Genetic polymorphism at *GSTM1* and *GSTT1* gene loci and susceptibility to oral cancer

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GSTs are phase II enzymes which are involved in the detoxification of active metabolites of many potential carcinogens from tobacco smoke and therefore may play an important role in modulating susceptibility to tobacco related cancers. This study evaluates the influence of genetic polymorphisms of *GSTM1* and *GSTT1* gene loci on susceptibility to oral cancer. The genotyping was based on multiplex PCR assay that identified the *GSTM1* and *GSTT1* null (-/-) genotypes but didn't distinguish homozygous wild type+/+ and heterozygous +/- individuals. Genomic DNA was isolated from cases with oral cancer (n=40) and normal controls (n=87). The prevalence of the *GSTM1* null genotypes was 29/87 (33.3%) and 21/40 (52.5%) in controls and oral cancer cases, respectively but the differences were not significant (OR=2.2; 95%CI=0.96–5.1; p=0.06). The frequency of homozygous *GSTT1* null genotype in cancer cases was 17/40 (42.5%) as compared to 13/87 (14.94%) in controls and the differences were highly significant (OR=4.2; 95%CI=1.64–10.9; p=0.0002). Oral cancer cases had higher proportion of both *GSTM1* and *GSTT1* null genotypes as compared to controls but the differences were not statistically significant (OR=2.9; 95%CI=0.71–11.9; p=0.17). When individuals were categorized into two groups, no differences were observed for GSTM1 null genotype frequencies in control and cancer cases (OR=2.9; 95%CI=0.9–9.6; p=0.08) (OR=1.6; 95%CI=0.44–6.1; p=0.58) in ≤50 yrs and >50 yrs of age groups. Significant differences between control and cancer cases were observed for *GSTT1* null genotypes both in ≤50 yrs and >50yrs of age groups (OR=4.0; 95%CI=1.1–15.0; p=0.03) (OR=4.5; 95%CI=0.97–22.29; p=0.05), respectively. The effect of smoking on *GSTM1* null individuals was not found significant (OR=1.0; 95%CI=0.19–4.86; p=0.75) but it was significant in case of *GSTT1* null individuals (OR=6.33; 95%CI=1.0–44.1; p=0.02). Our results thus suggest that *GSTT1* gene polymorphisms modulate susceptibility to tobacco-related cancer of the oral cavity.

Key words: oral squamous cell carcinoma, epidemiology, Indian population, glutathione s-transferase, GSTM1, GSTT1, genetic polymorphism

Oral cancer is a malignancy of the lip, mouth, or tongue and 90–95% of oral cancers are comprised of squamous cell carcinoma. The incidence of oral cancer varies considerably worldwide, with the highest levels in South-East Asia, especially India and the lowest in Western Europe and North America [1]. Like most other cancers, oral cancer also affects the individuals in higher age group, most of the patients being over the age of 40. Literature suggests that tobacco consumption in all its various forms (smoking, chewing & snuff dipping etc.) is the commonest etiological risk factor for the subsequent development of oral cancer [2].

In recent years, evidences have accumulated to support the hypothesis that genetic polymorphisms in carcinogen-metabolizing enzymes may be of importance in determining individual susceptibility to cancer [3, 4]. It is known that glutathione S-transferase (GST) and cytochrome P450 (CYP) are the candidate genes for susceptibility in cancer since they catalyze the detoxification of many relevant electrophiles [5]. GSTs are a family of enzymes that play an important role in the prevention of cancer by detoxifying numerous potentially carcinogenic compounds. GSTs catalyze the conjugation of glutathione to electrophilic compounds resulting in glutathione conjugates, which are less reactive and more easily ex-

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Abbreviations: GST – glutathione s – transferase; SCC – squamous cell carcinoma; PAH – polycyclic aromatic hydrocarbons; PCR – polymerase chain reaction; CI – confidence interval.

creted. GSTs are also important for maintaining cellular genomic integrity and hence, may play an important role in cancer susceptibility [6].

Based on sequence homology and immunological cross reactivity, human cytosolic GSTs have been grouped into seven families, designated as GST Alpha, Mu, Pi, Sigma, Omega, Theta and Zeta [7]. In view of the importance of GSTs in cellular detoxification of carcinogens, genetic variants of GSTs have attracted the attention of epidemiologists with respect to cancer risk, of which *GSTM1* and *GSTT1* have been the most commonly studied genes. The GSTM subfamily is encoded by a 100 kb gene cluster and 1p13.3 arranged as 5'-*GSTM4-GSTM2-GSTM1-GSTM5-GSTM3* '[8]. *GSTM1* is involved in the detoxification of polycyclic aromatic hydrocarbons (PAHs) and other mutagens, and cells from *GSTM1* null individuals are more susceptible to DNA damage caused by these agents [5].

The GST theta subfamily consists of two genes *GSTT1* and *GSTT2*, which are located at 22q11.2 and separated by about 50 kb [9]. The GST theta polymorphism accounts for the variation in GST catalyzed metabolism of halomethanes by human erythrocytes [10]. Individuals with homozygous deletions of either the *GSTM1* locus or the *GSTT1* locus have no enzymatic functional activity of the respective enzyme and they are known as $GSTM1^{*}0$ and $GSTT1^{*}0$ or null alleles, respectively. Polymorphisms of *GSTM1* and *GSTT1* have been associated with differences in susceptibility to various forms of cancers, particularly those caused by cigarette smoking [5] in resistance to chemotherapy treatment in drug response [11] and in disease susceptibility and outcome [12]. Geographical and ethnic variations exist in genotype frequencies of both *GSTM1* & *GSTT1* allele [13]. Data is inconsistent with some studies reporting weak to moderate association and others, finding no elevation in risk [14] to develop oral cancer. In the present study an attempt has been made to study an association between genetic polymorphism of *GSTM1* and *GSTT1* and the risk of developing oral cancer.

Material and methods

Selection of cases and controls. Biopsies of 40 histologically proven incident oral cancer cases were collected from the Department of Radiotherapy and ENT Surgery of Lok Nayak Hospital, New Delhi, India after appropriate informed consent prior to any chemotherapy/ radiotherapy during a period from March to November 2002. Case subjects were diagnosed as having squamous cell carcinoma of oral cavity. The biopsies of oral cancer were collected in cold phosphate buffer saline (PBS) at 4 °C. The age of the male patients ranged from 6–75 yrs. The mean age being 47.9±19.2 with a median of 50 yrs, where as the ages of the female patients ranged from 48–70 yrs. The mean age being 58.8±8.1 with a median of 59 yrs. Peripheral blood was collected in heparinized vials from the controls, selected for the study. They were normal volunteers, who were not related to the patients or normal healthy individuals visiting hospital with the patients or the healthy volunteers from the Institute only. They were approximately in the same range as that of cases and without any history of chronic disease. The mean ±S.D. of age was 43.5±9.7 of males and 44.6±9.02 of females and median age of both male and female controls was 41 yrs. Information on smoking and alcohol habits was obtained. Cases and controls were of same socio-economic background.

DNA isolation. High molecular weight DNA from peripheral blood and oral cancer biopsy samples was isolated using standard procedures of proteinase K digestion and phenol-chloroform extraction [15].

Genotyping assays. The homozygous null polymorphisms of *GSTM1* and *GSTT1* genotypes were determined by using three sets of primers to amplify a 215 bp sequence of the *GSTM1* gene, a 480 bp sequence of *GSTT1* and a 350 bp sequence of albumin gene fragment, which served as an internal positive control [16].

The PCR primers were as follows:
GSTM1,
5'-GAACTCCCTGAAAAGCTAAAGC-3' and
5'GTTGGGCTCAAATATACGGTGG-3'
GSTT1,
5'-TTCCTTACTGGTCCTCACATCTC-3' and
5'-TCACCGGATCATGGCCAGCC-3';
Albumin,
5'-GCCCTCTGCTAACAAGTCCTAC-3' and
5'-GCCCTAAAAAGAAAATCGCCAATC-3'.

PCRs were performed in 25 µl reaction volume containing 50–100 ng of genomic DNA, 50 mM KCl 2.5 mM $MgCl_2$, 200 mM Tris-HCl (pH 8.4), 200 mM of dNTPs, GSTM1 primers at 3 µg each, GSTT1 primers at 1 µg each, albumin primers at 600 ng/ml each, and 1.5 units of Taq DNA polymerase (Bangalore Genei) in a Perkin-Elmer thermal cycler. After an initial denaturation at 96 °C for 5 min, amplification was carried out for 35 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min followed by final elongation at 72 °C for 7 min. The amplicon products of multiplex PCR for *GSTM1*, *GSTT1* and albumin were separated by electrophoresis in 3% agarose gel stained with ethidium bromide. The *GSTM1* homozygous null was evidenced by the absence of a 215 bp amplicon and of *GSTT1* homozygous null by the absence of a 480 bp amplicon. The presence of 350 bp albumin amplicon was an indicator of a successful PCR.

Statistical analysis. The data was tabulated and analyzed. The mean ±S.D. were estimated for quantitative data. In order to test for the significance in the proportion of presence or absence of *GSTM1* and *GSTT1* genotypes and effect of other factors like sex, age, smoking and alcohol status, in cases and controls, chi-square test of significance and Fisher's exact test were employed. Probability value of <0.05 was considered for statistical significance. Odds ratio (OR) and 95% confidence intervals (95%CI) were also estimated.

Results

Overall prevalence of GSTM1 and GSTT1 null genotypes. The study consists of 40 oral cancer cases and 87 controls. The results of the present study are summarized in Tables 1, 2, 3. The cases and controls showed a wide range of age distribution. The median age for male and female cancer cases being 50 and 59 yrs, respectively and was 41 yrs for both males and females in control group.

The prevalence of the *GSTM1* null genotype in cancer cases was 52.5% (21/40) while that of controls was 33.33% (29/87) (Tab. 3). Although an increased risk for *GSTM1* null genotype for oral cancer cases was observed as compared to controls, but the differences were not statistically significant (p=0.06). The OR was found to be 2.2 (95%CI=0.9–5.1).

A total of 42.5% (17/40) of oral cancer cases were having homozygous deletion of *GSTT1* genotype as compared to 14.9% (13/87) of the controls (Tab. 3). The differences in the frequencies of the *GSTT1* null genotype were significant between cancer cases and controls (p=0.002). The OR was found to be 4.2 (95%CI=1.6–10.9). The proportion of the cases that had null genotype for both the genes i.e. *GSTM1* and *GSTT1* were 15% (6/40) and 5.75% (5/87) in cancer cases and controls, respectively. Though the results indicate a higher proportion of oral cancer cases with both *GSTM1* and *GSTT1* null genotypes as compared to controls the differences were not statistically significant (p=0.17) (OR=2.9, 95%CI=0.71–11.9).

Prevalence of GSTM1 and GSTT1 according to age and sex. The mean age of cases and control in males was found to be 47.9±19.2 and 43.4±9.6 respectively, whereas in females it was 58.83±8.1 and 44.6±9.03 for cases and controls, respectively. It was observed that the female cases were nearly more than one decade higher in age as compared to controls. Hence, a stratified analysis according to age group was carried out. The individuals were categorized into two groups, up to 50 yrs and above 50 yrs of age. The proportion of GSTM1 null genotype up to 50 yrs of age group was found to be higher in cancer cases 57.9% (11/19) as compared to controls 32.2% (19/59). The difference in the proportions of null genotype up to 50 yrs of age was not significant (p=0.08) (OR=2.9; 95%CI=0.9–9.6) (Tab. 2). Similarly, in age group of above 50 yrs, the proportion of GSTM1 null genotype was 47.6% (10/21) in oral cancer cases as compared to 35.7% (10/28) in controls. In this age group also, the differences in the proportion of the null genotype were not found to be statistically significant between cases and controls (p=0.58) (OR=1.6; 95%CI=0.44–6.1) (Tab. 2).

Statistically significant differences were detected when the data were compared in controls and cancer cases for *GSTT1* genotype according to age group. Up to 50 yrs of age, the

Table 1. Frequency distribution of several variables and risk factors

		Oral cancer patients		Controls	
		Number	%	Number	%
Sex	Male	34	85.0	55	63.2
	Female	6	15.0	32	36.8
Age (years)	≤ 50	19	47.5	59	67.8
	> 50	21	52.5	28	32.2
Smoking status	Non-smoker	22	55.0	65	74.7
	Smoker	4	10.0	20	23.0
	Heavy smoker	14	35.0	2	2.3
Alcohol status	Current	7	17.5	11	12.6
	Never	33	82.5	76	87.4

Table 2. GST genotype frequencies of cases and controls stratified by selected factors

		Total cases	Total controls	*GSTM1* null (%)			*GSTT1* null (%)		
				cases	controls	*p* value	cases	controls	*p* value
Sex	Male	34	55	18 (52.9)	17 (30.91)	0.06	15 (44.12)	8 (14.54)	0.004
	Female	6	32	3 (50.0)	12 (37.5)	0.44	2 (33.33)	5 (15.62)	0.30
Age (years)	≤ 50	19	59	11 (57.9)	19 (32.2)	0.08	8 (42.1)	9 (15.25)	0.03
	> 50	21	28	10 (47.6)	10 (35.7)	0.58	9 (42.9)	4 (14.3)	0.05
Smoking status	non-smoker	22	65	16 (61.54)	21 (32.31)	0.002	10 (38.46)	10 (15.38)	0.009
	smoker	4	–	–	–	–	–	–	–
	heavy smoker	14	22	5 (35.7)	8 (36.4)	0.8	7 (50)	3 (13.6)	0.04
Alcohol status	current	7	11	4 (57.14)	4 (36.36)	0.35	4 (57.14)	6 (54.54)	0.65
	former	–	–	–	–	–	–	–	–
	never	33	76	17 (51.51)	25 (32.89)	0.10	13 (39.39)	7 (9.21)	0.005

Table 3. Genotype frequency of *GSTM1* & *GSTT1* in cases with oral cancer and controls

Genotypes	Controls (N=87)	Oral cancer cases (N=40)	OR	95%CI	*p* value
GSTM1 0/0	29 (33.3)	21 (52.5)	2.2	0.9–5.1	0.06
GSTM1 +/+ or +/–	58 (66.7)	19 (47.5)	1.0		
GSTT1 0/0	13 (14.9)	17 (42.5)	4.2	1.6–10.9	0.0002
GSTT1 +/+ or +/–	74 (85.1)	23 (57.5)	1.0		
GSTM10/0&GSTT10/0	5 (5.7)	6 (15)	2.9[a]	0.7–11.9	0.17

Figures in parentheses indicate percentages. [a]The other groups comprising of GSTM1 and GSTT1+ve individuals formed as reference category.

prevalence of homozygous *GSTT1* null genotype was 42.1% (8/19) in oral cancer cases as compared to 15.25% (9/59) in controls (p=0.03) (OR=4.0; 95%CI=1.1–15.01) (Tab. 2). Similarly above 50 yrs of age, the proportion of *GSTT1* null genotype in patients with oral cancer was higher 42.9% (9/21) to that found in healthy controls 14.3% (4/28) (p=0.05), (OR=4.5; 95%CI=0.97–22.29) and it was marginally significant.

In present data the number of males is higher 34(85%) as compared to females 6(15%) in cancer cases (Tab. 1). The frequency of GSTM1 null genotype was 18/34 (52.9%) and 17/55 (30.91%) in males and 3/6 (50%) and 12/32 (37.5%) in females in oral cancer and controls, respectively, the differences were borderline significant in males (p=0.06) (OR=2.51; 95%CI=0.95–6.71) and not significant in females (p=0.44) (Tab. 2). Similarly, in case of GSTT1, the differences were significant (p=0.004) in males but not in females (0.30) (Tab. 2).

Relative risk associated with smoking level by GSTM1 and GSTT1 genotypes. Only 14 individuals from cancer cases were heavy smokers (>40 pack years) and 7 were alcohol consumers. Four individuals were occasional smokers and the rest were non-smokers. Three individuals were pan/tobacco chewers. In controls, 22 males were smokers and 11 were alcoholics (Tab. 1). No female was either smoker or pan/tobacco chewer both in controls and cases.

The *GSTM1* null genotype prevalence was 35.71% (5/14) in cases and 36.36% (8/22) in control smokers. The differences for *GSTM1* null genotype between controls and cancer cases were not significant (p=0.8) (OR=1; 95%CI=0.19–4.86), where as in case of *GSTT1*, the differences between oral cancer cases and control smokers were significant (p=0.04) (OR=6.33; 95%CI=1.0–44.1) (Tab. 2).

In this study, alcoholics constituted a very small percentage of subjects i.e. 7 individuals in oral cancer cases and 11 in controls. The differences for *GSTM1* null genotype in both the groups i.e. current smokers (p=0.35) and those who never consumed alcohol (p=0.10) (OR=2.17; CI=0.87–5.43) were not significant. Similar results were observed for *GSTT1* null genotypes in current alcohol consumers (p=0.65) but in case of individuals who never consumed alcohol, the differences in *GSTT1* null gene frequency were highly significant (p=0.005) (OR=6.41; CI=2.03–20.86).

Discussion

Recent results have suggested that genetic polymorphisms of the phase II detoxifying agents (GSTs) may be the risk factors in the pathogenesis of oral cancer, as GSTs are involved in the metabolism of many carcinogens and environmental pollutants [17]. The genes coding for the enzymes *GSTM1* and *GSTT1* are polymorphic and the prevalence of *GSTM1* and *GSTT1* null genotypes varies in different populations. Ethnic differences in the baseline frequencies of the *GSTM1* null genotypes have been reported to vary between 23–45% in Africans, 39–62% in Europeans and 33–63% in East Asian populations [18]. The *GSTT1* null genotype varies from 10–18% in Caucasians [19, 20] to 58% in China [21]. So far there are only a few reports on the prevalence of these genotypes in oral cancer cases from India and they too are inconsistent. In our study, we observed that the overall prevalence of GSTM1 null genotypes was 33.33% (Tab. 3), which was relatively lower than that (~50%) reported in Caucasian populations and higher than Indian population from Mumbai (17%) and Trivandrum (24%) [22, 23]. Further, gender wise analysis showed homozygous *GSTM1* null genotype as 30.91% and 37.5% in males and females respectively and the differences were not significant (Tab. 2). We have not found any significant correlation of homozygous *GSTM1* null genotypes in controls and oral cancer cases (p=0.06) (Tab. 3). Our results are comparable to majority of the studies that have examined the risk of oral cavity squamous cell carcinoma and have found no association with the *GSTM1* deletion genotype [24–30). However, there are few studies showing positive association between *GSTM1* null genotypes and oral cancer [31–34, 22, 23]. A significant association has also been reported between *GSTM1* null genotypes and risk of oral cancer in most of the Japanese studies and this increase in incidence has been explained by the high frequency (>50%) of homozygous *GSTM1* null genotypes in Japanese population [35–37] but others have failed to observe this association in same population [38, 28]. There are few conflicting reports also showing increased risk of oral cancer and laryngeal cancer associated to the *GSTM1* non-null genotype [39, 40].

In present study, the frequency of *GSTT1* null genotype was 14.9% (Tab. 3) in all the controls, which was within the range as in Caucasians. When further analyzed according to gender it was found to be 14.54%, and 15.62% in males and females, respectively. The prevalence of *GSTT1* null genotype was less in Indian population as compared to *GSTM1*, which has also been reported earlier [41]. We observed significant differences in the frequency of *GSTT1* null genotypes, between oral cancer cases and controls (p=0.002) (Tab. 3). Our results are comparable to those of other studies, which have reported an increased risk of oral cancer due to

the presence of *GSTT1* homozygous null genotype [27, 32, 23, 34, 42]. Whereas, lack of association between *GSTT1* genotype and oral cancer cases has been reported in different ethnic groups [22, 24, 29, 33, 36].

GISLER and OLSHAN [14] reviewed 14 studies with head and neck cancer, six of which suggested increased risk, while others did not show any relation with *GSTT1* null genotype. The results of the recently published Meta and pooled analysis supported the notion of a greater risk of head and neck cancer when genotypes at multiple GST loci are considered [43]. The data presented here indicate that the incidence of homozygous null genotype of *GSTT1* with OR of 4.2 serve as a critical factor in oral cancer development.

Furthermore, we found that the subjects with null genotype for both *GSTM1* and *GSTT1* were 15% (6/40) in cases and 5.7% (5/87) in controls, where as NAIR et al [23] did not find any subject with homozygous null genotype for *GSTM1* and *GSTT1* suggesting it rare in South Indian population.

As there was difference of more than a decade in age so stratified analysis was done and two groups were selected i.e. ≤50 yrs and >50 yrs of age. There were no significant differences in the frequencies of the homozygous *GSTM1* null genotypes between oral cancer cases and controls in both the age groups (p=0.08, 0.58) but in case of *GSTT1* null genotypes the differences were highly significant in both the groups (p=0.03, 0.05) in ≤50 yrs and >50 years, respectively.

Link between chronic consumption of tobacco and/or alcohol and oral cavity cancer has been observed for many years. This observation has been supported by numerous epidemiological studies indicating that heavy smoking or drinking increases the risk of oral cavity cancer [44]. In our study, 14 oral cancer cases were heavy smokers and 22 controls were smokers. Till now, there are not many studies on the role of GSTs in oral carcinogenesis in relation with smoking. The results available from these case control studies are inconsistent. We found significant correlation of homozygous null genotypes of *GSTT1* null genotypes between controls and oral cancer smokers (OR=6.33; p=0.024) but not in case of *GSTM1*, where the differences were not significant (p=0.8). Others have also observed that heavy tobacco chewing increased the risk of oral cancer in individuals with *GSTT1* homozygous null genotype only [36, 2]. They also reported the risk of leukoplakia to be increased in individuals who were *GSTM1* null genotype only and were having life time exposure to tobacco smoking [36, 2] whereas others [45, 46] reported higher prevalence of both *GSTM1* and *GSTT1* null genotypes in leukoplakia cases with habit of tobacco chewing.

There are few studies reporting a significantly increased risk [31, 33, 22] lower risk [13, 48] and no association [13] for oral cancer among GSTM1 null individuals who smoked and consumed alcohol. A few studies did not find significant risk of oral cancer in GSTT1 homozygous null individuals, but reported increase in cancer risk with increase in lifetime exposure to smokeless tobacco [33, 37].

The results of all the studies reviewed are inconsistent, with some studies reporting weak to moderate associations with *GSTM1* and *GSTT1*, some studies finding no elevations in risk and others with negative correlations. Thus the evidence for the role of *GSTM1* and *GSTT1* and the risk of oral cancer is still inconclusive. This may be attributed, to the fact that study groups were taken from different ethnic populations and to the differences in exposure of different carcinogens in different populations To conclude, the data presented here indicate that the incidence of homozygous *GSTT1* null genotype is significant in oral cancer cases as compared to controls, but *GSTM1* null genotype results showed that this is not a critical factor in oral carcinogenesis. For any conclusive report further studies are required with greater number of cases, with follow-ups, and also to examine polymorphisms of other detoxifying genes.

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