3.1 Background:

The cytochrome P-450 enzymes (CYPs) and glutathione S-transferases (GSTs) are important components of Phase I and Phase II enzyme system respectively, primarily responsible for detoxification of the carcinogens by converting them into water soluble and more excertable compounds [1-3]. The single nucleotide polymorphisms (SNPs) in these metabolic enzymes may alter their expression or function, change the activation and detoxification of carcinogenic compounds [3, 4]. Such SNPs might lead to significant inter-individual differences in metabolizing the carcinogens, increasing the susceptibility among the BN and tobacco users to various cancers.

The polymorphism in the genes of the detoxifying enzymes CYP and GST are susceptibility factors for HNC [5]. Studies have demonstrated the association of CYP1A1 and CYP2E1 in the risk of HNC, lung, cervical and esophageal cancer [2, 6- 9]. Similarly, polymorphism in GSTM1, GSTT1 and GSTP1 genes has been reported to regulate the risk for HNC in the tobacco users [10-12]. Meta-analysis of 31 published case-control studies supports modest association of GSTM1 and GSTT1 genotypes with HNC risk [13]. Few studies have been conducted in the Indian population to determine the association between CYP1A1, CYP2E1, GST (M1, T1, P1) gene polymorphism and risk of HNC, but the results are not consistent. A case-control study with smaller sample size in healthy tribal and non-tribal individuals of Assam demonstrated that the GSTT1 null genotype was associated with higher risk for developing leukoplakia [14]. Recent studies have shown that polymorphism in CYP1A1, GSTM1 and GSTT1 and their interaction with tobacco exposure might influence the risk for HNC in the NE population [15]. No reports are available on the role of polymorphism in CYP2E1 and GSTP1 and its interaction with BN or tobacco alone or in combination on the risk of HNC from the NE region of India. Though NE region has the highest incidence of HNC, very few studies have shown the association of BN with HNC risk. The role of polymorphism in CYPs and GSTs and their interaction with BN, tobacco and smoking on the risk of HNC is yet to be established.

In the present study, we investigated the distribution of polymorphic genotypes of CYP1A1, CYP2E1, GSTM1, GSTT1 and GSTP1 in HNC patients of NE region and their interactions with BN, tobacco and smoking leading to host susceptibility for HNC. The

identification of the susceptibility factors may assist in risk determination and early detection which might lead to the effective management of the disease.

3.2 Material and Methods

3.2.1 Case-control study

This case-control study comprised of 205 histologically confirmed cases of HNC patients recruited from the Dr. Bhubaneswar Borooah Cancer Institute (BBCI), Guwahati, India. The healthy volunteered controls (210 individuals) with same geographical area, age and gender were randomly enrolled from the same institute referred for routine examination and not related to cancer and were representative of the population in the region of study. The enrollment of the patients and healthy controls were carried out between October, 2011 to May, 2015. All the individuals who took part in this study signed an informed consent and the information was obtained on standardized questionnaire through personal interview. The data on demographic variables including vegetarian or non-vegetarian, BN, tobacco chewing, cigarette smoking habits etc. were collected for both cancer patients and healthy volunteer controls. This study was approved by the institutional ethical committee of BBCI, Guwahati, Assam.

3.2.2 Collection of blood samples and DNA extraction

Peripheral leukocyte was drawn from all participants of the study, collected in EDTA containing tubes and stored at -20°C, till further processing. Genomic DNA was isolated from blood lymphocytes using conventional phenol-chloroform extraction method [16], and was stored in TE (10 mM Tris-HCl, pH 8.0, 1mM EDTA) buffer at - 20°C and subsequently used for genotyping. The quality and quantity of the genomic DNA was verified by agarose gel electrophoresis followed by UV spectrophotometry at 260nm/280nm.

3.2.3 Genotyping analysis

The CYP1A1 MspI polymorphism was identified by PCR-restriction fragment length polymorphism (PCR-RFLP) [17]. PCR was performed in a total volume of 25 µl containing 100 ng DNA, 15 picomoles of both forward and reverse primers, 2.5 mM of PCR reaction buffer, 2.5 mM dNTPs each (New England biolabs, USA), 1.5 mM $Mgcl_2$, 1U of the Taq polymerases (New England biolabs, USA), The PCR product was digested

with MspI (New England Biolab, USA) for testing the substitution of CCGG for CTGG, and is shown in figure 6.

The genetic polymorphism in the 5' flanking region of CYP2E1 was determined by digestion of PCR product with RsaI (New England Biolab, USA) [18]. PCR was performed in a total volume 25 µl containing 100 ng DNA, 10 picomoles of both forward and reverse primers, 2.5 mM of PCR reaction buffer, 2.5 mM dNTPs each (New England biolabs, USA), 1.5 mM Mgcl2, 1U of the Taq polymerases (New England biolabs, USA). The predominant allele c1 was sensitive to RsaI digestion and the c2 allele was resistant to RsaI digestion as shown in figure 7.

Multiplex PCR was used to detect the presence and absence of GSTM1 and GSTT1 genes along with β-globin as shown in figure 8 [19]. The polymorphism of GSTP1 (Ile105Val) was studied by PCR-restriction fragment length polymorphism [20]. PCR was performed in a total volume 25 µl containing 100 ng DNA and same concentration of the above mention. The PCR product was digested with 0.5U Alw261 (New England Biolab, USA) and separated on 3.5% agarose gel and bands were visualized using ethidium bromide staining as shown in figure 9.

The details on primer sequences for CYP1A1, CYP2E1, and GSTP1 and PCR and post PCR conditions, fragment size details are mentioned in Table 7. 20 % results were cross checked and the results obtained were found to be 100 % concordant.

3.2.4 Statistical analysis

The $\chi 2$ test and Fisher's exact test was used to analyze the differences in the distribution of demographic characteristics between cases and healthy control. Genetic polymorphism in genes CYP1A1,CYP2E1,GSTM1,GSTT1 and GSTP1 and its association with occurrence of HNC in presence of risk factors such as BN, tobacco chewing and smoking habits were analyzed by calculating odd ratios (ORs), 95% confidence intervals (95% CI) and corresponding P- values using SPSS software version 20.0 and Epi-info Version 6 software. The P- value < 0.05 was considered as statistically significant. The univariate and multivariate Logistic regression (LR) analysis was used to examine the association between polymorphism and risk for HNC. Allele frequencies were calculated and tested for Hardy-Weinberg equilibrium within the cases and control.

Table 7: Sequence of oligonucleotide primers and reaction conditions used for CYP1A1, CYP2E1, GSTP1, GSTM1 and GSTT1 genotyping

Genotype	Primers (5' to 3')	Alleles (bp)	PCR Condition
CYP1A1 (rs4646903)	Sense -CAGTGAAGAGGTGTAGCCGCT Antisense -TAGGAGTCTTGTCTCATGCCT	m1/m1: 340 m1/m2: 340, 200, 140 m2/m2: 200, 140	94°C/5min, 35 cycles 94°C/1min 55°C/1min 72°C/1.5min 72°C/2min
CYP2E1 (rs2031920)	Sense –CCAGTCGAGTCTACATTGTCA Antisense '-TTCATTCTGTCTTCTAACTGG	c1/c1: 360, 50 c1/c2: 410, 360, 50 c2/c2:410	95°C/1min, 30 cycles 95°C/1min 50°C/ 1min 72°C/ 1min 72°C/ 4min
GSTP1 (rs2495636)	Sense – ACCCCAGGGCTCTATGGG AA Antisense –TGA GGGCACAAGAAGCCCCT	Ile/Ile- 176 Ile/Val- 176, 91, 85 Val/Val- 91,85	95°C/12min, 35 cycles 95°C/ 30 sec 58°C/ 30 sec 72°C/ 45 sec 72°C/ 10min
GSTM1	Sense: GAACTCCCTGAAAAGCTAAAGC Antisense: GTTGGGCTCAAATATACGGTGG	− Null genotype + Fragment of 219	94°C/ 5min , 35 cycles 94°C/ 2min 57°C/ 1min
GSTT1	Sense: TTCCTTACTGGTCCTCACATCTC Antisense: TCACCGGATCATGGCCAGCA	− Null genotype + Fragment of 480	72°C/ 1min 72°C/10min
β - Globin	Sense: -CAACTT CAT CCA CGTTCACC Antisense-GAAGAGCCAAGGACA GGTAC	Internal control, Fragment of 268	

3.3 Results

3.3.1. Demographic and clinico-pathological Characteristics

The demographic characteristics, lifestyle and other confounding factors of the subjects (cases 205 and control 210) who participated in the study are presented in Table 8. Among males, cases and controls represented as (76.59% and 76.19%) the mean age of cases were 53.65 ± 12.15 years and controls 52.10 ± 12.32 years respectively, and mostly belonged to rural part of the NE region. The association of HNC risk and food habits such as betel nut consumption [OR^a = 1.87, P< 0.03], tobacco chewing [OR^a = 4.47, P< 0.001] and smoking habits [OR^a = 1.92, P< 0.01] was found when compared with control (Table 8). The present results demonstrated the combined habits of BN and tobacco exhibit higher risk for HNC than the triple habits of BN, tobacco and smoking (Table 8).

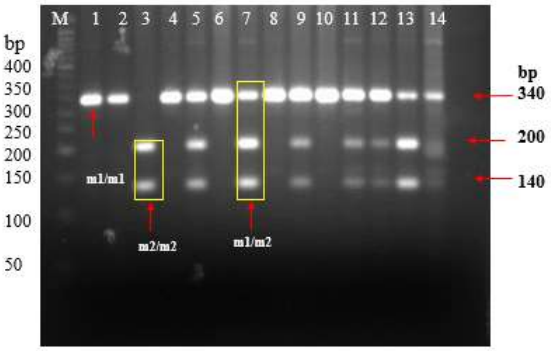

Figure. 6 CYP1A1 genotype analyzed by (PCR-RFLP) the PCR product digested with MspI from fourteen samples with three genotypes. The CYP1A1 (m1/m1) genotype (wild type) was characterized by 340 bp fragment homozygous CYP1A1 (m2/m2) genotype by 200 and 140 bp fragments, and heterozygous CYP1A1 (m1/m2) genotype by 340, 200, 140 bp fragments respectively. lane-M, represent 50 bp, ladder

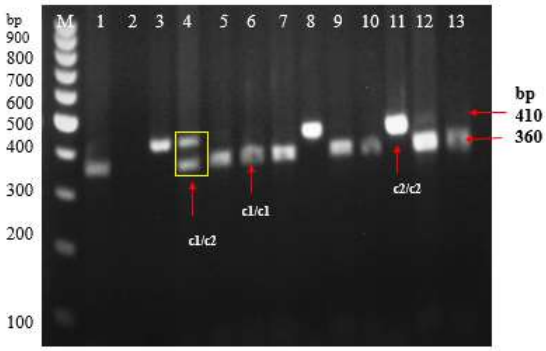

Figure 7 CYP2E1 genotype analyzed by (PCR-RFLP) the PCR product digested with RsaI from thirteen samples. The yield were fragments with 360 bp, and 50 bp for c1/c1; 410, 360, 50 bp, for c1/c2; and 410 bp for c2/c2 respectively. lane-M, represent 100 bp, ladder

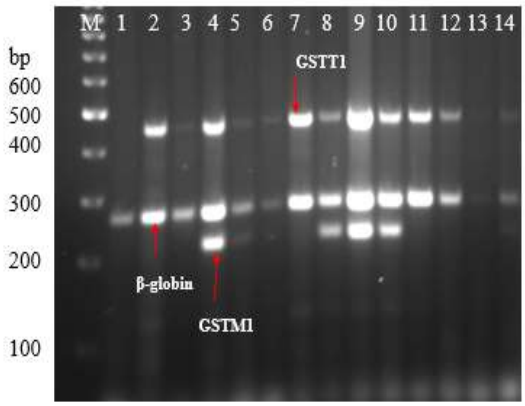

Figure. 8 Multiplex PCR patterns for GSTM1 and GSTT1 genes. The β –globin gene was used as an internal positive control. Lane M -100 bp ladder, lane 4 , 8, 9 and 10 represents genotype for both GSTM1 and GSTT1 genes, lane 2, 7, 11, 12 represents the GSTT1 genotype.

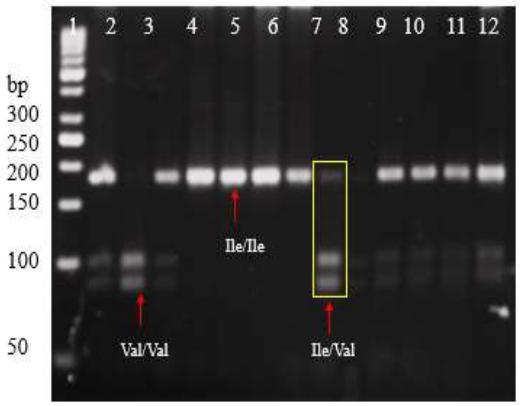

Figure. 9 GSTP1 genotype analyzed by (PCR-RFLP) the PCR product digested with BsmAI from twelve samples. The yield were fragments with 176 bp, for Ile/Ile; 176, 91,85 bp for Ile/Val and 91, 85 bp for Val/Val;, lane – 1 50 bp ladder

Table 8: Baseline characteristics of HNC cases and healthy control

Characteristic	Cases (n = 205) N (%)	Control (n = 210) N (%)	Crude OR [95%CI]	P-value	Adjusted OR [a] (95% CI)	P-value
Gender						
Female	48 (23.41)	50 (23.81)	**1.0 (Reference)**		**1.0 (Reference)**	
Male	157 (76.59)	160 (76.19)	1.02[0.63-1.65]	0.92	1.32 [0.78- 2.23]	0.29
Age						
≤50	91 (44.39)	93 (44.28)	**1.0 (Reference)**		**1.0 (Reference)**	
>50	114 (55.61)	117 (55.72)	1.00[0.66-1.49]	0.98	1.22 [0.78-1.92]	0.37
Education						
literate	168 (81.95)	179 (85.23)	**1.0 (Reference)**		**1.0 (Reference)**	
illiterate	37 (18.05)	31 (14.76)	1.27 [0.75- 2.14]	0.36	0.97 [0.51 -1.80]	0.90
Community						
Non- Tribal	167 (81.46)	188 (89.52)	**1.0 (Reference)**		**1.0 (Reference)**	
Tribal	38 (18.54)	22 (10.48)	1.94[1.07-3.56]	**0.02**	1.31 [0.65- 2.62]	0.43
Place of residence						
Urban + Semi Urban	41 (20)	94 (44.76)	**1.0 (Reference)**		**1.0 (Reference)**	
Rural	164 (80)	116 (55.24)	3.24[2.09-5.01]	**<0.001**	3.00 [1.84-4.89]	**<0.001**
Dietary habits						
Vegetarian	10 (4.88)	9 (4.28)	**1.0 (Reference)**		**1.0 (Reference)**	
Non -vegetarian	195 (95.12)	201 (95.72)	0.87[0.34-2.19]	0.77	0.71 [0.25-2.02]	0.53
Betel nut Chewing						
Non-chewers	23 (11.21)	60 (28.57)	**1.0 (Reference)**		**1.0 (Reference)**	
Chewers	182 (88.79)	150 (71.43)	3.16 [1.86-5.36]	**<0.001**	1.87 [1.04 -3.38]	**0.03**
Tobacco Chewing						
Non-chewers	73 (35.60)	158 (75.23)	**1.0 (Reference)**		**1.0 (Reference)**	
Tobacco- chewers	132 (64.40)	52 (24.77)	5.49[3.59-8.39]	**<0.001**	4.47 [2.80-7.13]	**<0.001**
Smoking						
Non- smokers	118 (57.57)	162 (77.14)	**1.0 (Reference)**		**1.0 (Reference)**	
Smokers	87 (42.43)	48 (22.86)	2.48 [1.62-3.80]	**<0.001**	1.92 [1.19-3.10]	**<0.01**
Both betel nut - Tobacco Chewing						
Non-chewers	83(40.48)	161 (76.67)	**1.0 (Reference)**		**1.0 (Reference)**	
chewers	122 (59.52)	49 (23.33)	4.83 [3.16- 7.38]	**<0.001**	4.72 [3.01-7.39]	**<0.001**
Betel nut-Tobacco Chewers-smokers						
Non -habits	153 (74.64)	188 (89.52)	**1.0 (Reference)**		**1.0 (Reference)**	
habits	52 (25.36)	22 (10.48)	2.90[1.68- 4.99]	**<0.001**	2.63 [1.49-4.64]	**<0.01**

P<0.05 is consider to be significance, OR (odds ratio), CIs (confidence Intervals), OR [a] (adjusted in multivariate logistic regression models) including, gender, age, education, community, place of residence, dietary habits, betel nut, tobacco, smoking.

3.3.2. Distribution of CYPs and GSTs genotypes in cases and controls

To understand the disease susceptibility influenced by inherent genetic constituent, genotypes of CYP, GST genes involved in xenobiotic metabolism were analyzed by PCR-RFLP method and are presented in Table 8. The genotype distribution in both cases and control were in consensus with Hardy-Weinberg equilibrium (both P>0.05, data not shown). Both univariate and multivariate analysis showed strong association of m1/m2 and m2/m2 mutant genotypes of CYP1A1 with HNC as compared to control (Table 9). Similarly, the c2/c2 [OR[a]= 4.50, P= 0.01] genotype of CYP2E1 exhibit highly significant

association with HNC. The polychotomized analysis exhibited that both mutantm1/m2 + m2/m2 have significant association with HNC as compared to healthy control (Table 9).

Differences in polymorphism in the subjects showed that GSTT1 null genotype was significantly associated with HNC risk compared with the healthy control (Table 9). Interestingly, the null genotype of both GSTM1/GSTT1 exhibited protective role [OR[a] =0.62, P=0.04]. The univariate and multivariate analysis exhibited that GSTP1 Ile/Val and Val/Val were also significantly associated with HNC risk The polychotomized analysis in Ile/Val + Val/ Val of GSTP1 [OR[a] = 2.32, 95 % CI 1.45-3.70, <0.001] have shown significant association with HNC risk (Table 9).

Table 9: Distribution and comparison of CYP1A1, CYP2E1, GSTM1, GSTT1, GSTP1 genotypes in HNC cases and healthy control

Genotypes	Case (n=205) N (%)	Control (n=210) N (%)	Crude OR [95% CI]	P- value	Adjusted OR [a](95% CI)	P-value
CYP1A1						
m1/m1	98 (47.80)	138 (65.72)	**1.0 (Reference)**		**1.0 (Reference)**	
m1/m2	80 (39.02)	61 (29.04)	1.84 [1.21- 2.81]	**<0.01**	1.90 [1.18-3.04]	**<0.01**
m2/m2	27 (13.17)	11 (5.24)	3.45[1.63-7.29]	**<0.01**	3.21 [1.41-7.29]	**<0.01**
m1/m2+ m2/m2	107 (52.19)	72 (34.28)	2.09 [1.40-3.10]	**<0.001**	2.11 [1.36-3.28]	**<0.01**
CYP2E1						
c1/c1	102 (49.75)	127 (60.47)	**1.0 (Reference)**		**1.0 (Reference)**	
c1/c2	79 (38.53)	74 (35.23)	1.32 [0.88-2.00]	0.17	1.21 [0.76-1.92]	0.41
c2/c2	24 (11.70)	9 (4.28)	3.32 [147.7.45]	**<0.01**	4.50 [1.83-11.07]	**0.01**
c1/c2+ c2/c2	103 (50.24)	83 (39.52)	1.54 [1.04-2.28]	**0.02**	1.50 [0.97-2.32]	**0.06**
GSTM1						
Present (+)	144 (70.24)	140 (66.66)	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	61 (29.75)	70 (33.34)	0.84 [0.56-1.28]	0.43	0.65 [0.40-1.04]	0.07
GSTT1						
Present (+)	97 (47.31)	131 (62.38	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	108 (52.68)	79 (37.62)	1.84 [1.24.2.73]	**<0.01**	1.69 [1.09- 2.62]	**0.01**
GSTM1/ GSTT1						
Present (+)	68 (33.18)	54 (26.67%)	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	137 (66.82%)	156 (73.33)	0.73 [0.48-1.11]	0.14	0.62 [0.38-0.99]	**0.04**
GSTP1						
Ile/Ile	122 (59.51)	153 (72.85%)	**1.0 (Reference)**		**1.0 (Reference)**	
Ile/Val	66 (32.19)	49 (22.86%)	1.68 [1.08-2.62]	**0.01**	2.21 [1.34-3.63]	**<0.01**
Val/Val	17 (8,29)	8 (4.28%)	2.66 [1.11-6.38]	**0.02**	2.94 [1.11-7.79]	**0.03**
Ile/Val + Val/Val	83 (40.48)	57 (27.14)	1.82 [1.20-2.75]	**<00.01**	2.32 [1.45-3.70]	**<0.001**

Reference group are the homozygous wild-type genotype for each gene (m1/m1, c1/c1, present (+), Ile/Ile) and P value was consider statically significance <0.05 by Chi-square ($\chi 2$) and Fisher's exact test, adjusted OR[a] multivariate logistic regression models including gender, age, betel nut, tobacco, smoking was employed

3.3.3. Interaction of CYP and GST genotype with BN consumption habits:

The risk of HNC for the CYP and GST genotypes with habits of BN consumption, tobacco chewing and smoking are presented in (Table 10). In the BN consuming cases, CYP1A1genotypes m1/m2 with moderate risk and homozygous polymorphism m2/m2 have shown strong association with higher risk. The combined polymorphic genotype (m1/m2 + m2/m2) of CYP1A1 was also found to have significant association. The minor allele genotype of CYP2E1, c2/c2 found to have higher risk with strong association [OR^a = 6.59, P< 0.01] and combined c1/c2 + c2/c2 [OR^a = 1.71, P< 0.02] of CYP2E1 showed strong association with increased risk of HNC.

The m1/m2 and m1/m2 + m2/m2 genotypes exhibit strong association with HNC risk in non-BN consumers also indicating important role of minor allele m2 in HNC. Among BN consuming cases null genotype of GSTT1 [OR^a = 1.73, P= 0.02], Val/Val [OR^a = 3.93, P= 0.01] and Ile/Val +Val/Val [OR^a = 2.25, P=<0.01] genotype of GSTP1 exhibited increased risk for HNC. Among, BN chewing cases the GSTM1/GSTT1 (null) genotype was showed protective role (Table 10).

3.3.4. Interaction of CYP and GST genotype with Tobacco chewing habits:

Role of interaction of CYP and GST genotypes with tobacco chewing habit on the risk of HNC are represented in (Table 11). Notably the m2/m2 [OR^a = 5.09, P= 0.03] genotype of CYP1A1, c1/c2 [OR^a = 4.86, P=<0.001], c2/c2 [OR^a 10.23, P< 0.03] and c1/c2 + c2/c2 [OR^a = 5.30, P< 0.001] genotypes of CYP2E1 showed strong association with HNC risk among tobacco chewing cases. Even in the non-tobacco chewing cases m1/m2, m1/m2+m2/m2 genotype of CYP1A1 and c1/c2, c2/c2 genotype of CYP2E1 exhibit significant association. Among GSTs, GSTT1 null allele was found to have significant association with HNC risk in the cases with tobacco chewing habits [OR^a =2.86, P<0.01]. However, among non-chewers, the null genotype of GSTM1+GSTT1 and Ile/Val, Ile/Val+ Val/Val genotype of GSTP1 also exhibit risk for HNC.

3.3.5. Interaction of CYP and GST genotype with Smoking habits:

The association and interaction of CYP and GST genotypes with smoking habits in the HNC cases are represented in (Table 12). From the present data, it is evident that in CYP1A1 genotype m1/m2, m2/m2 and m1/m2 + m2/m2 genotype of CYP1A1 and c2/c2 genotype of CYP2E1 exhibit strong risk for HNC even in non- smoking cases.

Interestingly, we didn't observe any significant association of these genotypes among smokers. Among smokers, the null genotype of GSTT1 was found to have significant association with high risk, the Ile/Val + Val/Val genotype of GSTP1 have shown strong risk association in smoking, whereas in non-smoking cases it represented as risk with significant statistical association in Ile/Val [OR^a=2.05, 95% CI, 1.13-3.79, P= 0.02] and Ile/Val + Val/Val [OR^a=2.03, 95% CI, 1.14-3.62, P= 0.01] genotypes.

3.3.6. Interaction of CYP and GST genotype with combined habits of BN, tobacco and smoking:

The interaction of CYP and GST genotypes with combined habits of BN consumption, tobacco chewing and smoking are presented in (Table 13). It is evident from the observation that the m2/m2 [OR^a = 4.58, P= 0.05] genotype of CYP1A1 exhibit higher risk for HNC when BN are consumed along with tobacco chewing habits. However, addition of smoking to this combined habits didn`t exhibit risk for HNC. The c1/c2 [OR^a = 4.75, P= 0.001] and c1/c1 + c1/c2 [OR^a = 4.96, P= 0.001] genotype of CYP2E1 showed strong association and represented as risk genotype for HNC in both dual habit of BN and tobacco. The addition of smoking to these dual habits increases the HNC risk by 2.20 and 1.99 fold in c1/c2 [OR^a = 10.43, P<0.01] and c1/c1+c1/c2 [OR^a = 9.86, P<0.001] genotypes, respectively. In the case of GSTs, only the GSTT1 null genotype increases risk for HNC in both dual habit of BN and tobacco [OR^a = 3.90, P< 0.001] and similarly, addition of smoking habit increases the risk by 1.82 fold [OR^a = 7.22, , P< 0.01]. However, the GSTM1 and GSTM1/GSTT1 genotype, had shown reduced effect in BN-tobacco and BN-tobacco-smoking habits.

3.4 Discussion

In the present study, we have investigated the association between polymorphism in xenobiotic metabolizing genes (CYP1A1, CYPE2, GSTP1, M1,T1) and their interaction with betel nut chewing, tobacco chewing and smoking habits towards the susceptibility of head and neck cancer from NE region of India. The rationale behind the present study is that the NE region of India encompasses huge ethnic variation and is habituated with various consumption habits of betel nut, tobacco and smoking as part of their cultural diversity [21, 22]. The prevalence and incidence of HNC is also found to be very high in

this region affecting the lives of thousands of people in the past few decades. Several recent studies have demonstrated that these exposure habits (BQ, tobacco and smoking) play a contributory role in the causation of HNC [15]. However, individual variation in the capacity of metabolizing the carcinogens and/or pro-carcinogens present in these habits may play a role in bridging the inherent genetic constitution in modulating the risk of cancer [23]. This study aims to determine the susceptible genotypes and their interaction with various habits in order to estimate the risk for HNC.

From our study, it is evident that most of the cases were from rural background. In spite of having good literacy rate, most of them have BN, tobacco chewing and smoking habits. The present study reported the association of betel nut i.e. 'Tamul' chewing habit as a risk factor for HNC. A case control study from Southeast Asia has reported that chewing betel quid without tobacco elevates the risks of various pre-cancers lesions [24]. To our knowledge, in addition to the above, we are the first to report that BN/tamul consumption is a major risk factor for HNC.

In this study we investigated the interaction between gene (CYPs and GSTs) and environment exposure (BN, tobacco and smoking habits) to explore the exact causation of HNC in NE populations. The CYP1A1 and CYP2E1 superfamily genes play critical role in the biotransformation of pre-carcinogens and environmental toxicants into more potent carcinogens and toxicants that are probably associated with cancer risk [25, 26]. Altered forms of enzyme are identified with increased activities and consequently, have been associated with various cancers [27].

We have investigated the individual/coordinated risk posed by the effect of CYP1A1, CYP2E1, GSTP1, GSTM1 and GSTT1 gene polymorphism and environmental factors on HNC risk. It was observed from our study that CYP1A1 and CYP2E1, particularly minor allele variants have positive correlation with elevated risk for HNC in North East region of India (Table 9). The earlier reports support our observation that CYP1A1 (m2) and CYP2E1 (c2) polymorphisms increases the risk for HNC [28, 29]. In particular, polymorphism in CYP1A1 gene have strong association in increasing risk for oral cancer in southern [30], and northern region of India [31]. Likewise, meta-analysis indicated CYP2E1 variants are associated with higher risk for oral cancer in Asian population [29].

The GSTM1 and GSTT1 superfamily enzyme play an important role in detoxification of pro-carcinogens like nitroamines, PAHs, and other chemical carcinogens present in tobacco [32]. Therefore, deletion or null genotype of GSTM1 and GSTT1 decreases the capacity of the detoxification process and induces carcinogenesis [15]. As the CYP1A1 and CYP2E1 genes are involved in activation of major pro-carcinogens and GSTs in neutralization, therefore, polymorphisms in these genes with activities contributed in increasing the risk for several types of cancers [33, 34].

Our findings confirm the predictions made in previous reports from various parts of the world. Conflicting reports exist regarding the association of CYP1A1, GSTM1, GSTT1 genetic variations and combined effect of GSTM1 and GSTT1 null genotype that claimed no association with HNC among Netherlands, Indian and Brazilian populations [11, 35, 36].

Surprisingly, some studies have shown inconsistent results and negative correlations [37, 38]. But, In contrast to other studies, our study shows decreased HNC risk associated with GSTM1 null genotype, attributable due to difference of age stratification or exposure difference in the population, though some study in HNC [39], and lung cancer [40], supports the protective role played of GSTM1 null genotype. Anantharaman *et al.* have demonstrated that the genetic variations in xenobiotic-metabolizing enzymes *viz.* GSTM1 and GSTT1 significantly increased risk for oral cancer [35]. In Assam region of NE India, the GSTT1 null genotype was shown to be significantly increases the risk for oral and gastric cancer [40]. We also observed similar findings for HNC [13], which is also supported by earlier studies in lung [41], breast cancers [42], and esophageal cancer [43].

In context of GSTP1, our study confirmed association between GSTP1 genotypes with increased risk for HNC. Similar findings have been reported that the GSTP1 polymorphism increases the risk for cancer of oral cavity in Brazilian population [44, 45].
Numerous previous studies have indicated the association between CYP1A1, CYP2E1, GSTM1 and GSTT1polymorphisms and tobacco chewing in increasing the risk for HNC in Indian population [15, 46].
Our investigation have demonstrated significant impact of m1/m2, m2/m2 genotypes of CYP1A1 and c2/c2 genotype of CYP2E1 on HNC risk in betel nut chewing cases, whereas

in non-BN chewing cases, the m1/m2 genotype of CYP1A1 was significantly associated with HNC risk. The risk associated with these genotypes for HNC is also similar in the case of tobacco chewers and no-chewers, supported by earlier findings [47]. The GSTM1 polymorphism appears to play an important role in esophageal cancer among betel nut chewers from NE region of India [48]. In contradictory to other studies, GSTM1 and both GSTM1/GSTT1 have a protective role in NHC among BN chewers belonging to NE India (Table 9). Notable observation from our study was GSTT1 null genotype have strong association in HNC among BN and tobacco chewing cases. The combined effect of GSTM1 and GSTT1 null allele showing protective role needs to be reanalyzed with bigger sample sizes. Naier *et al*. have suggest that of Indian population having GSTT1 and GSTM1 and exposed to carcinogenic intermediates derived from BQ/ tobacco have increased risk for oral leukoplakia cancer, more than additively for individuals who have both GSTT1 and GSTM1 null genotypes [49]. This has been supported by combined analysis of GSTM1 and GSTT1 polymorphism that demonstrated a statistically significant impact on HNC carcinogenesis [48, 50, 51]. A study in the German population showed the null genotype of GSTM1 and GSTT1 have increased risk for HNC among tobacco chewers [15, 52].

Reports from the NE region of India have identified smoking as one of highest risk factor for HNC [36, 50]. Tobacco smoke contains a mixture of more than 4000 compounds and more than 50 compounds are classified as carcinogens including polycyclic aromatic hydrocarbons (PAHs) [53].

The CYP1A1, GSTM1, GSTT1 and GSTP1 polymorphisms are associated with increased risks for HNC among smokers as reported previously [15, , 54, 55]. In our study the GSTT1 null genotype and GSTP1 combined genotype of Ile/Val + Val/Val showed an increased risk for HNC among smokers. Similarly, in support of our observation that the CYP1A1 and GSTP1 polymorphism conferred significant risk in the non-smokers subgroup among lung cancer in North East Indian population [56]. However, meta-analysis reported GSTM1 null genotype is associated with a higher risk for oral cancer in Asians, but not in Caucasians, and this might be due to modified smoking status [44].

Further, we also observed increased risk among mixed habit of betel nut, tobacco chewing and smoking and polymorphism in xenobiotic metabolizing genes. Previous studies have demonstrated that tobacco and betel quid chewing by subjects with CYP1A1 and GSTM1 genotypes have significant individual effect as well as synergistic effect on HNC risk in NE population of India confirming the fact that gene-environment interaction determines the susceptibility towards carcinogenesis [15]. For the first time, we have investigated and established the combined effect of BN, tobacco chewing and smoking habits increases the risk for HNC with polymorphic forms of CYP2E1 and GSTs genes. In the present study, the c1/c2 and c1/c2 + c2/c2 genotype of CYP2E1 and GSTT1 null genotype have strong synergistic impact on development of HNC among individuals with BN + tobacco and BN + tobacco + smoking .

In conclusion, the present study demonstrated for the first time that the polymorphisms in CYP and GST genes are associated with increased risk for HNC in the BN-tobacco chewing-smoking cases of NE region of India. Our finding also demonstrates the synergistic role of polymorphic CYP2E1 and GSTT1 genotypes with BN/tamul, tobacco chewing and smoking habits on the risk of HNC suggesting the putative gene and environmental factors interactions. Further, we suggests more studies with larger sample inclusions to investigated the detailed polymorphism of CYP and GST gene that might contribute to the increasing incidences of HNC in the population.

Table 10: Distribution and comparison of CYP1A1, CYP2E1, GSTM1, GSTT1, GSTP1 genotypes in betel nut and non-betel nut chewing cases of HNC and healthy control

	Betel nut					Non-betel nut				
Genotype	**Cases/Control (n=182/150)**	**Crude OR [95% CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**	**Cases/ Control (n=23/60)**	**Crude OR [95% CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**
CYP1A1										
m1/m1	89/98	**1.0 (Reference)**		**1.0 (Reference)**		9/40	**1.0 (Reference)**		**1.0 (Reference)**	
m1/m2	70/46	1.67 [1.04- 2.68]	**0.03**	1.71[1.03 -3.84]	**0.03**	10/15	2.96 [1.00-8.71]	**0.04**	6.27 [1.23 -31.75]	**0.02**
m2/m2	23/6	4.22 [1.64- 10.84	**<0.01**	3.41 [1.27-9.15]	**0.01**	4/5	3.55 [0.79-15.94]	0.09	1.76 [0.25-12.13]	0.56
m1/m2+ m2/m2	93/52	1.97 [1.26- 3.07]	**<0.01**	1.93 [1.19-3.12]	**<0.01**	14/20	3.11 [1.15- 8.41]	**0.02**	3.86 [1.01-14.73]	**0.04**
CYP2E1										
c1/c1	91/96	**1.0 (Reference)**		**1.0 (Reference)**		11/31	**1.0 (Reference)**		**1.0 (Reference)**	
c1/c2	72/50	1.51 [0.95-2.40]	0.07	1.36 []0.82 2.26]	0.22	7/24	0.82 [0.27-.2.43]	0.72	2.45 [0.49-12.21]	0.27
c2/c2	19/4	5.01 [1.64-15.29	**<0.01**	6.59 [2.03-21.36]	**<0.01**	5/5	2.81 [0.68-11.63]	0.15	2.90 [0.26-31.70]	0.38
c1/c2+ c2/c2	91/54	1.77 [1.14-2.76]	**0.01**	1.71 [10.5-2.76]	**0.02**	12/29	1.16 [0.44-3.05]	0.75	2.55 [0.57-11.42]	0.21
GSTM1										
Present (+)	126/97	**1.0 (Reference)**		**1.0 (Reference)**		18/43	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	56/53	0.81 [0.51-1.28]	0.37	0.62 [0.37-1.03]	0.06	5/17	0.70 [0.22-2.19]	0.54	2.27 [0.48 -10.79]	0.30
GSTT1										
Present (+)	87/94	**1.0 (Reference)**		**1.0 (Reference)**		10/37	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	95/56	1.83 [1.18- 2.84]	**<0.01**	1.73 [1.07 -2.80]	**0.02**	13/23	2.09 [0.78-5.54]	0.13	1.37 [0.37-4.95]	0.63
GSTM1/ GSTT1										
Present (+)	62/37	**1.0 (Reference)**		**1.0 (Reference)**		6/17	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	120/113	0.63 [0.39-1.02]	0.06	0.56 [0.33-0.94]	**0.02**	17/43	1.12 [0.37-3.32]	0.83	1.82 [0.36 -8.99]	0.46
GSTP1										
Ile/Ile	110/110	**1.0 (Reference)**		**1.0 (Reference)**		12/43	**1.0 (Reference)**		**1.0 (Reference)**	
Ile/Val	56/36	1.55 [0.94-2.55]	0.08	2.04 [1.18-3.52]	**0.01**	10/13	2.75 [0.97-7.82]	0.06	2.35 [0.57-9.71]	0.23
Val/Val	16/4	4.00 [1.29-13.34]	**0.01**	3.93 [1.19-12.94]	**0.02**	¼	0.89 [0.09-8.78]	0.09	0.73 [0.03-13.74]	0.83
Ile/Val + Val/Val	72/40	1.80 [1.12-2.87]	**0.01**	2.25 [1.34-3.77]	**<0.01**	11/17	2.31 [0.85- 6.25]	0.09	1.95 [0.50-7.46]	0.33

P value was consider statically significance <0.05 by Chi-square ($\chi 2$) and Fisher's exact test, OR (odds ratio), CIs (confidence Intervals) for genotype status interaction between betel nut and non- betel nut habits. Adjusted – age, sex, tobacco, smoking.

Table 11: Distribution and comparison of CYP1A1, CYP2E1, GSTM1, GSTT1, GSTP1 genotypes in tobacco and non- tobacco chewing cases of HNC and healthy control

	Tobacco					Non- Tobacco				
Genotype	**Cases/ Control (n=132/52)**	**Crude OR [95% CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**	**Cases/ Control (n=73/158)**	**Crude OR [95% CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**
CYP1A1										
m1/m1	63/33	**1.0 (Reference)**		**1.0 (Reference)**		35/105	**1.0 (Reference)**		**1.0 (Reference)**	
m1/m2	49/17	1.51 [0.75-3.02]	0.24	1.48 [0.73 -2.97]	0.26	31/44	2.11 [1.16-3.84]	**0.01**	2.51 [1.31-4.81]	**<0.01**
m2/m2	20/2	5.23 [1.15-23.79]	**0.03**	5.09 [1.01-22.93]	**0.03**	7/9	2.33 [0.80-6.73]	0.11	2.22 [0.69-7.12]	0.17
m1/m2+ m2/m2	69/19	1.90 [0.98- 3.67]	**0.05**	1.85 [0.95-3.60]	0.06	38/53	2.15 [1.22-3.78]	**<0.01**	2.46 [1.35-4.51]	**<0.01**
CYP2E1										
c1/c1	54/40	**1.0 (Reference)**		**1.0 (Reference)**		48/87	**1.0 (Reference)**		**1.0 (Reference)**	
c1/c2	65/11	4.37 [2.05- 9.34]	**<0.001**	4.86 [2.22-10.62]	**<0.001**	14/63	0.40 [0.20-0.79]	<**0.01**	0.39 [0.19-0.80]	**0.01**
c2/c2	13/1	9.63 [1.20-76.66]	**0.03**	10.23 [1.26-83.12]	**0.03**	11/8	2.49 [0.93-6.61]	0.06	2.83 [1.01-7.95]	**0.04**
c1/c2+ c2/c2	78/12	4.81 [2.31- 10.01]	**<0.001**	5.30 [2.48-11.31]	**<0.001**	25/71	0.63 [0.35-1.13]	0.12	0.65 [0.35-1.92]	0.16
GSTM1										
Present (+)	90/39	**1.0 (Reference)**		**1.0 (Reference)**		54/110	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	42/22	0.63 [0.32 -1.23]	0.18	0.63 [0.32 -1.23]	0.18	19/48	0.80 [0.43 -1.50]	0.49	0.76 [0.39-1.48]	0.43
GSTT1										
Present (+)	56/35	**1.0 (Reference)**		**1.0 (Reference)**		41/96	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	76/17	2.79 [1.42 -5.48]	**<0.01**	2.86 [1.44-5.68]	**<0.01**	32/62	1.20 [0.68-2.12]	0.50	1.18 [0.65-2.14]	0.58
GSTM1/ GSTT1										
Present (+)	40/14	**1.0 (Reference)**		**1.0 (Reference)**		28/40	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	92/38	0.84 [0.41-1.73]	0.65	0.82 [0.39-1.71]	0.60	45/118	0.54 [0.30-0.98]	**0.04**	0.52 [0.28-0.97]	**0.04**
GSTP1										
Ile/Ile	89/39	**1.0 (Reference)**		**1.0 (Reference)**		33/114	**1.0 (Reference)**		**1.0 (Reference)**	
Ile/Val	30/13	1.01 [0.47-2.14]	0.97	0.97 [0.46-2.13]	0.99	36/36	3.45 [1.89-6.31]	**<0.001**	2.23 [1.72-6.05]	**<0.001**
Val/Val	13/0	-	0.07	-	-	4/8	1.72 [0.48-6.09]	0.39	1.55 [0.41-5.77]	0.51
Ile/Val + Val/Val	43/13	1.16 [0.56 2.43]	0.67	1.43[0.68-2.98]	0.33	40/44	3.14 [1.76-5.59]	**<0.001**	2.91 [1.60-5.23]	**<0.001**

P value was consider statically significance <0.05 by Chi-square ($\chi 2$) and Fisher's exact test, OR (odds ratio) CIs (confidence Intervals) for genotype status interaction between tobacco and non- tobacco habits adjusted – age, sex, smoking.

Table 12: Distribution and comparison of CYP1A1, CYP2E1, GSTM1, GSTT1, GSTP1 genotypes in smoking and non- smoking cases of HNC and health control

	Smoking Habits					Non- Smoking Habits				
Genotype	**Cases/Control (n=87/48)**	**Crude OR [95% CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**	**Cases/ Control (n=118/162**	**Crude OR [95% CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**
CYP1A1										
m1/m1	46/31	**1.0 (Reference)**		**1.0 (Reference)**		52/107	**1.0 (Reference)**		**1.0 (Reference)**	
m1/m2	31/12	1.74 [0.77-3.90]	0.16	1.62 [0.70-3.74]	0.25	49/49	2.05 [1.22-3.44]	**<0.01**	2.36 [1.30-4.29]	**<0.01**
m2/m2	10/5	1.31 [0.42-4.32]	0.61	1.32 [0.39-4.37]	0.65	17/6	5.83 [2.17-15.65]	**<0.001**	4.90 [1.62 -14.79]	**<0.01**
m1/m2+ m2/m2	41/17	1.62 [0.78-3.35]	0.18	1.53 [0.73- 3.23]	0.25	66/55	2.31[1.38-3.87]	**<0.001**	2.68 [1.52-4.72]	**<0.01**
CYP2E1										
c1/c1	39/30	**1.0 (Reference)**		**1.0 (Reference)**		63/97	**1.0 (Reference)**		**1.0 (Reference)**	
c1/c2	38/15	1.94 [0.90-4.18]	0.08	1.78 [0.81-3.92]	0.15	41/59	1.07 [0.64-1.78]	0.79	1.02 [0.57-1.84]	0.92
c2/c2	10/3	2.56 [0.64-10.14]	0.18	2.37 [0.58-9.61]	0.22	14/6	3.59 [1.31-9.84]	**0.01**	6.05 [1.99-18.39]	**0.01**
c1/c2+ c2/c2	48/18	2.05 [0.99-4.21]	0.05	1.88 [0.89-3.95]	0.09	55/65	1.30 [0.80-2.10]	0.27	1.38 [0.80-2.38]	0.24
GSTM1										
Present (+)	61/24	**1.0 (Reference)**		**1.0 (Reference)**		83/116	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	26/24	0.42 [0.20-0.88]	**0.02**	0.37 [0.17-0.81]	**0.01**	35/46	1.06 [0.63-1.79]	0.81	1.03 [0.56-1.87]	0.92
GSTT1										
Present (+)	35/31	**1.0 (Reference)**		**1.0 (Reference)**		62/100	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	52/17	2.70 [1.30-5.62]	**<0.01**	2.39 [1.12 -5.09]	**0.02**	56/62	1.45 [0.90-2.35]	0.12	1.46 [0.84-2.53]	0.17
GSTM1/ GSTT1										
Present (+)	28/7	**1.0 (Reference)**		**1.0 (Reference)**		40/47	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	59/41	0.36 [0.14 – 0.90]	**0.02**	0.28 [0.10-0.75]	**0.01**	78/115	0.79 [0.47- 1.32]	0.38	0.88 [0.49-1.56]	0.66
GSTP1										
Ile/Ile	52/36	**1.0 (Reference)**		**1.0 (Reference)**		70/117	**1.0 (Reference)**		**1.0 (Reference)**	
Ile/Val	27/11	1.69 [0.74-3.85]	0.20	2.21 [0.91 -5.40]	0.08	39/38	1.71 [1.00-2.93]	**0.04**	2.05 [1.11-3.79]	**0.02**
Val/Val	8/1	5.53 [0.66-46.22]	0.11	6.83 [0.76 -60.96]	0.08	9/7	2.14 [0.76-6.02]	0.14	1.93 [0.59-6.28]	0.27
Ile/Val + Val/Val	35/12	2.01 [0.92- 4.41]	0.07	2.63 [1.13-6.12]	**0.02**	48/45	1.78 [1.07-2.94]	**0.02**	2.03 [1.14-3.62]	**0.01**

P value was consider statically significance <0.05 by Chi- square ($\chi 2$) and Fisher's exact test, OR (odds ratio) CIs (Confidence Intervals) for genotype status interaction between smoking and non- smoking habits. Adjusted – age, sex, tobacco

Table 13: Distribution and comparison of CYP1A1, CYP2E1, GSTM1, GSTT1, GSTP1 genotypes in different combination of betel nut, tobacco chewing and smoking in HNC cases and healthy control

	Betel nut- Tobacco					Betel nut -Tobacco –Smoking				
Genotypes	**Cases/Control (n=122/49)**	**Crude OR [95% CI]**	**P-value**	**Adjusted OR[a] [95% CI]**	**P- value**	**Cases /Control (n=52/22)**	**Crude OR [95% CI]**	**P-value**	**Adjusted OR[a] [95% CI]**	**P- value**
CYP1A1										
m1/m1	59/31	**1.0 (Reference)**		**1.0 (Reference)**		26 /14	**1.0 (Reference)**		**1.0 (Reference)**	
m1/m2	45/16	1.47 [0.72-3.02]	0.28	1.46 [0.99-2.99]	0.30	20/7	1.53 [0.52-4.52]	0.43	1.67 [0.55-5.02]	0.36
m2/m2	18/2	4.72 [1.02-21.71]	**0.04**	4.58 [0.99-21.18]	**0.05**	6/1	3.23 [0.35- 29.58]	0.29	2.90 [0.30-27.70]	0.35
m1/m2+ m2/m2	63/18	1.83 [0.93-3.63	0.07	1.80 [0.91 -3.57]	0.09	26/8	1.75 [0.62- 4.87]	0.28	1.83 [0.64 -5.21]	0.25
CYP2E1										
c1/c1	50/37	**1.0 (Reference)**		**1.0 (Reference)**		16/17	**1.0 (Reference)**		**1.0 (Reference)**	
c1/c2	63/11	4.23 [1.96-9.14]	**<0.001**	4.75 [2.15-10.49]	**<0.001**	31/4	8.23 [2.37-28.60]	**<0.01**	10.43 [2.74- 39.66]	**<0.01**
c2/c2	9/1	6.60 [0.80 -54.89]	0.07	7.28 [0.85-62.08]	0.06	5/1	5.31 [0.55- 50.55]	0.14	7.41 [0.69-78.88]	0.09
c1/c2+ c2/c2	*72/12*	4.40 [2.10-9.34]	**<0.001**	4.96 [2.29-10.70]	**<0.001**	36/5	7.65 [2.40-24.35]	**<0.001**	9.86 [2.80-34.68]	**<0.001**
GSTM1										
Present (+)	81/27	**1.0 (Reference)**		**1.0 (Reference)**		35/7	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	41/22	0.62 [0.31-1.22]	0.16	0.61 [3.11-1.22]	0.16	17/15	0.22 [0.07-0.65]	**<0.01**	0.22 [0.07-0.66]	**<0.01**
GSTT1										
Present (+)	50/35	**1.0 (Reference)**		**1.0 (Reference)**		14/16	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	72/14	3.60 [1.75- 7.37]	**<0.001**	3.90 [1.86-8.17]	**<0.001**	38/6	7.23 [2.36- 22.19]	**<0.01**	7.22 [2.32-22.44]	**<0.01**
GSTM1/ GSTT1										
Present (+)	35/14	**1.0 (Reference)**		**1.0 (Reference)**		13/2	**1.0 (Reference)**		**1.0 (Reference)**	
Null (-)	87/35	1.00 [0.47-2.07]	0.98	0.98 [0.46-2.07]	0.96	39/20	0.30[0.06-1.46]	0.13	0.26 [0.05-1.32]	0.10
GSTP1										
Ile/Ile	84/37	**1.0 (Reference)**		**1.0 (Reference)**		39/16	**1.0 (Reference)**		**1.0 (Reference)**	
Ile/Val	26/12	0.95 [0.43- 2.09]	0.90	0.96 [0.43 -2.12]	0.92	8/6	0.54 [0.16-1.83]	0.32	0.55 [0.16-1.86]	0.33
Val/Val	12/0	-	-	-	-	5/0	-	-	-	-
Ile/Val + Val/Val	38/12	1.39 [0.65-2.96]	0.38	1.39 [0.65-2.96]	0.38	13/6	0.88 [0.16- 1.83]	0.83	0.88 [0.16- 1.83]	0.83

P value was consider statically significance <0.05 by Chi- square ($\chi 2$) and Fisher's exact test, OR (odds ratio), CIs (confidence Intervals) for genotype status interaction between both betel nut- tobacco and betel nut- tobacco- smoking habits. Adjusted – age, sex, smoking**.**

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