5.1 Background

Epidemiologic studies have demonstrated that alcohol consumption is associated with the risk for cancers of the head and neck, esophagus, colon and breast [1, 2]. The World Health Organization (WHO) and International Agency for Research on Cancer (IARC) have recently classified ethanol and acetaldehyde associated with alcohol consumption as Group 1 human carcinogens [3]. The ethanol in alcoholic beverages are metabolized to acetaldehyde by alcohol dehydrogenase 2 (ADH2) and alcohol dehydrogenase 3 (ADH3) and subsequently acetaldehyde is metabolized to acetate by aldehyde dehydrogenase 2 (ALDH2) in the liver [4]. Genetic variations in the ability to metabolize alcohol are known to have association with cancer risk. The change of amino acid from arginine (CGC) to histidine (CAC) at codon 47 of exon 3 of ADH2 gene results in mutant allele ADH2*2/*2 that encodes super active subunit causing accumulation of acetaldehyde in the circulation [5, 6]. Likewise, the enzymes encoded by ADH3*1 has higher activity than that of ADH3*2, which is a result of substitution of amino acid isoleucine from valine in exon 8 [7]. These two allelic version of ADH2 and ADH3, *ie* ADH2*2/*2 and ADH3*1/*1 exhibit both protective role and risk against alcoholism [8, 9], depending upon the polymorphic status of ALDH2 which is responsible for metabolism of aldehyde. Similarly, the change of amino acid from glutamic acid (codon) to lysine (codon) at codon 487 of exon 12 of ALDH2 encodes mutant allele ALDH2*2, which has relatively lower activity as compared to ALDH2*1 allele, leading to accumulation of acetaldehyde [5, 10-12].The inactive ALDH2 encoded byALDH2*1/*2 and less active ADH2 encoded by ADH2*1/*1 are reported to be associated with the increased risk for esophageal cancer in East Asian drinkers [13-14]. Even though the toxic effects of alcohol are well documented, the mechanism by which alcohol and alcoholic drinks affects cancer risk is poorly understood. Furthermore, impact of alcohol drinking status, betel nut and tobacco chewing habits and their interactions with alcohol metabolizing genes are not yet determined.

The NE region has recorded the highest incidence of HNC and the epidemiological studies have demonstrated betel quid chewing with or without tobacco or other forms of tobacco consumption as major risk factor for the development of HNC in the region [15]. There are very few studies that have suggested the association of BN with HNC risk. [16]. Therefore, we conducted a case-control study to evaluate the impact of both individual and combined

effects of alcohol drinking status, betel nut and tobacco chewing habits on the risk of HNC in the population of Northeast India. We have also investigated distribution of ADH2, ADH3 and ALDH2 genotypes and their interactions with betel nut, tobacco and risk stratified by drinking status.

5.2 Material and Methods

The genomic DNA was extracted from the peripheral blood by phenol-chloroform method as described in section 3.2.2 and 4.2.

As there were several types of habits exist in NE population, we included alcohol consumption (whisky, wine, RUM, vodka and local rice beer) habits only. Further, the frequency of alcohol consumption was converted to a categorical variable that include never drinkers, ≤120 ml/ day as light drinkers, >120 ml/ day as heavy drinkers. We included alcohol consumption as whole amount irrespective types of liquor

5.2.1 DNA isolation

The genomic DNA was extracted from the peripheral blood by phenol-chloroform method as described in section 3.2.2.

5.2.2 Genotyping

The blood samples of the participants were collected for genotyping. Genomic DNA was extracted from leukocyte pellet following conventional phenol-chloroform/isoamylalcohol extraction and ethanol precipitation method and then stored in T.E buffer (10Mm Tris-HCl, pH 0.8, 1 Mm EDTA) at -20ºC for further used [17]. The genotyping of the ADH2, ADH3 and ALDH2 polymorphism were determined by PCR-RFLP method. The details on primer sequences for ADH2, ADH3 and ALDH2 PCR and post PCR conditions, fragment size details are mentioned in (Table 1). The ADH2 genotyping was carried out by duplex PCR with the confronting two-pair primers (PCR-CTPP) method The amplified product contain 219 base pair (bp) for 47Arg (ADH2*1), 280 bp for 47His (ADH2*2), as well as common bands with 459 bp for ADH2 [18].

For genotyping ADH3 the PCR product was digested with 5 U of SspI restriction enzyme (New England BioLab, USA) for overnight at 37 °C. The presence of SspI restriction site

resulted in three fragments of 67, 63 and 15 bp indicated ADH3*1/*1 allele, four fragments of 130, 67, 63 and 15 bp indicated the ADH3*1/*2 allele and two fragments of 130 and 15 bp for ADH3*2/*2 allele [19].

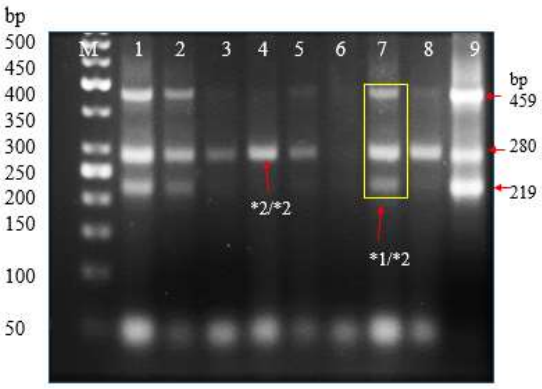
For the ALDH2 genotype analysis the PCR product was digested with the 5U of MboII restriction enzyme (New England BioLab, USA) for overnight at 37 °C. The presence of MboII restriction enzyme site resulted in two fragments of 125 and 9 bp indicated ALDH2*1/2*1 allele and one fragment of 134 bp indicated the ALDH2*1/2*2 or ALDH2*2/2*2 allele of ALDH2 gene [20]. All the amplification was performed with a thermal Cycler C1000 (Bio-Rad, CA, USA) and PCR-RFLP products were visualized in 2- 3 % agarose gel (Sigma, India) stained with ethidium bromide (SRL, India) in Gel Doc system G-Box (SYNGENE, USA). The 20 % results were cross checked and the results obtained were found to be 100 % concordant.

5.2.3 Statistical Analysis

To assess the association between ADH2, ADH3 and ALDH2 polymorphism and alcohol consumption with betel nut and tobacco habits in the risk of HNC, The details of the statistical tools and software used were mentioned in 3.2.4

Table 23: Primer sequences and reaction conditions used for ADH2, ADH3 and ALDH2 genotyping

Genotype	Primers (5' to 3')	Alleles (bp)	PCR Condition
ADH2	F1: 5′-GGG CTT TAG ACT GAA TAA CCT TGG-3’ R1: 5′-AAC CAC GTG GTC ATC TGT GC-3’ F2: 5′-GGT GGC TGT AGG AAT CTG TCA-3’ R2: 5′-AGG GAA AGA GGA AAC TCC TGA A-3’	ADH2 *1-219 bp ADH2 *2- 280 bp Common band – 459 bp	30 cycle 94°C/5 min, 95°C/65 sec 57 °C/90 sec 72°C/90 sec 72°C/5min
ADH3	5-‘ GCTTTAAGAGTAAATATTCTGTCCC –3’ 5-‘ AATCTACCTCTTTCCGAAGC –‘3	AH3 *1/*1- 67, 63, and 15 bp ADH3 *1/*2- 130, 67,63, 15 bp *ADH3*2/*2*- 130, 15bp ***SspI*, 5 U**	30 cycles 95°C/5min, 94°C/30 sec 50°C/60 sec 72°C/ 60 sec 72°C/ 10 min
ALDH2	5-‘CAAATTACAGGGTCAAGGGCT-3’ 5'-CCACACTCACAGTTTTCTCTT-3’	ALDH2*1/2 *1 - 125,9 bp ALDH2 *1/2*2 or *2/2*2 - 134 bp ***MboII 5 U***	35 cycles 95°C/ 5 min, 94°C/ 15 sec 52°C/ 90 sec 72°C/ 30 sec 72°C/ 5 min



Figures 16: Representative gels for alcohol dehydrogenase 2 (ADH2) Arg47His by polymerase chain reaction with confronting two-pair primers (PCR–CTPP). Lane M: 50 bp ladder. Lanes 1–9 are: *2/*2 were 280 bp , *1/*2 were 219 and 459 bp.

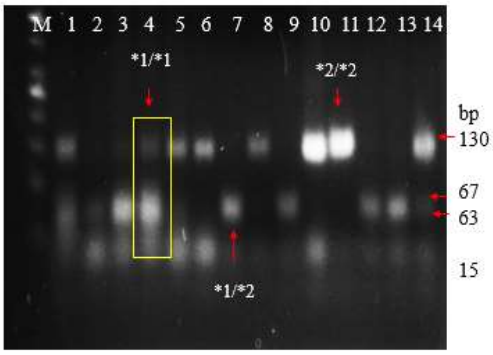


Figure 17: ADH3 genotype analyzed by (PCR-RFLP) the PCR product digested with SspI from fourteen samples. The yield were fragments with 130, 15 bp for *2/*2; 130 67, 63, 15 bp, for *2/*1 bp and 67, 63, 15 for *1/*1 lane M- 50 bp ladder

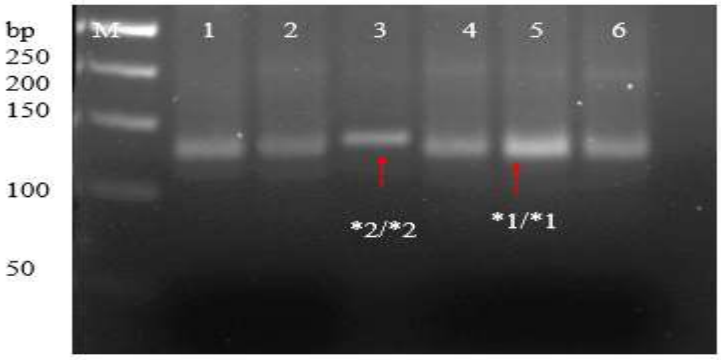


Figure 18: ALDH2 genotype analyzed by (PCR-RFLP) the PCR product digested with MboII from six samples and two genotypes were seen, the yield were fragments with 125 bp for *1/*1 and 134 bp, for *2/*2 lane M – 50 bp marker

5.3 Results

5.3.1 *Subject characteristics and risk estimates for demographic and lifestyle factors*

The distribution of demographic characteristics, lifestyle and other confounding factors of the cases (n= 205) and healthy control (n= 210) are summarized in (Table 24). The mean age of subjects participated in the study were 53.65 (±12.15) and 52.10 (±12.32) years for cases and controls respectively. There was no significant impact of non-vegetarian habits on the risk of HNC but consumption of packaged food were found to be strongly associated with the increased risk. We have also investigated the doses of BN, tobacco and smoking on the risk of HNC. The multivariate analysis exhibited significant impact of different doses BN consumption and tobacco chewing on the risk of HNC (Table 24).

The consumption of > 10 BN/day had higher impact and increased risk 2.88 fold [CI 1.05 – 7.88, P=0.03] for HNC. The impact of tobacco on the risk of HNC was independent of the doses. The smoking had lesser impact on the risk of HNC as compared to BN and tobacco (for >10 cigarettes/day OR^{a}, 2.32, CI 1.17-4.61, P= 0.01]. Higher consumption of alcohol >120 ml/day showed increased risk for HNC (OR^{a}, 2.12, CI 1.10-4.06, P<0.01) and BN and BN-tobacco synergistically increased the risk with higher alcohol intake (OR^{a}, 4.40, CI 2.33-8.31, P<0.001 and OR^{a}, 3.28, CI 1.61-6.65, P<0.01). Interestingly lower consumption of alcohol ≤120 ml/day exhibited strong protection against HNC risk in subjects (OR^{a}, 0.24, CI 0.10-0.56, P<0.01) and in the BN and BN- tobacco cases, lower intake of alcohol is associated with reduced risk for HNC though was not significant.

5.3.2 *Distribution of ADH2, ADH3 and ALDH2 genotypes and risk assessment for genes polymorphisms.*

The risk of HNC with ADH2, ADH3 and ALDH2 are presented in the (Table 25). The genotypes distributions were in Hardy-Weinberg equilibrium (P> 0.05) in both cases and control. The frequencies of ADH2*1/*2 and ADH2*1/*1 genotypes was found to be significant (OR^{a}, 1.75, 95% CI 1.16-2.64, P<0.01 and OR^{a}=3.13, 95%CI 1.40-6.98, P< 0.01) as compared to healthy control. Both univariate and multivariate analysis showed strong association of ADH2*1/*2 and ADH2*1/*1 genotypes with HNC risk. The polychotomized analysis exhibited that ADH2*1/*2 + ADH2*1/*1 (OR^{a}, 1.91, 95% CI 1.29-2.83, P< 0.01) had significant association with HNC risk. Even the genotype ADH3*2/*2 and polychotomized

analysis ADH3 *1/*2 + ADH3 *2/*2 was showed to have significant risk (OR^a, 2.07, 95%CI 1.08-3.94, P=0.02 and OR^a, 1.50, 95%CI 1.01-2.21, P=0.04) respectively, the genotypes ALDH2*1/*2 or ALDH2*2/*2The combined effects of ADH2, ADH3 and ALDH2 genotypes were also analyzed for their risk for HNC. The ALDH2*1/*2 or ALDH2*2/*2 genotypes in combination with ADH2 and ADH3 genotypes exhibited significant synergistic impact on the risk for HNC in the studied subjects. The ALDH2*1/*2 or ALDH2*2/*2 in combination with ADH2*1/*2 and ADH3 *1/*2 resulted in 3.96 fold (P< 0.04) and 3.15 fold (P< 0.01) increased risk for HNC. The combined analysis of ADH2 and ADH3 genotypes also demonstrated the synergistic impact on the risk for HNC. Interestingly the combination of ALDH2*1/*2 or ALDH2*2/*2 and ADH3*2/*2 genotypes with non-risk allele of ADH2 (ADH2*/*2) also exhibited significant impact on the risk for HNC.

5.3.3 *Interaction of ADH2, ADH3 and ALDH2 genotypes with alcohol*

The risk of HNC with ADH2, ADH3 and ALDH2 genotypes with habits of alcohol and non-alcohol habits are presented in (Table 26). In the cases with alcohol drinking habits, the ADH2 *1/*2, ADH2*1/*1, and ADH3*2/*2 (OR^a, 1.83, 95%CI 1.09-3.06, P=0.03; OR^a, 2.70 95%CI 1.06-6.88, P=0.03 and OR^a, 2.19 95%CI 1.01-4.74, P=0.04) genotypes exhibited strong impact on the risk for HNC. The multivariate analysis also demonstrated that among the combined genotypes only ADH2*1/*2 + ADH2*1/*1 (OR^a, 1.96, 95% CI 1.20-3.19, P<0.01) showed significant association with the increased risk for HNC. ALDH2 genotypes did not exhibited significant association with the HNC risk. In the cases with non-alcoholic habit, only ADH2*1/*1 (OR^a, 5.16, 95%CI 0.99-26.72, P=0.05) genotype was found to be associated with HNC risk, but are represented on the borderline.

5.3.4 *Interaction of ADH2, ADH3 and ALDH2 genotypes with betel nut and, tobacco habits and risk stratified by alcohol drinking status*

Here we have investigated the life style factors including chewing of BN, tobacco and alcohol intake and their interactions with ADH2, ADH3 and ALDH2 genotypes on risk of HNC. We have also investigated the role of alcohol consumption on the risk of HNC. When alcohol drinking status were analyzed alone heavy drinking status >120 ml/day showed 2.88

and 3.73 fold increased risk with ADH2 *1/*2 and ADH3*1/*2 genotypes (CI 1.30-6.34, P<0.01and CI 1.59-8.76, P= 0.02) respectively for HNC (Table 27).

Table 24: Predictor for head and neck patient and healthy control

Characteristic	Cases (n = 205)(%)	Control (n = 210) (%)	Crude OR[95%CI]	P value	Adjusted OR[a] [95% CI]	P-value
Age						
≤45	72 (35.12)	61 (29.75)	**1.0 (Reference)**		**1.0 (Reference)**	
>45	133 (64.87)	149 (70.95)	0.75 [0.50-1.14]	0.18	0.74 [0.43-1.27]	0.28
Education						
Graduate + Post graduate	7 (3.4)	43 (20.50)	**1.0 (Reference)**		**1.0 (Reference)**	
Illiterate	37 (18.04)	31 (14.76)	7.93[2.91-22.43]	**<0.001**	6.56 [2.20-19.53]	**<0.01**
Primary + Middle	131 (63.90)	79 (37.61)	9.95[4.05-25.57]	**<0.001**	8.89 [3.35-23.57]	**<0.001**
Secondary	30 (14.63)	57 (27.14)	3.23[1.21-8.97]	**<0.01**	2.75 [0.96-7.91]	**0.06**
Diet mainly including						
Never	154 (75.12)	177 (80.95)	**1.0 (Reference)**		**1.0 (Reference)**	
≤ 2packed food per week	40 (19.51)	30 (14.28)	1.53[0.91-2.57]	0.10	2.61 [1.26-5.42]	**0.01**
> 2 packed food per week	11 (5.36)	3 (1.42)	4.21[1.15- 15.3]	**0.02**	6.17 [1.29-29.38]	**0.02**
Non-veg intake (Fish, Meat)						
Never	10 (4.87)	9 (4.28)	**1.0 (Reference)**		**1.0 (Reference)**	
≤ 3 times per day	128 (8.78)	137 (65.23)	0.84 [0.33-2.13]	0.71	0.89 [0.29-2.68]	0.84
> 3 times per day	59 (28.78)	45 (21.42)	1.18 [0.44-3.14]	0.74	1.06 [0.33-3.41]	0.91
Occasional	8 (3.90)	19 (9.04)	0.37 [0..11-1.28]	0.12	0.16 [0.3-.076]	0.11
Betel nut						
Never	23 (11.21)	60(28.57)	**1.0 (Reference)**		**1.0 (Reference)**	
≤ 10 per day	152 (74.14)	138 (65.71)	2.87[1.68-4.89]	<**0.001**	1.17 [0.58-2.34]	0.64
>10 per day	30 (14.63)	12 (5.71)	6.52 [2.86-14.86]	<**0.001**	2.88 [1.05-7.88]	**0.03**
Tobacco						
Never	73 (35.60)	158 (75.23)	**1.0 (Reference)**			
≤ 10 per day	82 (40)	39 (18.57)	4.55[2.84- 7.29]	<**0.001**	4.19 [2.34-7.49]	<**0.001**
>10 per day	50 (24.39)	13 (6.19)	8.32 [4.25-16.27]	<**0.001**	5.49 [2.49-12.11]	<**0.001**
Smoking						
Never	118 (57.5)	162 (77.14)	**1.0 (Reference)**		**1.0 (Reference)**	
≤ 10 per day	40 (19.51)	28 (13.33)	1.96[1.14-3.35]	**0.01**	1.29 [0.64-2.58]	0.46
>10 per day	47 (22.92)	20 (9.52)	3.22 [1.81-5.73]	<**0.001**	2.32[1.17-4.61]	**0.01**
Alcohol drinking						
Never	129 (62.92)	146 (69.52)	**1.0 (Reference)**		**1.0 (Reference)**	
Light	15 (7.31)	43 (20.47)	0.39 [0.20- 0.74]	**<0.01**	0.24 [0.10-0.56]	**<0.01**
Heavy	61 (29.76)	21 (10)	3.28 [1.89- 5.69]	**<0.001**	2.12 [1.10-4.06]	**0.02**
Betel nut-Alcohol drinking						
Never	134 (63.36)	173 (82.38)	**1.0 (Reference)**			
Light	15 (7.31)	19 (9.04)	1.01 [0.49-2.08]	0.95	0.72 [0.32-1.62]	0.43
Heavy	56 (27.31)	18 (8.57)	4.01 [2.25-71.5]	**<0.001**	4.40 [2.33-8.31]	**<0.001**
Betel nut- tobacco - Alcohol drinking						
Never	158 (77.07)	184 (87.61)	**1.0 (Reference)**			
Light	10 (4.87)	11 (5.23)	1.05 [0.43-2.55]	0.89	0.56 [0.20-1.54]	0.26
Heavy	37 (18.04)	15 (7.14)	2.87 [1.52-5.42]	**<0.01**	3.28 [1.61-6.65]	**<0.01**

OR[a] adjusted in multivariate logistic regression models for age, education, diet mainly including, non-veg intake, betel nut, tobacco, smoking, and alcohol drinking, bold type indicates statistical significance (p<0.05)

Table 25: Distribution of polymorphisms and combined genotype of ADH2, ADH3 and ALDH2 genes in head and neck cancer patient and healthy control

Genotype	Case (n=205) (%)	Control (n=210) (%)	Crude OR [95%CI]	P- value	Adjusted OR[a] [95% CI]	P-value
ADH2						
*2/*2	84 (40.97)	119 (56.66)	**1.0 (Reference)**		**1.0 (Reference)**	
*1/*2	99 (48.29)	81 (38.57)	1.73 [1.55-2.59]	<**0.01**	1.75 [1.16 2.64]	<**.0.01**
*1/*1	22 (10.73)	10 (4.76)	3.11[1.40-6.92]	<**0.01**	3.13 [1.40-6.98]	<**0.01**
*1/*2 + *1/*1	121 (59.02)	91 (43.33)	1.88 [1.27-2.78]	<**0.01**	1.91 [1.29-2.83	<**0.01**
ADH3						
*1/*1*	87 (42.43)	110 (52.38)	**1.0 (Reference)**		**1.0 (Reference)**	
*1/*2*	88 (42.92)	81 (38.57)	1.37 [0.90-2.07]	0.13	1.37 [0.90-2.07]	0.13
*2/*2*	30 (14.63)	19 (9.04)	1.99 [1.05-3.78]	**0.03**	2.07 [1.08-3.94]	**0.02**
*1/*2+*2/*2*	118 (57.56)	100 (47.61)	1.49 [1.01-2.19]	**0.04**	1.50 [1.01-2.21]	**0.04**
ALDH2						
*1/2 *1*	174 (84.87)	182 (86.66)	**1.0 (Reference)**		**1.0 (Reference)**	
*1/2*2 or *2/2*2	31 (15.12)	28 (13.33)	1.15[0.66-2.01]	0.60	1.20 [0.68-2/09]	0.52
ALDH2+ADH2						
*1/2*1*+ *2/*2	73 (35.60)	113 (53.80)	**1.0 (Reference)**		**1.0 (Reference)**	
*1/2*1*+ *1/*2	87 (42.43)	62 (29.52)	2.17 [1.40-3.36]	<**0.01**	2.19 [1.403.43]	<**0.01**
*1/2*1 + *1/*1*	14 (6.82)	7 (3.33)	3.09 [1.19-8.03]	**0.02**	3.16 [1.21 -8.24]	**0.01**
*1/2*2 or *2/2*2 + *2/*2	11 (5.36)	6 (2.85)	2.93 [1.00-8.00]	**0.04**	2.91 [1.09-8.25]	**0.04**
*1/2*2 or *2/2*2 +* 1/*2	12 (5.85)	19 (9.04)	0.97 [0.44-2.13]	0.95	1.03 [0.47-2.28]	0.92
*1/2*2 or *2/2*2+ *1/*1*	8 (3.90)	3(1.42)	4.12 [1.06-16.06]	**0.04**	3.96 [1.01-15.52]	**0.04**
ALDH2+ADH3						
*1/2*1* + *1/*1	72 (35.12)	98 (46.66)	**1.0 (Reference)**		**1.0 (Reference)**	
*1/2*1*+ *1/*2	72 (35.12)	74 (35.23)	1.32 [0.84-2.06]	0.21	1.33 [0.85-2.08]	0.20
*1/2*1 + *2/*2*	30(14.61)	10 (4.76)	4.08 [1.87-8.88]	<**0.001**	4.20 [1.92-2.91]	<**0.001**
*1/2*2 or *2/2*2 + *1/*1	15 (7.31)	12 (5.71)	1.70 [0.75-3.85]	0.20	1.80 [0.78-4.11]	0.16
*1/2*2 or *2/2*2 + *1/*2	16 (7.80)	7 (3.33)	3.11 [1.21-7.95]	**0.01**	3.15 [1.22-8.01]	**0.01**
*1/2*2 or *2/2*2+ *2/*2*	0 (0.00)	9 (4.28)	-	-	-	-
ADH2+ADH3						
*2/*2 +*1/*1	27 (13.17)	65 (30.95)	**1.0 (Reference)**		**1.0 (Reference)**	
*2/*2 +*1/*2	52 (25.36)	54 (25.71)	2.31 [1.28-4.17]	<**0.01**	2.25 [1.24-4.06]	<**0.01**
*2/*2 +*2/*2	5 (2.43)	0(0.00)	-	-	-	-
*1/*2 +*1/*1	51 (24.87)	39 (18.57)	3.14 [1.70-5.80]	<**0.001**	3.12 [1.67-5.80]	<**0.001**
*1/*2 +*1/*2	25 (12.19)	23 (10.95)	2.61 [1.27-5.39]	<**0.01**	2.61 [1.26-5.40]	<**0.01**
*1/*2 +*2/*2	23 (11.21)	19 (9.04)	2.91 [1.36-6.20]	<**0.01**	2.94 [1.38- 6.28]	<**0.05**
*1/*1 +*1/*1	9 (4.39)	6(2.85)	3.61 [1.17-11.13]	**0.02**	3.36 [1.07-10.49]	**0.03**
*1/*1 +*1/*2	11 (5.36)	4 (1.90)	6.62 [1.93-22.63]	<**0.01**	6.78 [1.97-23.26]	<**0.01**
*1/*1 +*2/*2	2 (0.97)	0 (0.00)	-	-	-	-

OR[a] (Adjusted in multivariate logistic regression models) for age, gender. OR (odds ratio), CI (Confidence Intervals). Bold type indicates statistical significance ($p<0.05$).

Table 26: Genotype distribution among HNC Patients and healthy controls by alcohol and Non- Alcohol drinking

	Alcohol			Non-Alcohol		
Genotype	**Cases/ Control n=76/64**	**Crude OR [95%CI] P- value**	**Adjusted OR[a] (95% CI) P- value**	**Cases/Control n=129/146**	**Crude OR [95%CI] P- value**	**Adjusted OR[a] [95% CI] P- value**
ADH2						
*2/*2	30/35	**1.0 (Reference)**		54/84	**1.0 (Reference)**	
*1/*2	38/27	1.75 [1.06-2.90] **0.02**	1.83 [1.09-3.06] **0.03**	61/54	1.64 [0.82-3.28] 0.16	1.57 [0.77-3.18] 0.20
*1/*1	8/2	2.72 [1.07-2.92] **0.03**	2.70 [1.06-6.88] **0.03**	14/8	4.66 [0.91-23.68] 0.06	5.16 [0.99-26.72] **0.05**
*1/*2 + *1/*1	46/29	1.88 [1.16-3.04] 0.10	1.96 [1.20-3.19] **<0.01**	75/62	1.85 [0.94-3.63] 0.07	1.80 [0.91-3.57] 0.08
ADH3						
*1/*1	35/36	**1.0 (Reference)**		52/74	**1.0 (Reference)**	
*1/*2	32/23	1.37 [0.82-2.28] 0.22	1.37 [0.82-2.29] 0.22	56/58	1.43 [0.70-2.91] 0,32	1.42 [0.69-2.93] 0.33
*2/*2	9 /5	2.13 [0.99-4.58] **0.05**	2.19 [1.01-4.74] **0.04**	21/14	1.85 [0.56-6.07] 0.31	1.80 [0.53-6.07] 0.34
*1/*2+ *2/*2	41/28	1.52 [0.94-2.45] 0.08	1.52[0.94-2.46] 0.08	77/72	1.50 [0.77-2.94] 0.23	1.48 [0.75 -2.95] 0.20
ALDH2						
*1/2*1	65/53	**1.0 (Reference)**		109/129	**1.0 (Reference)**	
*1/2*2 or *2/2*2	11/11	1.39 [0.69-2.79] 0.35	1.41 [0.70-2.84] 0.33	120/17	0.81 [0.32-2.02] 0.66	0.86 [0.33-2.18] 0.75

OR (odds ratio), CIs (Confidence Intervals) for genotype status interaction between alcohol and non- alcohol consumption, OR[a] (Adjusted in multivariate logistic regression models) for age, gender, bold type indicates statistically significance (p<0.05)

The consumption of alcohol > 120 ml/day increased the risk 3.49 folds in the BN associated cases with ADH2*1/*2 genotype (95%CI, 1.50-8.70, P<0.01) respectively, as compared with the subjects that never consumed BN. Interestingly, ADH3 and ALDH2 genotypes were associated with the risk in the BN cases were found to have strong impact on the risk for HNC, with inclusion alcohol drinking status. The multivariate analysis exhibited 4.30 fold increase in the risk with ADH3*1/*2 (95% CI 1.84-10.01, P< 0.01) and 3.40 fold increase with ALDH2*1/2*2 or ALDH2*2/2*2 genotype (95 %CI, 1.02-11.35, P=0.04) amongst heavy drinkers (> 120 m/day of alcohol) in the BN chewing cases (Table 28). When alcohol drinking status were analyzed amongst the BN - tobacco chewing cases very strong impact on the risk of HNC were observed. The multivariate analysis exhibited 3.39 fold increase in the risk among the heavy drinkers (> 120 ml/day) with ADH2 *1/*2 genotype (95% CI, 1.31-8.75, P= 0.01) in BN - tobacco chewing cases. The synergistic impact on the risk for HNC were observed among the heavy drinkers with ALDH2*1/2*2

or ALDH2*2/2*2 genotypes (OR[a], 5.35, 95%CI 1.11-25.64, P= 0.03) in BN- tobacco cases as compared to BN only (Table 29).

5.4 Discussion

Here in the present study, we demonstrated the interaction of both individual and combined *ADH2, ADH3, ALDH2* polymorphism on HNC risk and their interaction with BN and tobacco chewing habits and alcohol drinking status. We have observed that higher intake of alcohol increased 2.12 fold and higher alcohol intake in combination with BN and BN-tobacco synergistically increased 4.40 and 3.28 fold risk for HNC respectively. Alcohol drinking at high dose is reported as risk factor for HNC and exposure of acetaldehyde, principle metabolite of alcohol is suggested to account for the increased risk [21]. Alcohol consumption coupled with other lifestyle factor is reported to synergistically increase the risk for esophageal cancer in the Japanese population [22]. Our study is first report that demonstrates the higher consumption of alcohol act synergistically with BN and tobacco on the risk of HNC in the NE region. However, the lower doses of alcohol intake exhibit protective role for HNC risk in our subjects. Several studies have also demonstratedthat the light to moderate drinking has health benefits i.e. protection against heart and stroke in the European population [14]. There are few reports with similar trend in East Asian population which needs further evaluation [14].

The genetic polymorphisms of alcohol dehydrogenase 2 (ADH2), alcohol dehydrogenase 3 (ADH3) and aldehyde dehydrogenase-2 (ALDH2) affects the metabolism of alcohol [14]. A case control study showed significant increase of risk for HNC in subjects with ADH2Arg/Arg and ALDH2 Glu/Lys polymorphisms in Japanese population and further demonstrated to be associated with significant gene-gene interaction ADH2 and ALDH2 as well as gene-environment interactions [1]. Recent studies have also demonstrated role ADH3*2 allele in conferring increased susceptibility to the effect of alcohol on OSCC risk [23]. A hospital based case-control study on the lifetime use of alcohol and presence of ADH3 ‘rapid allele’ (ADH3*1) on the risk for HNSCC showed no association with ADH3*1 polymorphism [24]. Sturgis et al. also found no evidence that supported the effect of ADH3 genotype or a combined effect of alcohol and ADH3 genotype on the risk of oral cavity or pharynx [25]. Ji et al. demonstrated the association of ADH2 (3170A>G) and

ADH3 (13044A>G) polymorphisms and the risk of HNSCC and could be used as biomarker for high risk group of HNSCC in Korean population [26].

Table 27: Distribution of ADH2, ADH3 and ALDH2 genotypes stratified with alcohol drinking status and HNC risk

alcohol						
Alcohol consumption	**Genotype**	**Cases/Control (n=205/210) (%)**	**Crude OR [95%CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**
ADH2 **Never**	*2/*2	54/84	**1.0 (referent)**		**1.0 (referent)**	
	*1/*2	61/54	1.75 [1.06-2.90]	**0.02**	1.82 [1.08-3.07]	**0.02**
	*1/*1	14/8	2.72 [1.07-6.92]	**0.03**	2.79 [1.08-7.23]	**0.03**
Light	*2/*2	26/0	-	-	-	-
	*1/*2	15/14	1.45 [0.64-3.24]	0.36	1.61 [0.71-3.68]	0.25
	*1/*1	2/1	0.77 [0.06-8.78]	0.83	0.72 [0.061-8.59]	0.79
Heavy	*2/*2	30/9	5.18 [2.28-11.76]	**<0.001**	4.74 [2.04-10.98]	**<0.001**
	*1/*2	24/12	3.11 [1.43-6.43]	**<0.01**	2.88 [1.30-6.34]	**<0.01**
	*1/*1	7/0	-	**-**	-	**-**
ADH3 **Never**	*1/*1	52/74	**1.0 (referent)**		**1.0 (referent)**	
	*1/*2	56/58	1.37 [0.82-2.28]	0.22	1.37 [0.81-2.31]	0.27
	*2/*2	21/14	2.13 [0.99-4.58]	**0.05**	2.31[1.05-5.07]	**0.03**
Light	*1/*1	6/25	0.34 [0.13.089]	**0.02**	0.35 [0.13-0.93]	**0.03**
	*1/*2	5/14	0.50 [0.17-1.49]	0.22	0.56 [0.18-1.68]	0.30
	*2/*2	4/4	1.42 [0.34-5.95]	0.62	1.58 [0.36-6.80]	0.53
Heavy	*1/*1	29/11	3.75 [1.72-8.17]	**<0.01**	3.61 [1.63-8.01]	**0.02**
	*1/*2	27/9	4.26 [1.85-9.82]	**<0.01**	3.73 [1.59-8.76]	**0.02**
	*2/*2	5/1	3.11 [0.80-62.70]	0.07	5.69 [0.61-52.85]	0.12
ALDH2 **Never**	*1/2*1	109/129	**1.0 (referent)**		**1.0 (referent)**	
	*1/2*2 or*2/2*2	20/17	1.39 [0.60-2.70]	0.35	1.71 [0.84-3.49]	0.13
Light	*1/2*1	15/37	0.48 [0.25-0.92]	**0.02**	0.52[0.27-1.02]	0.06
Heavy	*1/2*2 or*2/2*2	0/6	-	-	-	-
	*1/2*1	50/16	3.69 [1.99-6.86]	**<0.01**	3.36 [1.79-6.33]	**<0.001**
	*1/2*2 or*2/2*2	11/5	2.60 [0.87-7.72]	0.08	2.57 [0.84-7.82]	0.09

OR (odds ratio) CIs (Confidence Intervals) OR [a] (Adjusted in multivariate logistic regression models) for genotype status interaction between ADH2, ADH3, ALDH2 genotype and alcohol drinking in never, light and heavy adjust with age, gender and doses of smoking, bold type indicates statistically significance ($p<0.05$)

Table 28: Distribution of ADH2, ADH3 and ALDH2 genotypes stratified with betel nut, alcohol drinking status and HNC risk

		Betel nut- alcohol				
Alcohol drinking	**Genotype**	**Cases/Control (n=205/210)(%)**	**Crude OR [95%CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**
ADH2 **Never**	*2/*2	56/105	**1.0 (referent)**		**1.0 (referent)-**	
	*1/*2	64/59	2.03 [1.25-3.28	**<0.01**	2.13 [1.29-3.52]	**<0.01**
	*1/*1	14/9	2.91 [1.18-7.15]	**0.01**	3.05 [1.24-7.60]	**0.01**
Light	*2/*2	0/6	-	-	-	-
	*1/*2	14/12	2.18 [0.94-5.04]	0.06	2.60 [1.10-6.12]	**0.02**
	*1/*1	1/1	1.87 [0.11-30.54]	0.65	1.60 [0.08-29.40]	0.75
Heavy	*2/*2	28/8	6.56 [2.80.15.35]	**<0.001**	6.05 [2.53-14.50]	**<0.001**
	*1/*2	21/10	3.93 [1.73-8.93]	**<0.01**	3.49 [1.50-8.07]	**<0.01**
	*1/*1	7/0	-	-		
ADH3 **Never**	*1/*1*	55/92	**1.0 (referent)**		**1.0 (referent)**	
	*1/*2*	57/67	1.42 [0.87-2.31]	0.15	1.42 [0.86-2.35]	0.16
	*2/*2*	22/14	2.62 [1.24-5.55]	**0.01**	2.83 [1.31-6.11]	**<0.01**
Light	*1/*1*	6/10	1.00 [0.34-2.91]	0.99	1.11 [0.37-3.31]	0.84
	*1/*2*	5/5	1.67 [0.46-6.03]	0.43	1.81 [0.49-6.65]	0.37
	*2/*2*	4/4	1.67 [0.40-6.95]	0.47	1.88 [0.44-8.03]	0.39
Heavy	*1/*1*	26/8	5.43 [2.30- 12.84]	**<0.001**	4.99 [2.08-12.00]	**<0.001**
	*1/*2*	26/9	4.83 [2.11-11.06]	**<0.001**	4.30 [1.84-10.01]	**<0.01**
	*2/*2*	4/1	6.69 [0.72-0.61.39]	0.09	5.33 [0.54-52.61]	0.15
ALDH2 **Never**	*1/2*1*	113/153	**1.0 (referent)**		**1.0 (referent)**	
	*1/2*2 or *2/2*2	21/20	1.42 [0.73-2.78]	0.29	1.65 [0.84-3.24]	0.14
	*1/2*1*	15/15	1.35[0.63-2.88]	0.43	1.49 [0.69-3.21]	0.30
	*1/2*2 or*2/2*2	0/4	-	-	-	-
Light	*1/2*1*	46/14	4.44 [2.32-8.48]	**<0.001**	3.92 [2.02-7.58]	**<0.001**
	*1/2*2 or*2/2*2		3.38 [1.03-11.06]	**0.04**	3.40 [1.02-11.35]	**0.04**
Heavy		10/4				

OR (odds ratio) CIs (Confidence Intervals) OR[a] (Adjusted in multivariate logistic regression models for genotype status interaction between ADH2, ADH3, ALDH2 genotype and alcohol drinking in never, light and heavy adjust with age, gender and doses of smoking, bold type indicates statistically significance ($p<0.05$)

Table 29: Distribution of ADH2, ADH3 and ALDH2 genotypes stratified with betel nut, tobacco and alcohol drinking status and HNC risk

Betel nut – tobacco -- alcohol						
Alcohol consumption	**Genotype**	**Cases/Control (n=205/210) (%)**	**Crude OR [95%CI]**	**P- value**	**Adjusted OR[a] [95% CI]**	**P- value**
ADH2 **Never**	*2/*2	68/111	**1.0 (referent)**			
	*1/*2	72/64	1.83 [1.16-2.88]	<**0.01**	1.93 [1.20-3.10]	<**0.01**
	*1/*1	18/9	3.26 [1.38-7.67]	<**0.01**	3.26 [1.36-7.80]	<**0.01**
Light	*2/*2	0/0	-	-	-	-
	*1/*2	10/10	1.63 [0.64-4.12]	0.30	1.85 [0.72-4.76]	0.19
	*1/*1	0/1	-	-	-	-
Heavy	*2/*2	16/8	3.26 [1.32-8.03]	**0.01**	2.86 [1.12-7.31]	**0.02**
	*1/*2	17/7	3.96 [1.56-10.05]	<**0.01**	3.39 [1.31-8.75]	**0.01**
	*1/*1	4/0	-			
ADH3 **Never**	*1/*1*	63/99	**1.0 (referent)**			
	*1/*2*	70/70	1.57 [0.99-2.48]	**0.05**	1.57 [0.98-2.51]	0.06
	*2/*2*	25/15	2.61 [1.28-5.34]	<**0.01**	2.86 [1.36-5.98]	<**0.01**
Light	*1/*1*	4/4	1.57 [0.37-6.51]	0.53	2.02 [0.48-8.52]	0.33
	*1/*2*	4/3	2.09 [0.45-9.67]	0.34	2.25 [0.47-10.63]	0.30
	*2/*2*	2/4	0.78 [0.14-4.41]	0.78	0.77 [0.13-4.50]	0.77
Heavy	*1/*1*	20/7	4.49 [1.79-11.23]	<**0.01**	3.99 [1.56-10.21]	0.21
	*1/*2*	14/8	2.75 [1.09-6.93]	**0.03**	2.33 [0.90-6.00]	0.08
	*2/*2*	3/0	-	-	-	-
ALDH2 **Never**	*1/2*1*	136/162	**1.0 (referent)**			
	*1/2*2 or*2/2*2	22//22	1.19 [0.63-2.24]	0.58	1.37 [0.71-2.64]	0.33
Light	*1/2*1*	10/7	1.70 [0.63-4.59]	0.29	1.88 [0.68-5.15]	0.21
	*1/2*2 or*2/2*2	0/4	-	-	-	
Heavy	*1/2*1*	28/13	2.56 [1.27-5.14]	<**0.01**	2.12 [1.03-4.36]	**0.04**
	*1/2*2 or*2/2*2	9/2	5.36 [1.13-25.23]	**0.03**	5.35 [1.11-25.64]	**0.03**

OR (odds ratio) CIs (Confidence Intervals) OR [a] (Adjusted in multivariate logistic regression models) for genotype status interaction between ADH2, ADH3, ALDH2 genotype and alcohol drinking in never, light and heavy adjust with age, gender and doses of smoking, bold type indicates statistically significance ($p<0.05$)

Even though the role of ADH2, ADH3 and ALDH2 genotypes and alcohol on the risk of various cancer are well reported, the role of these genotypes and their combined effect coupled with alcohol on the risk of HNC have not been studies from the NE region of India. This is for the first time we have demonstrated that the interaction of ADH2, ADH3 and

ALDH2 genotypes with betel nut and tobacco chewing habits and risk for HNC stratified by the alcohol drinking status in the NE population.

Our case-control study from the subjects of NE region demonstrated significant association of ADH2*1/*2, ADH2*1/*1, ADH3*2/*2 and combination of ADH2*1/*2 + ADH2*1/*1 and ADH3*1/*2 + ADH3*2/*2 genotypes for the risk for HNC. Interestingly our study did not exhibited the association of ALDH2 genotype with the risk for HNC in the population of NE region. The combined analysis of the ADH2 and ADH3 genotypes showed synergistic impact on the risk for HNC. Here we also observed strong impact less active ALDH2 allele in combination with non-risk allele of ADH2 (ADH2*2/*2) on the risk for HNC (Table 2). This is the unique observation from our analysis on the subjects of NE region. This indicated the predominant role of ADH2 in determining the risk for HNC in the general population. The stronger impacts of ADH2 as compared to ALDH2 were also observed in case of esophageal cancer in Thai population [27].

On studying the role of ADH2, ADH3 and ALDH2 genotype distribution on HNC risk with alcohol drinking status, we found that ADH2*1/*2, ADH2*1/*1 and combination of ADH2*1/*2 + ADH2*1/*1 had strong modulatory effect in alcoholic cases, but in non-alcoholic cases only ADH2*1/*1 had borderline effect on the risk of HNC . Our observations also exhibited association of low activity ADH3*2/*2 allele on the risk for HNC in alcoholic cases which are also more prevalent in African and Caucasians than in Asians and European population [27, 28, 29]. The strong impact of ADH3 on the risk for HNC Was also reported from the western countries. Solomon et al. reported that ADH3*2/*2 genotype may have increased risk for OSCC in heavy drinker and less significant in moderate drinkers and negligible risk in light drinkers of southern region of Indian [30]. Our data on the role of low activity ADH3*2 allele on the risk of HNC is the first report to our knowledge from the NE India.

Our data further showed no association of the risk alleles ALDH2*1/2*2 or ALDH2*2/2*2 with HNC. A case-control study among Japanese men with oral and pharyngeal squamous cell carcinoma demonstrated significant independent risk factor for oral and pharyngeal squamous cell cancer among moderate to heavy drinkers with inactive ALDH2*1/*2, less

active ADH2*1/*1, frequent drinking of strong alcohol, smoking etc. [12].The earlier studies have shown that the presence of both ADH2 and ALDH2 risk alleles in the drinkers had contributed to a fourfold increased risk for esophageal squamous cell carcinoma as compared with drinkers without the presence of these alleles [31]. *Wu et al*. have suggested that polymorphisms of ADH2 and ALDH2 can change the impact of alcoholic consumption on esophageal cancer risk in Taiwan population [32]. Similar observations were reported among heavy drinkers with ADH2*1/*1 or ALDH2 *1/*2 alleles had increased risk for esophageal cancer in Thai population [27]. Hidaka et al suggested for understanding gene-environment interaction it is important to consider both alcohol consumption level and ADH3 and ALDH2 polymorphism [33]. A meta-analysis also demonstrated that ADH2*1 and ALDH2*2 allele can increase the risk of EC in China which can be modified by alcohol consumption. [34].

Meta-analysis of 11 case-control studies have demonstrated that ALDH2 polymorphism (Glu487Lys) and not ADH2 polymorphism, significantly associated with the risk for colorectal cancer in East Asians [35]. Similar meta-analysis by Zhao et al. suggested that ALDH2 Glu478Lys polymorphism may be associated with a decreased risk of colorectal cancer [36]. The discrepancy in the reported studies might due to dissimilarity in the drinking characteristics and the frequency of ADH2 and ALDH2 variant alleles across the population [37]. In our study we also could not observe association of ALDH polymorphism with HNC risk which could be dissimilarity in the frequency of variant allele in the population.

The combination of alcohol consumption, tobacco, smoking and the inactive heterozygous ALDH2 genotype (ALDH2*1/*2) and less active homozygous ADH2 genotype (ADH2*1/*1) increases risk for squamous cell carcinoma in the upper aero-digestive tract in multiplicative fashion in East Asians [38]. The previous study have reported that in addition to cigarette and areca chewing, alcohol consumption is one of risk factor for esophageal cancer in Taiwan. The subjects with alcohol drinking status and carried susceptibility genotypes of ADH2 and ALDH2 are reported to experience multiplicative increase in risk for esophageal cancer as compared to non-drinkers without the susceptibility genotypes [6]. In Japanese alcoholics, the oropharyngeal, esophageal and

stomach cancer were detected at extremely high rates and were proportional to amount of alcohol consumed [39]. *Lee et al.* have demonstrated that the ADH2 and ALDH2 genotypes, in combination with alcohol drinking habit along with continued tobacco consumption, plays an important pathogenic role in modulating esophageal squamous cell carcinoma risk [40]. A case-control study population of USA has also shown that the ADH3 polymorphism modifies the risk of squamous cell carcinoma of head and neck associated with alcohol and tobacco use [41]. However, there are no reports on the role of alcohol drinking status coupled with BN and tobacco and the risk alleles of ADH2, ADH3 and ALDH2 from the NE region of India.

Our study demonstrated that higher consumption of alcohol increased 3.49, 4.30 and 3.40 fold risk for HNC with risk alleles ADH2*1/*2, ADH3*1/*2 and ALDH2*1/2*2 or ALDH2*2/2*2 respectively in BN chewing subjects. When combination BN and tobacco chewing subjects were analyzed for the risk HNC with the alcohol drinking status, the higher intake demonstrated 3.39 and 5.35 fold increase risk respectively in the NE subjects. Interestingly our study indicated synergistic impact of higher consumption of alcohol in the BN-tobacco subjects with ALDH2*1/*2 or ALDH2*2/*2 allele as compared to BN subjects. Here in our study we could also see the role of ADH2*1/*1 and ADH3*2/*2 alleles on the HNC risk in alcoholic case, however when drinking status was stratified as heavy and light, no association were found, which might be due to smaller number of subjects in the stratified analysis. Further investigation must be undertaken with larger sample size to precisely establish association with the alcohol drinking status, betel nut, tobacco and HNC risk.

Our findings further suggest that the higher alcohol intake by an individual with risk alleles of ADH2, and ALDH2 possess risk for HNC in the NE population. The present study also indicated that higher intake of alcohol has synergistic impact on the risk for HNC in the subjects with low activity alleles of ALDH2 and betel – tobacco chewing habits. The present knowledge on the confounding risk factors in connection with ADH2 and ALDH2 risk alleles will enable in developing strategies/approaches aimed at prevention of HNC amongst the population of NE region of India

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