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Susceptibility to oral cancer by genetic polymorphisms at *CYP1A1, GSTM1* and *GSTT1* loci among Indians: tobacco exposure as a risk modulator

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Oral cancer is the leading cancer type among Southeast Asian men and is causally associated with the use of tobacco. Genetic polymorphisms in xenobiotic-metabolizing enzymes modify the effect of environmental exposures, thereby playing a significant role in gene–environment interactions and hence contribute to the high degree of variance in individual susceptibility to cancer risk. This study investigates the role of polymorphisms at *CYP1A1, GSTM1* and *GSTT1* to oral squamous cell carcinoma (OSCC) in a case–control study involving 155 patients with precancerous lesions, 458 cancer patients and 729 age and habit-matched controls. Genotypes at these loci were determined by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism performed on genomic DNA extracted from peripheral blood lymphocytes. Risk to oral cancer was estimated among different tobacco exposure groups and doses using logistic regression analysis. *GSTM1* null genotype conferred 1.29-fold increased risk [95% confidence interval (CI), 1.04–1.65] to OSCC. *GSTT1* null genotype, however, conferred 0.57 times reduced risk to OSCC (95% CI, 0.39–0.83), specifically among tobacco chewers (odds ratio 0.27; 95% CI, 0.14–0.53). This risk was further reduced to 0.13 times (95% CI, 0.04–0.46) with increase in lifetime exposure to tobacco. We also investigated risk conferred by these genotypes at two different intra-oral sites, buccal mucosa and tongue. We found increased susceptibility to buccal mucosa cancer among individuals carrying these genetic markers. These results support the finding that *GSTM1* null genotype is a risk factor to OSCC among Indian tobacco habits; *GSTT1* null genotype, however, emerged as a protective factor.

Introduction

Oral cancer is among the leading cancer type in South Central Asian men (1). In India, oral cancer is the leading cancer type among men and third most common cancer among women (2). Oral precancerous lesions (PCLs) such as leukoplakia and submucous fibrosis are early indicators of damage to the oral mucosa with a transformation rate of 2–12% to frank malignancies (3). The use of tobacco is an established etiological factor in the development of cancers of the oral cavity. Tobacco is consumed in both its smoking and smokeless form. The consumption of smokeless tobacco occurs with the concomitant use of several additives that can alter cancer risk (4). Tobacco smoke comprises nearly 60 carcinogenic compounds whereas its unburned form contains 16 identified carcinogens. Polycyclic aromatic hydrocarbons (PAHs), nitrosamines, aldehydes and ketones form the major carcinogens present in tobacco (5). However, the concentrations of these compounds vary depending upon the nature of tobacco use (4,6). Tobacco smoke contains pyrolysis products, which are generated due to high temperatures at the burning tip, whereas smokeless tobacco is rich in nitrosamines (5). The concomitant use of betel quid leads to a 50-fold increase in reactive oxygen species generated (7). Most of these carcinogenic moieties are metabolically processed by xenobiotic-metabolizing enzymes in two broad steps: phase I mediated by cytochrome p450s (CYPs) and phase II catalyzed by glutathione *S*-transferases (GSTs), *N*-acetyltransferases, etc. Phase I reactions expose functional groups of the substrates and therefore yield highly reactive intermediates. These intermediates form the substrates for phase II reactions that involve their conjugation with endogenous molecules such as glutathione (GSH) and thus facilitate their elimination. Hence, the coordinated expression and regulation of phase I and II enzymes determines the outcome of carcinogen exposure. Sequence variants or polymorphisms in these genes can alter the expression, function and/or activity of these enzymes and, in turn, cancer risk (8).

Abbreviations: CI, confidence interval; CYP, cytochrome p450; GSH, glutathione; GST, glutathione *S*-transferase; OR, odds ratio; OSCC, oral squamous cell carcinoma; PAH, polycyclic aromatic hydrocarbon; PCL, precancerous lesion; PCR, polymerase chain reaction; SCE, sister chromatid exchange.

The *CYP1A1* encodes an aromatic hydrocarbon hydroxylase enzyme that catalyzes the oxidation of PAHs to their phenolic metabolite or diol epoxide [e.g. Benzo [a] pyrene (B(*a*)P) to Benzo [a] pyrene -Diol-Epoxide (BPDE)] (8). A transition from T to C in the 3′ noncoding region results in the introduction of an MspI restriction site and is associated with increase in enzyme activity and hence cancer risk (9,10). GSTs are a superfamily of ubiquitous, multifunctional enzymes that facilitate detoxification thus protecting cells from oxidative stress. *GSTM1* catalyzes the conjugation of the tripeptide GSH to PAH diol epoxides whereas *GSTT1* participates in detoxification of monohalomethanes and reactive diol epoxides (11,12). A structural deletion in these genes represents a null genotype and has been associated with an increased cancer risk (13). Previous studies have evaluated the relationship between xenobiotic-metabolizing gene polymorphisms and oral cancer risk (14–17). However, these associations have been inconsistent (18–21). Even though the concept of gene–gene and gene–environment interactions to oral cancer risk has long been postulated, only a few studies have addressed this with reference to the nature and dose of tobacco exposure (14,19,20). Understanding this phenomenon in the Indian context where oral cancers are most predominant not only becomes significant but also particularly difficult as the consumption of tobacco occurs in several forms (use of smokeless tobacco with or without additives and smoking of cigarettes and/or bidis) and most often, as mixed habits. The present study therefore investigates the role of polymorphisms at *CYP1A1, GSTM1* and *GSTT1* gene loci and susceptibility to oral PCLs and cancer. In this report, we have analyzed gene–environment interactions among various tobacco habit groups and among different levels of tobacco exposure. The present study also explores the possibility of altered susceptibility among different intra-oral sites.

Materials and methods

Study subjects

The present case–control study comprises 155 patients with oral PCL (leukoplakia and submucous fibrosis), 458 oral cancer patients and 729 control subjects. After obtaining an informed consent from all participants in the study, personal details were recorded in a questionnaire upon interview. Information regarding age, gender, occupation and details about duration, frequency, nature of tobacco habit (smoking or smokeless) and alcohol consumption were recorded. Patients with PCLs such as leukoplakia and submucous fibrosis were enrolled from Tata Memorial Hospital and other nearby dental colleges. These were histopathologically confirmed. Cancer cases were patients with cancers of the oral cavity, i.e. buccal mucosa, alveolus, retromolar trigone ($n = 283$) and tongue ($n = 175$), who were admitted and underwent therapy and surgery in Tata Memorial Hospital, Bombay, from the period of 2001 to 2004. All cancers were confirmed by histopathology to be squamous cell carcinoma. Genetically unrelated healthy individuals who reported the absence of personal history of cancer of any organ were recruited from the blood bank of hospitals

and dental clinics formed the control subjects. Controls enrolled in this study were matched for age, gender and tobacco habits. Subjects were classified on the basis of their tobacco habits as exclusive chewers, exclusive smokers, mixed tobacco habits, others and habit-free individuals (Table I)

Genotyping

Five milliliters of blood was collected from all study subjects. DNA was isolated from peripheral lymphocytes and polymerase chain reaction (PCR) was performed to determine the polymorphic genotypes at *CYP1A1, GSTM1* and *GSTT1* gene loci as described previously (14,22). Amplifications were performed on PTC 100 thermal cycler (MJ Research, Waltham MA, USA) and MspI restriction analysis was performed for the *CYP1A1* gene. Products of PCR and PCR-restriction digestion were electrophoresed on 12% polyacrylamide gel and visualized after ethidium bromide staining. Amplified products (3%) were sequenced periodically on automated sequencer from Applied Biosystems (Foster City, CA, USA) (Avant 3100) for confirmation of genotype data.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 11.5). Frequency distribution of the various genotypes was examined among cases and controls and also among different habit groups. Risks were estimated by calculating the odds ratios (ORs) among cases and controls after age adjustment. Multivariate analyses were performed to understand gene–gene and gene–environment interactions.

Results

Cohort characteristics

In order to understand the disease susceptibility conferred by *CYP1A1, GSTM1* and *GSTT1* genes, oral cancer risk was estimated in the total study population. In order to analyze the interaction between the nature of exposure and genetic susceptibility factors, the study population was divided into different habit groups. The demographic characteristics of the study population are summarized in Table I. 'Exclusive chewers' (318 controls, 77 PCL patients and 216 cancer patients) comprised individuals who consumed tobacco only in the smokeless form either with or without additives such as betel quid and lime. Some individuals also consumed tobacco in the form of a dentifrice commonly referred to as mishri. 'Exclusive smokers' (52 controls, 12 PCL patients and 23 cancer cases) were individuals who smoked tobacco in the form of cigarettes or bidis. 'Mixed tobacco habits' (110 controls, 55 PCL patients and 85 cancer cases) were individuals who consumed tobacco in both the smokeless form and were smokers. Individuals who reported a lack of former or current consumption of tobacco and alcohol formed the 'habit-free group' (48 controls, 1 PCL patient and 67 cancer cases). 'Others' (201 controls, 10 PCL patients and 67 cancer cases) included individuals who reported either exclusive alcohol habit or the consumption of alcohol with tobacco. These were not analyzed as the influence of alcohol could modify the effects of tobacco. Lifetime exposure was calculated as chewing and smoking index among all tobacco users for both cases and controls as follows:

Chewing index = frequency of chewing events per day × duration in years
Median chewing index for both controls and cases = 60
Heavy chewer: >60; light chewer: ≤60
Smoking index = number of cigarettes/10× duration in years or number of bidis/25 × duration in years
Median smoking index for both controls and cases = 5
Heavy smoker: >5; light smoker: ≤5

The mean and the median exposure were determined. The mean lifetime exposure of smokeless tobacco users was comparable among all three groups: cases, PCLs and controls. However, among exclusive chewers, the PCL group reflected a lower median exposure. Dose was determined by subcategorizing the study group into above and below median exposures. Initially, the study groups were examined as four quartiles of exposures, two below median (low and low–moderate exposures) and two above median (moderate–high and high exposure) dose categories. Later, on the basis of trend and to strengthen statistical power, these categories were combined into above (high) and below (low) median exposures. All comparisons were made within these categories.

Genotype and allele frequency

The frequency distribution of *CYP1A1, GSTM1* and *GSTT1* genotypes among patients and controls are summarized in Table II. The frequency of *CYP1A1* polymorphism m1/m2 and m2/m2 was found to be 45 and 10% among PCL group, 44 and 10% among cancer cases and 47.5 and 7% in controls, respectively. The allele frequencies for *CYP1A1* m1 and m2 variants in the control population were found to

Table I. Demographic characteristics of the study group

Variable	Controls	PCL	Oral cancer
Age			
Mean ± SE	42.89 ± 0.42	41.65 ± 1.15	50.38 ± 0.60
Median	42	41	50
Range	19–84	18–77	18–81
Gender, *n* (%)			
Male	636 (87)	135 (87)	323 (70)
Female	93 (13)	20 (13)	135 (30)
Tobacco status, *n* (%)			
Exclusive chewing	318 (44)	77 (50)	216 (47)
Exclusive smoking	52 (7)	12 (8)	23 (5)
Mixed habits	110 (15)	55 (35)	85 (18)
Habit free	48 (6)	01 (1)	67 (15)
Others	201 (28)	10 (6)	67 (15)
Total	729	155	458
Chewing index			
All chewers			
Mean ± SE	124.0 ± 18.5	98.7 ± 10.5	121.6 ± 8.9
Median	60.00	56.00	64.00
Exclusive chewers			
Mean ± SE	97.9 ± 6.2	95.3 ± 14.1	111.1 ± 9.4
Median	60.00	42.50	60.00
Smoking index			
All smokers			
Mean ± SE	12.4 ± 1.4	9.9 ± 1.6	11.7 ± 1.6
Median	5.00	4.00	6.00
Exclusive smokers			
Mean ± SE	15.6 ± 2.2	14.7 ± 4.6	10.3 ± 2.6
Median	12.25	9.50	6.00

Table II. Distribution of *CYP1A1, GSTM1* and *GSTT1* genotypes among patients and controls

Genotype	Control, *n* (%)	PCL, *n* (%)	Cancer, *n* (%)
CYP1A1			
m1/m1	331 (45.5)	68 (45)	205 (46)
m1/m2	345 (47.5)	68 (45)	195 (44)
OR*		0.96 (0.67–1.39)	0.80 (0.63–1.06)
m2/m2	51 (7)	16 (10)	46 (10)
OR*		1.50 (0.81–2.79)	1.40 (0.90–2.20)
Total	727	152	446
GSTM1			
Not null	458 (63)	99 (64)	253 (56)
Null	269 (37)	55 (36)	198 (44)
OR*		0.94 (0.66–1.35)	1.29 (1.04–1.65), $P = 0.05$
Total	727	154	451
GSTT1			
Not null	612 (84)	143 (92)	411 (90)
Null	114 (16)	12 (8)	45 (10)
OR*		0.45 (0.24–0.84), $P = 0.01$	0.57 (0.39–0.83), $P = 0.004$
Total	726	155	456

OR* represents age-adjusted OR (95% CI).

be 69 and 31%, respectively. The calculated chi-square values for the *CYP1A1* genotype suggested that the control population was not in Hardy–Weinberg equilibrium (data not shown). The frequency of *GSTM1* null genotype was found to be 36% among PCL patients, 44% among cancer cases and 37% among controls. The frequency of *GSTT1* null genotype was found to be 8% in precancer, 10% in cases and 16% in controls. The proportion of *CYP1A1* m2/m2 variant and the *GSTM1* null genotype was higher among oral cancer patients as compared with controls. The frequency of *GSTT1* null genotype, however, was higher among controls as compared with patients (Table II). The distribution of these genotypes suggested a potential influence in the incidence of oral squamous cell carcinoma (OSCC).

Genotypes and squamous cell carcinoma incidence

Individually, the risk conferred by the three genes to OSCC was calculated using binary logistic regression model with *CYP1A1* m1/m1, *GSTM1* not-null and *GSTT1* not-null serving as the reference category. Single-gene associations are summarized in Table II. Interactions between tobacco exposure, genotype and cancer risk were examined by determining the frequency of *CYP1A1, GSTM1* and *GSTT1* genes among individuals exposed to different tobacco habit groups among cases and controls. The results of these interactions are summarized in Table III. No association was seen between the *CYP1A1* MspI genotypes and oral precancer or cancer (Table II). However, among the mixed habit group, *CYP1A1* MspI homozygous variant genotype (m2/m2) contributed a 3.2-fold increased risk to OSCC [95% confidence interval (CI), 1.10–10.28; $P = 0.05$] and 4.29 times increased risk to PCL (95% CI, 1.16–16.02; $P = 0.03$). No significant association was found in any other category (Table III). No association of the *GSTM1* null genotype was found to PCL; however, a 1.29 times greater risk was conferred to OSCC (95% CI, 1.04–1.65; $P = 0.05$) (Table II). The overall tobacco chewers group did not show any association. Initial dose–response analysis showed greater proportion of *GSTM1* null genotype among the moderate to high exposure group (56% in cases versus 33% in controls). Estimation of ORs reflected a 2.64 times greater risk (95% CI, 1.17–5.95; $P = 0.02$) to OSCC in this quadrant alone. However, upon dichotomizing the tobacco chewers' category into above and below median exposures, no significant association was found (Table III). *GSTT1* null genotype on the other hand offered protection to both PCL and OSCC. Individuals carrying this genotype were at 0.5 times reduced risk to PCL (95% CI, 0.24–0.84; $P = 0.01$) and cancer conditions (95% CI, 0.39–0.83; $P = 0.004$) (Table II). Among tobacco chewers, *GSTT1* null genotype offered protection by decreasing the risk to OSCC by 0.27-fold (95% CI, 0.14–0.53; $P = 0.0001$). Dose relationship was associated with higher chewing index, in this category; OSCC risk was further reduced to 0.13 times. No significant association was found in any other group.

Gene interactions and tobacco exposure

It has been postulated that certain genotype combinations can increase risk to cancer by acting synergistically. For example, enhanced activation by phase I enzymes accompanied by reduced or loss of phase II function can lead to greater risk than attributed by single-gene variant alone. We tested this hypothesis in the present study in the context of different types and doses of tobacco exposure. Gene interactions within various exposure groups are summarized in Table IV. We found that the *CYP1A1* homozygous variant or *GSTM1* null genotype combination increased oral cancer risk by 1.62-fold (95% CI, 1.15–2.28; $P = 0.006$). A marginal increase in risk was observed to 1.83-fold (95% CI, 1.10–3.05; $P = 0.02$) among chewers, and a further increase to 2-fold risk was seen among the high exposure category (95% CI, 1.00–4.03, $P = 0.05$). Individuals carrying *GSTM1* and *GSTT1* null genotypes showed a trend toward protection, this observation, however, lacked statistical power. Among exclusive chewers, on the other hand, a significant protection was seen. This category was at 0.3-fold reduced risk to OSCC (95% CI, 0.12–0.83; $P = 0.02$). This protective effect was pronounced among the high exposure category, 0.1 (95% CI, 0.12–0.80; $P = 0.03$). Individuals carrying *GSTM1* null and *GSTT1* positive genotypes conferred 1.35 times increased risk to OSCC (95% CI, 1.03–1.76, $P = 0.03$). Individuals carrying *GSTT1* null and *GSTM1* positive genotypes were significantly protected from oral precancerous conditions. Among exclusive chewers, this genotype combination reduced oral precancer and cancer risk to 0.26- and 0.3-fold, respectively. The protective effect of this genotype combination to oral cancer risk increased with lifetime exposure to smokeless tobacco (Table IV).

The pattern of interaction between *CYP1A1* and *GSTT1* was similar to that observed between *GSTM1* and *GSTT1*. Smokeless tobacco users carrying *GSTT1* null genotype and/or *CYP1A1* variant genotypes were significantly protected from OSCC (data not shown). In this study, the presence of null allele at the *GSTT1* locus was a strong

Table III. Relative risk associated among different tobacco exposures in cases and controls

Genotype	Tobacco exposure/dose	Control genotype/ref	PCL genotype/ref	OR* (95% CI)	Oral cancer genotype/ref	OR* (95% CI)
CYP1A1 m2/m2	Chewers	21/142	8/35	1.49 (0.62–3.68)	20/98	1.42 (0.71–2.81)
	≤60 CY	12/65	6/18	1.83 (0.62–5.61)	7/50	0.79 (0.28–2.26)
	>60 CY	9/77	2/17	1.00 (0.19–5.21)	13/48	2.34 (0.91–5.98), $P = 0.08$
	Smokers	5/28	NR	—	NR	—
	Mixed habits	5/50	7/20	4.29 (1.16–16.02), $P = 0.03$	12/43	3.2 (1.10– 10.28), $P = 0.05$
	No habit	3/22	NR	—	6/29	1.26 (0.22–7.10)
GSTM1 null	Chewers	129/188	32/44	1.03 (0.62–1.73)	97/117	1.20 (0.85–1.76)
	≤60 CY	67/93	21/28	1.03 (0.54–1.97)	49/57	1.10 (0.70–1.92)
	>60 CY	62/95	11/16	1.07 (0.43–2.39)	48/60	1.30 (0.76–2.20)
	>60 ≤ 145 CY (intermediate–high)	23/46	6/4	2.9 (0.80–11.0)	25/20	2.64 (1.17–5.95), $P = 0.02$
	Smokers	18/34	2/10	0.4 (0.1–1.7)	14/9	2.31 (0.79–6.77)
	Mixed habits	36/74	18/37	0.57 (0.19–1.16)	31/51	1.35 (0.62–3.14)
	No habit	14/34	NR	—	31/36	2.00 (0.90)–4.40), $P = 0.08$
GSTT1 null	Chewers	54/263	7/69	0.51 (0.20–1.15)	12/202	0.27 (0.14–0.53), $P = 0.0001$
	≤60 CY	22/138	6/43	0.76 (0.29–1.97)	9/98	0.40 (0.17–0.94)
	>60 CY	32/125	1/26	0.18 (0.02–1.36)	3/104	0.13 (0. 04–0.46), $P = 0.001$
	Smokers	6/47	NR	—	3/20	1.4 (0.28–6.90)
	Mixed habits	16/94	3/52	0.44 (0.09–2.17)	12/73	1.1 (0.48–2.57)
	No habit	4/44	NR	—	8/59	1.4 (0.30–6.47)

OR* represents age-adjusted OR (95% CI), and NR denotes no representation of the variant genotype.

Table IV. Gene interactions among cases and controls

Genotype combinations	Tobacco exposure/dose	Control genotype/ref	PCL genotype/ref	OR* (95% CI)	Cancer genotype/ref	OR* (95% CI)
CYP1A1 m2/m2 or *GSTM1* null	Total†	149/213	36/44	1.17 (0.72–1.91)	124/109	1.62 (1.15–2.28), $P = 0.006$
	Chewers	67/90	23/18	1.64 (0.82–3.31)	63/48	1.83 (1.10–3.05), $P = 0.02$
	≤60 CY	36/37	13/9	1.50 (0.57–3.95)	33/23	1.6 (0.73–3.50)
	>60 CY	31/53	10/9	1.86 (0.66–5.24)	30/25	2.0 (1.00–4.03), $P = 0.05$
	Smokers	14/17	NR	—	5/4	1.16 (0.23–5.92)
	Mixed habits	21/32	10/15	1.10 (0.4–2.9)	24/26	1.40 (0.64–3.15)
	No habit	8/16	NR	—	18/14	2.18 (0.4–11.2)
CYP1A1 m2/m2 and *GSTM1* null	Total†	20/213	4/44	0.91 (0.65–2.34)	16/109	1.34 (0.64–2.79)
	Chewers	6/90	2/18	1.45 (0.27–7.9)	6/48	2.20 (0.65–7.50)
	≤60 CY	4/37	2/9	2.05 (0.32–12.9)	NR	—
	>60 CY	2/53	NR	—	6/25	7.42 (1.36–40.40), $P = 0.02$
	Smokers	2/17	NR	—	NR	—
	Mixed habits	2/32	2/15	2.19 (0.28–17.2)	4/26	2.10 (0.34–13.60)
	No habit	1/16	NR	—	3/14	0.66 (0.4–11.20)
GSTM1 null and *GSTT1* null	Total†	39/382	6/93	0.64 (0.26–1.56)	14/221	0.56 (0.29–1.07), $P = 0.08$
	Chewers	23/157	5/42	0.81 (0.28–2.32)	5/112	0.30 (0.12–0.83), $P = 0.02$
	≤60 CY	11/82	4/24	1.15 (0.33–4.0)	4/54	0.59 (0.16–0.2.08)
	>60 CY	12/75	1/18	0.42 (0.05–3.5)	1/58	0.1 (0.12–0.8), $P = 0.03$
	Smokers	1/30	NR	—	1/7	3.10 (0.15–61.6)
	Mixed habits	4/62	NR	—	2/41	0.74 (0.12–4.41)
	No habit	2/32	NR	—	2/30	1.29 (0.10–16.6)
GSTM1 null and *GSTT1* not-null	Total†	229/382	49/93	0.87 (0.60–1.28)	183/221	1.35 (1.03–1.76), $P = 0.03$
	Chewers	106/157	27/42	0.94 (0.54–1.62)	91/109	1.26 (0.85–1.85)
	≤60 CY	56/82	17/24	1.10 (0.52–2.17)	45/51	1.31 (0.75–2.3)
	>60 CY	50/75	10/18	0.76 (0.32–1.83)	46/58	1.21 (0.70–2.1)
	Smokers	17/30	2/10	0.35 (0.68–1.80)	13/7	2.56 (0.80–8.14)
	Mixed habits	31/62	18/34	1.06 (0.52–2.17)	29/41	1.33 (0.68–2.57)
	No habit	13/32	NR	—	29/30	1.67 (0.64–4.5)
GSTM1 not-null and *GSTT1* null	Total†	75/382	6/93	0.33 (0.14–0.78), $P = 0.01$	31/221	0.71 (0.45–1.13)
	Chewers	31/157	2/42	0.26 (0.06–1.14), $P = 0.07$	7/109	0.30 (0.13–0.73), $P = 0.008$
	≤60 CY	11/82	1/24	0.31 (0.42–2.6)	5/51	0.64 (0.19–2.19)
	>60 CY	20/75	1/18	0.21 (0.03–1.70)	2/58	0.13 (0.03–0.59), $P = 0.008$
	Smokers	5/30	NR	—	2/7	1.97 (0.28–14.16)
	Mixed habits	12/62	3/34	0.48 (0.13–1.83)	10/41	1.45 (0.56–3.76)
	No habit	2/32	1/6	—	6/302	1.95 (0.29–13.17)

Total†: reflects sum total of all groups (chewers + smokers + mixed habits + habit-free individuals + others.
OR* represents age-adjusted OR (95% CI).
NR denotes no representation of the variant genotype.

protective factor for OSCC irrespective of the variants in the other two loci (*CYP1A1* and *GSTM1*). Overall, the protective effect of *GSTT1* null genotype was pronounced and specific to tobacco chewers.

Differential susceptibility among intra-oral sites

We explored the possibility of altered predisposition among different intra-oral sites, i.e. buccal mucosa and tongue. A distinct susceptibility pattern emerged for the two sites. Analyses of gene interactions indicated that genetic factors conferred an overall greater risk to buccal mucosa than tongue cancers. The patterns of interactions of single genes to OSCC in the total study group and also among specific tobacco exposure categories were replicated at the buccal mucosa alone. Similar results were observed for gene interactions as well. Results are summarized in Table V. These differences are reflective of altered risk to the two intra-oral sites and not a consequence of sample size (supplementary table). *GSTM1* null genotype conferred 1.35-fold increased risk (95% CI, 1.01–1.81; $P = 0.04$) specifically to cancer of the buccal mucosa. No association was seen among any other category (Table V). *GSTT1* null genotype conferred protection to buccal mucosa cancers alone by reducing oral cancer risk by 0.47 times. Among chewers, further decrease in risk was observed to 0.23-fold. This protection increased with increase in lifetime exposure. No other exposure group revealed an association. Gene interactions between *CYP1A1* and *GSTM1* genotypes equally predisposed both the buccal mucosa and tongue to cancer risk. However, the risk conferred by this genotype combination, *CYP1A1* homozygous variant or *GSTM1* null genotype was modulated by exposure to smokeless tobacco only at the buccal mucosa.

All the three genes were analyzed with respect to different stages of differentiation as well, moderate and poorly differentiated tumors. No significant difference in distribution of genotypes was seen (data not shown).

Discussion

In the present study, we examined the association between *CYP1A1* MspI, *GSTM1* and *GSTT1* null polymorphisms to oral PCLs and cancer among various tobacco exposure groups. This is the largest study till date to investigate the role of metabolic gene variants in oral cancer susceptibly. The results indicate that the *CYP1A1* MspI polymorphism does not independently confer risk to OSCC, whereas *GSTM1* null polymorphism confers a 1.3-fold increased risk to oral cancer. *GSTT1* null polymorphism, however, protects against oral precancerous conditions and oral cancer. The results of this study also indicate differences in gene–environment interactions. The *CYP1A1* homozygous variant genotype conferred risk only in the presence of tobacco as an environmental exposure factor, i.e. among mixed tobacco habits. *GSTM1*, however, emerged as an independent risk factor to OSCC without an association to any specific tobacco exposure group. The protection conferred by *GSTT1* null genotype to oral precancer and cancer was consistent and specific to smokeless tobacco exposure. The findings summarized herein support the hypothesis that site-specific differences in susceptibility to carcinogens may exist.

Table V. Risk genotypes: analysis with respect to two intra-oral sites

Genotype	Tobacco exposure/dose	Control genotype/ref (n = 729)	Buccal mucosa genotype/ref (n = 283)	OR* (95% CI)	Tongue genotype/ref (n = 175)	OR* (95% CI)
CYP1A1 m2/m2	Total†	51/331	27/134	1.30 (0.80–2.16)	19/71	1.70 (0.90–3.10)
	Chewers	21/142	15/73	1.43 (0.70–2.99)	5/25	1.40 (0.47–4.10)
	≤60 CY	12/65	4/33	0.61 (0.17–2.15)	3/17	1.04 (0.25–4.30)
	>60 CY	9/77	11/40	2.50 (0.93–6.60)	2/8	2.10 (0.38–11.3)
	Smokers	5/28	NR	—	NR	—
	Mixed habits	5/50	9/31	3.70 (1.04–12.90), P = 0.04	3/12	2.90 (0.58–14.90)
	No habit	3/22	1/11	1.2 (0.10–14.3)	5/18	1.55 (0.25–9.70)
GSTM1 null	Total†	269/458	123/153	1.35 (1.01–1.81), P = 0.04	75/100	1.26 (0.90–1.78)
	Chewers	129/188	75/87	1.23 (0.83–1.84)	22/30	1.12 (0.62–2.05)
	≤60 CY	67/93	33/38	1.19 (0.66–2.17)	16/19	1.35 (0.63–2.93)
	>60 CY	62/95	42/49	1.28 (0.75–2.18)	6/11	0.85 (0.30–2.42)
	Smokers	18/34	6/3	3.60 (0.75–17.3)	8/6	2.10 (0.60–7.41)
	Mixed habits	36/74	22/35	1.20 (0.60–2.40)	9/16	1.12 (0.45–2.80)
	No habit	14/34	8/7	2.78 (0.90–9.00)	23/29	1.90 (0.90–4.40)
GSTT1 null	Total†	114/612	24/258	0.47 (0.29–0.76), P = 0.002	21/153	0.71 (0.43–1.19)
	Chewers	54/263	8/155	0.23 (0.11–0.51), P = 0.0003	4/47	0.39 (0.14–1.14)
	≤60 CY	22/138	5/67	0.44 (0.15–1.31)	4/30	0.82 (0.25–2.70)
	>60 CY	32/125	3/88	0.13 (0.04–0.4), P = 0.001	NR	—
	Smokers	6/47	1/8	1.14 (0.10–12.65)	2/12	1.32 (0.21–8.30)
	Mixed habits	16/94	9/51	1.17 (0.49–2.95)	3/22	0.88 (0.23–3.32)
	No habit	4/44	1/14	0.80 (0.10–6.00)	7/45	1.70 (0.52–6.00)
GSTM1 null and *GSTT1* null	Total†	39/382	6/135	0.38 (0.16–0.96), P = 0.04	8/86	0.86 (0.38–1.93)
	Chewers	23/157	3/85	0.23 (0.06–0.79), P = 0.02	2/27	0.50 (0.11–2.27)
	Smokers	1/30	NR	—	1/5	4.30 (0.20–89.43)
	Mixed habits	4/62	1/27	0.48 (0.05–4.70)	1/14	1.19 (0.12–11.60)
	No habit	2/32	NR	—	2/24	1.70 (0.13–22.80)
GSTM1 null and *GSTT1* not-null	Total†	229/382	116/135	1.43 (1.04–1.95), P = 0.026	67/86	1.29 (0.89–1.87)
	Chewers	106/157	71/82	1.27 (0.83–1.93)	20/27	1.16 (0.60–2.20)
	Smokers	17/30	6/2	4.90 (0.83–28.9), P = 0.08	7/5	2.00 (0.53–7.80)
	Mixed habits	31/62	21/27	1.50 (0.71–3.2)	8/14	1.10 (0.42–2.90)
	No habit	12/32	8/6	3.61 (0.91–14.2), P = 0.06	21/24	1.49 (0.52–4.30)
GSTM1 not-null and *GSTT1* null	Total†	75/382	18/135	0.65 (0.37–1.16)	13/86	0.76 (0.40–1.45)
	Chewers	31/157	5/82	0.29 (0.11–0.79), P = 0.016	2/27	0.36 (0.80–1.59)
	Smokers	5/30	1/2	3.30 (0.21–52.8)	1/5	1.18 (0.10–14.23)
	Mixed habits	12/62	8/27	1.82 (0.84–5.17)	2/14	0.80 (0.16–4.02)
	No habit	2/32	1/6	1.91 (0.09–39.40)	5/24	2.50 (0.35–17.31)
CYP1A1 m2/m2 or *GSTM1* null	Total†	149/213	80/70	1.66 (1.11–2.47), P = 0.013	44/39	1.67 (1.02–2.14), P = 0.04
	Chewers	67/90	48/34	1.93 (1.10–3.39), P = 0.02	15/14	1.57 (0.70–3.53)
	Smokers	14/17	3/1	3.34 (0.30–37.00)	2/3	0.53 (0.06–4.73)
	Mixed habits	21/32	18/18	1.53 (0.64–3.68)	6/8	1.27 (0.38–4.30)
	No habit	8/16	6/6	2.73 (0.5–14.79)	12/8	2.19 (0.48–1.01)
CYP1A1 m2/m2 and *GSTM1* null	Total†	20/213	9/70	1.39 (0.59–3.20)	7/39	1.47 (0.54–3.99)
	Chewers	6/90	5/34	2.80 (0.75–16.20)	1/14	1.26 (0.14–11.2)
	Smokers	2/17	NR	—	NR	—
	Mixed habits	2/32	3/18	2.72 (0.39–8.95)	1/8	1.57 (0.11–21.50)
	No habit	1/16	NR	—	3/8	1.23 (0.72–21.07)

Total†: reflects sum total of all groups (chewers + smokers + mixed habitués + habit free individuals + others.
OR* represents age adjusted odds ratio (95% CI).
NR denotes no representation of the variant genotype.

In the present study, the allele frequencies of *CYP1A1* m1 and m2 alleles among the control group were found to be 0.68 and 0.32, respectively, which is in agreement with previous reports (23). In this study population, the frequency of *GSTM1* and *GSTT1* null genotypes among control subjects are 37 and 16%, respectively, which lie within the range of 17–38% and 8–27% reported among Indian population (7,14,20,24–26).

The role of *CYP1A1* in metabolism of tobacco is well established (8). Tissue-specific expression of this isoform has been demonstrated in buccal cells of the oral cavity (27). The contribution of GSTs to detoxification of carcinogens is also well documented (11). Hence, several studies have examined the risk of OSCC conferred by *CYP1A1, GSTM1* and *GSTT1* null genotypes and these are summarized in a recent meta-analysis (15). Conflicting reports exist regarding the association of *CYP1A1* polymorphism and oral cancer risk. In the meta-analysis, the polymorphism resulting in a isoleucine to valine substitution has been reported to increase oral cancer risk modestly (15). However, relatively fewer studies exist on the association of the MspI polymorphism and oral cancer risk. Among the total six studies that have investigated the contribution of the *CYP1A1* polymorphism to oral cancer risk (10,19,23,28–30), only two reported a risk association (10,19). The lack of consistency in these reports warrants further studies to elucidate the role and degree of contribution of this variant to oral cancer risk. In the present study, we find an association to risk, conferred by the homozygous variant (m2/m2) to oral cancer. We also observed a further increase in risk among mixed habits. In this category of exposure, the genetic determinant, *CYP1A1*, conferred risk to both oral precancer and cancer. This risk was greater than that of the total study group, possibly indicating an interaction with tobacco exposure. *CYP1A1* is known to metabolize PAHs generated from tobacco smoke. However, in the present study, we could not determine the specific interaction between this genotype among

exclusive smokers due to the lack of representation of the homozygous variant genotype in this category. This can be attributed to the small sample size of exclusive smokers in our study.

Among the 31 studies reviewed in the meta-analysis for the association of *GSTM1* null genotype and head and neck squamous cell carcinoma risk, an overall OR of 1.23 (95% CI, 1.06–1.62) has been reported (15). Of the remaining five studies published thereafter, two reported increased risk (16,31), two showed no association (18,32) and one study in Puerto Rico found a protective effect of the null allele to head and neck squamous cell carcinoma (21). In the present study, *GSTM1* null genotype emerged as a risk factor for OSCC, which is in agreement with previous reports (14,15). No significant interaction was observed in any of the other exposure categories analyzed. Further, an increase in oral cancer risk with increase in lifetime exposure to tobacco has been demonstrated (14). Although the data summarized herein indicates a significant association in the moderate–high exposure category among the smokeless tobacco group, neither the above median exposure category nor the overall chewers category reflected any risk association. Further, a trend toward risk was observed in the no-habit group. GSTs are known to conjugate several endogenous and environmentally derived substrates. Hence, it is postulated that lack of these enzymes, as a consequence of the null genotype, could compromise metabolism and hence contribute to cancer risk. It has been demonstrated that smokers with *GSTM1* null genotype have higher sister chromatid exchange (SCE) and CA levels than *GSTM1* positive smokers (33). However, data on habit-free individuals are scant. A few studies have reported increased SCE levels among *GSTM1* null compared with *GSTM1* positive individuals (34). These results could imply that *GSTM1* null genotype can serve as an independent risk factor. The results in the present study support such observations reported previously.

Of the 27 studies that have examined risk to OSCC conferred by *GSTT1* null allele, 21 have been reviewed and an overall OR of 1.17 (95% CI, 0.98–1.4) has been reported (15). Of the remaining six studies published thereafter, three found a positive association (17,31,35), two found no association (20,21) and one study supports our finding of *GSTT1* null genotype and protection to head and neck squamous cell carcinoma (18). The present study is the first report indicating a protective effect of the *GSTT1* null allele to oral PCLs and oral cancer. This protective effect increases with lifetime exposure to chewing tobacco. Similar findings have been reported among smokers (18) and bladder cancer patients (12). A study by Garcia Closas et al. (36) revealed a decreased risk to breast cancer among premenopausal women with *GSTT1* null genotype. Although *GSTT1* is predominantly involved in detoxification of xenobiotics, certain compounds are reported to be bioactivated via the *GSTT1* pathway (37,38). It has been demonstrated that the activation of halogenated compounds by the *GSTT1* pathway is the mechanism of genotoxicity in mice (37). It has been postulated that a similar mechanism may be operational among *GSTT1* positive individuals. Exposure to tobacco in any form occurs as a complex mixture of compounds, which may simultaneously be metabolized by various enzymes with overlapping substrate specificities. Moreover, the concentration of carcinogens extracted from smokeless tobacco is known to vary contingent upon concomitant use of betel quid and/or lime as additives (6). Subjects enrolled in the present study report overlapping tobacco exposures. Although substrates for *GSTT1* in smokeless tobacco have not yet been identified, a decreased risk conferred by the null allele in this study could imply that *GSTT1* activates certain known or yet to be identified procarcinogens present in chewing tobacco. This hypothesis is also supported by the observation that no protection was offered to habit-free individuals.

Relatively fewer studies have analyzed combined gene effects. To date, few studies have examined the risk conferred by the combination of *CYP1A1* MspI, *GSTM1* and *GSTT1* null polymorphisms (19,20). Gene interactions in the present study reflected a pattern of susceptibility similar to independent gene effects. Overall, combination of *CYP1A1* homozygous variant with *GSTM1* null genotype increased oral cancer risk. We observed that the presence of a variant either at *CYP1A1* or *GSTM1* locus increased oral cancer risk by 1.6-fold. Exposure to smokeless tobacco increased the risk conferred by these genotype combinations. Further, an increase in risk with increase in lifetime exposure was observed. Greater than 7-fold risk was observed specifically among above median exposure category of smokeless tobacco group in the presence of both variants at *CYP1A1* and *GSTM1* gene loci. This finding supports the hypothesis that combined gene variants can lead to increased risk by acting synergistically. This observation is supported by similar reports from other populations (10,19).

In the present study, we observe a protection to oral cancer risk in the presence of *GSTT1* null allele, irrespective of any other risk genotype. This association was specific to tobacco chewers and increased with increase in lifetime exposure. This finding differs from reports on increased risk conferred by *GSTM1* and *GSTT1* null combination (7,31). The increased risk contributed by the *GSTM1* null and *GSTT1* not-null genotypes reflects the contribution by *GSTM1* null genotype. No significant differences were observed in distribution of all three genes variants in combination. This lack of association is in agreement with previous reports (28,29)

It has long been postulated that decreased detoxification by phase II enzymes increases susceptibility to cancers (13). Analyses of gene–environment interactions by studying cytogenetic end-points support this hypothesis. It has been shown that smokers with *GSTM1* null genotype have higher SCE frequencies and higher levels of chromosomal aberrations (39). *GSTT1* null genotype, on the other hand, increases the baseline frequency of SCE indicating the presence of a common environmental genotoxicant or a specific endogenous substrate (40,41). Levels of reduced GSH and activity of GSTs are known to be up-regulated in cancer cells and in certain studies this is shown to correlate with tumor stage and differentiation (42,43). Further, recent evidence points to the role of GSTs in tumor resistance to radiation and chemotherapy. GSTs (pi and theta) are known to regulate Mitogen-Activated Protein (MAP) kinase activities (44). They also regulate post-translational glutathionylation reactions (45). These aspects have recently been reviewed (46). Both *GSTM1* and *GSTT1* null genotypes are associated with prognosis (32). In the light of these evidences, the function of GSTs seems best explained in a biphasic model. The principle function of GSTs is to regulate the antioxidant status of the cell. Deregulation of the same in the stage of initiation can lead to tumorigenesis. However, deregulation post-initiation might render a survival advantage by blocking apoptosis. Hence, GSTs exhibit a dual role depending on the stage of carcinogenesis.

In the present study, we found differential susceptibility among the two intra-oral sites, buccal mucosa and tongue. Such differences in genetic susceptibility to cancers have been previously reported within sites of oral cavity (10). It is known that differences exist in the incidence of cancers among various regions of the oral cavity and these are in part explained by differences in biological function of these organs (47). The degree of aggression is also known to vary between tongue and buccal mucosa cancers. These are explained on the basis of differences in structure and function; tongue being vascular by nature, cancers of this site are highly metastatic as compared with buccal mucosa. Altered profile of structural proteins such as cytokeratins is also reported (48). Cytokeratins are known to be involved in carcinogenesis (49). Among various sites of head and neck cancers, different genetic alterations have been reported. This might indicate altered molecular signature patterns in the process of carcinogenesis in these regions (50). It is known that tissue-specific differences in expression and regulation of phase I and phase II enzymes occur (51). These differences can alter cancer risk attributed by carcinogens that form their substrates. The profile of expression of phase I and phase II enzymes in the buccal cells has been investigated and the expression of *CYP1A1, GSTM1* and *GSTT1* has been demonstrated (27). However, data on tissue-specific expression in tongue are scant. In this study, we found a significant difference between the risk to buccal mucosa and tongue cancers in the presence of genetic susceptibility factors: *CYP1A1, GSTM1* and *GSTT1* null polymorphisms. These differences could imply altered gene–environment interactions. This

speculation is supported by the observation of differences among the various habit groups analyzed and dose categories investigated. The pattern of interactions that we found among oral cancers was replicated in the case of buccal mucosa but not tongue cancers. Thus, we report for the first time a differential risk to oral cancer, specifically buccal mucosa cancers in the presence of *CYP1A1* homozygous variant and *GSTM1* and *GSTT1* null allele. However, further validation by larger studies would reveal the true meaning of such associations.

We conclude that the three xenobiotic-metabolizing enzymes reported in the present study, *CYP1A1, GSTM1* and *GSTT1*, significantly alter oral cancer risk singly and in combination. Further, specific tobacco exposures appear to modulate this risk. The present study reinstates the complexity of the interplay between genetic and environmental factors as determinants of oral cancer risk.

Supplementary material

Supplementary table can be found at http://carcin.oxfordjournals.org/.

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