Copy number polymorphism of glutathione-S-transferase genes (*GSTM1 & GSTT1*) in susceptibility to lung cancer in a high-risk population from north-east India

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Background & objectives: Genetic polymorphisms in glutathione-S-transferase genes (*GSTM1* and *GSTT1*) have been studied intensively for their potential role in lung cancer susceptibility. However, most of the studies on association between the polymorphisms and lung cancer do not distinguish between genotypes with one or two copies of the genes. The present study investigates the gene dosage effects of *GSTT1* and *GSTM1* copy number and their environmental interactions to examine the association of lung cancer risk with trimodular genotypes of the *GSTs* in a high-risk population from north-east India.

Methods: A total of 154 lung cancer cases and 154 age and sex matched controls from the high risk region of north-east India were analyzed by multiplex real-time PCR to determine the trimodal genotypes (+/+, +/- and -/-) in both the genes (*GSTM1* and *GSTT1*).

Results: No significant association and gene dosage effect of *GSTM1* gene copy number with lung cancer risk (P_{trend}=0.13) were found. However, absence of *GSTT1* conferred 68 per cent (OR=0.32;95%CI=0.15-0.71;P=0.005) reduced risk compared to the two copy number of the gene. There was evidence of gene dosage effect of *GSTT1* gene (P_{trend}=0.006). Tobacco smoking was a major environmental risk factor to lung cancer (OR=3.03;95%CI=1.73-5.31;P<0.001). However, its interaction with null genotype of *GSTT1* conferred significant reduced risk to lung cancer (OR=0.30;95%CI=0.10-0.91;P=0.03). Further in only tobacco smokers, null genotype was associated with increased reduced risk [0.03(0.001-0.78)0.03; P_{trend}=0.006]. No effect modification of *GSTM1* was observed with lung cancer risk by environmental risk factors.

Interpretation & conclusions: The results suggest that absence of *GSTT1* null genotype may be associated with a reduced risk of lung cancer and the effect remains unchanged after interaction with smoking.

Lung cancer is the leading cause of cancer-related deaths worldwide[1]. In India, it is the most frequent cancer amongst men[2]. In north eastern (NE) part of India lung cancer is among the ten leading sites of cancer, with the highest age-adjusted incidence rate (AAR) in Mizoram State (24.5 in males and 26.3 in females). Aizwal district alone showed an AAR of 36.0 in males and 38.7 in females which is almost three to ten times higher than Delhi[3]. High incidence rates suggest role of both genetic as well as environmental factors such as smoking, tobacco use and dietary carcinogen consumption.

Tobacco smoking remains the primary aetiological factor associated with the development of lung cancer accounting for nearly 80-90 per cent of the disease. Polycyclic aromatic hydrocarbons (PAHs) particularly benzo[a]pyrene (BaP) and nitrogen containing nitrosamines and aromatic amines are main carcinogens present in tobacco smoke that are implicated in lung carcinogenesis. Non-smoking tobacco and betel quid have also been implicated in lung carcinogenesis probably due to their accompanied consumption with smoking[4]. Deleterious effects of tobacco carcinogens are primarily mediated through DNA adduct formations following their activation in the detoxifying pathways. Activated PAHs and N-nitroso compounds produced by phase I xenobiotic metabolizing enzymes are substrates for the glutathione-S-transferase M1 and T1 (*GSTM1* and *GSTT1)* phase II enzymes.

Evidences are available in literature on association of *GSTM1* and *GSTT1* genotypes with lung cancer[5,6]. Most of these studies have compared the "null" genotype with the "non-null" genotype and thus do not distinguish between one and two copy number of the genes. However, studies have reported a trimodal phenotype distribution for both *GSTM1* and *GSTT1* identifying homozygous wild type (+/+), hemizygous (+/-) and null (-/-) genotypes of the genes[7,8]. These studies suggest a gene dosage effect with three alleles corresponding to fast, intermediate and slow enzyme activity. Enzymatic activity of *GSTT1* has been reported to be varying with the copy number of the gene[9]. Sprenger *et al*[7] in their genotype-phenotype comparison showed correlation of significantly increased enzyme activity in individuals with two copy number of the *GSTT1* compared to those with one copy number. Roodi *et al*[10] showed that the relative risk of breast cancer increased with the present allele (+/- and +/+ genotypes) compared with -/- genotype, however, this trend was not significant.

Several methods (standard and long-range PCR) in the past have been used for distinguishing *GSTM1*[10,11] and *GSTT1*[7,11,12] alleles into three genotypes. These methods were primarily based on the fact that the two genes are flanked by highly homologous regions. Recent studies have used Taqman based real-time PCR assays to discriminate between the wild-type, hemizygous deletion, and homozygous deletion of the *GSTM1* and *GSTT1* genes[13,14]. In our previous report on association of GST polymorphisms, comparing the null genotype (-/-) with combined non-null genotype (+/- and +/+) using traditional multiplex PCR-gel electrophoresis method in high risk north-east Indian population, we showed a significant protective effect of *GSTM1* and *GSTT1* null genotypes in lung cancer[15]. The present study was conducted to examine the relationship between *GSTM1* and *GSTT1* genes and lung cancer risk by assessing potential gene dosage effects and gene-environment interactions.

Material & Methods

Study subjects: This study consisted of 154 histopathologically diagnosed lung cancer cases registered at Dr. Bhubaneswar Borooah Cancer Institute, Guwahati, Assam and Civil Hospital, Aizawl, Mizoram and Sir Thutob Namgyal Memorial Hospital, Gangtok, Sikkim, the collaborating centers in north east India. Incident cases with lung as primary site of cancer, during the period of December 2006 to 2009 and willing to participate in the study were included. An equal number of voluntary, age and sex matched individuals with no history of any obvious disease were selected as controls (n=154) from the unrelated attendants who accompanied cancer patients. This provided a readily available and cooperative source of controls from the same socio-economic background as the cases reducing confounding biases.

The sample size was determined based on power calculation methods from evidences provided by our research group on association between GSTs and lung cancer[15], the minimum sample size calculated was 144 at 5 per cent level of significance and 80 per cent power. Demographic data and characteristics such as age, sex, smoking habit, usage of tobacco, betel quid and alcohol, were obtained from subjects in a standard questionnaire used for all the centers, in an in-person interview by a trained data collector. A majority of cases and controls were literate with full primary schooling and some upto the college level. The occupational history of the study participants revealed that most of them were farm labourers or engaged in petty jobs with

no exposure to any occupational hazards. Patients with only lung as their primary site of cancer were included. Any subject with history of familial malignancy or pulmonary infectious disease was excluded both from case and control. Final selected controls were included on the basis of no history of any obvious disease and those not taking any medication at the time of recruitment. All subjects provided written informed consent for participation in this research which was done under a protocol approved by the institutional ethics committee of Regional Medical Research Centre, North East Region, Guwahati (Indian Council of Medical Research). Smokers, chewers and drinkers were classified into two categories ever and never. For smoking, an individual who had never smoked or smoked less than 100 cigarettes in his/her lifetime and was not smoking at the time of reporting was considered never smoker or non-smokers. Ever smokers or smokers category included current smokers, and those who had quit within <1 year of reporting[16]. Blood sample (5 ml) was collected in EDTA vials and stored under -70^{0}C until processed.

DNA isolation: Genomic DNA was isolated using Qiagen Blood DNA Isolation kit (Qiagen GmbH, Germany) and stored at -30^{0}C till further analysis. DNA quantification was done using Nano-drop ND-1000 Full–spectrum UV/Vis spectrophotometer (NanoDrop Technologies, USA); 5 μl (20 ng) of DNA solution was used for each real-time PCR reaction.

Quantitative real-time TaqMan PCR for GSTM1 and GSTT1 copy number determination: *GSTM1* and *GSTT1* genotyping was performed using Taqman Gene Copy Number Assays purchased from Applied Biosystems (Foster City, California, USA). TaqMan gene copy number assays (*GSTM1*:Hs no Hs02595872_cn and *GSTT1*: Hs no Hs00817631_cn) were run simultaneously with a TaqMan Copy Number reference assay (RNase P: Part No. 4403326) in a duplex real-time PCR. Each 20 μl assay mixture containing 20 ng of genomic DNA (5 μl) was prepared according to protocols developed by Applied Biosystems for copy number detection. Real-time PCR reactions were run on ABI PRISM 7000 Sequence Detection System (Applied Biosystems CA, USA) Each reaction was run in duplicates. In addition, a no-template control was also included in each run to rule out any contamination. Universal thermal cycling conditions were used, *i.e* 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Real-time data were collected by the SDS 1.0 software (Applied

Biosystems, CA, USA). The number of copies of the target sequence in each test sample was determined by relative quantitation (RQ) using the comparative CT (ΔΔCT) method[17]. This method measures the CT difference (ΔCT) between target and reference gene, and then compares the ΔCT values of test samples to a calibrator sample which is known to have two copies of the target sequence. The copy number of the target is calculated to be two times the relative quantity. A sample homozygous for *GSTM1* and *GSTT1* wild type allele (2 copy number) was used as calibrator. The samples employed as calibrators were previously confirmed to have two copies of the genes examined.

Validations of copy number by PCR method: For validation of copy number estimation done through real-time PCR, 30 per cent of the samples were reanalyzed through PCR-gel electrophoresis methods. Copy number detection of *GSTM1* was done through a two-step method. Samples showing presence of *GSTM1* gene in multiplex PCR described in our previous report[15] were reanalyzed for detection of null allele using primer described for *GSTM1* null allele by Buchard *et al*[11]. Samples showing amplification of the 4748 bp null allele were considered as hemizygous genotype (1 copy) and those with no amplification were considered carrying two copies of *GSTM1* gene. A sample with null *GSTM1* genotype was included as positive control in each PCR. Genotypes of *GSTT1* were detected through multiplex PCR described by Naito *et al*[12]. Amplification products of 566 and 458 bp represented null and present alleles of *GSTT1* respectively. The results from PCR-gel electrophoresis method were in complete concordance with those from real-time PCR.

Statistical analysis: The association of *GSTM1* and *GSTT1* genotypes with lung cancer was evaluated by multivariate conditional logistic regression. The association of tobacco smoking, tobacco chewing, betel quid chewing, and alcohol intake with disease development was assessed by chi square/Fisher's exact test. Estimates of cancer risk imparted by *GSTM1* and *GSTT1* genotypes and other covariates such as tobacco smoking, chewing, betel quid chewing, and alcohol were determined by deriving the odds ratio (OR) and corresponding 95 per cent confidence intervals (95% CIs) using univariate and multivariate conditional logistic regression models. To evaluate the putative modifying effects of the GST genotypes on the effects of environmental factors, stratified analysis was performed for subjects positive for

individual risk factors. For all the tests, a two-sided $P<0.05$ was considered significant. The data analysis was performed on STATA 8.0 software (State-Corp LP, College Station, TX, USA).

Results

A total of 154 lung cancer cases and 154 controls were successfully genotyped for polymorphism in *GSTT1* and *GSTM1*.The distribution of gender and ethnicity was similar for cases and controls. Males were overrepresented in the study compared to females (M/F ratio: 3.05). Mean ages of cases and controls were 59.16 ± 9.95 (range 35-80 yr) and 60.39 ± 10.43 (range 38-85 yr), respectively (Table I). Distribution of both *GSTM1* and *GSTT1* genotypes was in agreement with Hardy-Weinberg equilibrium (HWE) in controls ($P>0.05$), however, significant deviation of *GSTT1* genotypes from HWE was seen in cases ($P=0.01$).

Association of genetic and environmental factors with lung cancer risk: The distribution of environmental risk factors and genotypes in cases and controls and their association with lung cancer risk are summarized in Table II. Risk habits such as smoking, tobacco chewing and betel quid chewing were more common among the cases compared to controls. Smokers constituted 68.2 per cent of the cases and 46.1 per cent of the controls, thus smoking was associated with a significant risk of lung cancer (OR=3.03; 95%CI=1.73-5.31; $P<0.001$). Betel quid chewing was present in 77.3 per cent of the cases and 57.1 per cent of controls, with the habit conferring greater than 2 fold risk to chewers compared to non-chewers (OR=2.39; 95% CI=1.38-4.16; $P=0.002$).

Frequency of *GSTM1* wild-type and null alleles in the control population was 0.35 and 0.64, respectively. Distribution of wild type (+/+) two copy, hemizygous deletion (+/-) one copy and homozygous deletion (-/-) null copy of *GSTM1* genotypes was 20.8, 42.2 and 37.0 per cent in cases and 14.3, 42.9 and 42.9 per cent in controls (Table II). Compared to individuals with two copy (+/+) genotype, the relative risk of lung cancer was 0.73 (95% CI = 0.37-1.44; $P = 0.37$) for the hemizygous genotype (+/-) and 0.62 (95% CI=0.31-1.23; $P=0.17$) for the null genotype (-/-). There was no evidence of gene dosage effect for *GSTM1* ($P_{trend}=0.13$). In contrast *GSTT1* the wild type (+/+) two copy number and hemizygous one copy number genotype was more frequent in cases than controls (27.3 vs 19.5% and 58.4 vs 53.2%, respectively). Patients with null genotype conferred 68 per cent (OR = 0.32; 95% CI = 0.15-0.71;

Table I. Distribution of demographic variables for lung cancer patients and controls

	Cases N (%)	Controls N (%)
Sex(M/F)	38/116	38/116
Age (yr)		
Mean age (±SD)	59.16 ± 9.95	60.39 ± 10.43
Range	(35-80)	(38-85)
Ethnicity		
Guwahati	100 (64.93)	100 (64.93)
Sikkim	29 (18.83)	29 (18.83)
Aizawal	25 (16.23)	25 (16.23)
Smoking status*		
Non-smokers	49 (31.8)	83 (53.9)
Smokers	105 (68.2)	71 (46.1)
Tobacco chewing		
Non-chewers	74 (48.1)	82 (53.2)
Chewers	80 (51.9)	72 (46.8)
Betel quid chewing*		
Non chewers	35 (22.7)	66 (42.9)
Chewers	119 (77.3)	88 (57.1)
Alcohol consumption		
Non-alcoholic	114 (74.0)	109 (70.8)
Alcoholic	40 (26.0)	45 (29.2)

*$P<0.001$ cases vs controls

$P=0.005$) reduced risk compared to patients with two copy number of *GSTT1*. When risk associated with null genotype was compared with one copy number (hemizygous) of the gene it reduced to 51 per cent (OR = 0.49; 95% CI = 0.25-0.95; $P = 0.03$) (data not shown). Decreasing copy number of *GSTT1* gene showed a positive dose relationship with lung cancer ($P_{trend}=0.006$). A comparison of our data according to the classical 'null' versus the 'non null' genotype have attenuated the protective effect of *GSTT1* null genotype from 68 to 45 per cent (OR = 0.55; 95% CI = 0.30-1.00; $P=0.05$) (Table II).

Gene-gene interaction: To elucidate gene-gene interactions associated with lung cancer, we investigated the role of these polymorphisms in combination (Table III). Interaction of all three *GSTM1* genotypes with one or no copy of *GSTT1* gene conferred reduced risk to lung cancer. However, most of these interactions were not significant. Only significant reduced lung cancer risk was observed for individuals with the combined *GSTM1* and *GSTT1* null genotype (OR=0.23; 95%

Table II. Association of *GSTM1* and *GSTT1* genotypes and environmental risk factors with lung cancer susceptibility

	Cases N (%)	Controls N (%)	OR1 (95% C.I.), P value[b]	OR2 (95% C.I.), P value[c]
GSTM1				
+/+	32 (20.8)	22 (14.3)	Ref	Ref
+/-	65 (42.2)	66 (42.9)	0.68 (0.36-1.29), 0.24	0.73 (0.37-1.44), 0.37
-/-	57 (37.0)	66 (42.9)	0.60 (0.32-1.14), 0.12	0.62 (0.31-1.23), 0.17
P_{trend}				0.13
Non-null	97 (62.9)	88 (57.1)	Ref	Ref
Null	57 (37.0)	66 (42.9)	0.79(0.50-1.23)0.30	0.77(0.47-1.25)0.29
GSTT1 [a]				
+/+	42 (27.3)	30 (19.5)	Ref	Ref
+/-	90 (58.4)	82 (53.2)	0.77 (0.44-1.35), 0.37	0.66 (0.36-1.22), 0.19
-/-	22 (14.3)	42 (27.3)	**0.35 (0.17-0.73), 0.005**	**0.32 (0.15-0.71), 0.005**
P_{trend}				0.006
Present	132 (85.7)	112(72.7)	Ref	Ref
Null	22 (14.3)	42(27.3)	**0.65(0.48-0.87)0.005**	**0.65(0.47-0.91)0.01**
Smoking status*				
Non-smokers	49 (31.8)	83 (53.9)	Ref	Ref
Smokers	105 (68.2)	71 (46.1)	**3.14 (1.83-5.39), <0.001**	**3.03 (1.73-5.31), <0.001**
Tobacco chewing				
Non-chewers	74 (48.1)	82 (53.2)	Ref	Ref
Chewers	80 (51.9)	72 (46.8)	1.25 (0.78-2.01), 0.33	0.98 (0.58-1.66), 0.95
Betel quid chewing*				
Non chewers	35 (22.7)	66 (42.9)	Ref	Ref
Chewers	119 (77.3)	88 (57.1)	**2.63 (1.58-4.39), <0.001**	**2.39 (1.38-4.16), 0.002**
Alcohol consumption				
Non-alcoholic	114 (74.0)	109 (70.8)	Ref	Ref
Alcoholic	40 (26.0)	45 (29.2)	0.83 (0.48-1.41), 0.49	0.67 (0.38-1.19), 0.175

*P<0.05
OR1 (95% C.I.), P value: Odd ratio, 95% Confidence interval obtained from univariate conditional logistic regression models
[c]OR2 (95% C.I.), P value: Odd ratio obtained from multivariate conditional logistic regression models
Bold number indicates significant P value

CI = 0.06-0.80; *P*=0.02) which becomes insignificant after Bonferroni correction (P $_{Bonferroni\ correction}$=0.12).

Gene-environment interaction: To evaluate the potential modifying effect of *GSTM1* and *GSTT1* genes on risk factors, stratified analysis was performed. Genotype distribution and association of *GSTM1* and *GSTT1* in cases and controls positive for smoking, tobacco chewing, betel quid chewing and alcohol consumption are given in Table IV. Interaction of risk factors with *GSTM1* and *GSTT1* genes imparted reduced risk with deletion in functional allele (+/- and -/-) compared to individuals with the presence of both allele (+/+).

Smokers carrying *GSTT1* null genotype showed significantly reduced risk (OR=0.30; 95%CI=0.10-0.91; *P*=0.03). Moreover, after stratifying the data further between exclusive smokers and betel nut chewers, protective effect of *GSTT1* null genotype was more pronounced in smokers only (OR=0.030; 95% CI=0.001-0.78; *P*=0.03, P$_{trend}$=0.006) (Table V). Similar interaction was observed for alcohol, individuals with *GSTT1* null genotype showed significantly reduced risk (OR=0.04; 95% CI=0.003-0.61; *P*=0.02) (Table IV). Interaction of risk habits with *GSTM1* and *GSTT1* genotypes showed carcinogen specificity. It was interesting to note that even though most of the results

GSTM1	GSTT1	Cases N (%)	Controls N (%)	OR1 (95% C.I.) *P* value	OR2 (95% C.I.) *P* value
+/+	+/+	7 (21.8)	4 (18.1)	1.0	1.0
	+/-	17 (53.1)	12 (54.5)	0.38 (0.06-2.40), 0.30	0.99 (0.20-4.92), 0.99
	-/-	8 (25.0)	6 (27.2)	0.12 (0.007-2.32), 0.16	0.70 (0.11-4.39), 0.70
+/-	+/+	21 (32.3)	16 (24.2)	1.0	1.0
	+/-	37 (56.9)	37 (56.0)	0.80 (0.31-2.04), 0.64	0.52 (0.21-1.27), 0.15
	-/-	7 (10.7)	13 (19.6)	0.73 (0.19-2.72), 0.64	0.31 (0.09-1.09), 0.07
-/-	+/+	14 (24.5)	10 (15.1)	1.0	1.0
	+/-	36 (63.1)	33 (50.0)	0.79 (0.26-2.34), 0.67	0.67 (0.24-1.84), 0.44
	-/-	7 (12.2)	23 (34.8)	0.30 (0.07-1.17), 0.08	**0.23 (0.06-0.80), 0.02**

Table III. Joint effect of *GSTM1* and *GSTT1* genotypes and lung cancer risk

OR1 (95% C.I.), *P* value: Odd ratio, 95% Confidence interval obtained from univariate conditional logistic regression models
OR2 (95% C.I.), *P* value: Odd ratio, 95% Confidence Interval obtained from multivariate conditional logistic regression models
Bold number indicates significant *P* value

were not significant, yet interactions of risk habits with null genotype almost always yielded more protective effects than interactions with one copy number genotypes for both *GSTM1* and *GSTT1*.

Discussion

Worldwide numerous studies have been conducted on the association between *GSTT1* and *GSTM1* polymorphism but with conflicting results. Although findings from India showed no significant association of *GSTM1* and *GSTT1* with lung cancer, but some studies suggest a possible interaction with smoking. Sreeja *et al*[18] showed significant risk associated with *GSTT1* null polymorphism. Similarly, Kumar *et al*[19] have also found marginally significant risk associated with *GSTT1* null genotype. However, these studies have investigated the risk for lung cancer in individuals with null genotype (no allele) compared with a combined group of individuals with either one or two functional alleles, thus underestimating the risk without accounting the effect of gene dosage of allele.

In the present study, there was no overall effect of *GSTM1* polymorphism on lung cancer. For *GSTT1* gene, the risk of lung cancer significantly decreased with the deletion of the functional allele *i.e.* from single copy of the gene (0.66) to the null genotype (0.32) compared to the two copy number of gene. However, the defined classical functions of *GSTM1* and *GSTT1* do not support protective roles for the null genotypes, but there has been precedent in studies on lung cancer particularly from outside India[20-27]. Risch *et al*[20] reported that *GSTT1* null genotype was underrepresented among squamous cell carcinomas. Similarly in two other studies,

GSTM1 null genotype was associated with reduced risk of lung cancer particularly in younger patients of 50-60 yr and in squamous cell carcinomas[21,22]. Some other studies have also found moderately decreased although non-significant, risk associated with *GSTT1* null genotype[23-25]. In the light of the above results it is easy to speculate a dual role of the GSTs depending upon the conditions of stress. Studies have explained this duality on the basis of metabolite toxicity[26] and population genetics[10].

When analyzing joint effects of the two GST genes, combination of null genotypes of both the genes imparted significant reduced risk though insignificant after Bonferroni correction. This suggests that an increased glutathione conjugation by the present alleles of both genes imparts increased risk to cancer. Combined conjugation activities by GSTs deplete the level of glutathione in the cell impairing xenobiotic defense and thereby exposing it to oxidative damage and induced mutagenesis[27]. The formation of glutathione conjugates generally causes the electrophiles to be less toxic and readily excreted. However, this conjugation might also act as transporter molecule by releasing reversibly bound electrophilic compounds.

Tobacco smoking was a strong risk factor in the study. Tobacco is consumed both in smoking and smokeless forms. In India, tobacco is smoked as cigarettes or in the form of *bidi*. Tobacco smoke comprises nearly 60 carcinogenic compounds whereas its unburned form contains 16 identified carcinogens[28]. Among these, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons

Table IV. Effect modification of *GSTM1* and *GSTT1* genotypes and environmental risk factors on lung cancer risk

Genotypes	Smoking		Tobacco		Betel quid		Alcohol	
	Cases/Controls N (%)	OR (95%CI) P value	Cases/Controls N (%)	OR (95%CI) P value	Cases/Controls N (%)	OR (95%CI) P value	Cases/Controls N (%)	OR (95%CI) P value
GSTM1								
+/+	22(21.0)/12(16.9)	1.0	14(17.5)/7(9.7)	1.0	23(19.3)/14(15.9)	1.0	7(17.5)/9(20.0)	1.0
+/-	46(43.8)/28(39.4)	0.84(0.34-2.09), 0.71	36(45.0)/34(47.2)	0.52(0.13-2.11), 0.36	51(42.9)/39(44.3)	0.81(0.32-2.04), 0.67	18(45.0)/18(40.0)	2.31(0.48-11.06), 0.29
-/-	37(35.2)/31(43.7)	0.75(0.30-1.87), 0.54	30(37.5)/31(43.1)	0.44(0.11-1.75), 0.24	45(37.8)/35(39.8)	0.76(0.27-1.98), 0.63	15(37.5)/18(40.0)	1.58(0.32-7.76), 0.56
GSTT1								
+/+	28(26.7)/11(15.5)	1.0	19(23.8)/15(20.8)	1.0	25(21)/19(21.6)	1.0	10(25.0)/5(11.1)	1.0
+/-	62(59.0)/41(57.7)	0.59(0.24-1.43), 0.25	49(61.3)/35(48.6)	1.37(0.53-3.55), 0.51	76(63.9)/51(58.0)	1.18(0.54-2.57), 0.66	27(67.5)/28(62.2)	0.75(0.17-3.29), 0.71
-/-	15(14.3)/19(26.8)	**0.30(0.10-0.91), 0.03**	12(15.0)/22(30.6)	0.64(0.20-2.05), 0.46	18(15.1)/18(20.5)	0.62(0.22-1.79), 0.38	3(7.5)/12(26.7)	**0.04(0.003-0.61), 0.02**

OR (95% C.I.), *P* value: Odd ratio, 95% Confidence interval
Bold number indicates significant *P* value

Gene	Group	Betel (74)			Smoker (43)		
		ratio	OR (95% C.I.)p	OR1 (95% C.I.) *P*	ratio	OR2 (95% C.I.) *P*	OR (95% C.I.) *P*
GSTM1	+/+	7/6	Ref	Ref	6/4		
	+/-	11/18	0.81(0.04-15.36)0.89	1.08(0.003-29.9)0.96	6/7	1.08(0.18-6.40)0.92	1.05(0.14-7.31)0.98
	-/-	15/17	6.88(0.44-106.9)016	9.37(0.36-243.8)0.17	7/13	0.16(0.001-1.80)0.13	0.07(0.004-1.23)0.07
P$_{trend}$			0.88				0.19
GSTT1							
	+/+	6/10	Ref	Ref	9/2	Ref	Ref
	+/-	21/23	1.84(0.40-8.47)0.43	1.70(0.36-7.89)0.49	7/13	0.22(0.02-2.20)0.19	0.14(0.001-1.93)0.14
	-/-	6/8	2.71(0.14-49.68)0.50	3.24(0.09-116.2)0.52	3/9	0.05(0.003-0.97)0.004	**0.03(0.001-0.78)0.03**
P$_{trend}$			0.74				**0.006**

Table V. Effect modification of *GSTM1* and *GSTT1* genotypes in only smokers and betel chewers

OR1 (95% C.I.), *P* value: Odd ratio, 95% Confidence interval obtained from univariate conditional logistic regression models
OR2 (95% C.I.), *P* value: Odd ratio obtained from multivariate conditional logistic regression models (Adjusted for alcohol and tobacco chewing)
Bold number indicates significant *P* value

(PAH) are considered to be the most important causative agents for the development of lung cancer. PAH require metabolic activation and subsequent binding to DNA (forming bulky "PAH-DNA adducts") to exert their carcinogenic action[29]. Similar activation of one of the N-nitrosamines, NNK, by the P450 system produces metabolites that form methyl and pyridyloxobutyl DNA adducts. Detoxification of these toxic metabolites occurs via the action of multiple Phase II enzymes, most notably the glutathione-S-transferases.

In the present study, interaction of smoking with *GSTT1* +/- and -/- genotypes compared to the +/+ genotype was protective in nature indicating a genetic modulation of the risk imparted by smoking. Similar result was reflected in a meta-analysis by Raimondi *et al*[5], where a negative trend of the odds ratios for *GSTT1* null allele was observed with increasing amount of lifetime smoking for both Caucasians and Asians subjects. Further, studies have indicated adverse association between smoking and lung cancer among individuals with *GSTT1* null genotype particularly in non-smokers. Alexandrie *et al*[24] found that *GSTT1* null genotype was associated with a decreased risk for lung cancer in heavy smokers. Although not significant, Wenzlaff *et al*[30] also found that never smokers with *GSTT1* null genotype with no household environmental tobacco smoke were at one-third the risk of lung cancer compared with *GSTT1* present genotype. Further, a possible protective effect of being *GSTT1* null in non-smoker has also been reported[31].

The discrepancy in results could be explained by different ethnic population, differences in categorization of smokers and different habit of dietary compounds. As there are several dietary compounds, particularly intake of crucifreous vegetables that need to be controlled to fully elucidate true gene-environment interactions related to lung caner risk. London *et al*[32] found that individuals with detectable level of isothiocyanate (ITC) were at reduced risk of lung cancer with the null genotype of both *GSTM1* and *GSTT1*. Isothiocyanates found in cruciferous vegetables, are substrates for GSTs and are associated with reduced cancer risk. The present study lacks information on both dietary status and the pack years of the smokers.

There might be some more limitations to this study. The sample size of our study was relatively small. *GSTT1* genotypes showed deviation from HWE in lung cancer cases. After ruling out false positive associations and genotyping errors perhaps population stratification, could be a reason for this deviation. However, the cases were incident, and thus, the data did not show report or recall bias. Also case-control matching was done in reference to age, gender, and ethnicity, thereby controlling for any confounding effect accounted by these variables. Estimation of interactive OR in this study in some cases yielded small subgroup sizes which limit the reliability of estimating gene-environment effects. Thus these results should be considered empirical observations for further studies on larger number of samples. In summary, trimodular genotypes of *GSTM1* and *GSTT1* were determined and

gene dosage effect was observed with *GSTT1* copy number. Our results indicated that null genotype of *GSTT1* might be associated with a reduced risk of lung cancer risk. Furthermore, protective effect of *GSTT1* was strongly associated with smokers only.

Our results were in contrast with some previous reports on lung cancer[14,22] which observed no significant association of hemizygous and homozygous genotypes of *GSTM1* and *GSTT1* when compared with homozygous wild type genotype. This might be due to the difference in sample size, however, in contrast to many of these studies, the homogeneity of our population from an ethnically isolated north-eastern part of India allowed the detection of small inherited variations in metabolism. Thus, the present results indicate that ethnicity and carcinogen exposure along with trimodal distribution of GST enzymes can be a major determinant of risk of lung cancer.

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