7.1 Background

Human Papillomavirus (HPV) was advocated first time by Syrjanen et al (1983) as a risk factor especially for oropharyngeal and oral cancer [1]. In subset of HNC cases, HPV association have been acknowledged in younger age group (<50 yrs) patients without habit of tobacco and alcohol consumption [2, 3]. The HPV genomic DNA was mostly detected by PCR based method and studies have shown that up to 60% of HNC cases may be HPV positive [4, 5]. The causal association between HPV and head and neck cancer remains contradictory due to conflicting evidences [6, 7]. HPV association with HNC has assumed significance due to the findings that HPV positive HNC cases have good prognosis as compared to the HPV negative cases [8-12]. The possible routes of transmission of HPV in HNC may be oral sexual behavior in adults and perinatal transmission in the neonatal children. In particular, the oral cavity, pharynx and larynx, epithelial cells are more susceptible to HPV infection [13-17]. HPV types are classified as high-risk (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and low-risk (HPV-26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85) on the basis of their carcinogenic potential. HPV-16 and HPV-18 high-risk type have been considered as major contributory genotypes in HNC [18, 19].

The master cell cycle regulators, p53, pRB and p16 are important tumor suppressor genes having significant role in cell cycle regulatory pathway and cancer [20]. These genes play important role in maintaining genomic integrity and cell cycle, and control of apoptosis [21, 22]. The High Risk HPV (hr-HPV) types 16 and 18 principally exercise their carcinogenic potential through the expression of E6 and E7 oncoprotein. The E6 oncogene activation leads to degradation of p53 through its interaction with the E3 ubiquitin ligase E6AP [22, 23]. The active E7 degrades the retinoblastoma tumor suppressor protein (pRb) due to which transcription factor E2F is stimulated and results in overexpression of p16 INK4A, a cyclin-dependent kinase inhibitor [22, 23]. By evading the above mentioned master guardians of cell cycle, HPV genes take control over the cellular proliferation that leads to uncontrolled cell division.

In the present study, E6 nested multiplex PCR method was used for the sensitive and type-specific detection of HPV infections based on the amplification of the viral E6/E7 oncogene as described previously [19, 24]. The Hybrid Capture 2 (HC2) test, which is

FDA-USA approved and WHO recommended for detection of HPV in clinical specimens of cervical intraepithelial lesions (CINs) have been applied for the first time for hr-HPV detection in HNC specimens of NE India.

7.2 Materials and Methods

7.2.1 Patients Characteristics

A total of 106 HNC patients were enrolled in this study (male 73; female 33). Tumor tissue specimens were obtained from the Head and Neck oncology surgery unit of Dr. Bhubaneswar Borooah Cancer Institute (BBCI), Regional Cancer Center, Guwahati, India. The histopathologically confirmed HNC cases were enrolled during the period of October, 2011 to September, 2013. The demographic and lifestyle information were collected using a pre-designed questionnaire through personal interview. The information collected about demographic variables included age, gender, ethnicity, education, socio-economic status, life style factors of alcohol, cigarette smoking, betel nut chewing and other food habits. The American Joint Committee on Cancer's TNM staging was used for staging and diagnosis. The patients were informed about the study and written consent was obtained prior to collection of the specimens. This study was approved by the institutional ethics committee of Dr. Bhubaneswar Borooah Cancer Institute (BBCI), Guwahati, Assam (India). Genomic DNA isolation from tumor tissue.

Tissue biopsy specimens were collected in 1mL volume of phosphate buffered saline (PBS) and the genomic DNA was extracted from the homogenized tissue by using QIAamp DNA mini kit (Qiagen, Germany) following the manufacturer's instructions. The quantity and quality of the isolated genomic DNA was confirmed by Nano-spectrophotometer (Bio-photometer, Eppendorf, Germany) and agarose gel electrophoresis respectively.

7.2.2 High -risk HPV DNA detection by Hybrid Capture 2 (HC2) Assay

Genomic DNA isolated from biopsy samples of the patients were suspended in the specimen transport medium (STM) (Qiagen, Germany) and the presence of HPV was detected by HC2 High-Risk HPV DNA Test™ kit (Qiagen, Germany) as per the instructions of the manufacturer. In the patient STM samples, chemiluminescent detection

of the 13 most common hr-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was performed using automated DML 2000 Luminometer system (Digene, USA). Light signals were measured as relative light units (RLUs) with light intensity indicating the presence or absence of target DNA in the tested specimen. The mean RLU value for high risk HPV calibrator of positive control samples was considered as positive cutoff (CO) value. Patients specimen with RLU/CO value ratio -1 with high-risk HPV probe were considered "positive" for any of the hr-HPV types (16,18,31,33,35,39,45,51,52,56,58,59 and 68). The absence of hr-HPV DNA below the detection limit was denoted in sample with RLU/ CO <1. When RLU = CO it confers presence of approximately 5000 virus copies in the specimen. Two confirmed cases of cervical cancer with hr-HPV positivity were also used in each test run and carrier DNA as negative control [25, 26]. The test reports were generated as per the format of the Hybrid Capture TM software ver. 2.0.

7.2.3 High -risk HPV DNA detection by nested multiplex PCR (NMPCR)

All the samples were tested twice and samples with hr- HPV positive outcome in both test run were only scored as hr- HPV positive. E6 nested multiplex PCR (NMPCR) for HPV genotyping. The samples tested positive for hr-HPV DNA in the HC2 assay were further analyzed for HPV genotyping. The nested E6 PCR reaction 1st round was done in 20 μL volume using 25–100 ng genomic DNA, 1X Maxima Hot Start PCR buffer, 1.5–2.0 mM $MgCl_2$ (Thermo Scientific, USA), 250 nM of each primers (Metabion, Germany), 250 μM of each dNTPs and 0.5 U Maxima Hot Start Taq DNA Polymerase (Thermo Scientific, USA). The thermal cycling condition for 1st round of E6 PCR was as: Denaturation- 95°C- 5 min, 38 amplification cycles with Denaturation- 95°C- 30 sec, Annealing- 55°C- 45 sec, Extension-72°C- 90 sec, and final Extension- 72°C for 7 min. The 1st round E6 PCR product (630bp) was used as a template (1 μL) for the 2nd round of nested reaction for HPV genotyping using multiplex set of HPV type specific primers. The reaction volume and constituents were similar as mentioned above. The 2nd round nested PCR condition for HPV genotyping was similar to 1st round with changes only in Annealing- 55°C- 40 sec and Extension-72°C- 45 sec [24]. All PCR products were visualized in 2% agarose gel (Amresco, USA) stained with ethidium bromide (Amresco, USA) in Gel Doc

XR™ system (Bio-Rad, USA), as shown in figure 23.a. The hr-HPV positive cervical squamous cell carcinoma samples DNA was used as positive control in each PCR run. The primer sequences are described in Table 36.

7.2.4 Immunohistochemistry

The tumor tissues samples collected from the HNC patients were processed for immunohistochemistry analysis following standard method as described by [27]. Briefly, paraffin embedded sections were sliced (5 µm thickness) and fixed on microscopic slides. The fixed sections were deparaffinized, rehydrated and incubated in methanol containing 10 % H_2O_2 to reduce the endogenous peroxide activity. The section were kept in microwave for antigen retriever in citrate buffer, pH 6.0 and thereafter kept in blocking buffer solution (1X PBS + 30X H_20_2). The sample were then incubated at 4°C overnight with mouse monoclonal p16 antibody (Santa Cruz, USA) diluted (1:50) in 20 mM Tris-buffered saline (pH 7.4) containing 2mM CaCl2 solution, Bovine serum albumin (BSA). The samples were incubated with HRP conjugated secondary antibody, followed by DAB working solution, and finally counterstained with hematoxylin [27]. Stained tumor sections were analyzed by light microscope (Carl Zeiss, Germany) and images were captured at 400X magnifications. Immunoreactivity (represented by brown staining) was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), as shown in figure 24.

7.2.5 Statistical Analysis

The data was represented as mean ± SD or in frequencies (%). The χ^2 test and Fisher exact test were employed to identify the association between clinico-pathological characteristics and hr- HPV status. Univariate analysis was done by calculating the odds ratios (ORs) along with 95% confidence intervals (CIs) and $P < 0.05$ was considered to be the significance level. Epi-info Version 6 software was employed for the statistical analysis

Table 36: HPV type-specific nested PCR Primer Sequences

	Primers	Primers (5' to 3')	Amplicon size (bp)
	GP-E6-3F	Sense: GGG AGG TAC TGA AAT CGG T	630
	GP-E6-6B	Antisense: TCC TCT GAG TCG CCT AAT TGC TC	
Multiplex Primer set	**High risk-HPV genotype**	**Primers (5' to 3')**	
I	16	Sense: CAC AGT TAT GCA CAG AGC TGC	457
		Antisense: CAT ATA TTC ATG CAA TGT AGG TGT A	
	18	Sense: CAC TTC ACT GCA AGA CAT AGA	322
		Antisense: GTT GTG AAA TCG TCG TTT TTC A	
	31	Sense: GAA ATT GCA TGA ACT AAG CTC G	263
		Antisense: CAC ATA TAC CTT TGT TTG TCA A	
	59	Sense: CAA AGG GGA ACT GCA AGA AAG	215
		Antisense: TAT AAC AGC GTA TCA GCA GC	
	45	Sense: GTG GAA AAG TGC ATT ACA GG	151
		Antisense: ACC TCT GTG CGT TCC AAT GT	
II	33	Sense: ACT ATA CAC AAC ATT GAA CTA	398
		Antisense: GTT TTT ACA CGT CAC AGT GCA	
	52	Sense: TAA GGC TGC AGT GTG TGC AG	229
		Antisense: CTA ATA GTT ATT TCA CTT AAT GGT	
	56	Sense: GTG TGC AGA GTA TGT TTA TTG	181
		Antisense: TTT CTG TCA CAA TGC AAT TGC	
	58	Sense: GTA AAG TGT GCT TAC GAT TGC	274
		Antisense: GTT GTT ACA GGT TAC ACT TGT	
III	35	Sense: CAA CGA GGT AGA AGA AAG CAT C	358
		Antisense: CCG ACC TGT CCA CCG TCC ACC G	
	42	Sense: CCC AAA GTA GTG GTC CCA GTT A	277
		Antisense: GAT CTT TCG TAG TGT CGC AGT G	
	43	Sense: GCA TAA TGT CTG CAC GTA GCT G	219
		Antisense: CAT GAA ACT GTA GAC AGG CCA AG	
	44	Sense: TAA ACA GTT ATA TGT AGT GTA CCG	163
		Antisense: TAT CAG CAC GTC CAG AAT TGA C	
IV	39	Sense: GAC GAC CAC TAC AGC AAA CC	280
		Antisense: TTA TGA AAT CTT CGT TTG CT	
	51	Sense: GAG TAT AGA CGT TAT AGC AGG	223
		Antisense: TTT CGT TAC GTT GTC GTG TAC G	
	66	Sense: TTC AGT GTA TGG GGC AAC AT	172
		Antisense: AAA CAT GAC CCG GTC CAT GC	
	68	Sense: GCA GAA GGC AAC TAC AAC GG	333
		Antisense: GTT TAC TGG TCC AGC AGT GG	

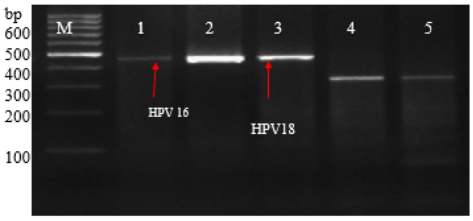

Figure 23: (a) Representative image of agarose gel (2%) showing HPV Type-specific E6 Nested Multiplex PCR (NMPCR) Product. Lane 1, 2, 3: HPV-16 HNC cases and lane 4, 5: HPV -18 HNC cases, M= 100 bp DNA Ladder. Amplified NMPCR product size were 457 bp (HPV 16), 322bp (HPV18). **Figure 23 (b)** represents of HPV positive and HPV negative cases and **Figure 23 (c)** represents p16 IHC expression

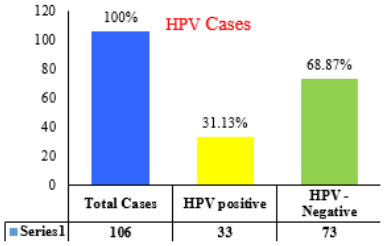

Figure 23: (b)

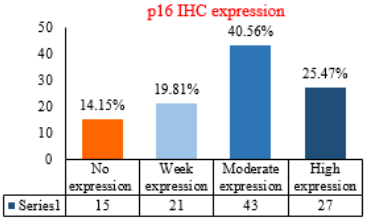

Figure 23: (c)

P16INK4A immunohistochemistry (IHC)

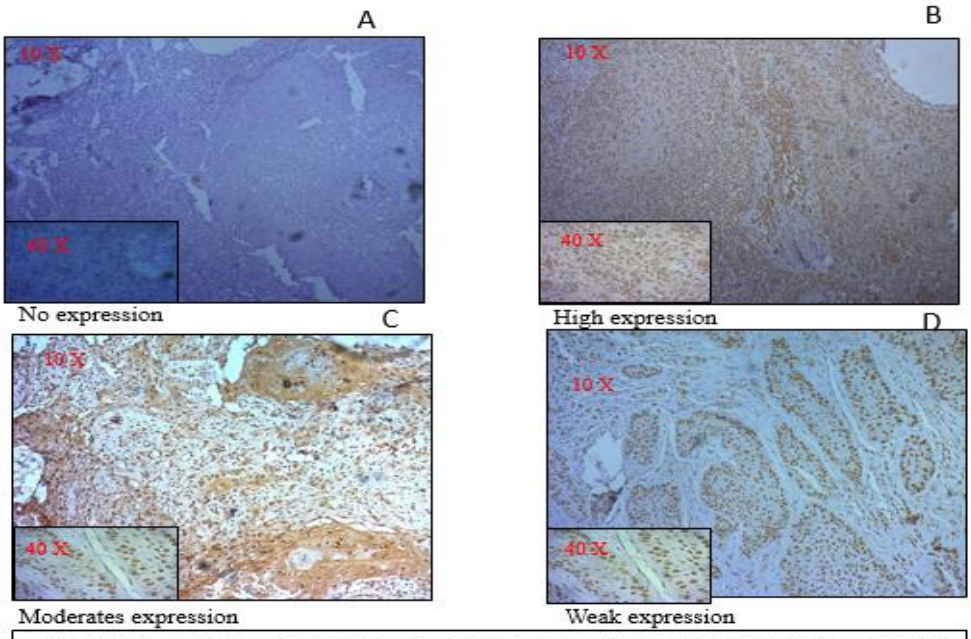

Figure 24. IHC for p16 showing (A) a negative case in tumor tissue and (B) a positive case with diffuse, strong, nuclear and cytoplasmic staining in tumor cells (10X,40X), (C) a moderate expression and (D) is weak expression in tumor tissue. The brown color reflects the reaction of diaminobenzidine (DAB) substrate and thus antibody binding to particular protein in sections. The blue is hematoxylin counter stain.

7.3 Results

7.3.1 Characteristics of the Patient

In the study population overall (n=106), 68.86 % of patients (n=73) were males and 31.13% were (n=33) of females. Patients' age ranged from 25 to 80 years (Mean= 53.5 years). More than three fourths (80 %) of patients were from rural areas, rest (20%) being from semi urban and urban areas. Non-vegetarian dietary habit was reported by >92% of the patients (Table 37). The study population was grouped into either of 'hr-HPV positive' (n=33) or 'HPV negative' (n=73) based on the presence of hr-HPV DNA. Demographic characteristics of the patients and their dietary habits are mentioned in Table 2. The demographic factors were not significantly associated with hr-HPV infection ($p>0.05$). The anatomical site among clinico-pathological characteristics of the patient are, out of 106 cases, of oral cavity 81 % (n=86), oropharynx 4.7 % (n=5), hypopharynx 2.8 % (n=3), larynx 8.5 % (n=9) in (Table 38).Patients with advanced stages III-stage 24.5% (n=26), IVA- 55.6 % (n=59) and IVB 5.6 % (n=6) had been diagnosed in HNCs patients. Squamous cell carcinoma was predominant 85.8 % (n=91) histological type in the present study population.

7.3.2 Hr-HPV detection and its association with Demographic and Clinico-Pathological characteristic

The high risk HPV DNA was detected by nested multiplex PCR (NMPCR) in 31.13% (n = 33) and hybrid capture in 24.52% (n = 26) of the HNC patients. The sensitivity and negative positive predictively was found to be 100% each; the specificity and positive predictive value (PPV) was observed as 91.19% and 78.78% respectively. This test classified 93.29% of the cases correctly and the area under the curve was found as 89.50% ($p<0.001$) with 95%CI (81.30% - 97.80%). To confirm the biologically active HPVs, p16 expression was analyzed in both HPV positive and HPV negative cases, and over expression of p16 was found in HPV positive cases which is an indirect indicator for the presence of HPV (data not shown). In HC2 tested samples, mean RLU value was in the range of 99–3466, and the mean positive cut off (CO) value for hr-HPV was 347 (Qiagen, Germany) as per the instruction. The High risk HPV positive cases were genotyped for HPV subtypes using E6 nested multiplex PCR. The primer details used for the E6 nested multiplex PCR [29], are mentioned in the (Table 36).

The hr-HPV positive cases were further univariate analyzed for life style habits and significant association was found between hr-HPV positivity and alcohol consumption (OR = 7.56; 95% CI. 2.64–22.19, p <0.001) as indicated in (Table 37). The alcohol drinker samples have shown to bear hr-HPV infection compared to non-drinkers. To look out for the synergistic or complementary effect between tobacco and alcohol habits, we observed significant association with hr-HPV positivity (OR = 3.09; 95% CI. 1.00–9.65, p = 0.02), but when cases of smokers and alcohol habits were together analyzed and calculated we found that there was no significant association with hr- HPV positivity. The betel nut chewers also had shown more hr-HPV infection. Among hr- HPV positive cases (n = 33), 39.40% (n = 13) patients were tobacco non-chewers and 60.60% (n = 20) were tobacco chewers. Non- smokers group 69.69% (n = 23) have more likelihood of hr-HPV infection compared to smokers 30.31% (10). Patients residing in rural areas have more hr-HPV positivity 87.88% (n = 29) compared to semi urban 9.09% (n = 3) and urban 3.03% (1%) area residents. There was no significant association found between hr-HPV infection and gender, betel quid, tobacco chewers, smoking habit, dietary habit, age and place of residence (Table 37). Among hr-HPV positive cases (n = 33), oral cavity 72.72% (n = 24), larynx 6.06% (n = 2), hypopharynx 6.06% (n = 2) and oropharynx 15.15% (n = 5) had hr-HPV DNA (Table 38). There was significant association between hr-HPV infection and oropharyngeal tumor site (p = 0.01). Majority of the hr-HPV positive cases 90.90%, (30 of 33) were presented with advanced stage III, IVA or IVB disease. Histologically, 90.90% hr-HPV positive cases were squamous cell carcinoma, 6.06% were verrucous carcinoma and 3.03% were other carcinomas (Adenoid cystic carcinoma, spindle cell malignant) (Table 38). There was no significant association of TNM tumor stage, histology grade and morphology with hr- HPV positivity (p>0.05). When HNC cases were analyzed with lifestyle factors, alcohol consumption was found to be significantly associated with hr-HPV infection (p<0.001) (Table 38).

Next we analyzed hr-HPV status in oral cavity cancers alone (Table 39) and compared with smoking, tobacco, alcohol, betel nut use and found that there was significant association of hr-HPV positivity and alcohol consumption (OR = 2.98; 95% CI. 0.95–9.43, p = 0.03). To find the synergistic or complementary effect of tobacco and alcohol habits, from univariate analysis it was found that there is significant association of hr-

HPV positivity with tobacco and alcohol habits(OR = 2.94; 95% CI. 0.95–8.88, p = 0.05) (Table 39). But when analyzed the case of smokers and alcohol habits together, no significant association was found with hr-HPV positivity.

Table 37: Demographic profiles and association with hr- HPV Positivity- Hr- HPV detection and its association with Demographic and Clinico- Pathological Character

Characteristic	Total Cases (n=106) N (%)	hr-HPV positive (n = 33) N (%)	HPV - Negative (n=73) N (%)	OR [95%CI]	P–value
Gender					
Male †	73 (68.86)	23 (69.70)	50 (68.49)	1.0 (referent)	
Female	33 (31.13)	10 (30.30)	23 (31.51)	0.95 [0.35-2.51]	0.90
Betel quid					
Non-chewers†	9 (8.18)	03 (9.10)	06(8.22)	1.0 (referent)	
Chewers	97 (91.82)	30 (90.90)	67 (91.78)	0.90 [0.18-4.89]	1.00
Tobacco- chewers					
Non-chewers†	35 (33.02)	13 (39.40)	22 (30.13)	1.0 (referent)	
Chewers	71 (66.98)	20 (60.60)	51 (69.87)	0.66 [0.26 -1.71]	0.34
Smoking habit					
Non- smokers†	83 (78.30)	23 (69.69)	60 (82.19)	1.0 (referent)	
Smokers	23 (21.70)	10 (30.31)	13 (17.81)	2.01 [0.70-5.77]	0.14
Alcohol intake					
No-intake†	78 (73.58)	15 (45.45)	63 (86.30)	1.0 (referent)	
Intake	28 (26.42)	18(55.55)	10 (13.70)	7.56 [2.64 –22.19]	**<0.001**
Both Tobacco-Alcohol habits					
Non-Intake †	87 (82.07)	23 (69.69)	64 (87.67)	1.0 (referent)	
Intake	19 (17.93)	10 (30.31)	9 (12.33)	3.09 [1.00- 9.65]	**0.02**
Both Smoking -Alcohol habits					
Non- smokers†	94 (88.68)	27 (81.82)	67 (91.78)	1.0 (referent)	
Smokers	12 (11.32)	6 (18.18)	6 (8.21)	2.48 [0.63- 9.74]	0.18
Dietary habits					
Vegetarian †	8 (7.54)	3 (9.09)	5 (6.49)	1.0 (referent)	
Non -vegetarian	98(92.45)	30 (90.91)	68 (93.50)	0.74 [0.14-4.20]	0.70
Place of residence					
Urban†	7 (6.36)	1 (3.03)	6 (8.21)	1.0 (referent)	
Semi urban	14 (13.20)	3 (9.09)	11 (15.06)	1.64 [0.10-51.13]	1.00
Rural	85 (80.18)	29 (87.88)	56 (76.71)	3.11 [0.34-71.78]	0.42
Age					
≤50 years †	52(49.05)	16 (48.48)	36 (49.31)	1.0 (referent)	
>50 years	54(50.95)	17 (51.52)	37 (50.69)	1.03 [0.42-2.55]	0.93

*Statistically Significant; OR = odds ratio; CI = confidence interval; †Reference group for OR calculation, since only one variable is significant that will be significant in multivariate analysis also

Table 38: Relationship between clinico-pathological characteristics and hr- HPV Positivity

Characteristic	Total Cases (n = 106) N (%)	hr- HPV positive (n = 33) N (%)	hr-HPV Negative (n = 73) N (%)	OR [95%CI]	P -value
Oral cavity †	86 (81.13)	24 (72.72)	62 (84.93)	4.73[0.17-1.28]	1.4
Laryngeal	9 (8.49)	2 (6.06)	7 (9.58)	0.61[0.11-3.10]	0.55
Hypopharyngeal	3(2.83)	2 (6.06)	1 (1.37)	4.64[0.40-53.1]	0.18
Oropharyngeal	5 (4.71)	5 (15.15)	0 (0.00)	-	**<0.01**
Nose and PNS	3 (2.83)	0 (0.00)	3 (4.10)	-	0.24
TNM Tumor stage					
II †	15 (14.15)	3 (9.09)	12 (16.45)	**1.0 (referent)**	
III	26 (24.52)	6 (18.18)	20 (27.39)	1.20 [0.20-7.55]	1.0
IVA	59 (55.66)	24 (72.73)	35 (47.94)	2.74[0.62-13.81]	0.13
IVB	6 (5.66)	0 (0.00)	6 (8.21)	-	0.52
Histology					
Verrucous †	8 (7.54)	2 (6.06)	6 (8.21)	**1.0 (referent)**	
Carcinoma Squamous Cell	91(85.85)	30 (90.91)	61 (83.56)	1.48[0.25-11.31]	1.00
Others	7 (6.60)	1 (3.03)	6 (8.21)	0.50[0.01-11.02]	1.00
Morphology					
Well Differentiated †	74 (69.81)	21 (63.63)	53 (72.60)	**1.0 (referent)**	
Moderate Differentiated	22 (20.75)	8 (24.24)	14 (19.17)	1.44[0.47-4.38]	0.47
Poor Differentiated	10 (9.44)	4 (12.12)	6 (8.21)	1.68[0.35-7.74]	0.38

*****Statistically Significant OR = odds ratio; CI = confidence interval; †Reference group for OR calculation

7.3.3 HPV-16, 18 types association with Demographic and Clinico- Pathological characteristics

Among hr- HPV positive cases, HPV-16 was present in 82% (n=27) and HPV-18 in 18% (n=6) of the cases. The tobacco chewers had significantly increased risk of HPV-16 infection (p=0.02) but not of HPV-18. The risk of HPV-16 being positive is 11.88 times more in chewers as compared to non-chewers with 95% CI (1.01-317.2). However the smoking habit does not show any significance compared to non-smokers (OR- 0.14) with 95% CI (0.01-1.28). The HPV-18 positivity was found in 5 cases (83%) of non-chewers compared to chewers. HPV-16 infection in smokers was found to be higher (p=0.05) compared to HPV-18 (Table 40), Though hr-HPV DNA was found to be significantly associated with alcohol consumption (Table 37) but with reference to HPV -16 alone or, HPV type 18 alone, there was no such association found with it. The oral cavity 70% (n=19), larynx 7% (n=2), hypopharynx 7% (n=2) and oropharynx 15% (n=4) cancer patients have HPV-16 as most predominant hr-HPV type whereas HPV -18 was found only in oral cavity 83% (n=5) and oropharynx 17% (n=1). In histological subtype squamous cell carcinoma, the HPV-16 was found in 25 cases (92%) and HPV-18 in 5 cases (83%) was detected. HPV-16 was predominant in males 67% (n=18) as well as

proportionately high in female 33% (n=9), whereas HPV-18 was more in males 83% (n=5) compared to female 17% (n=1) cases. Betel quid chewing, alcohol consumption, age, gender did not show any significant association with the presence of HPV-16, 18 types (p>0.05) (Table 40).

Table 39: Relationship of betel nut, tobacco, smoking, and alcohol and status of hr-HPV in Oral cavity patients only

Characteristic	Oral cavity Cases (n = 86) N (%)	hr-HPV positive (n = 24)N (%)	HPV - Negative (n=62)N (%)	OR [95%CI]	P –value
Betel quid					
Non-chewers†	9 (10.46)	6 (25)	3 (4.84)	**1.0 (referent)**	
Chewers	77 (89.54)	18 (75)	59 (95.16)	0.15[0.03-0.79]	0.01
Tobacco- chewers					
Non-chewers†	30 (34.88)	10 (41.67)	20 (32.25)	**1.0 (referent)**	
Chewers	56 (65.12)	14 (58.33)	42 (67.75)	0.67[0.23-1.96]	0.41
Smoking habit					
Non- smokers†	66 (76.74)	20 (83.33)	46 (74.19)	**1.0 (referent)**	
Smokers	20 (23.26)	4 (16.67)	16 (25.81)	0.57[0.14-2.17]	0.36
Alcohol intake					
No-intake†	64 (74.42)	14 (58.33)	50(80.64)	**1.0 (referent)**	
Intake	22 (25.58)	10 (41.67)	12 (19.36)	2.98[0.95-9.43]	**0.03**
Both Tobacco-Alcohol habits					
Non-Intake †	69 (80.23)	16 (66.67)	53 (85.48)	**1.0 (referent)**	
Intake	17 (19.77)	8 (33.33)	9 (14.52)	2.94 [0.97- 8.88]	**0.05**
Both Smoking -Alcohol habits					
Non- smokers†	77 (89.54)	20 (83.33)	57 (91.93)	**1.0 (referent)**	
Smokers	9 (10.46)	4 (16.66)	5 (8.06)	2.28 [0.55- 9.33]	0.25

*****Statistically Significant**,** OR = odds ratio; CI = confidence interval; †Reference group for OR calculation

7.3.4 Expression profile of p16 gene in HNC

The expression levels of p16 tumor suppressor gene in tumor samples were analyzed by immunohistochemistry. As shown in Figure 24. We have observed high level of expression in 27 Nos. of hr-HPV positive cases; however, the remaining 4 hr-cases exhibit moderate level of expression. Out of the 68 Nos. of hr-HPV negative, 39 and 21 cases demonstrated moderate and weak expression, respectively, as shown in figure 23 (b and c). The remaining 15 Nos. of hr-HPV negative cases didn't show p16 expression.

Table 40: Relation of HPV -16 and HPV-18 with clinico-pathological characteristics

Characteristic	Total HPV positive (n = 33) N (%)	HPV-16 Positive (n=27) N (%)	HPV-18 Positive (n=6) N (%)	OR [95%CI]	P- value
Gender					
Male †	23 (69.69)	18 (66.66)	5 (83.33)	1.0 (referent)	
Female	10 (30.30)	9 (33.44)	1 (16.67)	2.5[0.21- 15.52]	0.64
Age					
≤50 years †	16 (48.48)	12 (44.44)	4 (66.67)	1.0 (referent)	
>50 years	17 (51.51)	15 (55.56)	2 (33.33)	2.50 [0.30- 24.23]	0.40
Betel quid					
Non-chewers†	3 (9.09)	1 (3.70)	2 (33.34)	1.0 (referent)	
Chewers	30 (90.91)	26 (96.29)	4 (66.66)	13.0[0.66- 483.12]	0.08
Tobacco-chewers					
Non-chewers†	13 (39.39)	8 (29.63)	5 (83.33)	1.0 (referent)	
Chewers	20 (60.60)	19 (70.37)	1 (16.67)	11.88[1.01-317.21]	**0.02**
Smoking habit					
Non- smokers†	23 (69.69)	21(77.78)	2 (33.33)	1.0 (referent)	
Smokers	10 (30.30)	6 (22.22)	4 (66.66)	0.14 [0.01- 1.28]	**0.05**
Alcohol intake					
No-intake†	15 (45.45)	13(48.15)	2 (33.33)	1.0 (referent)	
Intake	18 (54.54)	14 (51.85)	4 (66.66)	0.54[0.06- 4.43]	0.51
Sites					
Oral cavity †	24 (72.72)	19 (70.37)	5 (83.33)	1.0 (referent)	
Laryngeal	2 (6.06)	2 (7.41)	0 (0.00)	-	0.48
Hypopharyngeal	2 (6.06)	2 (7.41)	0 (0.00)	-	0.48
oropharyngeal	5 (15.15)	4 (14.82)	1 (16.66)	1.05[0.07- 30.65]	0.96
TNM Tumor ***stage***					
II †	3 (9.09)	3 (11.11)	0 (0.00)	-	
III	6 (18.18)	4 (14.81)	2 (33.33)	-	0.44
IVA	24 (72.72)	20 (74.08)	4 (67.67)	-	
Histology					
Squamous Cell †	30 (90.90)	25 (92.59)	5 (83.34)	1.0 (referent)	
Verrucous Carcinoma	2 (6.06)	1 (3.70)	1 (16.66)	0.20[0.01- 8.93]	0.24
Adenoid Cystic Carcinoma	1 (3.03)	1 (3.70)	0 (0.00)	0.64[0.02-18.0]	0.65
Morphology					
Well Differentiated †	21 (63.63)	18 (66.67)	3 (50)	1.0 (referent)	
Moderate Differentiated	8 (24.24)	6 (22.22)	2 (33.33)	0.50[0.05- 5.69]	0.49
Poor Differentiated	4 (12.12)	3 (11.11)	1 (16.66)	0.50[0.02- 17.07]	0.59

*****Statistically Significant**,** OR = odds ratio; CI = confidence interval; †Reference group for OR calculation

7.4 Discussion

The Human papilloma virus (HPV) has been identified as an etiological factor in subset of head and neck cancer especially oropharyngeal cancer [28]. Most of the HPV positive associated oropharyngeal carcinoma patients were reported to be younger in age and mostly have no tobacco/alcohol use history [29]. Some research groups have advocated that HPV positive and HPV negative HNC cases should be considered as two distinct clinical, pathological entities and treatment regimen need to be devised accordingly [28-30]. The tumor hypoxia fraction and proliferation abilities were significantly decreased in HPV positive tumor cells after irradiation in comparison to HPV negative cells [31]. It was demonstrated that HPV positive locally advanced HNC patient have shown significantly higher pathological complete response as compared to HPV negative cases undergoing chemotherapy regimen [32]. Numerous studies have reported HPV DNA detection in HNSCC with varying rates, the detection techniques included in situ hybridization and southern blot hybridization (lower sensitivity methods) or PCR (high sensitivity methods) [33]. Moreover, methods used for sampling and storage also vary. The lack of universally acceptable standardized and clinically relevant procedure for HPV detection has resulted in concerns about their application in routine clinical setting. Our study has first time assessed the applicability of HC2 assay in clinical specimen of HNC cases from North-East India. We propose that Hybrid Capture 2 (HC2) assay, which is approved by Food and Drug Administration (FDA, USA), for hr-HPV detection in clinical cervical specimens, can also be used for hr-HPV detection in HNC specimens. Due to semi-quantitative nature of HC2 assay it may have application for prognostic assessment of hr-HPV in HNC patients.

We used E6 nested multiplex PCR (NMPCR) for HPV genotyping, and found HPV-16 in 82 % (n=27) and HPV-18 in 18% (n=6) of the hr-HPV positive (n=33) cases, which is 31.13% of HNC patients (33/106). Our study data is supported by previous studies from India which had found HPV DNA in the range of 22 % to 73% in oral cancer cases [43-45]. Among (n=33) hr- HPV cases, we found HPV 16 in 58%, 12%, 6%, 6% and HPV-18 in 15%, 3%, 0%, and 0% in oral cavity, oropharyngeal, laryngeal and hypopharyngeal cancer patients respectively. Our study shows higher HPV-16, 18 infection rate compared to earlier studies from India [34- 37]. We found that oropharyngeal cancer site compared

to other tumor sites has shown significant risk for high-risk HPV infection. Our data is supported by earlier study, in which it was found to have association of oropharyngeal cancer with high- risk HPV infection [38]. Epidemiology of HPV positive oropharyngeal squamous cell carcinoma (OPSCC) are distinct from HPV negative ones and are characterized by younger age at one set male predominance and strong association with sexual behavior. HPV associated oropharyngeal cancer (HPV-OPC) is growing in incidence and has distinct clinical, pathological, molecular are epidemiologic features [29]. The difference between our observations with other studies can be attributed to different methodologies, ethnic variation, tumor sites etc. [39]. Our data show that betel nut chewing, smoking may not have significant association with hr-HPV infection. The hr-HPV positivity in the HNC cases was small which may confound the role of betel nut, smoking as risk factors for HPV infection. It was demonstrated by Ang et al. that HPV positive oropharyngeal cancer progression may not be strongly associated with tobacco-smoking [38]. But other studies have shown tobacco association with HPV infection [40].

We observed in our data that tobacco chewers have significantly higher hr-HPV infection, posing tobacco chewing as a risk factor for hr- HPV infection. The concentration of nicotine and its exposure duration to the cell may alter the antigen mediated signaling pathways [41]. Alteration in the antigen mediated signaling event in the immune cell may be the mechanism by which nicotine or other constituent of cigarette inhibits the response of cell for bacterial/ viral infection [41]. We suggest that tobacco associated carcinogens may induce alterations in genetic events which may lead to molecular changes making the individual susceptible to hr HPV infection.

In the present study hr-HPV infection was found to be significantly associated with alcohol consumption in HNC cases. Oh et al. recently demonstrated that hr-HPV load and alcohol consumption may have synergistic effect on hr-HPV infection and its persistence in cervical cancer [42]. Another recent study has shown that alcohol intake was associated with significantly increased risks for HPV infection in men [43], which supports our observation that alcohol intake, may act as risk factor for HPV infection.

Ethanol, a major constituent of alcoholic beverages, is oxidized to acetaldehyde by alcohol dehydrogenases (ADH). Acetaldehyde is metabolized to acetate by aldehyde

dehydrogenases (ALDH). Poor oral hygiene and smoking can change the oral bacterial flora which can lead to increased acetaldehyde production [44]. In animal model, acetaldehyde has been proved as carcinogenic and mutagenic [45]. Genetic factors ADH, ALDH2 polymorphism, lack of ALDH2 or low levels of expression of ALDH2 and alcohol drinking habits were reported to be associated with higher risk of head and neck cancer [46, 47].

Alcohol can also modify innate immune response in dose dependent manner which can result in altered inflammatory response to infection [57]. Since immune response plays major role in HPV infection, alcohol consumption may help in evading the immune response for HPV infection. Systemic immunity components such as cytokine serum level have been found to be increased in persistent HPV infection cases [48].

Our study provides evidence about increase in vulnerability for hr-HPV infection in tobacco chewers and alcohol drinkers mostly in oral cavity cancer patients. Our observation is well supported by previous HNC studies from India and worldwide [49]. From the total number of cases when tobacco chewing and smoking habits were analyzed with HPV, the associations were not significant (Table 37), as major proportion of population were HPV negative and HPV positive cases were less. But when the chewing habits were taken along HPV positive cases alone, significant association was found (Table 39) proving that tobacco chewing and alcohol consumption act as risk factor HPV infection. More number of oropharyngeal cancers in our study might have brought a better assessment of alcohol and tobacco association with hr-HPV in these cancers. We focused primarily on hr- HPV DNA detection by employing newer methodology such as HC2 assay, E6-NMPCR which is less used in HNC. The alcohol drinking and tobacco chewing/smoking are widespread habit in North-East Indian population. We feel that mass sensitization through screening cum awareness programme about HNC risk factors can help in good capacity in reduction of HNC incidence/burden in future in NE population.

We conclude from our present study data that hr-HPV infection may be more prevalent in tobacco chewers, alcohol drinkers and they act as risk factors for HPV infection in HNC cases of North-East India. Hybrid Capture 2 (HC2) assay due to its high negative

predictive value, specificity and sensitivity can be applied for hr- HPV detection in HNC clinical specimens.

7.5 Bibliography

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