GLUTATHIONE *S*-TRANSFERASE *M3* (*A/A*) GENOTYPE AS A RISK FACTOR FOR ORAL CANCER AND LEUKOPLAKIA AMONG INDIAN TOBACCO SMOKERS

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Polymorphism in glutathione *S*-transferase (*GST*) genes, causing variations in enzyme activities, may influence susceptibility to oral cancer and leukoplakia in smokers and/or smokeless tobacco users. In this case-control study consisting of 109 leukoplakia and 256 oral cancer patients and 259 controls, genotype frequencies at *GSTM1*, *GSTT1*, *GSTM3* and *GSTP1* loci were determined by polymerase chain reaction-restriction fragment length polymorphism methods and analyzed by multiple logistic regression to determine the risks of the diseases. There were no significant differences in the distributions of *GSTM1*, *GSTM3* and *GSTT1* genotypes in patients and controls when all individuals were compared. In contrast, frequencies of *ile/ile* genotype at codon 105 and variant *val-ala* haplotype of *GSTP1* was significantly higher (OR = 1.5; 95% CI = 1.0–2.0) and lower (OR = 1.4; 95% CI = 1.0–1.9) in oral cancer patients compare to controls, respectively. The impacts of all genotypes on risks of oral cancer and leukoplakia were also analyzed in patients with different tobacco habits and doses. Increased risks of cancer and leukoplakia were observed in tobacco smokers with *GSTM3* (*A/A*) genotype (OR = 2.0, 95% CI = 1.0–4.0; OR = 2.0, 95% CI = 1.0–4.4, respectively). So, *GSTM3* (*A/A*) genotype could become one of the markers to know which of the leukoplakia would be transformed into cancer. Heavy tobacco chewing (> 124 chewing-year) increased the risk of cancer in individuals with *GSTT1* homozygous null genotype (OR = 3.0; 95% CI = 1.0–9.8). Furthermore, increased lifetime exposure to tobacco smoking (> 11.5 pack-year) increased the risk of leukoplakia in individuals with *GSTM1* homozygous null genotype (OR = 2.4; 95% CI = 1.0–5.7). It may be suggested that polymorphisms in *GSTP1*, *GSTM1*, *GSTM3* and *GSTT1* genes regulate risk of cancer and leukoplakia differentially among different tobacco habituals.

Key words: GST *polymorphism; oral cancer; leukoplakia; Indian population*

Tobacco chewing and smoking have been reported as major risk factors for oral cancer and leukoplakia in India. But tobacco smoking and alcohol consumption have been identified as main risk factors for oral cancer in Western population.[1] 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 concentrations of carcinogenic tobacco-specific nitrosoamines and reactive oxygen species (ROS) in mouth.[2] As an early sign of damage to oral mucosa, tobacco smokers and chewers often develop precancerous lesion such as leukoplakia.

Oral leukoplakia, a common premalignant lesion among smokers, is defined as "a chronic white mucosal maculae 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."[3] Different types of leukoplakia are classified as homogeneous, ulcerated, nodular and verrucous in order of their increasing severity. This lesion is easily accessible to diagnosis and can be considered as indicators of oral cancer risk. In a 10-year prospective study, carried out in several geographical areas of India with various kinds of tobacco habits, annual age-adjusted incidence rates of leukoplakia per 1,000 individuals per year varied from 1.1 to 2.4 among men and 0.2 to 1.3 among women.[4] About 2–12% of leukoplakia cases becomes malignant within several years.[5] Oral cancer incidence ranks fifth in the global cancer burden, but in India it ranks first in males and third in females among all cancer cases in many regions. In India, age-standardized incidence rates of oral cancer ranged from 7 to 17/100,000 persons/year, which is higher than the Western rate of 3–4/100,000 persons/year.[6] The overall age-standardized (world population) incidence rates for all cancers combined is 102.1/100,000 males and 114.6/100,000 females in Kolkata, which is located in the eastern part of India. In this city, the most frequently reported malignancy in males is head and neck cancer (18.5%), followed by cancer in lung (16.3%). But the most frequently reported cancer site in females is breast (22.7%), followed by uterine cervix (17.5%), gall bladder (6.4%), ovary (5.8%) and head and neck (5.2%). The incidence of oral cavity cancer in both men and women in Kolkata is low compared to other regions in India. This may be due to lower prevalence of tobacco chewing in Kolkata (27.5% adult men and 18% women) compared to other regions in India: 56% in Mumbai and 40% in the state of Tamil Nadu.[7]

Molecular epidemiologic studies have now provided evidence that an individual's susceptibility to leukoplakia and cancer is modulated 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 environmental procarcinogens require metabolic activation for conversion into their respective reactive electrophilic intermediates. Cytochrome P450s (*CYPs*) are mostly the phase I enzymes in the activation pathway. *CYP1A1*, one of the various forms of *CYP* enzymes, is considered to play an important role in the activation of PAHs such as benzo-α-pyrene (BP).[8,9] Glutathione *S*-transferases (*GSTs*), one group of phase II enzymes in detoxification pathway, detoxify many electrophilic substrates by conjugation with reduced glutathione. Four members of the *GST* genes, *GSTM1*, *GSTT1*, *GSTP1* and *GSTM3*, display polymorphisms that have been associated with increased risk for certain cancers.[10–14] Absence of *GSTM1* activity, a mu-class *GST* enzyme that detoxifies the reactive metabolites of BP and other PAHs, is due to homozygous inherited deletion of the gene.[15] A similar deletion polymorphism in the *GSTT1* gene, encoding a theta–class enzyme,

is also known. *GSTT1* metabolizes various potential carcinogens such as monohalomethanes and ethylene oxide present in tobacco smoke and some endogenously generated reactive products derived from lipid peroxides.[16,17] *GSTM3* gene has 2 alleles identified so far: *GSTM3*A* and *GSTM3*B*, of which the latter has a 3 bp deletion in intron 6, known as a recognition motif for the YY1 transcription factor. *GSTM3*B* allele, having increased transcription potential, enhances detoxification activity of *GSTM3*-encoded protein.[12] This allele has also been linked to decreased risk of laryngeal carcinoma.[13] *GSTP1* is most abundant among *GST* enzymes and expressed in most human tissues, including oral cavity. This enzyme is found in greatest concentration in the oral and pharyngeal mucosa of head and neck compared with the other *GST* enzymes.[18] Apart from the wild-type *GSTP1*A* allele, 3 variant alleles (*GSTP1*B*, *GSTP1*C* and *GSTP1*D*) of *GSTP1* have been described. These 4 different alleles are constituted from a combination of polymorphisms present at codons 105 and 114 of the enzyme. An A/T-to-G/C transition at codon 105 of *GSTP1* results in change from isoleucine (*ile*) to valine (*val*), which changes the substrate–binding region of the enzyme. In addition to this transition at codon 105, C/G-to-T/A transition is also observed at codon 114, resulting in change of alanine (*ala*) to valine (*val*). These 4 alleles have been designated as the following haplotypes: *GSTP1*A* (105*ile*.114*ala*), *GSTP1*B* (105*val*.114*ala*), *GSTP1*C* (105*val*.114*val*) and *GSTP1*D* (105*ile*.114*val*). Codon 105 polymorphism has been reported to be associated with increased susceptibility to bladder, testicular, breast and lung cancer.[19,20] Similarly, codon 114 polymorphism was also reported to influence lung, colorectal and oral cancers in Caucasians and African Americans.[21,22]

Studies examining GST and CYP genotypes as determinants for oral leukoplakia and cancer risk have been described.[6,23–25] In the present case-control study, containing 109 leukoplakia and 256 cancer patients and 259 controls of an Indian population, we explored the potential relationships between genotypes for 4 *GST* enzymes and risk of oral leukoplakia and cancer. We have also examined the relationship between tobacco dose and polymorphisms for the risk of the diseases.

MATERIAL AND METHODS

Patients, controls and tobacco habit

Unrelated patients diagnosed with leukoplakia or primary squamous cell carcinoma (SCC) in the oral cavity were recruited during 1999 to 2002 from the R. Ahmed Dental College and Hospital (Kolkata, India). For all patients, the department of pathology from the same hospital performed histopathologic confirmation of the lesions. Unrelated controls who came for treatments of dental ailments but without any previous and present lesions in oral cavity were recruited from outpatient department of the same hospital. *GST* polymorphism might have effects on the incidences of some diseases, so to avoid selection bias, individuals with any prior or present diagnosis of lung, colon, gastric and bladder cancers or respiratory ailments were also excluded from the controls. After obtaining informed written consent, all individuals were personally interviewed using a questionnaire. Information on age, sex, occupation, alcohol consumption, type of tobacco habit, daily tobacco use frequency, duration of habits and economic status was recorded. Data pertaining to histopathologic diagnosis and clinical staging were obtained from the pathologic reports of the biopsy materials.

All patients and controls were ethnically similar, called *Bengalee*, and living in and around the city of Kolkata, located in the eastern region of India. Most of the patients and controls belonged to low-income group (family income < $100 USD 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, car driving, private sector office and small business. Most of the females were housewives and doing only household jobs.

Patients and controls reported tobacco habits such as smoking of *bidi* and/or cigarette and chewing of tobacco in different forms. In India, the prevalent tobacco chewing habits involve use of *betel quid* (betel leaf with tobacco, areca nut, lime), *gutkha* (dried mixture of *betel quid* and tobacco sold in attractive pouches), *mawa* and *zarda* (flavored tobacco), or *khaini* (crude form of dried and ground tobacco with lime). Some patients and controls reported dual habits comprising both smoking and chewing of tobacco, while the majority had single habit. Information, provided by the tobacco chewers regarding the amount of tobacco used per chew, was not reliable. Hence, lifetime tobacco exposure was measured in terms of the frequency of chewing per day multiplied by the duration of habit. This is termed as chewing-year (CY; taking chewable tobacco once in a day for 1 year = 1 CY). Chewers who were cancer patients were classified as light (< 124 CY) and heavy (> 124 CY) where the median dose of all chewers in cancer patient and control groups is 124 CY. But in the case of leukoplakia, light and heavy chewers had the habit of chewing < 104 and > 104 CY, respectively, where the median dose of all chewers in leukoplakia patient and control groups is 104 CY. Tobacco was smoked as cigarettes and/or in the form of *bidi*, a native cigarette-like stick of coarse tobacco hand-rolled in a dry *tembuhurni leaf* and the tobacco content of 1 cigarette is nearly equal to that present in 2 *bidies*. Dose of tobacco smoking was measured as pack-years (PY): 1 packet per day for 1 year = 1 PY (1 pack = 20 cigarettes or 40 bidies). Smokers were classified as light (< 11.5 PY) and heavy smokers (> 11.5 PY) where the median dose of all smokers in patients and controls is 11.5 PY.

Sample collection and processing

About 5 ml blood was collected by vein puncture from all patient and control individuals and stored at −20°C until DNA isolation. Genomic DNA was isolated from whole blood by salt precipitation method.[26] Biopsy materials collected from the patients were used to study histology and stage of differentiation of cells.

Genotyping assays

GSTM1 *and* GSTT1 *gene*. Homozygous null deletion polymorphisms in *GSTM1* and *GSTT1* genes were determined using a modified multiplex PCR approach for simultaneous amplification of both genes.[6] Coamplification of an albumin gene fragment served as an internal control for a successful amplification reaction. *GSTM1* and *GSTT1* homozygous null genotypes were evidenced by the absence of 215 and 480 bp fragments, respectively. The presence of the 350 bp albumin fragment in the gel was indicative of a successful PCR.

GSTM3 *gene*. The presence of the *GSTM3* 3 bp deletion polymorphism was screened by PCR and polyacrylamide gel analysis using exon 6 and exon 7 primers.[27] *GSTM3*A* and *GSTM3*B* alleles were detected as 273 and 270 bp DNA bands in a polyacrylamide gel, respectively.

GSTP1 *gene*. The presence of the *GSTP1* codon 105 polymorphism was screened by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis using primers homologous to exon 4 and intron 5 of the *GSTP1* gene to generate a 568 bp fragment similar to that described previously.[19] PCR products were digested with *Bsm*AI and electrophoresed in 2% agarose gels. In addition to the polymorphic *Bsm*AI site at codon 105, 2 additional monomorphic *Bsm*AI sites are present within the 568 bp PCR amplified product, which served as internal control for restriction enzyme digestion. *GSTP1* (*ile/ile*) genotype corresponded to 125, 138 and 305 bp bands. *GSTP1* (*ile/val*) genotype corresponded to 83, 125, 138, 222 and 305 bp bands. *GSTP1* (*val/val*) variant genotype corresponded to 83, 125, 138 and 222 bp bands.

For the *GSTP1* codon 114 polymorphism, primers homologous to intron 5 and exon 6 sequences in the *GSTP1* gene were used to generate a 170 bp PCR fragment as described above[19] with a change in annealing temperature (48°C). PCR products were di-

gested with *BstU*I for 16 hr at 60°C and electrophoresed in 3% agarose gels. PCR-RFLP banding patterns for *GSTP1* codon 114 polymorphism were as follows: 26 and 144 bp bands corresponded to the *GSTP1* (*ala/ala*) genotype; 26, 144 and 170 bp bands corresponded to the *GSTP1* (*ala/val*) genotype and 170 bp band corresponded to the *GSTP1* (*val/val*) variant genotype.[22]

PCR product sequencing

Few PCR products (6%) were sequenced (ABI prism 3100; Applied Biosystem, Foster City, CA) to confirm the genotypes at all loci, which were determined by PCR and PCR-RFLP methods.

Statistical analysis

Age-, sex- and tobacco habits-adjusted risk of oral cancer and leukoplakia was calculated as odds ratios (ORs) and 95% confidence intervals (CIs) for genotypes in all patients and stratified samples by multiple logistic regression analysis using SPSS statistical package. Chi-square test with Yates' correction was used for comparison of proportions. Genotype-environment interactions were evaluated for smoking and chewing doses. Frequencies of the haplotypes resulting from 2 polymorphisms in *GSTP1* gene were estimated using HAPLOFREQ software.[28]

RESULTS

A total of 109 leukoplakia, 256 oral cancer patients and 259 controls were included in this study and the patients were almost similar in mean age with respect to controls (48 ± 10.9, 55 ± 11.6 and 53 ± 11.0 years, respectively). Individuals in study population were divided into 3 habits groups: tobacco chewers, smokers and mixed habits (Table I). The exclusive smokers comprised 60 leukoplakia, 45 cancer patients and 99 controls. The exclusive chewers were 15 in leukoplakia, 144 in cancer patient and 104 in control groups, respectively, while 34 leukoplakia, 67 cancer patients and 56 controls smoked and chewed tobacco simultaneously (*i.e.*, mixed habits). To increase the number of individuals in the tobacco habit group, exclusive smokers and mixed habituals were pooled as smokers in leukoplakia, cancer and control groups (94, 112 and 155 in the respective groups). Similarly, exclusive chewers and mixed habituals were pooled as chewers in leukoplakia, cancer and control groups (49, 211 and 160 in the respective groups). About 85% of smokers had habits of both cigarettes and *bidis*, so *bidi* and cigarettes smokers were not analyzed separately. In both patient groups, only few (< 5%) had occasional alcohol-drinking history and none of the controls had alcohol-drinking habit. So, alcohol consumption was not considered in statistical analysis. The ranges of lifetime smoking exposures in cancer patients and controls were similar (1–90 PY and 1–92.5 PY, respectively), whereas leukoplakia patients had less range of smoking exposure (1–50 PY). But the mean smoking exposures in all groups were similar (cancer, 12.4 PY; leukoplakia, 12.7 PY; controls, 14.7 PY). The ranges of lifetime chewing exposures were similar in patients and controls (cancer, 10–1,200 CY; leukoplakia, 6–900 CY; controls, 10–1,250 CY), but mean chewing exposures were less in leukoplakia patients (105 CY) than cancer patients and controls (181 and 187 CY, respectively).

The sites of oral cavity affected by leukoplakia were buccal mucosa and commissure area (80%), buccal mucosa and alveolar sulcus (16%) and tongue (4%). Most of the patients suffered from ulcerative (62%) followed by homogeneous (35%) and nodular (3%) types of leukoplakia. Fifty-two percent of the cancer sites were buccal mucosa and alveolar sulcus and the remaining sites were distributed equally between lip, tongue, retromolar area and buccal sulcus. Histologically, all malignancies were diagnosed SCC of oral cavity. These were classified as well-differentiated (62%), moderately differentiated (21%) and poorly differentiated (17%) SCC.

Genotypes (6%) determined by sequencing method were identical to those done by PCR or PCR-RFLP methods at all loci. So, the remaining genotypes were determined by PCR or PCR-RFLP methods. The age-, sex- and tobacco habit-adjusted distributions of *GSTM1*, *GSTT1*, *GSTM3* and *GSTP1* (codon 114) genotypes were not significantly different in leukoplakia, cancer patients and controls (Tables II and III). But the cancer patients with predominant homozygous *ile/ile* genotype at *GSTP1* (codon 105) had significantly increased risk of the disease (Table III; OR = 2.0; 95% CI = 1.0–2.0). The frequency of *val-ala* haplotype (*i.e.*, *GSTP1*B* allele), estimated from the genotypes at codons 105 and 114 of *GSTP1* gene, was significantly lower in cancer patients than in controls (17% *vs.* 23%, respectively). But the frequencies of the remaining 3 haplotypes were not significantly different in leukoplakia, cancer patients and controls (Table IV). The distributions of all *GST* genotypes according to different types of habits (*i.e.*, exclusive smoking, exclusive chewing and mixed) were not significantly different in leukoplakia, cancer and control groups (data not shown). But a significant association between the *GSTM3* (*A/A*) genotype and risks of both oral cancer and leukoplakia was observed in smokers (Table V, OR = 2.0, 95% CI = 1.0–4.0; Table VI, OR = 2.0, 95% CI = 1.0–4.4, respectively). But no significant association was noticed when the smokers were again divided into light- and heavy-smoking-habit groups (Tables V and VI).

For dose-response relationship study, we have calculated pack-year and chewing-year of all individuals. The smokers were classified as light (< 11.5 PY, same median value for leukoplakia, cancer and control) and heavy (> 11.5 PY, same median value for leukoplakia, cancer and control) smokers to calculate relationships between the dose of smoking, genotype and the risk of the diseases. Similarly, chewers were classified as light (< 104 CY, median for leukoplakia and controls; < 124 CY for cancer and control) and heavy (> 104, median for leukoplakia and control; > 124 CY for cancer and control) chewers to calculate relationships

TABLE I – CHARACTERISTICS OF PATIENTS AND CONTROLS

Subjects and tobacco habits		Cancer (256) *n*	Controls (259) *n*	Leukoplakia (109) *n*
Sex	Male	168	183	96
	Female	88	76	13
Age (years)	Mean ± SD	55 ± 11.6	53 ± 11.0	48 ± 10.9
Smokers	Exclusive smoking habit	45	99	60
	Mixed habits[a]	67	56	34
	Total (individuals)	112	155	94
	Lifetime smoking range (PY)	1–90	1–92.5	1–50
	Mean smoking dose ± SD (PY)	12.4 ± 12.2	14.7 ± 12.1	12.7 ± 8.9
Chewers	Exclusive chewing habit	144	104	15
	Mixed habits	67	56	34
	Total (individuals)	211	160	49
	Lifetime chewing range (CY)	10–1,200	10–1,250	6–900
	Mean chewing dose ± SD (CY)	181 ± 193	187 ± 188	105 ± 114

[a]Smoking and smokeless tobacco habits simultaneously.

TABLE II – DISTRIBUTION OF *GSTM1* NULL, *GSTT1* NULL AND *GSTM3* (*AA*) GENOTYPES IN LEUKOPLAKIA AND CANCER PATIENTS AND CONTROLS

Subjects (n)	*GSTM1* (%)	OR[a] (95% CI)	*GSTT1* (%)	OR (95% CI)	*GSTM3* (%) (*AA*)	OR (95% CI)
Cancer (256)	84 (33)	1.05 (0.7–1.5)	42 (16)	1.4 (0.9–2.4)	206 (80)	1.4 (0.9–2.1)
Control (259)	85 (32)		32 (12)		193 (74)	
Leukoplakia (109)	41 (38)	1.2 (0.7–2.0)	15 (14)	1.2 (0.6–2.4)	91 (83)	1.7 (0.9–3.1)

[a]Genotypes of patients and controls were compared. Age-, sex- and tobacco habit-adjusted OR.

TABLE III – DISTRIBUTION OF HOMO- AND HOMO- AND HETEROZYGOUS GENOTYPES AT POLYMORPHIC SITES (CODONS AT 105 AND 114) OF *GSTP1* GENE, RESPECTIVELY, IN LEUKOPLAKIA AND CANCER PATIENTS AND CONTROLS

Subjects (*n*)	*GSTP1* codon 105 (*ile/ile*) (%)	OR[a] (95% CI)	*GSTP1* codon 114 (*ala/val* + *val/val*) (%)	OR (95% CI)
Patients (256)	149 (58)	2.0[b] (1.0–2.0)	32 (13)	1.2 (0.4–4.0)
Controls (259)	128 (49)		23 (9)	
Leukoplakia (109)	59 (54)	1.0 (0.6–1.5)	6 (6)	0.6 (0.3–1.7)

[a]Genotypes of patients and controls were compared. Age-, sex- and tobacco habit-adjusted OR.–[b]$p = 0.04$. Polymorphisms at codons 105 and 114 are *ile* to *val* and *ala* to *val,* respectively.

TABLE IV – ESTIMATED FREQUENCIES OF HAPLOTYPES DUE TO POLYMORPHISMS AT CODONS 105 (*ILE* TO *VAL*) AND 114 (*ALA* TO *VAL*) OF *GSTP1* GENE

Subjects and number of chromosomes	Allele or haplotype frequencies			
	GSTP1 *A or *ile-ala* (%)	*GSTP1* *B or *val-ala* (%)	*GSTP1**C or *val-val* (%)	*GSTP1* *D or *ile-val* (%)
Cancer (512)	397 (78)[a]	88 (17)[b]	26 (5)	1 (< 1)
Leukoplakia (218)	160 (73)	52 (24)	6 (3)	0 (0)
Control (518)	378 (73)[a]	117 (23)[b]	21 (4)	2 (< 1)

Alleles or haplotypes in patients and controls were compared.–[a]$p = 0.10$; crude OR = 1.27; 95% CI = 0.95–1.71.–[b]$p = 0.03$; crude OR = 1.40; 95% CI = 1.0–1.9.

TABLE V – DISTRIBUTION OF *GSTM3* (*AA*), *GSTT1* AND *GSTP1* (CODON 105) GENOTYPES IN CANCER PATIENTS AND CONTROLS WITH RESPECT TO DOSE OF SMOKING (PY) AND CHEWING (CY)

Genotype	Tobacco dose (PY/CY)	Cancer genotype/*n* (%)	Control genotype/*n* (%)	Crude OR (95% CI)
GSTM3 (*AA*)	< 11.5 PY	58/65 (89)	55/70 (79)	2.5 (0.9–7.1)
	> 11.5 PY	38/47 (81)	61/85 (72)	1.5 (0.6–4.1)
	Total	96/112 (86)	116/155 (75)	2.0 (1.0–4.0)[a]
GSTT1 null	< 124 CY	19/109 (17)	13/80 (16)	1.0 (0.5–2.5)
	> 124 CY	17/101 (17)	5/79 (6)	3.0 (1.0–9.8)[b]
	Total	36/210 (17)	18/159 (11)	1.6 (0.8–3.1)
GSTP1 (*ile/ile* at codon 105)	< 124 CY	70/109 (64)	38/80 (48)	2.0 (1.0–3.7)[c]
	> 124 CY	50/101 (50)	44/79 (56)	0.84 (0.4–1.6)
	Total	120/210 (57)	82/159 (52)	1.25 (0.8–1.9)

11.5 PY and 124 CY are median values in total cohorts of cancer patients and controls Heavy and light doses mean the chewing/smoking doses greater and less than the corresponding median doses, respectively.–[a]$p = 0.04$.–[b]$p = 0.05$.–[c]$p = 0.03$.

between the dose of chewing, genotype and risk of the diseases. The difference in distributions of *GSTT1* homozygous null genotype among heavy chewers of cancer patients and controls was significant (Table V; OR = 3.0; 95% CI = 1.0–9.8), thus indicating the risk of cancer among heavy chewers with this homozygous null genotype. Although *ile/ile* genotype at codon 105 of *GSTP1* increased the risk of cancer (Table III), distributions of this genotype were similar in light and heavy smokers (data not shown). But light tobacco chewers with this genotype had increased risk of oral cancer (Table V; OR = 2.0; 95% CI = 1.0–3.7) in the population. The difference in distributions of *GSTM1* homozygous null genotype among heavy smokers of leukoplakia patients and controls was significant (Table VI; OR = 2.4; 95% CI = 1.0–5.7), thus indicating the risk of leukoplakia among heavy smokers with this homozygous null genotype. Distributions of *GSTM1* and *GSTP1* (codon 114) genotypes, according to different PY and CY doses, were not significantly different in cancer and control groups (data not shown). Distributions of *GSTT1* and *GSTP1* genotypes according to different PY and CY doses were also not significantly different in leukoplakia and control groups (data not shown). We did not find any significant increase in risk of cancer when 2 genotypes from *GSTM1*, *GSTM3*, *GSTP1* and *GSTT1* were combined and compared in cancer patients and controls (data not shown). But increased risk of leukoplakia was observed among exclusive and heavy smokers with the *GSTM1 null-GSTM3* (*A/A*) genotypes (Table VII, OR = 2.8, 95% CI = 1.0–8.5; OR = 4.0, 95% CI = 1.0–15.5, respectively).

Calculated chi-square values (*GSTM3* = 0.85; *GSTP1*/codon 105 = 1.6; *GSTP1*/codon 114 = 0.54) for genotypes, at *GSTM3* and *GSTP1*, suggested that control population was in Hardy-Weinberg equilibrium.

DISCUSSION

Epidemiologic studies have strongly implicated tobacco smoking and chewing as etiologic agents in the development of oral cancer and leukoplakia.[1,2,25] Most of the carcinogens are lipophilic and have a tendency to be converted into water-soluble hydrophilic compounds for easy removal from the body through the excretory

TABLE VI – DISTRIBUTION OF *GSTM1* HOMOZYGOUS NULL AND *GSTM3* (*AA*) GENOTYPES IN LEUKOPLAKIA PATIENTS AND CONTROLS WITH RESPECT TO DOSE OF SMOKING (PY)

Genotype	Smoking dose (PY)	Leukoplakia genotype/*n* (%)	Control genotype/*n* (%)	Crude OR (95% CI)
GSTM1 null	< 11.5	17/53 (32)	30/70 (43)	0.6 (0.3–1.4)
	> 11.5	18/41 (44)	21/85 (25)	2.4 (1.0–5.7)[a]
	Total	35/94 (37)	51/155 (33)	1.2 (0.7–2.1)
GSTM3 (*AA*)	< 11.5	45/53 (85)	55/70 (79)	1.4 (0.5–3.9)
	> 11.5	36/41 (88)	61/85 (72)	2.8 (0.9–9.3)
	Total	81/94 (86)	116/155 (75)	2.0 (1.0–4.4)[b]

11.5 PY is median value in total cohorts of leukoplakia patients and controls. Heavy and light smoking doses mean the doses greater and less than 11.5 PY, respectively.–[a]$p = 0.03$.–[b]$p = 0.04$.

TABLE VII – SUMMARY OF RESULTS SHOWING INCREASED RISK OF CANCER/LEUKOPLAKIA DUE TO POLYMORPHISMS IN DIFFERENT GENES (ALONE OR IN COMBINATION) AND TOBACCO HABITS

Genotypes of the genes	Patients and tobacco dose				
	All	Light smokers	Heavy smokers	Light chewers	Heavy chewers
GSTP1 (*ile/ile* at codon 105)	Cancer; OR = 1.5; 95% CI = 1.0–2.0			Cancer; OR = 2.0; 95% CI = 1.0–3.7	
GSTM1 homozygous null			Leukoplakia; OR = 2.4; 95% CI = 1.0–5.7		
GSTT1 homozygous null					Cancer; OR = 3.0; 95% CI = 1.1–9.8
GSTM3 (*A/A*)		All smokers of cancer and leukoplakia; OR = 2.0, 95% CI = 1.0–4.0 and OR = 2.0, 95% CI = 1.0–4.44, respectively			
GSTM1-M3 (homozygous null and *A/A*)		Exclusive smokers of leukoplakia; OR = 2.8; 95% CI = 1.0–8.5			
GSTM1-M3 (homozygous null and *A/A*)			Leukoplakia; OR = 4.0; 95% CI = 1.0–15.5		

Heavy and light tobacco chewers of cancer patients had chewing dose greater and less than 124 CY, respectively. Heavy smokers of leukoplakia patients had smoking dose greater than 11.5 PY.

system. This conversion or detoxification of carcinogens is achieved by the addition of glutathiones to the carcinogenic compounds and brought about by the several isozymes of GST known as one of the phase II detoxification enzymes.

In this study, we examined association between polymorphisms in 4 *GST* genes and risk of oral leukoplakia and cancer among Indian tobacco users. The patients and controls of this study were similar in ethnicity and nutrition (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. In this study, cancer patients and controls had similar ranges of lifetime chewing and smoking exposures. But may be due to low sample size, these exposure ranges in leukoplakia patients were fewer than controls (Table I). Mean smoking exposures were similar in leukoplakia, cancer and control individuals (12.7, 12.4 and 14.7 PY, respectively). But mean chewing exposures in leukoplakia patients (105 CY) were lower than cancer patients and controls (181 and 187 CY, respectively). The controls had either similar or more tobacco exposures than patients. So they served as a good control since they remained healthy even after sufficient tobacco exposures. A salient feature of our study, however, is that we attempted to examine the link between risks of oral leukoplakia and cancer and different *GST* genotypes in the presence of different types of tobacco habit using satisfactory measurements of exposure.

Ethnic differences in the prevalence of the *GSTM1* homozygous null genotype have been reported to vary between 22–35% in Africans, 38–67% in Caucasians and 33–63% in East Asian population.[29] The Pacific Islanders (Oceania) have the highest reported frequency of *GSTM1* homozygous null genotype, ranging from 64% to as high as 100% in Kiribati natives.[6] The frequency of *GSTT1* homozygous null genotype varies from 10–18% in Caucasians to 58% in Chinese.[29] African Americans have frequencies ranging from 22% to 29%, while those of Hispanic origin carry 10–12% *GSTT1* homozygous null deletion.[29] The frequencies of homozygous null genotypes at *GSTM1* and *GSTT1* loci in another Indian population were reported as 25% and 13%, respectively,[30] which were similar to our observations (32% and 12%, respectively) in the control population (Table II). The frequencies of *GSTM3* (*A/A*) genotype in Caucasians and African Americans were reported as 85% and 54%, respectively.[27] The frequency of *GSTM3* (*A/A*) genotype in another Indian population[30] was 82%, which was close to our observation (74%) in this study (Table II). The genotypes at polymorphic codons 105 and 114 were expressed as *GSTP1*A*, **B*, **C* and **D* alleles to compare with those in other populations. The frequencies of *GSTP1*A* allele were 55% and 64% among African Americans and Caucasians, respectively, and those of *GSTP1*B* allele were 44% in African Americans and 28% in Caucasians. The frequencies of 2 other alleles, *GSTP1*C* and *GSTP1*D*, were less than 7% in both populations.[22] In our control population, the frequencies of *GSTP1*A*, *GSTP1*B*, *GSTP1*C* and *GSTP1*D* alleles were 73%, 23%, 4% and < 1%, respectively (Table IV). Due to nonavailability, we cannot compare our data to any other Indian population, but the frequencies of *GSTP1* alleles in our control population were similar to those in Caucasians.

There are reports of both positive and negative associations between *GSTM1* deletion genotype and risk of oral cancer in different worldwide populations.[27,31–35] It is worth noting, however, that among the Japanese, the majority of oral cancer studies

have observed positive association between risk of cancer and presence of *GSTM1* deletion genotype.[24,36,37] This phenomenon has been explained by the high frequency (> 50%) of homozygous null genotype in this population. In a north Indian population, one study[30] showed significant association between *GSTM1* homozygous null genotype and risk of oral cancer with a total sample size of 747 (297 patients and 450 controls). But in a south Indian population, another study[38] could not notice any association between risk of oral cancer and *GSTM1* homozygous deletion genotype with a total sample size of 158 (98 patients and 60 controls). These 2 samples were collected from 2 different regions of India. Our samples were collected from a different geographical region of India (east) and are ethnically different from the above-mentioned Indian populations. The absence of positive association between risk of cancer and *GSTM1* deletion genotype observed in this study (Table II) may be due to the effect of small sample size or a real lack of association, as has also been observed in other populations.[31,33] In this study, we observed positive association between risk of leukoplakia and *GSTM1* homozygous null genotype among heavy smokers only (Table VI), which was also observed in a south Indian population[6] and a report on cancer in the African American population.[27]

Few studies also demonstrated an increased risk of oral SCC and leukoplakia due to the presence of *GSTT1* homozygous deletion genotype,[6,34,39] while others could not show the same.[31,32,36] These discrepancies in results have been attributed to small sample sizes and differences in ethnicities of the populations studied. Similar to the result of one Indian study,[30] we also did not observe significant risk of oral cancer in *GSTT1* homozygous null individuals (Table II). But dose–response relationship exhibited an increase in cancer risk with increase in lifetime exposure (*i.e.*, heavy chewers) to smokeless tobacco (Table V). The increase in risk of cancer among heavy chewers with *GSTT1* homozygous null deletion may be linked to increased ROS-mediated damage in these patients.[40] One study on Indian samples[6] had shown positive association between leukoplakia and *GSTT1* homozygous null genotype, but we did not observe any significant increase in risk of leukoplakia due to *GSTT1* homozygous null genotype in our study population, which is ethnically different.

There have been relatively few studies on both positive and negative associations of *GSTM3* genotype with altered risk of head and neck cancer.[13,27,34,39] One report from India[30] did not observe any association between *GSTM3* genotype and risk of oral cancer. When all patients and controls were considered for analysis, no association between the *GSTM3* genotype and oral cancer risk was observed in this study, which is in agreement with those reported in other worldwide populations.[27,34] But we have demonstrated a positive association between *GSTM3* (*A/A*) genotype and risk of cancer and leukoplakia among smokers of both patients (Tables V and VI). This observation substantiates the report that *GSTM3 A* allele decreases synthesis of the protein and hence increases risk of both cancer and leukoplakia due to less detoxification of BP, PAH, *etc*., present in tobacco smoke.[12] Both *GSTM3* (*A/A*) and *GSTM1* homozygous null genotypes increased the risk of leukoplakia among smokers. We also observed that *GSTM1* homozygous null and *GSTM3* (*A/A*) genotypes, not only individually but also in combination, increased the risk of leukoplakia among exclusive and heavy smokers of the population (Table VII). Combined as well as individual effects of these 2 mu-class enzymes on the risk of leukoplakia support the observation that both the enzymes might have overlapping substrate specificities for detoxification.[16]

Among *GST* enzymes, *GSTP1* is most abundant in the oral cavity. Due to the presence of codon 105 polymorphism, there is a change in amino acid from *ile* to *val*. In the present study, we could demonstrate positive association between the homozygous *GSTP1 ile/ile* genotype (at codon 105) and increased risk of oral cancer (Table III). Other reports on Chinese and Japanese populations[41,42] also found significant positive association between *ile/ile* genotype at codon 105 of *GSTP1* and risk of SCC in esophagus among smokers and drinkers. Stratifying the tobacco habits, it was noticed that homozygous *ile/ile* genotype at *GSTP1* codon 105 polymorphic site increased risk of cancer among light but not among heavy chewers (Table V). The effect of low-dose tobacco product and *GSTP1* variant genotype on cancer susceptibility was also observed in Caucasian and African American smokers.[22] This observation among light chewers may be due to overwhelming effect of heavy chewing dose on this genotype.[25] The *ala*-to-*val* polymorphism at codon 114 of *GSTP1* gene did not influence susceptibility to cancer and leukoplakia in this population (Table III). But a high risk was reported in Caucasians and African Americans with the variant genotypes, at both 105 and 114 codons of *GSTP1* gene.[22] This difference in results between this and that of the above study may be attributed to differences in ethnicity. Haplotypes were estimated from these 2 polymorphisms of *GSTP1* gene and it was observed that *val-ala* haplotype (*i.e.*, *GSTP1*B* allele) was significantly less frequent in cancer patients (Table IV). One report[43] examined the kinetics of conjugation between reduced glutathione (GSH) and anti-1,2-dihydroxy-3,4-oxy-1,2,3,4-tetrahydrochrysene (anti-CDE), the activated form of the widespread environmental pollutant *chrysene*, by *GSTP1* enzyme and found that the val^{105} variant allele can offer a more effective conjugation than the ile^{105} allele. Since *chrysene* belongs to the PAH family, the main content of tobacco carcinogen, so some tobacco-related carcinogens may be preferentially detoxified by *GSTP1* having val^{105} allele. In other words, the *ile/ile* genotype might increase the risk of oral cancer when the individual is exposed to tobacco carcinogens that have been observed in this study (Table III). This observation is also supported by another report that ile^{105} allele may be less active toward carcinogenic diol-epoxides and benzo-α-pyrenes compared with val^{105} allele.[44] It also explains our observation that susceptible *ile-ala* haplotype was more frequent and resistant *val-ala* haplotype was less frequent in cancer patients than controls and as a result patients became susceptible to tobacco carcinogens (Table IV). Conversely, It was also demonstrated that *GSTP1* with val^{105} allele possessed significantly decreased activity against PAH and 1-chloro-2,4-dinitrobenzene.[45] So the real mechanism by which *GSTP1* polymorphism modulates the risk of oral cancer in different populations remained to be elucidated.

In conclusion, susceptibility to oral SCC was modulated by polymorphism at codon 105 of *GSTP1* gene in this study population (Table VII). The risk of cancer was also increased by *GSTT1* polymorphism among heavy chewers and *GSTM3* polymorphism among smokers. The *GSTM1* homozygous null and *GSTM3* (*A/A*) genotypes, both individually and in combination, increased risk of leukoplakia in different grades of smokers. In this study, there is a commonality in susceptibility to leukoplakia and cancer by *GSTM3* (*A/A*) genotype among smokers. This observation leads to a suggestion that some of the leukoplakia patients who are affected by smoking and *GSTM3* (*A/A*) had a high chance to suffer from cancer if not treated at the leukoplakia stage. So, *GSTM3* (*A/A*) genotype of the leukoplakia patient might become a possible marker in the process of development of cancer from leukoplakia. These findings also indicate that multiple low-risk drug-metabolizing genes are involved, individually and in combination, in the initial processes of tobacco-related oral carcinogenesis. Further study is required with greater sample size to investigate the full spectrum of gene-environment and gene-gene interactions and understand the initiation of the carcinogenic process.

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