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Polymorphism at *GSTM1*, *GSTM3* and *GSTT1* gene loci and susceptibility to oral cancer in an Indian population

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This study evaluates the influence of genetic polymorphism at *GSTM1*, *GSTM3* and *GSTT1* gene loci on oral cancer risk among Indians habituated to the use of, smokeless tobacco, bidi or cigarette. DNA extracted from white blood cells of 297 cancer patients and 450 healthy controls by the proteinase K phenol–chloroform extraction procedure were analyzed by the polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) analyses. Lifetime tobacco exposure was evaluated as a risk factor in relation to the polymorphism at the GST gene loci using logistic regression analysis. There was no significant difference in the distribution of the *GSTM3* and *GSTT1* genotypes between oral cancer patients and controls. In contrast, a significant 3-fold increase in risk was seen for patients with the *GSTM1* null genotype (age adjusted OR = 3.2, 95% CI 2.4–4.3). The impact of the *GSTM1* null genotype on oral cancer risk was also analyzed in separate groups of individuals with different tobacco habits. The odds ratio associated with the GSTM1 null genotype was 3.7 (95% CI 2.0–7.1) in tobacco chewers, 3.7 (5% CI 1.3–7.9) in bidi smokers and 5.7 (95% CI 2.0–16.3) in cigarette smokers. Furthermore, increased lifetime exposure to chewing tobacco appeared to be associated with a 2-fold increase in oral cancer risk in *GSTM1* null individuals. The results suggest that the *GSTM1* null genotype is a risk factor for development of oral cancer among Indian tobacco habitues.

Introduction

Oral cancer is a significant public health problem in many parts of the world and particularly so in South East Asia. It is the leading cancer among males in India (1), the proportion of oral cancer cases attributable to smokeless tobacco use and smoking habits being 81% in males and 36% in females (2–4). Most tobacco carcinogens are metabolized via complex enzymatic mechanisms involving both activation and detoxification reactions. Glutathione *S*-transferases (GSTs) are a very important family of enzymes that catalyze the detoxification of a wide variety of active metabolites of tobacco carcinogens such as benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons, monohalomethanes etc. (5,6). Therefore, variations in the expression of GSTs due to heritable genetic polymorphism probably modulate the process of carcinogenesis by altering the exposure levels of tobacco-derived carcinogens.

Several studies have demonstrated that the *GSTM1* null genotype is a risk factor for the development of lung cancer among cigarette smokers (7). Furthermore, a meta-analysis has confirmed that this polymorphism confers a modest but highly significant risk of lung cancer (8). Inheritance of the *GSTT1* null genotype too was found to be associated with several types of malignancies including colorectal and bladder cancer (9,10). Further, a polymorphism resulting in the generation of a recognition sequence for the YY1 transcription factor in the *GSTM3* gene, is reportedly involved in the modulation of risk for environmentally induced cancers (11,12). Despite the strong biological plausibility for the role of polymorphism at *GSTM1*, *GSTM3* and *GSTT1* gene loci in altering individual susceptibility to oral cancer, a relatively small number of studies have evaluated this relationship. Evidence of such an association has been reported in a few studies among white Americans, Europeans and Japanese populations, exposed chronically to cigarette smoke (13–17). However, in India, the high incidence of the most common oral cancer, squamous cell carcinoma (SCC) of the buccal mucosa is related to the use of smokeless tobacco as well as smoking of bidi or cigarette (18). The present study therefore investigates the influence of polymorphism at *GSTM1*, *GSTM3* and *GSTT1* gene loci on susceptibility to cancer of the buccal mucosa among Indian tobacco chewers and bidi and cigarette smokers.

Materials and methods

Subjects

The present case control study comprised of 297 cases with histopathologically confirmed SCC of the buccal mucosa, and 450 healthy unrelated controls. Both incident and prevalent cases of cancer of the oral cavity were included in this study. The inclusion criterion for the cases was the presence of histopathologically diagnosed SCC of the buccal mucosa. All the patients were recruited from the outpatient department of the Tata Memorial Hospital (TMH), Mumbai, Maharashtra from November 1995 to January 1997. In this study all 297 cases had SCC of the buccal mucosa.

The inclusion criterion for the controls was absence of prior history of cancer or pre-cancerous lesions. Unrelated healthy controls were recruited from blood donors who accompanied patients seeking treatment at TMH and from members of different community centers between March 1996 and September 1998. People from all parts of India migrate to Mumbai for employment but continue to maintain close regional association through respective community centers. Thus, cases and controls were matched for the region of origin through the cooperation of the community centers and blood donors. After obtaining informed consent, all individuals were personally interviewed using a questionnaire. Information on age, gender, occupation, region of origin, type of tobacco habit, daily habit frequency and duration was recorded. Data pertaining to histopathological diagnosis and clinical staging were collected from the hospital records.

The cases reported habits such as smoking of bidi or cigarette, chewing of tobacco with lime or with betel quid. While the majority had a single habit, some cases reported dual habits comprising of different combinations of bidi/cigarette smoking and chewing of tobacco with lime or with betel quid. Information provided by the smokeless tobacco habitues 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

Abbreviations: CI, confidence interval; GST, glutathione *S*-transferase; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; OR, odds ratio; SCC, squamous cell carcinoma.

the duration of habit. To facilitate a comparison of the amount of tobacco exposure among individuals in the different habit groups, exposure to bidis and cigarettes was also expressed as lifetime tobacco exposure instead of the conventional index of pack years.

Genotyping

Two milliliters of blood was collected in sterile EDTA vials from all the subjects. DNA was extracted from white blood cells using the proteinase K phenol–chloroform extraction procedure (19). Polymorphism at the *GSTM1*, *GSTM3* and *GSTT1* gene loci was examined using the polymerase chain reaction (PCR), or PCR-restriction fragment length polymorphism (RFLP) analysis. The reaction mix consisted of 50–100 ng of genomic DNA, 50 pm of each primer (Life Technologies, Scotland, UK), 200 µM dNTPs (Amersham, Bucks, UK) and 0.5 U *Taq* DNA polymerase (Life Technologies and Perkin Elmer, UK). A hot-start step was included in all the PCR assays and all reactions were carried out in a Perkin Elmer 2400 thermal cycler. Restriction digestion was carried out overnight at 37°C. PCR and PCR-RFLP products were electrophoresed on 12% polyacrylamide gels using appropriate molecular weight markers and were visualized after staining with ethidium bromide. Every precaution was taken to prevent cross contamination between samples during DNA extraction and PCR assays. Sequencing was carried out periodically using the *T7 Sequenase* direct PCR product sequencing kit (Amersham).

GSTM1

The PCR employed a common sense primer and different antisense primers to amplify a 270 bp region between exons 4 and 5 of the *GSTM1* gene and a 160 bp region between exons 6 and 7 of the GSTM2 gene (20). The protocol was modified by the addition of 8% DMSO, 3.3 mM $MgCl_2$ and a change in the annealing temperature to 59°C. The null genotype was represented by the absence of the 270 bp GSTM1 fragment and the presence of the 160 bp GSTM2 band.

GSTM3

A PCR-RFLP strategy was used to identify A and B alleles of the *GSTM3* gene. Primers to exon 7 were used to amplify a 273/270 bp product using the method of Inskip *et al.* (11). In order to reduce non-specific amplification, $MgCl_2$ concentration was changed to 2.0 mM, annealing was carried out at 57°C, and the extension step was omitted in all the cycles. The products were digested at 37°C with *Mnl*I. *GSTM3* A homozygotes were identified by band sizes of 11, 51, 86 and 125 bp due to an additional *Mnl*I site, while fragments of 11, 125 and 134 bp represented *GSTM3* B homozygotes.

GSTT1

Primers to the region containing the deletion in the *GSTT1* gene were used to amplify a 480 bp product. The *GSTM2* gene was co-amplified as an internal control. The method of Pemble *et al.* (21) was modified by a change in the annealing temperature to 64°C and addition of 8% DMSO. Absence of the 480 bp band indicated the *GSTT1* null genotype while the 160 bp *GSTM2* band was seen in all the samples.

Statistical analysis

Differences in genotype prevalence between the case and control groups were assessed by the chi-squared test; the Student's *t*-test was employed for comparing frequency, duration and lifetime exposure in cases and controls. Odds ratios (ORs) associated with a genotype and its 95% confidence intervals (CIs) were obtained by summarizing data over three tobacco habit strata and four age strata by the Mantel–Haenszel method. Further, lifetime tobacco exposure was dichotomized as above and below the median exposure level for each type of tobacco habit. Age adjusted OR and 95% CIs associated with the putative at-risk GST genotype were calculated by unconditional logistic regression analysis using the SPSS statistical analysis software, version 9.0 (SPSS, IL, USA). The fit of the models was evaluated using the Hosmer–Lemeshow likelihood ratio test.

Results

The study population consisted of 297 cases with oral cancer and 450 controls who were regular heavy users of tobacco in one form or another. Individuals were divided into three habit groups: those who chewed tobacco, those who smoked tobacco and those who had both habits. The tobacco chewers comprised of 120 cases (105 used tobacco with lime and 15 used tobacco with betel quid) and 172 controls (161 used tobacco with lime and 11 used tobacco with betel quid). There were 100 smokers (75 bidi smokers and 25 cigarette smokers) in the case group and 143 smokers (45 bidi smokers and 98 cigarette smokers) in the control group. It was difficult to assess lifetime tobacco exposure among individuals who smoked as well as chewed tobacco (77 cases and 135 controls). Reliable information on habit frequency and duration was available in nearly 95% of cases ($n = 285$) and controls ($n = 426$). Table I presents information on the mean frequency, duration and lifetime exposure in each habit group. Among cigarette smokers and tobacco chewers, the lifetime exposure level was significantly higher in cases than in controls ($P < 0.001$). As there were only two controls that chewed tobacco with betel quid, it was not possible to undertake comparative analysis with the 15 cases that had a similar habit.

Forty-one percent of the cases presented with stage 4 tumors of the buccal mucosa, the remaining were distributed equally in stages 1, 2 and 3. Histologically, all the malignancies were diagnosed as SCC of the buccal mucosa. Of these, 55.3% were classified as well differentiated, 28.3% were moderately differentiated and 16.4% were poorly differentiated SCC. There was no association between the distribution of any of the polymorphic GST genotypes and tumor grade and stage.

The frequency distribution of various GST genotypes in 297 buccal mucosa cancer patients and 450 controls is presented in Table II. The distribution of the *GSTM3* and *GSTT1*

Table I. Frequency, duration and lifetime exposure in cases and controls[a]

Habit	Cases ($n = 285$)	Controls ($n = 426$)	*P* value
Age	52.2 ± 0.69	42.9 ± 0.53	NS
Bidi smoking	(74)	(41)	
Frequency	19.2 ± 1.8	23.1 ± 3.1	NS
Duration	19 ± 1.2	18.8 ± 1.5	NS
Lifetime exposure[b]	361.64 ± 38.3	493.15 ± 98.6	NS
Cigarette smoking	(23)	(88)	
Frequency	15.1 ± 3.0	9.8 ± 1.2	NS
Duration	18.5 ± 1.7	13.4 ± 1.0	NS
Lifetime exposure[b]	328.76 ± 93.1	128.76 ± 16.16	<0.001
Tobacco chewing	(105)	(161)	
Frequency	11.2 ± 0.6	9.5 ± 0.7	NS
Duration	23.8 ± 1.1	12.2 ± 0.7	<0.001
Lifetime exposure[b]	268.5 ± 1.8	131.5 ± 14.2	<0.001
Betel quid chewing	(15)	(2)	
Frequency	12.9 ± 2.6	2.0, 20.0[c]	ND
Duration	17.3 ± 2.3	10.0, 15.0[c]	ND
Lifetime exposure[b]	224.65 ± 52.1	20, 300[c]	ND

[a]The body of the table shows the values in terms of mean ± SE.
[b]Lifetime tobacco exposure is given as the product of habit frequency/day and duration in years.
[c]The actual values are give exposure data available from 95% of the subjects, hence numbers in the table do not match those mentioned in the text.
NS, not significant; ND, not determined.

Table II. Frequency distribution of *GSTM1*, *GSTM3* and *GSTT1* genotypes in cases and controls

Genotype	Cases *n* (%)	Controls *n* (%)	Age adjusted OR (95% CI)
GSTM1 +ve	151 (50.8)	339 (76.0)	
Null	146 (49.2)	111 (24.0)	3.2 (2.4–4.3)
GSTM3 A/A	240 (81.0)	369 (82.0)	
A/B	51 (17.0)	72 (16.0)	1.07 (0.7–1.8)
B/B	6 (2.0)	9 (2.0)	1.1 (0.8–2.9)
GSTT1 +ve	243 (81.7)	395 (87.7)	
Null	54 (18.3)	55 (12.3)	1.6 (1.04–2.6)

Table III. *GSTM1* genotype frequency in cases and controls with different tobacco habits and cancer risk associated with lifetime exposure to tobacco modulated by the *GSTM1* null genotype

Habit cases/controls	Cases		Controls		OR (95% CI)
	GSTM1 null no. (%)	GSTM1 +ve no. (%)	GSTM1 null no. (%)	GSTM1 +ve no. (%)	
Bidi smoking 74/41	37 (50)*	37 (50)	10 (24)	31 (76)	3.7 (95% CI 1.3–7.9)[a]
⩽Median 40/19	21 (52.5)	19 (47.5)	3 (15.8)	16 (84.2)	ND
Greater than median 34/22	16 (47.0)	18 (53.0)	7 (31.8)	15 (68.2)	ND
Cigarette smoking 23/88	14 (61)*	9 (39)	21 (24)	67 (76)	5.7 (95% CI 2.0–16.3)[a]
⩽Median 7/49	4 (57.0)	3 (43.0)	15 (30.6)	34 (69.4)	ND
Greater than median 16/39	11 (68.7)	5 (31.3)	6 (15.4)	33 (84.6)	ND
Tobacco chewing 105/161	53 (50.5)*	52 (49.5)	34 (21)	127 (79)	3.7 (95% CI 2.0–7.1)[a]
⩽Median 23/110	10 (43.5)	13 (56.5)	25 (22.7)	85 (77.3)	2.5 (95% CI 0.9–7.1)[b]
Greater than median 82/51	43 (52.4)	39 (47.6)	9 (17.6)	42 (82.4)	4.6 (95% CI 1.9–11.4)[b]

[a]ORs adjusted over age (continuous variable) and exposure levels (two categories: ⩽ median, greater than median).
[b]ORs adjusted over age (continuous variable).
ND, not determined.

genotypes was similar in cases and controls (Table II). However, the frequency of the *GSTM1* null genotype in the patient group was markedly higher than in the control group (49.2 versus 24.0%, $P < 0.001$) and was associated with a 3-fold increase in risk for cancer of the buccal mucosa (OR 3.2; 95% CI 2.4–4.3).

The association between genetic susceptibility and exposure to the primary environmental risk factor for oral cancer—tobacco was also investigated. Information on lifetime tobacco exposure was available in 95% of cases and controls, the remaining cases and controls were not included in these analyses. Table III shows the OR associated with the *GSTM1* genotype, adjusted for age and lifetime tobacco exposure (⩽ median level versus greater than median level) within the three tobacco habit groups namely bidi smokers, cigarette smokers and tobacco chewers. Unconditional logistic regression analysis revealed that in bidi smokers the OR was 3.7 (95% CI 1.3–7.9), in cigarette smokers it was 5.7 (95% CI 2.0–16.3) and in tobacco chewers it was 3.7 (95% CI 2.0–7.1). The interaction *P* value for the regression analyses was $P < 0.06$.

Modification of oral cancer risk by exposure level could be studied for tobacco chewers only. As is seen in Table III, cancer risk associated with the *GSTM1* null genotype increased from 2.5 (95% CI 0.9–7.1) among chewers with less than median lifetime exposure to 4.6 (95% CI 1.9–11.4) in chewers with exposure greater than median lifetime exposure. The bidi and cigarette smoker groups could not be analyzed by logistic regression analysis with reference to the level of tobacco exposure, as the sample size was inadequate (Table III). From multivariate analyses, the interaction between any of the three polymorphisms was not found. This observation may be the result of a true lack of interaction or due to the effect of the sample size being reduced due to stratification.

Discussion

In this study, we examined the link between polymorphism at the *GSTM1*, *GSTM3* and *GSTT1* gene loci and susceptibility to oral cancer among Indian tobacco users. The results showed that the *GSTM3* and *GSTT1* genotypes did not influence susceptibility to cancer of the buccal mucosa, while the *GSTM1* null genotype emerged as a significant risk factor among Indian tobacco chewers as well as bidi and cigarette smokers.

To date 14 studies have examined the association of head and neck cancers with the *GSTT1* null genotype. As reviewed by Geisler and Olshan (22), six of these suggested an increase in cancer risk, with ORs ranging from 1.4 to 2.6, while the remaining reported ORs in the range of 0.5 to 1.2. There have been relatively fewer studies on the association of *GSTM3* genotypes with altered risk for head and neck cancers (13,23,24). No association was observed in those studies between the *GSTM3A* or *GSTM3B* allele and alteration in cancer risk. The lack of association between the GSTM3 alleles and oral cancer risk observed in this study is thus in agreement with that reported among other ethnic populations in the world.

Of the studies that examined the risk of head and neck cancers conferred by the *GSTM1* null genotype (as reviewed in ref. 22), 13 reported ORs of between 0.9 and 1.3, while the other eight reported ORs of between 1.4 and 3.9 (22). In the present study the *GSTM1* null genotype emerged as a significant risk factor for oral cancer with an OR of 3.2. A salient feature of our study, however, is that it attempted to examine the link between oral cancer risk conferred by the *GSTM1* null genotype in the presence of different types of tobacco exposure, and using satisfactory measurements of exposure. Among tobacco chewers, the risk conferred by the *GSTM1* null genotype was 3.7, and evaluation of a dose–response relationship, revealed a near 2-fold increase in cancer risk with increase in lifetime exposure to smokeless tobacco. Although, the substrates for the GSTM1 enzyme, in smokeless tobacco have not yet been identified, the significant increase in cancer risk implies that as in the case of tobacco smoke, the GSTM1 enzyme also inactivates carcinogens present in smokeless tobacco. Our results are in concordance with a recent study conducted in Thailand that reported an OR of 2.6 (95% CI 1.04–6.5) after adjusting for tobacco habits such as chewing of betel quid with tobacco (25). The ORs in the present study are however, much higher than those reported in studies on other ethnic groups in the world, and may be attributed to the ethnic differences in the frequency of the GSTM1 null genotype.

The frequency of GSTM1 null individuals (24%) obtained in this study is lower than that in Caucasian Americans (26)

and Japanese populations (26). Our finding is supported by similar frequencies (17, 33 and 36%) reported in Indians by other workers (26–28). A study on Indian betel quid/tobacco users has also suggested that the *GSTM1* null genotype is associated with an increased risk for the development of oral leukoplakia, a potentially malignant lesion (27). However, the present study is the only study that examines the effect of the *GSTM1* null genotype on the modulation of oral cancer risk in subjects who chewed tobacco without betel quid or smoked Indian bidis or cigarettes.

Our results also revealed that among the *GSTM1* null cigarette smokers, the risk for cancer of the buccal mucosa was ~6-fold higher than in those with the protective GSTM1 positive genotype. Lower risk with ORs ranging from 1.6 to 3.0 has been reported among African-American (14) and Japanese cigarette smokers (16). The higher risk seen in the present study appears to be related to the presence of higher amounts of benzo[*a*]pyrene in Indian bidis and cigarettes as compared with that in Western cigarettes (29,30).

Hung *et al*. (31) have demonstrated a dose–response relationship for oral cancer risk with respect to the *GSTM1* null genotype among subjects who chewed betel quid as well as smoked cigarettes. However, in those studies, risk assessment was based on exposure to smokeless tobacco only. We did not employ a similar approach to estimate oral cancer risk in individuals with both tobacco chewing and smoking habits as it does not provide a realistic estimate of the total exposure to tobacco. Nevertheless, the analysis of oral cancer risk conferred by the *GSTM1* null genotype in subjects with mixed habits requires the development of a suitable measure of total tobacco exposure.

To conclude, this is the first study that has identified the *GSTM1* null genotype as a risk factor for the development of oral cancer among Indian tobacco chewers and smokers. In a multifactorial disease like cancer, more than one genetic parameter would influence cancer causation by environmental agents. We are currently investigating the role of polymorphism in other metabolic and DNA repair genes in modulating oral cancer susceptibility in this population.

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