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Full Length Research Paper

GST genotypes in head and neck cancer patients and its clinical implications

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Polymorphisms of carcinogen-metabolizing enzymes, known to be involved in metabolism of carcinogens found in tobacco smoke, are relatively common in most populations. Cigarette and bidi smoking has been demonstrated to increase the risk of head and neck cancers in our study group. This study evaluated the risk of head and neck cancers (HNC) in relation to two deletion polymorphisms of the glutathione *S*-transferase family *GST M1* and *GST T1*. We found that the null GST M1 and GST T1 genotypes were associated with an increased risk of developing head and neck cancers. We found smokers, with null genotypes of GST M1 (68.3%) and GST T1 (57.5%) were at a significantly higher risk of head and neck cancers. It is possible that the decreased activity of GST affects various functions like the mechanisms of DNA damage, including those mediated by tobacco and oxidative stress. The present study investigated whether homozygous gene deletions of *GST M1* and *GST T1* increases the incidence of head and neck cancers and explored the relationship between the *GST* genotype patients to clarify the multistep pathogenesis of these cancers based on this possible genetic predisposition. Based on these results it is also concluded that genotyping of GSTs would be a useful biomarker for determining the risk of head and neck cancers in bidi and cigarette smokers.

Key words: Polymorphisms, genotypes, glutathione S-transferases, head and neck cancer.

INTRODUCTION

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, monohalometahnes, etc. Subsequently, epoxide intermediates are formed, for example benzo (a) pyrenediol epoxide, which release free radicals and may bind and modify DNA. Therefore, variations in the expression of GSTs due to heritable genetic polymorphisms probably modulate the process of carcinogenesis by altering the exposure levels of tobacco-derived carcinogenesis. Five different groups of GSTs have been described with in the GST super family; α(A), μ(M), θ (T), π(P), and ζ(Zeta). Genetic polymorphisms relevant to altered GST expression have been demonstrated in GST M1, GSTM3, GST T1 and GSTP1 genes (Hecht et al., 1999; Ketterer et al., 1988; Bongers et al., 1995). GSTs conjugate glutathione to DNA-damaging electrophiles which render them hydrophilic and nontoxic.

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent diseases among the population throughout the world. Head and neck cancer (HNC) is commonly associated with tobacco use alone and also in combination with alcohol consumption. Genetic predisposition may play an important role for the development of cancer of the head and neck that is not fully understood at the present time (Notani, 2000). Environmental factors may interact with the host's genetic material resulting in an accumulation of a series of detrimental genetic alteration that lead to invasive cancer.

It is reported that approximately 50% of Caucasians (range 38-67%) lack the functional GST M1 allele (GST M1 null genotype). It is hypothesized that individuals with the GST M1 null genotype have an impaired ability to

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detoxify carcinogens resulting in a high risk for the development of head and neck cancer. A link between GST M1-null and laryngeal cancer was first suggested by Lafuente et al. (1993). Positive associations between GST M1-null genotypes and head and neck cancers have also been reported in subsequent case-control studies, the majority of which were conducted in Japan. Moderate relative risks that ranged from 1.5-3.0 over healthy controls were observed in their studies. In contrast, multiple other studies primarily in Caucasians, have failed to demonstrate an association between GST M1 and head and neck cancer. Racial differences appear to play an important role in determing the contribution of certain genotypes in genetic susceptibility for head and neck cancer. A relationship between GST M1-null and oral cancer was described in African-Americans but not in Caucasians in one study (Cotton et al., 2000; Rebbeck et al., 1999). Thus, there is a greater possibility that GST M1 and T1 null genotypes may result in a synergetic risk for carcinogenesis (Rebbeck et al., 1997).

Therefore the objective of this study was to assess the role of GSTs in head and neck cancers. Secondly, our aim was also to determine the genotype frequency of these two genetic polymorphisms (GST M1 and T1) and to assess their roles in relation to its susceptibility to head and neck cancer. In order to get a better insight into the association between the null genotypes of GST M1 and T1 and susceptibility to head and neck cancer, the frequencies of GST M1, and T1 null genotypes among Indian head and neck cancer patients were determined and compared with those of healthy control group.

MATERIALS AND METHODS

Enrolment of cases and controls

The present case control study comprised 120 cases with histopathological confirmed SCC of the head and neck cancers, 125 smokers, and 130 healthy controls. Both incident and prevalent cases of head and neck cancers were included in this study. All the patients were recruited from the out-patient department of the ENT-Hospital, Hyderabad from Jan 2006 to Dec 2007; in this study all 120 cases had SCC of the various head and neck parts.

Inclusion and exclusion criteria

The inclusion criterion for the cases was the presence of histopathologicaly diagnosed SCC of the head and neck cancers. Information on smoking and alcohol habits were obtained in a structured questionnaire and tabulated. 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 patient seeking treatment at the hospital, and from members of different community centers. After obtaining informed consent, all individuals were personally interviewed using the questionnaire. Information on age, gender, occupation, region of origin, type of tobacco used, daily frequency and duration of use was recorded. Data pertaining to histopathological diagnosis and clinical staging of the disease was collected from the hospital records. The cases reported habits such as smoking of bidi or cigarettes. The majority had a single habit some cases reported dual habits comprising different combinations of smoking and drinking.

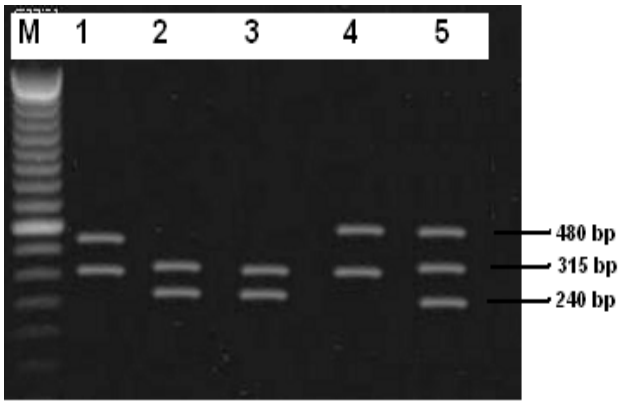

Figure 1. PCR products of GST M1 and GST T1 genotypes on gel electrophoresis. M shows 100 bp ladder. Samples 5 show T1M1, samples 1 and 4 show T1M0, samples 2 and 3 show T0M1.

Collection of blood samples

Venous blood samples (5 ml) were collected in syringes (heparinised) from patients and control groups (smokers and non-smokers). Immediately after collection, genomic DNA was extracted and used for polymerase chain reaction (PCR) to determine the genotypes.

Genotyping

The PCR conditions for determining GST M1 and GST T1 genotypes were the same as those reported previously by us and others (Haranatha and Kaiser, 2006; Pemble et al., 1994). For evaluating GST M1 and GST T1 polymorphisms, the amplified products were analyzed by gel electrophoresis (1.6% agarose). To test for contamination, negative controls (tubes containing the PCR mixture without the DNA template) were included in every run. A 240 bp fragment was amplified by PCR with the GST M1 primers while a 480 bp fragment was amplified with the GST T1 primers. The absence of an amplified product was consistent with the null genotypes. Successful amplification by β-globulin-specific primers confirmed the proper function of the PCR reaction (Figure 1).

The homozygous and null polymorphism of GST M1 and GST T1 were determined using a modified multiplex PCR approach for simultaneous replication of both genes for molecular analysis (Abdel-Rahman et al., 1996). The co-amplification of a 315 bp β-globulin gene fragment served as an internal positive control for a successful amplification reaction. The sequences for the primer pairs were as follows: GST M1, 5'-GAACTCCCTGAAAAGCTAAAGC-3'and 5'-GTTGGGCTCAAATATACGGTGG-3'.
GST T1, 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCC-3'. B-globulin, 5'GCCCTCTGCTAACAAGTCCTAC-3' and 5'-GCCCTAAAAAGAAAATCGCCAATC-3'
PCR was performed in a 50 µl reaction volume containing 50 mM KCL, 2.5 mM MgCl2, 200 mM Tris-HCL (pH 8.3), 200 mM dNTPs, GST M1 primers at 3 µg each, GST M1 primers at 1 µg, globulin primers at 600 ng/ml each and 50-100 ng of genomic DNA. Other conditions for running the PCR were the same as described by us earlier (Haranatha and Kaiser, 2006).

Statistical analysis

Allele frequencies were calculated by gene counting methods. Presence of the particular allele was designated as wild genotype and absence or deletion of the allele was designated as null genotype.

The association between GST M1 and GST T1 genotypes in head and neck cancer patients was analyzed using odds ratio (OR) and 95% confidence intervals (CI) derived from logistic regression analysis using EPI6 software (Epiinfo6 CDC). Because GST M1 and GST T1 genotypes may interact with each other in the development of head and neck cancer, further analysis combining the GST M1 and GST T1 genotypes was also carried out and their significance was determined by calculating the *p* values.

RESULTS

The category of the disease of the patients with head and neck cancers as per the diagnosis of the oncologists is given in Figure 2. It is seen from this data that within this study group we found that about 29% were suffering from cancer of Larynx followed by Hypopharynx 23%; Tongue about 17%; Epiglottis 8%; oropharynx 8%; Nasopharynx 7% and only 4% suffered with cancer of oral cavity and Paranasal sinuses. This distribution pattern may not be applicable globally, but in our study group, this pattern was observed for head and neck cancers (Figure 2). It is seen from Figure 2 that maximum cases were suffering from cancer of Larynx, (29%) followed by Hypopharynx (23%) and Tongue cases (17%) while the other types were 4-8%, as shown above.

Characteristics of the study and control group are presented in Table 1. From this data it was observed that the patients were mostly males between the age group of 28 to 80 years. The cigarette smokers were (53%) in both patient and non-patient category. The details of their personal smoking habits- Bidi or cigarettes are given in Table 1.

In Table 1 we present some of the characteristics of cases and controls. Besides age match, we also present smokers in both controls and cases; however there was only a small percentage of female participation in this study. We have also recorded the number of cigarettes/ bidi's the group was using, in both controls and cases (Table 1). This data have some correlation with genotypes; hence we have included the details.

Genotyping of the GST M1 and GST T1

The genotype distribution as analyzed by PCR, is presented in Table 2. We also analyzed the genotype frequencies with respect to the smoking habits of the patients and the controls. These tables show the comparative distribution of the genotypes of GSTs between patients and controls. It is seen that there is a marked significant difference between patients and controls in the GST M1 genotypes, but not in the GST T1 genotypes. It is seen from the data presented in these tables that similar results were obtained when we compared the patients with smokers.

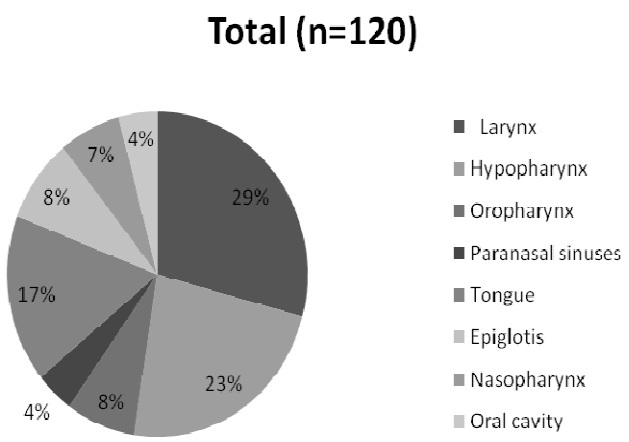

Figure 2. Regional distribution of the disease of patients with head and neck cancers as per diagnosis.

Table 1. Characteristics of the study and control group (smokers).

Characteristics	Patients	Controls
Age in years	55.2±0.8	51.3±0.72
Male/female	113/7	122/3
Bidi smoking	67	70
Frequency	16.2±0.8	19.2±0.8
Duration in years	15.9±01.1	14.5±1.5
Cigarette Smoking	53	55
Frequency	14.1±2.0	10.5±0.7
Duration in years	22.7±1.3	15.1±0.9

The frequencies of GST M1 and GST T1 variants (Table 2) are presented in Yates corrected Chi-square and p-values. The frequency of the cases and controls in wild type and null genotypes in the total population of GST M1 and GST T1 were determined. In null type, the frequency of GST M1 and GST T1 in patients was 82 (68.3%) and 69 (57.5%) and in smokers 70 (56%) and 59 (47.2%), respectively.

In Table 2a, the genotype frequency of smoking controls (of GST M1 and GST T1) is compared to patients with head and neck cancers. It was found that 25 (20%) were GST M1 and 20 (15.38%), were GST M1 healthy controls. These patients had much higher frequency of the disease compared to the non-smoking controls (68.3% and 57%) for GST M1and GST T1, respectively.

The distribution pattern of genotypes between non-smoking and smoking controls is presented in Table 2b. It is seen that null type frequency in GST M1 is higher (56%) when compared to healthy controls. Whereas there were less number of GST T1 null genotypes (47.2 and 15.4%) compared to the wild type genotypes (53 and 84%).

Table 3a shows the OR associated with the GST M1 genotypes, adjusted for age and lifetime tobacco expo-

Table 2. Distribution of GST M1 and GST T1 null genotypes in head and neck cases and controls with smoking habits.

Genotype	Patients (%) n = 120	Smokers (%) n = 125	OR (95% CI)	χ2	p- value
GST M1					
H/H	38(31.6)	55(44)	1.0 (NA)	NA	NA
Null	82 (68.3)	70(56)	1.70 (0.97-2.96)	3.95	0.04674
GST T1					
H/H	51(42.5)	66 (52.8)	1.0 (NA)	NA	NA
Null	69 (57.5)	59 (47.2)	1.5 (0.89-2.57)	2.60	0.1

OR, Crude odds ratios; CI, confidence interval with 95% of probability; NA, not applicable.

Table 2a. Difference in genotypes between patients and non-smoking controls.

Genotype	Patients (%) n = 120	Non-smokers (%) n = 130	OR (95% CI)	χ2	p - value
GST M1					
H/H	38 (31.6)	105 (80.7)	1.0 (NA)	NA	NA
Null	82 (68.3)	25 (19.3)	9.06 (4.88-16.96)	61.45	0.0000
GST T1					
H/H	51 (42.5)	110 (84.1)	1.0 (NA)	NA	NA
Null	69 (57.5)	20 (15.38)	7.44 (3.93-14.19)	48.0	0.0000

OR, Crude odds ratios; CI, confidence interval with 95% of probability; NA, not applicable.

Table 2b. Difference between the control genotypes with smoking and nonsmoking habits.

Genotype	Smokers (%) n = 125	Non-smokers (%) n = 130	OR (95% CI)	χ2	p - value
GST M1					
H/H	55 (44)	105 (80.7)	1.0 (NA)	NA	NA
Null	70 (56)	25 (19.3)	5.35 (2.94-9.76)	36.86	0.0000
GST T1					
H/H	66 (52.8)	110 (84.1)	4.92 (2.62-9.3)	NA	NA
Null	59 (47.2)	20 (15.38)		30.17	0.0000

OR, crude odds ratios; CI, confidence interval with 95% of probability; NA, not applicable.

sure (≤ median level versus greater than median level) within the two tobacco habit groups namely bidi smokers and cigarette smokers. Unconditional logistic regression analysis revealed that for GST M1 in bidi smokers, the OR was 2.43 and in cigarette smokers it was 1.10, respectively. Modification of head and neck cancer risk by exposure level could be determined for bidi smokers only. As seen in Table 3a cancer risk associated with GST M1 null genotype decreased from 3.29 (95% CI 1.21 to 9.08) among bidi smokers with less than median life time exposure 0.88 (95% CI 0.31-5.45). The cigarette smokers group could not be analyzed by logistic regression analysis because of less sample size.

Table 3b also shows the OR associated with the GST T1 genotypes, adjusted for age and lifetime tobacco expo- sure (≤ median level versus greater than median level) within the two tobacco habit groups. Unconditional logistic regression analysis revealed that for GST T1 in bidi smokers the OR was 1.98 and in cigarette smokers it was 1.08, respectively. As seen in Table 3b cancer risk associated with GST T1 null genotype increased from 1.47 (95% CI 0.56-3.87) among bidi smokers with less than median life time exposure 2.97 (95% CI 0.86 - 10.58). In this also cigarette smokers group could not be analyzed by logistic regression analysis because of small sample size.

Combined effect of GST M1 and GST T1 null genotypes in head and neck cases and smokers was determined. Our results show a significant odds ratio (2.82) in patients with combined deletions (GST M1 Null -- GST T 1Null) of both GSTs when compared with single gene deletion (GST M1 null -GST T1 H/H and GST M1 H/H-GST T1 null) for that the OR was 2.53 and 2.35, respectively (Table 4).

Table 3a. GST M1 genotype frequencies among cases (bidi and cigarette) and control smokers.

Habits cases/controls	Cases (n=120)		Smokers (n=125)		OR (95% CI)	p- value
	Null no (%)	+ ve no	Null no (%)	+ ve no		
Bidi smoking (67/70)	49 (40.8)	18 (15)	37 (29.6)	33 (26.4)	2.43 (1.12-5.30)	0.01
≤Median (37/45)	23 (19.1)	14 (11.6)	15 (12)	30 (24)	3.29 (1.219.08)	0.009
Greater than median (30/25)	26 (21.6)	4 (3.3)	22 (17.6)	3 (2.4)	0.88 (0.31-5.45)	0.88
Cigarette Smoking (53/55)	33 (27.5)	20 (16.6)	33 (26.4)	22 (17.6)	1.10 (0.47-2.57)	0.80
≤Median (33/42)	20 (16.6)	13 (10.8)	24 (19.2)	18 (14.4)	1.15 (0.41-3.24)	0.76
Greater than median (20/13)	13 (10.83)	7 (5.83)	9 (7.2)	4 (3.2)	ND	ND

OR, Crude odds ratios; CI, confidence interval with 95% of probability; ND, not determined.

Table 3b. GST T1 genotype frequencies among cases (bidi and cigarette) and control smokers.

Habits cases/controls	Cases (n=120)		Non-cancerous smokers (n=125)		OR (95% CI)	p- value
	Null no (%)	+ ve no	Null no (%)	+ ve no		
Bidi smoking (67/70)	41 (34.1)	26 (21.6)	31 (24.8)	39 (31.2)	1.98 (0.95-4.16)	0.047
≤Median (37/45)	20 (17.5)	17 (14.1)	20 (16)	25 (20)	1.47 (0.56-3.87)	0.386
Greater than median (30/25)	20 (17.5)	10 (7.5)	11 (8.8)	14 (11.2)	2.97 (0.86-10.5)	0.051
Cigarette Smoking (53/55)	28 (23.3)	25 (20.8)	28 (22.4)	27 (21.6)	1.08 (0.47-2.47)	0.84
≤Median (33/42)	17 (14.1)	16 (13.3)	18 (14.4)	24 (19.2)	1.42 (0.51-3.93)	0.455
Greater than median (20/13)	11 (9.1)	9 (7.5)	10 (8)	3 (2.4)	ND	ND

OR, Crude odds ratios; CI, confidence interval with 95% of probability; ND, not determined.

Table 4. Combined effects of GST M1 and GST T1 null genotypes in Head and neck cases and smoker controls.

Genotype	Patients (%) n = 120	Non-cancerous smokers (%) n = 125	OR (95% CI)	$\chi 2$	p- value
GST M1 H/H -GST T1 H/H	17 (14.2)	38 (30.4)	NA	NA	NA
GST M1 Null - GST T 1 Null	48 (40)	37 (29.6)	2.82 (1.31-6.15)	8.37	0.003
GST M1 Null - GST T 1 H/H	34 (28.3)	30 (22.4)	2.53 (1.08-5.79)	6.79	0.01
GST M1 H/H - GST T1 Null	21 (17.5)	20 (16)	2.35 (0.94-5.93)	4.05	0.04

OR, Crude odds ratios; CI, confidence interval with 95% of probability; NA, not applicable.

DISCUSSION

Tobacco smoking is the strongest risk factor for head and neck cancers (HNC) besides lung cancer. Similarly alcohol consumption is also linked to an increase in HNC. GSTs are a family of enzymes that play an important role in the detoxification of carcinogens found in tobacco smoke. Metabolism of these components from smoke involves a balance activation steps that produce reactive intermediates, activation is generally mediated by P-53 pathway and can result in the compounds that may bind to DNA covalently forming adducts. Subsequently accumulation of these adducts at critical loci like P-53, or oncogenes can lead to mutations and disrupt the cell cycle.

The present study was conducted to look into the relationship between the genetic polymorphism of glutathione S-transferase, which is involved in the metabolism of genotoxins like polycyclic aromatic hydrocarbons which are highly prevalent in cigarette and bidi smokers. There are some studies on the association of GST polymorphism within the literature with respect to their association with head and neck cancers; but more interestingly, no report from India has been published. It is therefore evident that patients or controls that do not have the ability to produce the GST M1 enzyme are at a higher risk for head and neck cancers. The difference between the two GSTs (M1 and T1) is their metabolizing capacity. GSTM conjugates predominantly epoxies, quinones (Vineis et al., 1999; Landi et al., 2000) and polycyclic aromatic hydrocarbons, which are common carcinogens, found in tobacco smoke, food, and combustion fumes. GST T1 metabolizes epoxybutanes, monohalomethanes, and certain alkyl halides (Hayes et al., 1995; Bruhn et al.,

1998).

GST M1 appears to be is more important in the cancer progression of the head and neck cancers. However we did not find significant difference in the genotypes between the bidi or cigarette smoking individuals. Genetic polymorphisms of the genes coding for the enzymes involved in the metabolism of genotoxins result in different phenotypes with respect to their ability to detoxify these agents.

The μ (GST M1) and θ (GST T1) members of the GST multigene family, which are polymorphic in humans, are involved in detoxifying mutagenic electrophilic compounds, and an increased frequency of these GST gene deletions have been associated with several malignancies (Salagovic et al., 1998; Bell et al., 1993; Lin et al., 1998). The polymorphism in the GST T1 and GST M1 gene loci is caused by the gene deletion. This results in virtual absence of enzyme activity in individuals with the GST T1 and GST M1 null genotypes. This has been confirmed by phenotype assays that have demonstrated 94% or more concordance between genotype and phenotype (Zhong et al., 1991; Bruhn et al., 1998). The polymorphisms affecting the activity of this enzyme may affect the processes contributing to the development of the disease.

This investigation has analyzed in depth the genotype frequencies with respect to several parameters as described in the series of tables presented here. The results indicated head and neck cancer patients had higher frequencies of null genotypes (of both GSTs M1 and T1) as compared to non-smoking controls, but the smokers (control group) were at higher risk to the disease. When we compared the smokers and non-smokers controls, we found that the GST deletion mutations were very high, indicating their risk factor (2 times higher). As tobacco smoking is known to induce DNA damage, the relationship between polymorphism of enzymes involved in the metabolism of genotoxins with development of head and neck cancers have been specifically evaluated in this study.

To date 15 studies have examined the association of head and neck cancer with the GST T1 null genotype. As reviewed by Geisler and Olshan (Geisler et al., 2001) 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 GST M3 genotypes with altered risk for head and neck cancers. This investigation also determined the difference between the 2 types of smoking materials i.e. the cigarettes and bidi's (which are raw tobacco rolled in tobacco leaf). It was seen that bidi smokers were at a greater risk for getting the disease, compared to cigarette smokers.

The higher risk seen in the present study appear to be related to the presence of higher amount of benzo[a]pyrene in Indian bidi and cigarette as compared with that in western cigarettes (Pakhales et al., 1900; Sanghvi et al., 1980). However the present study is the only study that examines the effect of the GST M1 and GST T1 null genotypes on the modulation of head and neck cancer in subjects who smoked Indian bidis or cigarettes.

Of the studies that examined the risk of head and neck cancers conferred by the GST M1 null genotypes (Harada et al., 1992), 13 reported ORs of between 0.9 to 1.3 while the other eight reported ORs of between 1.4 and 3.9 (Harada et al., 1992). In the present study the GST M1 null genotypes emerged as a significant risk factor for head and neck cancer with 1.70 OR. A salient feature of our study is that it attempted to examine the link between head and neck cancer risk conferred by the GST M1 null genotype in the presence of different types of tobacco exposure. Among bidi smokers, the risk conferred by the GST M1 null genotype was 3.29 and evaluation of a close response relationship, revealed a 1.79 fold increase in cancer risk. The ORs in the present study were, also higher than those reported in other studies on other ethnic groups in the world, and may be attributed to the ethnic differences in the frequency of the GST T1 and GST M1 null genotypes.

In a multifactorial disease like cancer, more than one genetic parameter would influence cancer causation by environmental agents. So we studied combined effect of the deletions in GST M1 and GST T1, which showed that the OR was 2.82 and individual incidence of deletions in GST M1 and GST T1 genes showed ORs of 1.7 and 1.5 respectively in the head and neck cancer patients (Table 2). These results revealed a significantly elevated risk of developing head and neck cancers in individuals with combined GST M1 and GST T1 gene deletions (Table 4). Because tobacco exposure involves multiple chemical substrates of both GSTs, the possibility should be considered that combined deletions of GST M1 and GST T1 interact to produce greater risk of head and neck cancers. Our results showed a significant odds ratio (2.82) in patients with combined deletions of both GSTs.

This is the first report that has identified the GST M1 and GST T1 null genotypes as a risk factor for the development of head and neck cancers in Indian bidi and cigarette smokers. Therefore genotyping of GST-polymorphisms could be useful biomarker in determining the risk factor for head and neck cancers.

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