individuals who had exclusive chewing as well as smoking and chewing habits simultaneously (i.e. mixed habits). The patients and controls had lifetime 6–900 and 7–900 CY, respectively. Using these CY estimates, chewers were classified as light (<104 CY) and heavy (>104 CY) chewers as 104 CY is median dose of all chewers in patients and controls. The only few patients had irregular alcohol drinking habit, they were excluded from this study and none of the controls had alcohol drinking habit.

2.1.1. DNA isolation

DNA was isolated from venous blood by incubating blood samples overnight with proteinase K (0.1 mg/ml) in 1% sodium dodecyl sulfate at 37°C followed by precipitating the DNA with ethanol as previously described [35].

2.2. Genotyping assay

2.2.1. CYP1A1 gene

2.2.1.1. MspI site. The PCR products (1078 bp) were digested with *MspI* enzyme to detect substitution of CTGG allele (lack of *MspI* site, i.e. *m1* allele) by CCGG allele (presence of *MspI* site, i.e. rare *m2* allele) at the polymorphic site [11]. The digested products were electrophoresed in 1.5% agarose and stained with ethidium bromide to count the genotypes of all individuals (*m1m1* = 1078 bp, *m1m2* = 1078, 878, 200 bp and *m2m2* = 878, 200 bp).

2.2.1.2. Ileu/Val site. Genotypes at this polymorphic site (i.e. A/G polymorphism) within exon 7 of *CYP1A1* locus were detected by PCR amplification followed by digestion with *Hinc*II enzyme according to the procedure described in Ref. [36]. The digested products were electrophoresed in 8% acrylamide gel

and stained with ethidium bromide to count genotypes (*Ileu/Ileu* = 139, 32 bp, *Ileu/Val* = 139, 120, 32, 19 bp and rare *Val/Val* = 120, 32, 19 bp) of individuals based on the absence or presence of polymorphic *Hinc*II recognition site, respectively.

2.2.2. CYP2E1 gene

2.2.2.1. PstI and RsaI polymorphic sites. The PCR products (410 bp) were divided and separately subjected to *PstI* and *RsaI* restriction enzyme digestions [22]. The digested samples were then analyzed by electrophoresis in a 2% agarose gel. The presence of restriction sites on both chromosomes yielded two fragments of 120 and 290 bp for the *PstI* and 360 and 50 bp for the *RsaI* digests.

2.2.2.2. DraI polymorphic site. The amplified DNA (373 bp) was digested with the *DraI* restriction endonuclease and subjected to electrophoresis in a 2% agarose gel [37]. In this analysis, the presence of an undigested 373 bp fragment (due to the absence of a restriction site on both chromosomes) and the presence of digested fragments of sizes 240 and 133 bp (due to restriction sites on both chromosomes) were indicative of the *CC* and *DD* genotypes, respectively. The presence of 373, 240 and 133 bp DNA fragments identified the *CD* genotype.

In *RsaI* and *DraI* polymorphism analysis, the undigested PCR product indicated the homozygous variant/rare genotype, whereas in *PstI* polymorphism analysis, undigested PCR product indicated the homozygous wild-type genotype.

2.2.3. Statistical analysis

Age- and sex-adjusted risk of leukoplakia was calculated as odds ratios (ORs) and 95% confidence intervals (CIs) for *CYP1A1* and *CYP2E1* genotypes in total cohort by logistic regression analysis [38]. Genotype–environment interactions were evaluated for smoking and chewing doses. Due to the low frequency of rare genotypes at all polymorphic sites, homozygous and heterozygous individuals for the rare allele were grouped together for calculation of OR. Chi-square (χ^2) tests (with d.f. = 2) were also performed to check whether the populations were in Hardy–Weinberg (HW) equilibrium.

3. Results

A total of 99 leukoplakia patients and 227 controls were included in this study. The mean age of cases and controls was 47 and 54 yr, respectively. Females represented 11 and 31% of patients and controls, respectively (Table 1). Approximately 55% of patients and 35% of controls were exclusively smokers. Some patients (13%) and controls (42%) had "exclusive" tobacco-chewing/smokeless tobacco habits, while 32% of patients and 23% of controls smoked and used chewing/smokeless tobacco (i.e. "mixed" habit) simultaneously. The patients and controls had similar lifetime 2–90 and 1–90 PY smoking and 6–900 and 7–900 CY chewing habits, respectively. To increase the number of individuals, "exclusive" smokers and "mixed habituals" were pooled as "smokers" for patient and control groups (87 and 58% in the respective groups). The "smokers" were classified as light (<21 PY) and heavy (>21 PY) smokers to calculate relationships among the extent of smoking, genotype and the risk of the disease. Similarly, "exclusive" chewers and "mixed habituals" were pooled as "chewers" for patient and control groups (45 and 65% in the respective groups). The "chewers" were classified as light (<104 CY) and heavy (>104 CY) chewers to calculate relationships among the extent of chewing, genotype and risk of the disease. The frequencies of sites affected by leukoplakia were in the following decreasing order: buccal mucosa and commissure area (77%), buccal mucosa and alveolar sulcus (17%) and tongue (6%). Most of the patients suffered from ulcerative (60%) followed by homogeneous (37%) and nodular (3%) types of leukoplakia.

Genotype frequencies at two polymorphic sites in *CYP1A1* gene were similar in patient and control groups (Table 2). Comparison of combined rare and heterozygous genotypes at these two sites did not show any significant differences among patients and controls (data not shown). Distributions of *CYP1A1* genotypes according to PY and CY doses were also not significantly different in patient and control groups (data not shown).

The frequency of the c1/c1 genotype at the *PstI* and *RsaI* sites in *CYP2E1* gene was almost equal in patients and controls. Only four subjects in the control group had the c1/c2 genotype (Table 3). Other "variant" alleles at the *PstI* and *RsaI* sites were absent

Table 1
Description of patients and controls

Demography and tobacco habits		Leukoplakia (99) *N* (%)	Control (227) *N* (%)
Sex	Male	88 (89)	157 (69)
	Female	11 (11)	70 (31)
Age (mean ± SD) in years		47 ± 10.7	54 ± 11.0
Smokers	Exclusive smoking habit	54 (55)	80 (35)
	Mixed habit (smoking and other tobacco habits simultaneously)	32 (32)	51 (23)
	Total	86 (87)	131 (58)
Chewers	Exclusive chewing and smokeless (i.e. keeping in mouth) tobacco habit	13 (13)	96 (42)
	Mixed habit	32 (32)	51 (23)
	Total	45 (45)	147 (65)

in our study population. Overall, no significant differences in *CYP2E1* genotype frequencies at these three sites (*PstI*, *RsaI* and *DraI*) were observed in patient and control groups (Table 3). However, the frequency of the combined rare and heterozygous (*CC* + *CD*) genotypes at *DraI* site was significantly higher in patients than controls (age-adjusted OR = 2.02, 95% CI = 1.21–3.35, sex-adjusted OR = 1.79, 95% CI = 1.09–2.94) which was also reflected in the frequency distribution of the "*C*" (i.e. *DraI*[−]) allele in patients and controls (0.27 vs. 0.19, respectively, $P = 0.032$).

Distributions of *CYP2E1*/*DraI* genotypes according to the PY and CY doses of patients and controls are shown in Table 4. The difference in the distribution of the combined rare and heterozygous genotypes (i.e. *CC* + *CD*) among smokers of patients and controls was significant (OR = 1.91, 95% CI = 1.05–3.46). To estimate the interaction between genotype and dose, tobacco smoking was divided into two categories (below and above the median PY): light "smokers" (<21 PY) and heavy "smokers" (>21 PY). High risk was observed among light "smokers" with the *CYP2E1* (*CC* + *CD*) genotypes (crude OR = 2.88, 95% CI = 1.16–7.22) when the "smokers" of the patients and controls were compared. No risk was demonstrated for heavy "smokers" due to polymorphism in *CYP2E1* gene (OR = 1.35, 95% CI = 0.58–3.13). No significant difference in distribution of (*CC* + *CD*) genotypes was observed (data not shown) when the "exclusive smokers" in patients and controls were compared.

The difference in the distribution of (*CC* + *CD*) genotypes among "chewers" of patients and controls was significant (crude OR = 2.66, 95% CI = 1.27–5.57). The chewing doses (CY) were divided into two categories (below and above the median CY): light (<104 CY) and heavy (>104 CY) "chewers".

Table 2
Distribution of *CYP1A1* genotypes at *MspI* and *Ileu/Val* polymorphic sites in patients and controls

Subjects (*N*)	*MspI* site				*Ileu/Val* site			
	m1/m1 (%)	*m1/m2* (%)	*m2/m2* (%)	χ^2 (HW)	*Ileu/Ileu* (%)	*Ileu/Val* (%)	*Val/Val* (%)	χ^2 (HW)
Leukoplakia (99)	53 (54)	38 (38)	8 (8)	0.16	72 (73)	23 (23)	4 (4)	2.65
Controls (227)	104 (46)	97 (43)	26 (11)	0.17	177 (77)	46 (20)	4 (3)	0.42
OR[a] (95% CI)		0.79 (0.48–1.29)		–		1.58 (0.89–2.8)		–

m2/m2: rare genotype (presence of *MspI* restriction enzyme site); *Val/Val*: rare genotype; χ^2 values for HW equilibrium test, non-significant.

[a] Age-adjusted OR was calculated combining rare and heterozygous genotypes.

Table 3
Distribution of *CYP2E1* genotypes at *PstI*, *RsaI* and *DraI* polymorphic sites in patients and controls

Subjects (*N*)	*PstI/RsaI* site			*DraI* site		
	c1/c1 *N* (%)	c1/c2 *N* (%)	(*CC* + *CD*) *N* (%)	OR[a], 95%CI	*DD N* (%)	χ^2 (HW)
Leukoplakia (99)	99 (100)	0 (0)	(4 + 45) 49 (49)	2.02, 1.21–3.35	50 (51)	2.37
Controls (227)	223 (98)	4 (2)	(7 + 73) 80 (35)		147 (65)	0.27

c1 = *PstI*[−].*RsaI*[+] haplotype; c2 = *PstI*[+].*RsaI*[−] haplotype; *CC*: rare genotype (i.e. absence of *DraI* restriction enzyme site); χ^2 values for HW equilibrium test, non-significant.

[a] Age adjusted OR, "*C*"-allele frequency in patients (0.27) was significantly higher from that of controls (0.19) ($P = 0.032$).

The light "chewers" with (*CC* + *CD*) genotypes showed high risk of leukoplakia (OR = 2.94, 95% CI = 1.15–7.65) whereas heavy "chewers" did not exhibit risk of the disease (OR = 1.16, 95% CI = 0.30–4.27). No significant difference in distribution of (*CC* + *CD*) genotypes was observed (data not shown) among the "exclusive chewers" in patients and controls as the sample sizes become small. Some of the patients (32%) and controls (23%), as shown in Table 1, had smoking and chewing habits simultaneously (i.e. mixed habits). The distribution of (*CC* + *CD*) genotypes in these "mixed habit" individuals of patients and controls was significantly different (OR = 3.86, 95% CI = 1.37–11.0).

Calculated χ^2 values (Tables 2 and 3) for genotypes, at *MspI* and *Ileu/Val* sites in *CYP1A1* and *DraI* site in *CYP2E1*, suggested that both the patient and control populations were in HW equilibrium.

4. Discussion

The patients and controls were similar in ethnicity and nutritionally (as they belonged to low-income group). Occupationally neither the patients nor the controls were exposed to any specific toxic chemicals. So, the effects, if any, of confounding factors such as ethnicity, diet and occupation would be similar in patients and controls. Since both the controls and patients are in HW equilibrium, it suggests that the population had undergone random mating. In our populations, the numbers of "exclusive" tobacco smokers were 55% in patients and 35% in controls, respectively. In this study, the patients and controls had similar ranges of lifetime smoking and chewing habits. About 32% patients and 23% controls had "smoking and other tobacco habits simultaneously" (i.e. mixed habit). In some analysis of the genotype data, these individuals were pooled to increase the number of "smokers" among patients (87%) and

Table 4
Distribution of *CYP2E1 DraI* (*CC* + *CD*) genotypes in patients and controls with respect to dose of smoking and chewing

Genotypes	Dose of tobacco (PY/CY)	Leukoplakia, *N*/all genotypes (%)	Controls, *N*/all genotypes (%)	OR[a] (95% CI)
DraI (*CC* + *CD*)	<21 PY	21/46 (46)	14/62 (23)	2.88 (1.16–7.22)
	>21 PY	22/40 (55)	31/69 (45)	1.35 (0.58–3.13)
	All smokers	43/86 (50)	45/131 (34)	1.91 (1.05–3.46)
	<104 CY	20/33 (61)	23/67 (34)	2.94 (1.15–7.65)
	>104 CY	5/12 (42)	24/80 (30)	1.16 (0.30–4.27)
	All chewers	25/45 (56)	47/147 (32)	2.66 (1.27–5.57)
	All "mixed" tobacco users	19/32 (59)	14/51 (27)	3.86 (1.37–11.0)

All "smokers" include individuals who had "exclusively" smoking and "mixed" (i.e. smoking and chewing) habits. The patients and controls had lifetime 2–90 and 1–90 PY smoking habit, respectively (PY = number of packs, of 10 cigarettes/20 *bidi*, in a day × duration of habit in year). All "chewers" include individuals who had exclusively chewing and "mixed" habits. The patients and controls had lifetime 6–900 and 7–900 CY, respectively (CY = frequency of chewing–keeping/day × duration of habit in year). All "mixed" tobacco users had smoking and chewing/keeping habits, simultaneously.

[a] Crude OR.

controls (58%) (Table 1). Females in India are traditionally not smokers but both males and females use smokeless tobacco. The high prevalence (89%) of leukoplakia among males could be explained by the fact that the majority of the patients (87%) were male "smokers". Similarly "exclusive chewers" and "mixed habituals" were pooled to increase the numbers of "chewers" in patients and controls (45 and 65%, respectively).

Frequency of rare homozygote (*m2m2*) at *MspI* site on *CYP1A1* locus was 11% in this control population (Table 2) which had been reported to be as 8% and less than 1% in Japanese and Caucasian populations, respectively [10,39]. Frequency of rare homozygote (*Val/Val*) at *Ileu/Val* site on *CYP1A1* locus is 3% in this population, which has been observed to be 3% in Caucasian and 5% in Japanese populations [9,36]. The frequency of combined rare homozygous and heterozygous genotypes (*Val/Val* and *Ileu/Val*) in controls of this study was similar to that reported in a South Indian population (23 vs. 17%, respectively) [12]. Although these two polymorphic sites on *CYP1A1* are of critical importance for activation for PAHs and nitrosamines, frequencies of combined rare and heterozygous genotypes at these sites did not differ significantly between patients and controls in this study. Reports on Caucasian and Japanese populations described association between polymorphism in *CYP1A1* gene and increased risk of oral SCC [10–12,14]. Park et al. [14] also demonstrated positive association between *Ile/Val* polymorphism and risk of oral cancer but could not notice the effect of smoking dose on the association. The lack of association was also observed in Japanese [40] as well as Caucasian populations [41]. The lack of association between risk of leukoplakia and *Ile/Val* polymorphism of *CYP1A1* gene, even among the smokers in this study, is consistent with a report demonstrating that expressed "*Val*" and "*Ile*" forms of *CYP1A1* enzyme exhibit similar metabolic activities toward benzo-(a)-pyrene, a tobacco carcinogen [42]. Our results indicate that genotypes at these two polymorphic sites in *CYP1A1* gene do not modulate risk of leukoplakia in this study population. But study with more sample sizes is to be done to validate this observation.

At the *PstI* and *RsaI* polymorphic sites, the frequency of the "c2" allele was 0.9% in controls of this study but it was reported to be 2–8% in Caucasians and African-Americans and higher in an Asian population [43,44]. We did not observe an association between genotype at the *PstI–RsaI* sites and the risk of leukoplakia in this population that contradicts reports on oral cancer in Chinese [31] and Caucasian and African-American populations [32]. The difference in ethnicity may account for difference in these observations. The absence of the "variant" c3 and c4 alleles in our population might be due to selection pressure by the environment on this gene.

At the *DraI* site, differences in distributions of *CC*, *CD* and *DD* genotypes in patients and controls were not significant. But the frequency of the combined rare and heterozygous ($CC + CD$) genotypes was significantly more in patients than controls (49 vs. 35%, Table 3). This finding is consistent with reports on upper aerodigestive tract [30] and lung cancers [37], but contrasts with a Finnish report on Caucasians [44]. The "*C*"-allele frequency in patients (0.27) was significantly higher than that of controls (0.19) ($P = 0.032$). But the frequency of the "rare" *C* allele in controls of this study was much higher (0.19) than in Caucasians (0.06), but similar to the Japanese population [30,37].

When "smokers" with combined ($CC + CD$) genotypes from the patient and control groups were compared (Table 4), the difference in frequency (50 vs. 34%, respectively) was significant (OR = 1.91, CI = 1.05–3.46). The extent of smoking also affected leukoplakia differentially. Light "smokers" (<21 PY) with the ($CC + CD$) genotypes had more risk of leukoplakia (OR = 2.88, 95% CI = 1.16–7.22), an observation also noticed with lung and oral cancer patients [32,37]. One-third of these light "smokers" had also tobacco "chewing" habit. So the increased risk of leukoplakia among light "smokers" may be due to dual effects of tobacco chewing and smoking. Since "smokers" included "exclusive smokers" and "mixed tobacco" users, we also checked, separately, risks of leukoplakia among these groups. Although the sample sizes become low, "mixed tobacco" users with ($CC + CD$) genotypes exhibited increased risk of leukoplakia (Table 4) whereas "exclusive tobacco" smokers with ($CC + CD$) genotypes did not show any risk of leukoplakia (data not shown). Like the

"smokers", difference in distributions of (*CC* + *CD*) genotypes among "chewers" in patients and controls (Table 4) was significantly different (OR = 2.66, 95% CI = 1.27–5.57). The effect is more pronounced at "light" chewing dose (<104 CY). Two-third of these light "chewers" had also tobacco "smoking" habit. So this increased risk of leukoplakia among light "chewers" may be due to dual effects of tobacco chewing and smoking. Since "chewers" included "exclusive chewers" and "mixed tobacco" users, we also checked, separately, risks of leukoplakia among these two groups. Although the sample sizes become low, "mixed tobacco" users with (*CC* + *CD*) genotypes exhibited increased risk of leukoplakia (Table 4) whereas "exclusive tobacco" chewers with (*CC* + *CD*) genotypes did not show any risk of leukoplakia (data not shown). So the above-mentioned results suggest that, rather than "exclusive" smokers and chewers, "mixed tobacco" users with (*CC* + *CD*) genotypes had high risk of leukoplakia. The "mixed tobacco" users in patients and controls had similar lifetime smoking (8–80 and 5–90 PY, respectively) and chewing (6–900 and 10–900 CY, respectively) habits, concurrently. So, the dual dose of tobacco might have affected the patients simultaneously. Nevertheless, analysis of risk in subjects with mixed habits requires the development of suitable procedure to measure total tobacco exposure.

Therefore, the presence of at least one "rare" *C* allele in "mixed tobacco" users may have predisposed individuals to leukoplakia. The "mixed tobacco" users are exposed to carcinogens from both the smoking and chewing products. Since *CYP2E1* is involved in the metabolism of a number of carcinogens present in tobacco, the present findings indirectly support the hypothesis that environmental exposure to carcinogens plays a role in the etiology of oral leukoplakia. In this context, nitrosamines should be considered as important carcinogens because some of the nitrosamines are activated primarily by *CYP2E1* [7]. In addition to activation of nitrosamines, *CYP2E1* may also catalyze the oxidation and DNA adducts formation of many low molecular weight carcinogens and increase the production of reactive oxygen species that may cause DNA damage. So this knowledge and our findings indicate a biological plausibility that the rare "*C*" allele of *CYP2E1* may be a risk factor for leukoplakia. Since the "rare" *C* allele is associated with enhanced transcription of the *CYP2E1* gene [37], more toxic products may have been synthesized to cause leukoplakia in these patients. How this "rare" *C* allele in intron 6 affects the activity of the *CYP2E1* enzyme in vivo remains to be determined.

Such a high effect of low-dose tobacco habit has been explained by individual susceptibility and formation of higher DNA–carcinogen adducts due to polymorphisms in some metabolic genes (e.g. *CYP1A1*, *CYP2D6*, *CYP2E1*) [45]. Although these data suggest that "*C*" allele at *DraI* site of *CYP2E1* may confer increased risk for oral leukoplakia among "mixed tobacco" users, attributable risk should be assessed in a larger case–control study exposed to low tobacco dose. The apparently absence of leukoplakia risk in heavy tobacco users may be due to overwhelming effect of heavy tobacco use on genotype in small molecular epidemiology study or due to the effect of small sample size being reduced due to stratification. This lack of association at high tobacco dose also suggests that study of polymorphism in other metabolic and/or DNA repair genes may provide important findings to explain this phenomenon.

From the above observations, it may be concluded that the wild-type "c1/c1" genotype at *PstI*/*RsaI* site did not increase risk of leukoplakia in low-dose tobacco users which was not in accordance with a report on oral cancer [32]. But the "rare" *C* allele for the *DraI* site predisposes light tobacco users to leukoplakia even in the presence of wild-type alleles at the *PstI* and *RsaI* sites. So, the *CYP2E1* gene might play important roles in the process of leukoplakia and oral cancer.

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