Increased Risk of Oral Cancer in Relation to Common Indian Mitochondrial Polymorphisms and Autosomal *GSTP1* Locus

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BACKGROUND. Polymorphisms at mitochondrial (mt) loci could modulate the risk of diseases including cancers. Here the mtDNA polymorphisms at 12,308 nucleotide pairs (np), 11,467 np, 10,400 np, and 10,398 np were studied to examine the association with the risk of oral cancer and leukoplakia, alone and in combination with polymorphisms at the *GST* loci.

METHODS. Polymorphisms at mt loci were screened in 310 cancer, 224 leukoplakia, and 389 control individuals by polymerase chain reaction (PCR) restriction length polymorphism (RFLP) and most of the *GST* genotype data were taken from previously published reports. Data were analyzed to determine the risk of the diseases.

RESULTS. The major allele, *A*, at 12,308 np on $tRNA^{Leu}$ (CUN), increased the risk of cancer (odd ratio [OR] of 1.7; 95% confidence interval [95% CI], 1.1–2.6) but not that of leukoplakia. The same allele also appeared to increase the risk of cancer in smokers (OR of 4.0; 95% CI, 1.1–14.4), who are mostly males (OR of 1.8; 95% CI, 1.1-3-2), but not in smokeless tobacco users, who are mostly females. The major allele *A* at 11467 np demonstrated identical results as the major allele, *A*, at 12,308 np. The major alleles *G* at 10,398 np and *T* at 10,400 np (ie, M-haplogroup) increased the risk of cancer significantly in smokers (OR of 2.6; 95% CI, 1.2–5.7 and OR of 2.4; 95% CI, 1.1–5.1, respectively). The risk-risk genotype-allele combination at *GSTP1* and mt12308 np loci increased the risk of cancer (OR of 2.6; 95% CI, 1.4–4.9) when compared with the nonrisk-nonrisk combination in leukoplakia patients.

CONCLUSIONS. Polymorphisms at the mt loci alone and in combination with the risk genotype at *GSTP1* increased the risk of oral cancer. Thus, risk genotypes from 2 different organelles may work in combination to increase the risk of oral cancer. ***Cancer*** **2007;110:1991–9.**

KEYWORDS: tobacco use, oral cancer risk, mitochondrial loci, *GSTP1*, polymorphisms.

The human mitochondrial (mt) genome is a circular DNA of 16,569 nucleotide pairs (np) and each cell contains 50 to 1000 copies of mitochondria. Instability at mtDNA, including mutations and deletions, has been reported in neurodegenerative diseases, sudden infant death syndrome, aging and longevity, and cancer.[1–6] Apart from mutations, polymorphisms at mtDNA also act as modulating factors for aging and longevity and risk of various diseases including cancer.[7–9] Mitochondrial polymorphisms could be designated as different related haplogroups, which are population-specific. Of the 4 African haplogroups L0, L1, L2, and L3, L3 is the only haplogroup that has spread outside Africa and all the modern-day

haplogroups are derived from L3. The L3 has been divided into 2 macro haplogroups, M and N. The N haplogroup is identified by the loss of a *DdeI* site at 10,397 because of *G10398A* transition. The M haplogroup, mostly observed in the Indian population, is identified by 2 transitions, *A10398G* and *C10400T*, which result in the creation of an *AluI* site in the ND3 gene. The second most common haplogroup found among Indians is haplogroup U, which belongs to the N haplogroup lineage. The U haplogroup is identified by *A12308G* transition in $tRNA^{Leu}$ (CUN). A transition in the ND4 gene at *A11467G*, which causes loss of the *TruI1* site, has also been used as a U haplogroup marker.

The human mt genome encodes 13 of more than 80 polypeptide subunits of the mt respiratory chain complexes and contains 24 additional genes for RNAs required for mt protein biosynthesis. These include 2 rRNA genes and 22 tRNA genes, 1 for each of 18 amino acids, and 2 each for $tRNA^{Leu}$ (which read UUR and CUN codons) and $tRNA^{Ser}$ (which read UCN and AGY codons).[10] Of the 22 mitochondrial tRNAs, the single most used tRNA (≈14.9%) for mitochondrial protein synthesis is $tRNA^{Leu}$ (CUN).[11] The most extensively studied polymorphism at the mt $tRNA^{Leu}$ (CUN) locus is the $A > G$ polymorphism, at 12,308 np, in the variable loop (Fig. 1), because to our knowledge, apart from this single nucleotide polymorphism (SNP), there is no report of polymorphism at the > 5% level at $tRNA^{Leu}$ (CUN).[10] The *G* allele at 12,308 np has been reported to be associated with a decreased risk of Parkinson disease and increased risk of stroke and prostate and renal cancers.[8,12,13] Because the $A > G$ at 11,467 np and $C > T$ at 10,400 np polymorphisms do not change the amino acid sequence in the protein, these polymorphisms were not given much importance in the estimation of disease risk except in the tracking of human migration. The *G10398A* polymorphism in the ND3 gene results in substitution of alanine to threonine. Cells harboring polymorphisms at mtDNA might show reduced activity of oxidative phosphorylation complexes[14] and increased generation of reactive oxygen species (ROS), which plays an important role in carcinogenesis.[9]

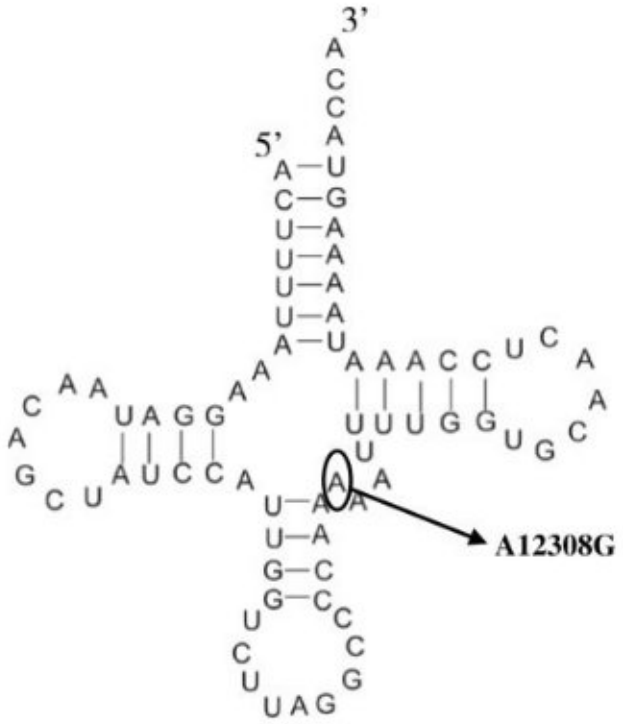

FIGURE 1. Theoretical cloverleaf structure of $tRNA^{Leu}$ (CUN) as deduced from the RNA sequence.[11]

Tobacco chewing and smoking have been identified as the major risk factors for oral cavity precancer and cancer in India.[15,16] Different classes of GST enzymes, which are mostly present in cytosol, generally detoxify tobacco carcinogens and polymorphisms at *GSTM1*, *GSTP1*, and *GSTM3* have been shown to be associated with increased risk of cancers in different populations.[17–21] A few recent reports also demonstrated localization of GSTP1, GSTA1, GSTK1, and GSTM1 enzymes in the inner membrane of mitochondria in human, rat, and mice.[22] The function of these proteins has been attributed to the inactivation of ROS generated in mitochondria. Thus, GSTs may play important roles not only in the detoxification of carcinogens in cytosol but also ROS inactivation in both cytosol and mitochondria. Therefore, individuals carrying risk alleles at different GST loci might also have altered GST activity in mitochondria and, subsequently, might become vulnerable to more doses of ROS.

In the current study, we conducted a case-control study to examine whether polymorphisms at the mt loci could increase the risk of oral cancer and leukoplakia in an Indian population. In addition, we also examined whether risk-risk genotype/allele combinations at *GST* and mt loci, respectively, could impart more risk of cancer, using our published data on *GSTs*.[23,24]

MATERIALS AND METHODS

Patients, Controls, and Tobacco Habits

Unrelated patients diagnosed with leukoplakia or primary squamous cell carcinoma (SCC) in the oral cavity were recruited during 1999 to 2005 from the R. Ahmed Dental College and Hospital, a primary refer-

ral center at Kolkata, India. For all patients, the Department of Pathology from the same hospital performed histopathologic diagnosis of the lesions. A small fraction of the patients (≈3%) without any tobacco habits were excluded from this study. Unrelated controls who came for treatment of dental ailments but without any previous and present lesions in the oral cavity were recruited from the same hospital. Initially, 340 cancer patients were approached, 326 of whom (96%) agreed to participate. Finally, 310 cancer patients (95% of the participants) donated blood for the study. In the case of 318 leukoplakia patients, 264 (83%) agreed to participate in this study and 224 leukoplakia patients (85% of the participants) agreed to donate blood. Approximately 665 controls were approached, 525 of whom (79%) agreed to participate in this study and 389 individuals (74% of the participants) came forward for blood donation. After obtaining informed written consent, all patients and controls were personally interviewed to obtain information regarding age, sex, occupation, alcohol consumption, type of tobacco habits, daily tobacco use frequency, duration of habits, economic status, place of job, and food habits.

All controls in this study were current tobacco users but all patients had tobacco habits before diagnosis. Some patients and controls reported tobacco habits such as smoking cigarettes and/or *bidis*, a native cigarette-like stick of coarse tobacco hand-rolled in a dry *tembuhurni* leaf. Individuals with only a smoking habit are termed smokers. Some of the patients and controls had a habit of smokeless tobacco in the form chewing or dipping.[23] Individuals having only a tobacco chewing/dipping habit are termed smokeless tobacco users. The remaining patients and controls had both smoking and chewing/dipping habits simultaneously and are termed 'mixed' habituees. Lifetime smokeless tobacco exposure was measured in terms of the frequency of chewing/dipping per day multiplied by the duration of habit. This is termed the chewing-year (CY; taking smokeless tobacco once a day for 1 year = 1 CY). Similarly, the dose of tobacco smoking was measured as pack-years (PY): 1 pack per day for 1 year = 1 PY (1 pack = 20 cigarettes or 40 *bidis*, because the tobacco content of 1 cigarette [700–1000 mg] is nearly equal to that present in 2 *bidis* [850–1050 mg]).

Sample Collection and Processing

Approximately 3.0 mL of blood was collected by vein puncture from patients (310 cancer patients and 224 patients with leukoplakia) and controls (n = 389) and stored at −20°C until DNA was isolated by the salt precipitation method.[25] DNA was also isolated from the affected tissues of a subset of leukoplakia (n = 30) and cancer (n = 30) patients. This preparation contained both nuclear and mtDNA. Biopsy materials collected from all leukoplakia and cancer lesions were processed for histopathology.

Genotyping at mt and Autosomal Loci

G10398A and C10400T

DNA samples were polymerase chain reaction (PCR)-amplified using primers located between 10,284–10,306 np (forward) and 10,484–10,459 np (reverse) followed by digestion with *DdeI* and *AluI* at 37°C, separately. On resolving the *DdeI*-digested products in a 2% agarose gel, the 10,398*A* allele gave rise to bands of 128 base pairs (bp) and 73 bp, whereas the 10,398*G* allele demonstrated bands of 90 bp, 73 bp, and 38 bp. On resolving the *AluI*-digested products, the *10400C* allele demonstrated a single band of 201 bp, whereas the *10400T* allele demonstrated bands of 115 bp and 86 bp.

A11467G

DNA samples were PCR-amplified using primers located between 11,319–11,338 np (forward) and 11,963–11,944 np (reverse) followed by digestion with *TruI1* at 65°C. On resolving the digested products in a 2% agarose gel, the 11,467*G* allele demonstrated bands of 538 bp, 86 bp, and 21 bp, whereas the 11,467*A* allele demonstrated bands of 412 bp, 126 bp, 86 bp, and 21 bp.

A12308G

DNA samples were PCR-amplified using forward primer 5′-CTC AAC CCC GAC ATC ATT ACC-3′ (12,104–12,124 np) and reverse primer 5′-ATT ACT TTT ATT TGG AGT TGC ACC AAg ATT-3′ (12,338–12,309 np), in which 'g' is the mismatched base. This mismatch created an *HinfI* site if the *G* allele is present at this SNP.[26] The PCR products were digested with *HinfI* and resolved in 2% agarose gel. The 12,308*A* allele gave rise to 168 bp and 67 bp and the 12,308*G* allele gave rise to 138 bp, 67 bp, and 30 bp DNA bands.

GSTM1, GSTM3, and GSTP1 (codon 105)

Previously, 310 cancer patients, 197 leukoplakia patients, and 348 control individuals from the present sample pools were genotyped at these loci.[23] In this study, an additional 27 leukoplakia patients and 41 controls were recruited and genotyped using the same methods.

TABLE 1
Demography and Tobacco and Alcohol Exposures of Patients and Controls

Subjects and habits		Controls N = 389 (%)	Leukoplakia N = 224 (%)	*P* (Leukoplakia vs Control)	Cancer N = 310 (%)	*P* (Cancer vs Control)
Sex	Male	302 (78)	196 (87)	.004	198 (64)	<.0001
	Female	87 (22)	28 (13)		112 (36)	
Age, y	Mean ± SD	49 ± 12	47 ± 10	.03	55 ± 11	<.0001
	Range	25–80	25–75		25–88	
Tobacco smoking habit	Smokers	145 (37)	133 (60)	.0001	53 (17)	<.0001
	Lifetime smoking range, PY	2–90	2–90		2–75	
	Mean smoking dose ± SD, PY	31 ± 18	24 ± 16	.0006	32 ± 14*	NS
Smokeless tobacco/ chewing habit	Smokeless tobacco users	169 (44)	32 (14)	<.0001	176 (57)	.001
	Lifetime smokeless tobacco using range, CY	12–925	12–420		4–1250	
	Mean smokeless tobacco dose ± SD, CY	183 ± 145	64 ± 174	.0001	182 ± 162	NS
Mixed habits	Smoking as well as smokeless tobacco habit	75 (19)	59 (26)	.05	81 (26)	.04
	Lifetime smoking, range in PY	2–90	2–80		2–120	
	Mean smoking dose ± SD, PY	22 ± 14	26 ± 19	NS	25 ± 16*	NS
	Lifetime smokeless tobacco use, range, CY	10–600	10–600		10–640	
	Mean smokeless tobacco dose ± SD, CY	100 ± 91	58 ± 103	.01	106 ± 88	NS
Alcohol consumer		14 (4)	10 (4)	NS	15 (5)	NS

SD indicates standard deviation; PY, pack-year; NS, not significant; CY, chewing-year.
* $P = .009$.

Risk Genotypes/Alleles

The *GSTM1* homozygous deletion, *GSTM3* (*A/A*) and *GSTP1 Ile/Ile* genotypes were considered as risk genotypes because these genotypes increased the risk of leukoplakia and cancer in different populations.[18–21,23,24] Contrary to reports,[7,9,12,13] the major *A* allele at 12,308 np, *G* allele at 10,398 np, and *T* allele at 10,400 np were considered risk alleles because they increased the risk of cancer in this population.

Sequencing of PCR Products

Genotypes and alleles at *GST* and mt loci, respectively, determined by PCR restriction length polymorphism (RFLP) or PCR methods were cross-checked in DNA samples isolated from the blood of 35 controls, 22 leukoplakia patients, and 28 cancer patients by resequencing (ABI 3100 Genetic Analyzer; Applied Biosystem, Foster City, Calif) using the respective GST[24] and mt primers. However, a different set of mt primers (forward primer: 12,117-12,138 np, reverse primer: 12,553-12,533 np) was used for resequencing the PCR product for the *A12308G* locus. In addition, PCR products synthesized by the mt primers from DNA isolated from 6 leukoplakia and 15 cancer tissues were also resequenced to check for somatic mutation.

Statistical Analysis

The risks of oral cancer and leukoplakia were calculated as odds ratios (ORs) with 95% confidence intervals (95% CIs) for mt alleles in all and stratified samples by binary logistic regression, adjusting for age, sex, and tobacco dose, using the SPSS statistical software package (SPSS Inc, Chicago, Ill). Chi-square tests were used for comparison of genotype/allele frequencies between 2 groups in 2 × 2 tables (*degree of freedom [df]* = 1). The risk of the cancer was also determined comparing the different risk/nonrisk genotype/allele combinations at *GSTs* and mt loci in patients and controls.

RESULTS

On interview, it was revealed that the majority of the patients and controls (>96%) were ethnically Bengalee and belonged to a low-income group (family income < U.S. $100 per month). All males were engaged in diverse occupations but with no exposure to toxic chemicals in the workplace. Most sampled females were housewives but some also worked as housemaids. Demographic characteristics and tobacco and alcohol habits of patient and control populations are summarized in Table 1. Because approximately 85% of smokers in our samples used both cigarettes and *bidis*, data concerning *bidi* and cigarette smokers were not analyzed separately. In control and patient groups, only a few (4%–5%) individuals consumed alcohol occasionally. Therefore, alcohol consumption was also not considered in the analysis.

The sites of oral cavity affected by leukoplakia were buccal mucosa and commissure area (74%), buccal mucosa and alveolar sulcus (21%), and ton-

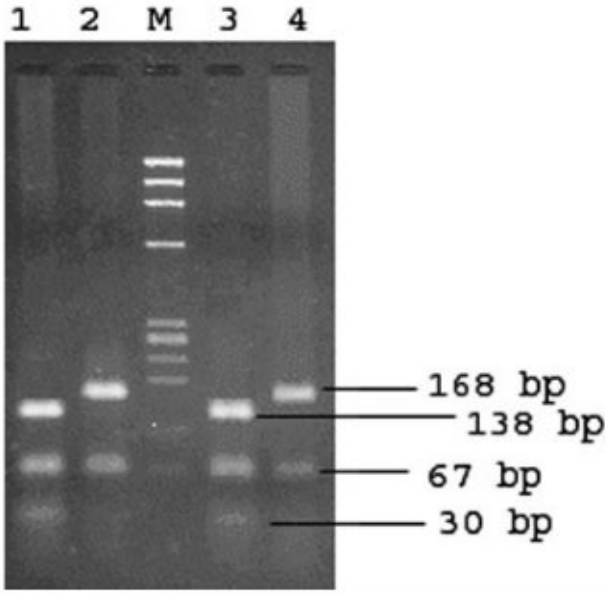

FIGURE 2. Representative restriction fragment length polymorphism (RFLP) pattern of G12308A polymorphism. Banding patterns in Lanes 1 and 3 represent G allele in blood and tissue DNA, respectively, from the same cancer patient. Similarly, banding patterns in Lanes 2 and 4 represent A allele in blood and tissue DNA, respectively, from another cancer patient. Lane M in HaeIII digest of phi X174 for size standard. bp indicates base pairs.

gue (5%). The majority of the patients had ulcerative (60%) followed by homogeneous (37%) 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 almost equally among the lip, tongue, retromolar area, and buccal sulcus. Histopathologically, all malignancies were diagnosed as SCC of the oral cavity. These could be classified as well (65%), moderately (17%), and poorly (18%) differentiated SCC.

In 8% to 10% of samples, alleles and genotypes, determined by PCR or PCR-RFLP, were also cross-checked by resequencing and there were no mismatches. In addition, mtDNA polymorphisms were also screened by PCR-RFLP in DNA isolated from affected tissues in a subset of patients. The SNP alleles detected in the tissue DNA were identical to those detected in the blood DNA (Fig. 2). A few mt PCR products obtained from tissue DNA (6 leukoplakia and 15 cancer) were also resequenced and no somatic mutation was detected. Thus, the possibility of somatic mutation at these loci was excluded. All 3 populations exhibited good fit to Hardy-Weinberg equilibrium at *GSTM3* ($P = .5$ for controls, .6 for leukoplakia, and .7 for cancer) and *GSTP1* ($P = .7$ for controls, .9 for leukoplakia, and .5 for cancer).

The major 12,308*A* allele increased the risk of oral cancer when compared with controls (OR of 1.7; 95% CI, 1.1–2.6) and leukoplakia patients (OR of 2.2; 95% CI, 1.3–3.7) (Table 2). However, no significant risk of leukoplakia was observed when the frequencies of this allele between leukoplakia patients and controls were compared (OR of 0.8; 95% CI, 0.52–1.18). This allele also increased the risk of cancer in males (OR of 1.8 [95% CI, 1.1–3.2] and OR of 2.4 [95% CI, 1.3–4.4]) when compared with those in controls and leukoplakia patients, respectively. Stratification of patients and controls by types of tobacco habit also revealed that this allele increased the risk of cancer among smokers when compared with controls (OR of 4.0; 95% CI, 1.1–14.4) and leukoplakia patients (OR of 6.3; 95% CI, 1.8–22.6), but not in smokeless tobacco users and mixed habituees. However, no dose-response correlation between the risk of cancer and smoking doses was observed when the cancer patients and controls with a smoking habit were divided into 2 groups with low (<19 PY) and high (>19 PY) smoking doses on the basis of median PY of controls. The reason for this could be the small sample sizes because of stratification of smoking doses.

Similar to the major 12,308*A* allele, the major *A* allele at 11,467 np also demonstrated identical results when data of patients and controls were compared (data not shown). This indicates complete linkage disequilibrium between these 2 alleles as has been reported in other Indian populations.[27] Major allele, *G*, at 10,398 np increased the risk of cancer marginally in overall samples (OR of 1.4; 95% CI, 1.0–1.9) but significantly (OR of 2.6; 95% CI, 1.2–5.7) in smokers when compared with those of controls (Table 3). Similarly, the major *T* allele at 10,400 np, the M-haplogroup marker, increased the risk of cancer marginally in overall samples (OR of 1.4; 95% CI, 1.0–2.0) but significantly (OR of 2.4; 95% CI, 1.1–5.1) in smokers when compared with those in controls. However, neither of the *10398G* and *10400T* alleles appeared to increase the risk of cancer in smokeless tobacco users and mixed habituees.

Among all *GST* loci, the risk-risk genotype-allele combination at *GSTP1* and mt12308 np loci, respectively, increased the risk of cancer significantly (OR of 2.6; 95% CI, 1.4–4.9) in comparison with leukoplakia patients carrying a nonrisk-nonrisk (genotype-allele) combination (Table 4). Similarly, the risk-risk (genotype-allele) combination at *GSTP1* and 10,398 np loci, respectively, increased the risk of cancer (OR of 1.8; 95% CI, 1.1–3.0) in comparison with leukoplakia patients carrying the nonrisk-nonrisk (genotype-allele) combination at these loci.

TABLE 2
Distribution of *A* and *G* Alleles at mt12308np in Patients and Controls and Risk of Cancer

Category of samples	Allele	Control No. (%)	Cancer No. (%)	OR (95% CI)	Allele	Leukoplakia No. (%)	Cancer No. (%)	OR (95% CI)
All samples	G	71 (18)	37 (12)	Reference	G	50 (22)	37 (12)	Reference
	A	312 (82)	271 (88)	1.7 (1.1–2.6), $P = .02$	A	173 (78)	271 (88)	2.2 (1.3–3.7), $P = .004$
	Total	383	308		Total	223	308	
Sex								
Male	G	57 (19)	23 (12)	Reference	G	45 (23)	23 (12)	Reference
	A	241 (81)	173 (88)	1.8 (1.1–3.2) $P = .03$	A	150 (77)	173 (88)	2.4 (1.3–4.4) $P = .005$
	Total	298	196		Total	195	196	
Female	G	14 (16)	14 (13)	Reference.	G	5 (18)	14 (13)	Reference
	A	71 (84)	98 (87)	1.5 (0.7–3.5)	A	23 (82)	98 (87)	1.9 (0.6–6.1)
	Total	85	112		Total	28	112	
Tobacco habit								
Smokers	G	26 (18)	3 (6)	Reference	G	35 (26)	3 (6)	Reference
	A	119 (82)	50 (94)	4.0 (1.1–14.4) $P = .03$	A	98 (74)	50 (94)	6.3 (1.8–22.6) $P = .004$
	Total	145	53		Total	133	53	
Smokeless tobacco users	G	28 (17)	24 (14)	Reference	G	5 (16)	24 (14)	Reference
	A	139 (83)	151 (86)	1.3 (0.7–2.4)	A	27 (84)	151 (86)	1.3 (0.4–3.8)
	Total	167	175		Total	32	175	
Mixed habits	G	17 (24)	10 (12)	Reference	G	10 (17)	10 (12)	Reference
	A	54 (76)	70 (88)	2.1 (0.9–5.1)	A	48 (83)	70 (88)	1.1 (0.4–3.1)
	Total	71	80		Total	58	80	

OR indicates odds ratio; 95% CI, 95% confidence interval. The OR and 95% CI were adjusted for age, sex (except when the samples were stratified by sex), and tobacco dose. Few DNA samples from patients and controls failed to amplify upon repeated attempts so they were excluded.

TABLE 3
Distribution of *A* and *G* Alleles at mt 10398np in Patients and Controls and Risk of Cancer

Category of samples	Allele	Control No. (%)	Cancer No. (%)	OR (95% CI)	Allele	Leukoplakia No. (%)	Cancer No. (%)	OR (95% CI)
All samples	A	144 (38)	93 (30)	Reference	A	89 (40)	93 (30)	Reference
	G	239 (62)	215 (70)	1.4 (1.0–1.9) $P = .05$	G	134 (60)	215 (70)	1.5 (1.0–2.2) $P = .05$
	Total	383	308		Total	223	308	
Tobacco habit								
Smokers	A	54 (37)	10 (19)	Reference	A	58 (44)	10 (19)	Reference
	G	91 (63)	43 (81)	2.6 (1.2–5.7) $P = .02$	G	75 (56)	43 (81)	3.6 (1.6–8.3) $P = .002$
	Total	145	53		Total	133	53	
Smokeless tobacco users	A	63 (38)	54 (31)	Reference	A	15 (47)	54 (31)	Reference
	G	104 (62)	121 (69)	1.4 (0.9–2.2)	G	17 (53)	121 (69)	2.1 (0.9–4.5)
	Total	167	175		Total	32	175	
Mixed habits	A	27 (38)	29 (36)	Reference	A	16 (28)	29 (36)	Reference
	G	44 (62)	51 (64)	1.0 (0.5–2.0)	G	42 (72)	51 (64)	0.5 (0.2–1.2)
	Total	71	80		Total	58	80	

OR indicates odds ratio; 95% CI, 95% confidence interval.
The OR and 95% CI were adjusted for age, sex, and tobacco dose.

DISCUSSION

In India males use both smoking and smokeless tobacco, whereas females use mostly smokeless tobacco. Although smokers and smokeless tobacco users are equally affected by leukoplakia, comparatively more male patients, who are mostly smokers, were present in the leukoplakia rather than the cancer population (Table 1). The reason might be that leukoplakia is not life-threatening initially, so most likely the females (mostly smokeless tobacco users) preferred to avoid the lengthy procedures to report to the hospital. As a result, females are less represented in the leukoplakia population.

Unlike earlier studies, in which the minor *G* allele at mt 12,308 np (ie, U-haplogroup) was positively associated with various disease phenotypes,[12,13] our

TABLE 4
Distribution of Genotype/Allele Combinations at *GSTP1* and mt12308np Loci and Risk of Cancer

Risk/Nonrisk (Genotype/Allele) combinations at	Control No. (%)	Cancer No. (%)	Crude OR (95% CI)	Leukoplakia No. (%)	Cancer No. (%)	Crude OR (95% CI)
GSTP1- mt *12308*						
Nonrisk/Nonrisk	28 (7)	20 (7)	Reference	28 (13)	20 (7)	Reference
Nonrisk/Risk	144 (38)	106 (35)	1.0 (0.5–1.9)	82 (37)	106 (35)	1.8 (0.9–3.4)
Risk/Nonrisk	42 (11)	17 (5)	0.6 (0.2–1.3)	22 (10)	17 (5)	1.1 (0.5–2.5)
Risk/Risk	167 (44)	164 (53)	1.4 (0.7–2.5)	89 (40)	164 (53)	2.6 (1.4–4.9)*
Total	381	307		221	307	

OR indicates odds ratio; 95% CI, 95% confidence interval.

* $P = .005$.

Few samples had to be excluded because, in some individuals, data regarding both the loci were not available. Therefore, the sample sizes are different from Table 3.

study revealed that the major *A* allele is positively associated with oral cancer risk when compared with controls and leukoplakia patients (Table 2). A recent study also reported a negative association between minor *G* allele and the risk of stroke in a Caucasian population.[28] Thus, it is observed that both the '*A*' and '*G*' alleles at 12,308 np could act as risk factors for different diseases. In this population, major allele *G* at 10,398 np increased the risk of oral cancer (Table 3). In another study,[9] minor '*A*' allele at 10,398 np (ie, N-haplogroup) increased the risk of breast cancer in an African-American population but somatic mutation from *A* to *G* allele was also observed in thyroid carcinoma from a European population.[29] Thus, these observations suggest that both alleles at few SNP sites could also act as risk factors for different diseases. The *A*12,308*G* polymorphism is located in the variable loop next to the anticodon stem of $tRNA^{Leu}$ (CUN) (Fig. 1). A report also suggested that mutation at a nearby position, 12,311 np, in the variable loop of $tRNA^{Leu}$ (CUN) could be a causative factor for chronic progressive external ophthalmoplegia in Japanese patients,[30] so single nucleotide change in the variable loop may affect the efficient functioning of $tRNA^{Leu}$ (CUN). Thus, a slight alteration in the efficiency of $tRNA^{Leu}$ (CUN) may cause disruption in the mitochondrial protein synthesis leading to a decrease in oxidative phosphorylation and an increase in ROS production. Currently, we do not have any biological evidence or explanation for how these major alleles at 12,308 np and 10,398 np loci could increase the risk of oral cancer, but it needs to be clarified. Alternatively, it could also be possible that these major alleles may be a surrogate marker of other risk allele/s which is/are in linkage disequilibrium. Apart from the major *12308A* and *10398G* alleles, the M-haplogroup (ie, a combination of *10398G* and *10400T* alleles) also increased the risk of cancer in this population. One interesting feature is that the risk of cancer is always more when the data of cancer patients are compared with those of leukoplakia patients instead of controls (Tables 2 and 3). Leukoplakia is a precancerous lesion and only 2% to 10% of leukoplakia progress to cancer,[15] so the *12308A* and *10398G* alleles and M-haplogroup may be a few of those germline polymorphisms that increase the chance of leukoplakia developing into cancer.

One interesting observation is that the major *12308A* allele increased the risk of cancer in males who had mostly a smoking habit (70%, 96%, and 66% of males in the control, leukoplakia, and cancer populations, respectively) but not in females, who had mostly a smokeless tobacco habit (98%, 89%, and 99% of females in the control, leukoplakia, and cancer populations, respectively), although the distribution of *A* and *G* alleles at 12,308 np is similar in males and females of the control population (Table 2). These observations suggest that smokers, but not smokeless tobacco users, are susceptible to cancer in the presence of the *12308A* allele. However, this observation should be reconfirmed in large Indian male samples with smoking or smokeless tobacco habits and European populations in which both males and females are mostly smokers. One of the reasons why the *12308A* allele that increased the risk of oral cancer in males persisted in this population may be that males are susceptible to this allele but females, who are not susceptible, transmit mtDNA. Hence, the *12308A* allele was insulated against selection in the males. Mt alleles did not increase the risk of cancer in mixed habituees (Tables 2 and 3), although they had both tobacco smoking and chewing habits. Because the types and amounts of carcinogens present in tobacco used for smoking and chewing are different,[31] we could not compare the total dosage of carcinogens in smokers

and mixed habituees. Moreover, the risk of cancer in smokeless tobacco users/chewers is not modified by these mt alleles. Therefore, a possible explanation of why mt alleles could not modify the risk of cancer in mixed habituees could be less smoking dose in mixed habituees in comparison to smokers ($P = .009$ for mean smoking doses between mixed habituees and smokers of cancer patients, Table 1).

The GSTM1, GSTP1, and GSTM3 enzymes could detoxify polyaromatic hydrocarbons present mostly in tobacco smoke. If the smokers of the 3 populations were stratified as carriers and noncarriers of at least 1 risk genotype at any of the *GSTM1*, *GSTM3*, or *GSTP1* loci, then distribution of these smokers among the control, leukoplakia, and cancer populations was not found to be significantly different ($P = .06$). However, analysis of the stratified smokers, who were carrying at least 1 risk genotype at any of the *GSTM1*, *GSTM3*, or *GSTP1* loci, as well as the mt *12308A* allele, demonstrated a risk of cancer when compared with the respective leukoplakia patients (OR of 5.8; 95% CI, 1.6–25.3). Thus, the observed risk of cancer among smokers might be because of the simultaneous presence of both *12308A* allele and at least 1 risk genotype at any of the 3 *GST* loci rather than the presence of the mt *12308A* allele alone (as shown in Table 2).

It has been reported that the GSTP1 enzyme could also move to mitochondria and inactivate ROS.[22] Therefore, individuals carrying a risk genotype at *GSTP1* will detoxify fewer carcinogens in cytosol and less ROS in mitochondria. In this study, the *GSTP1* risk genotype alone could not increase the risk of oral cancer (OR of 1.0; 95% CI, 0.9–1.0) but the major *A* allele at 12,308 np increased the risk of cancer in overall samples (Table 2). Again, a risk-risk (genotype-allele) combination at *GSTP1* and 12,308 np loci exhibited slightly more (≈18%) risk of cancer in comparison to major *A* allele at 12,308 np alone (an OR of 2.2 in Table 2 increased to 2.6 in Table 4). Similarly, a risk-risk (genotype-allele) combination at *GSTP1* and 10,398 np loci also exhibited slightly more (≈20%) risk of cancer in comparison with *G* allele at 10,398 np alone. Therefore, it may be possible that the mt risk alleles and *GSTP1* risk genotype might have increased the risk of cancer jointly.

In this hospital-based study, controls had dental ailments such as caries and gingivitis, so selection bias may occur. Although tobacco dose was adjusted whenever it was required, secondhand smoke exposure, diet, and other environmental exposures were not adjusted in our logistic regression models because of incomplete and missing information. Although the overall sample sizes were large, there were comparatively fewer females in the control and leukoplakia patient populations. Therefore, it is necessary to repeat a similar study with more female samples in cases and controls. To our knowledge, this is the first study to date to demonstrate an association between the risk of oral cancer and a major allele at the mt tRNA locus independently and also in combination with polymorphism at nuclear DNA.

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