List of Publications

1. **Seram Anil Singh** and Sankar Kumar Ghosh. Metabolic Phase I (CYPs) and Phase II (GSTs) gene polymorphisms and their interaction with environmental factors in nasopharyngeal cancer from the ethnic population of northeast India. (Communicated)
2. **Seram Anil Singh** and Sankar Kumar Ghosh. Polymorphisms of DNA repair genes (*XRCC1* and *XRCC2*) and interaction with environmental factors influence the risk of nasopharyngeal carcinoma in northeast India. (Communicated)
3. **Seram Anil Singh**, Javed Hussain Choudhury, Sharbadeb Kundu, et al. Influence of *CYP1A1* T3801C polymorphism on tobacco and alcohol-induced head and neck cancer susceptibility from Northeast India. Asian Pacific Journal of Cancer Prevention. 2015 (Accepted)
4. Javed Hussain Choudhury, **Seram Anil Singh**, Sharbadeb Kundu, et al. Tobacco carcinogen-metabolizing genes *CYP1A1*, *GSTM1*, and *GSTT1* polymorphisms and their interaction with tobacco exposure influence the risk of head and neck cancer in Northeast Indian population. Tumour Biol. 2015 Feb; DOI 10.1007/s13277-015-3246-0
5. Ruhina S Laskar, Fazlur R Talukdar, Javed H Choudhury, **Seram Anil Singh**, et al. Association of HPV with genetic and epigenetic alterations in colorectal adenocarcinoma from Indian population. Tumour Biol. 2015 Feb; DOI 10.1007/s13277-015-3114-y
6. **Seram Anil Singh** and Sankar Kumar Ghosh. Association of Epstein Barr virus and lifestyle on nasopharyngeal cancer risk among the ethnic population of northeast India. Science and Technology Journal. Vol. 2 (2), pp.95-102, 2014.
7. Sankar Kumar Ghosh, **Seram Anil Singh**, Rosy Mondal, et al. Dysfunction of mitochondria due to environmental carcinogens in nasopharyngeal carcinoma in the ethnic group of Northeast Indian population. Tumour Biol. 2014 Jul; 35 (7): 6715-24. DOI: 10.1007/s13277-014-1897-x.
8. Rosy Mondal R, Sankar Kumar Ghosh, Javed Hussain Choudhury, **Seram Anil Singh**, et al. (2013) Mitochondrial DNA Copy Number and Risk of Oral Cancer: A Report from Northeast India. PLoS ONE 8(3): e57771. DOI:10.1371/journal.pone.0057771

Research papers presented in scientific conferences

1. **Seram Anil Singh** and Sankar Kumar Ghosh. "Polymorphism in xenobiotic metabolism genes modulates the risk of tobacco induced nasopharyngeal cancer susceptibility in northeast India". *International Conference On Biotechnological Advances in EHBC & 39th Annual Meeting of EMSI*. Manipur University, 21st-23rd May, 2015
2. **Seram Anil Singh** and Sankar Kumar Ghosh. "Etiological factors of nasopharyngeal cancer in Northeast Indian population". *National Seminar on Advances in biotechnology research: Current trends and future prospects*. Assam University, 25th-26th March, 2014

RESEARCH ARTICLE

Influence of the *CYP1A1* T3801C Polymorphism on Tobacco and Alcohol-Associated Head and Neck Cancer Susceptibility in Northeast India

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Abstract

Background: Tobacco and alcohol contain or may generate carcinogenic compounds related to cancers. *CYP1A1* enzymes act upon these carcinogens before elimination from the body. The aim of this study was to investigate whether *CYP1A1* T3801C polymorphism modulates the relationship between tobacco and alcohol-associated head and neck cancer (HNC) susceptibility among the northeast Indian population. Materials and Methods: One hundred and seventy histologically confirmed HNC cases and 230 controls were included within the study. The *CYP1A1* T3801C polymorphism was determined using PCR-RFLP, and the results were confirmed by DNA sequencing. Logistic regression (LR) and multifactor dimensionality reduction (MDR) approaches were applied for statistical analysis. Results: The *CYP1A1* CC genotype was significantly associated with HNC risk (P=0.045). A significantly increased risk of HNC (OR=6.09; P<0.0001) was observed in individuals with combined habits of smoking, alcohol drinking and tobacco-betel quid chewing. Further, gene-environment interactions revealed enhanced risks of HNC among smokers, alcohol drinkers and tobacco-betel quid chewers carrying *CYP1A1* TC or CC genotypes. The highest risk of HNC was observed among smokers (OR=7.55; P=0.009) and chewers (OR=10.8; P<0.0001) carrying the *CYP1A1* CC genotype. In MDR analysis, the best model for HNC risk was the three-factor model combination of smoking, tobacco-betel quid chewing and the *CYP1A1* variant genotype (CVC=99/100; TBA=0.605; P<0.0001); whereas interaction entropy graphs showed synergistic interaction between tobacco habits and *CYP1A1*. Conclusions: Our results confirm that the *CYP1A1* T3801C polymorphism modifies the risk of HNC and further demonstrated importance of gene-environment interaction.

Keywords: Head and neck cancer - *CYP1A1* T3801C polymorphism - tobacco - alcohol - northeast India

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Introduction

Head and neck cancers (HNC) are a heterogeneous group of cancers that include cancers of the skin of the head and neck, nasal cavity, paranasal sinuses, nasopharynx, lip, oral cavity, oropharynx, larynx, hypopharynx, trachea, neck, salivary glands and the parapharyngeal region (Siegel et al., 2014). Incidence of HNC has increased at an alarming rate for the past 10 years. It is the fifth most prevalent among all cancers throughout the world with estimated death of 355 per 100,000 (Masood et al., 2014). These cancers occur more frequently in men (male: female ratio of 4-5:1). In India, HNC acquires 30-40% cancers of all sites and is the sixth most common cause of death in males and seventh in females (Bhattacharjee et al., 2006).

Tobacco smoking and betel quid chewing with or without tobacco are major risk factors to be associated with HNC (Mondal et al., 2013; Amtha et al., 2014; Choudhury et al., 2014; Choudhury and Ghosh, 2014). Alcohol habit is another risk factor associated with HNC (Risch et al., 2003; Hashibe et al., 2009; Ghosh et al., 2014b). The magnitude of risk conferred by the interaction between tobacco, and alcohol is still unclear. Studies have implicated that many carcinogens present in tobacco, and alcohols are metabolized to active forms that have deleterious effects in our body. These activated substances can cause oxidative reactions in tissues, and initiate reactions to produce free radicals (Cury et al., 2012). The presence of reactive oxygen species (ROS) can cause damage to cellular biomolecules, including protein and DNA, consequently resulting in carcinogenesis (Mondal and Ghosh, 2013; Kumar and Muniyandi, 2015).

Metabolic enzymes that are potentially involved in either the activation (phase I) or detoxication (phase II) of chemical carcinogens have received a great deal of attention recently as possible genetic susceptibility factors

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for a variety of cancers (Olshan et al., 2000). Human cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*) is one of the major phase I enzymes, involved in the activation of carcinogens related to head and neck cancers, such as polycyclic aromatic hydrocarbon (PAH), heterocyclic amines, N-nitrosamine, alkaloids, polyphenols and tannins present in tobacco smoke and betel quid (Wu et al., 2004; Sharan et al., 2012). Ethanol and acetaldehyde of alcoholic drinks are important chemical agents related to various cancers (Lachenmeier et al., 2012). Therefore, modulation of *CYP1A1* activity may be important in the aetiology of HNC. Both environmental and genetic factors influence the activity of *CYP1A1*. Tobacco smoking and betel quid consumption increases *CYP1A1* activity in humans. *CYP1A1* activity is also modulated by specific polymorphisms in the *CYP1A1* gene. One of its common polymorphisms *CYP1A1* T3801C (*CYP1A1*-MspI or *CYP1A1**2A or rs4646903) has been associated with higher induction of *CYP1A1*. The higher enzyme activity would result in increased levels of carcinogenic intermediates, leading to greater risk of cancer development. The associations of these *CYP1A1* single nucleotide polymorphisms (SNPs) with cancers have been well documented (Jiang et al., 2014; Liu et al., 2014; Lu et al., 2014).

The aim of this study was to investigate the frequency of *CYP1A1* T3801C polymorphism in patients with HNC and its role in modu¬lating the relationship between tobacco and alcohol consumption, and HNC risk. Furthermore, multifactor dimensionality reduction (MDR) approach was used to investigate the high-degree gene-environmental interaction in NPC carcinogenesis.

Materials and Methods

Study population

The present study consists of 170 histologically confirmed, untreated HNC cases (diagnosed between December, 2009 and July, 2013) and 230 healthy controls (without family history of cancer). The oral swab and/or peripheral blood of the participating subjects were collected upon written consent. Controls were individually matched to cases in sex, age, ethnicity and neighborhood. Further, a pre-designed questionnaire was used to collect demographic information including family history of cancer, habits of tobacco, and alcohol consumption as well as their frequencies. Tobacco habits in our study include smoking (cigarettes and beedi), tobacco chewing (khaini, zarda, gutkha), and betel quid chewing (betel leaf along with areca nut, lime with or without tobacco). The study was approved by the Institutional Ethics Committee, Assam University, Silchar. Precautions were taken to avoid contamination while collecting and processing the samples.

DNA extraction and genotyping

Genomic DNA was isolated from the collected blood or oral swabs by phenol/chloroform/ isoamylalcohol (Ghosh and Mondal, 2012) method, and then stored in T.E buffer (10Mm Tris-HCl, pH 0.8, 1 Mm EDTA) at -20ºC for further used. The polymorphisms of *CYP1A1* T3801C gene was analysed by polymerase chain reaction restriction length polymorphism (PCR-RFLP) method (Ng et al., 2005). PCR amplification of a 343-base DNA fragment containing MspI restriction site was performed, using the primers 5/-TAGGAGTCTTGTCTCATGCCTT-3/ and 5/-CAGTGAAGAGGTGTAGCCGCT-3/. PCR programme was performed at 95°C for 5 min for the initial denaturation, following 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 45 s, extension at 72°C for 30 s and final extension at 72°C for 5 mins. For the *CYP1A1* T3801C genotype analysis, the PCR product was digested with the MspI restriction enzyme (New England BioLabs, USA); a single 343 bp fragment represents the wild-type allele (TT), three fragments of 343, 200 and 143 bp indicates for the heterozygous (TC) and two fragments of 200 and 143 bp for the variant allele (CC). The RFLP results were confirmed by sequencing 10% of randomly selected samples from both cases and controls by Sanger sequencing using Genetic Analyzer 3500, Applied BioSystems (Molecular Medicine Lab, Department of Biotechnology, Assam University, Silchar, India).

Statistical analysis

Statistically significant differences between cases and controls for demographic characteristics were assessed by χ^2 test. The association between smoking, alcohol drinking and tobacco-betel quid chewing and *CYP1A1* T3801C polymorphism in HNC risk were analyzed by calculating odds ratios (ORs), 95% confidence intervals (95% CI), and their corresponding P-values. A P-value of <0.05 were considered to indicate statistically significant. Departures from Hardy-Weinberg equilibrium for *CYP1A1* T3801C genotype were evaluated by comparing the observed genotype frequencies with expected frequencies using χ^2 tests. Statistical analyses were performed using SPPS software, version 16 for Windows.

Multifactorial dimensionality reduction analysis

The MDR analysis is an advanced non-parametric approach (www. multifactordimensionalityreduction. org) used to detect the gene-environment interactions associated with disease risk (Hahn et al., 2003). The advantage of using MDR is it overcomes the sample size limitations often encountered by parametric approaches (example: logistic regression analysis) while studying high-level interaction. The best prediction model was selected on the basis of maximum training balance accuracy (TrBA), testing balance accuracy (TBA) and cross validation consistency (CVC). The best model with highest TrBA, TBA and CVC was tested by 1000 fold permutation testing and χ^2 test at 0.05 significance levels during MDR analysis.

Interaction entropy graphs

The interaction entropy graphs were constructed using MDR results to determine synergistic and non-synergistic interactions among the variables (Lavender et al., 2009; Yu et al., 2014). The graphs comprise of nodes containing percentage entropy of each individual variables and connections joining them pairwise showing entropy of interaction between them. Positive entropy signifies

synergy and negative entropy indicate redundancy, whereas, zero entropy indicates independence.

Results

Relationship between tobacco habits and alcohol drinking and the risk for HNC

The present study includes 170 cases and 230 controls. There were no significant differences between cases and controls in terms of gender and age. 67.6 % cases and 60.9 % controls were male; 44.7% cases and 47.4% controls were ≤50 years of age (P=0.1976 and 0.6629, respectively). Smokers were significantly higher among cases than controls (P<0.001). Differences in tobacco-betel quid chewing and alcohol intake status was observed between cases and controls (P<0.05). Of the 180 cancer patients, 63 (37.1%) had oral, 60 (35.3%) had nasopharyngeal, 24 (14.1%) had laryngeal, 10 (5.9%) had pharyngeal, and 13 (7.6%) had cancer in other head and neck region. We analyzed HNC risk in relation to smoking, alcohol drinking and tobacco-betel quid chewing, with adjustment for possible confounding factors (Table 1). Tobacco habits showed a dose-dependent increased risk of HNC. Heavy smokers (OR=2.9, 95% CI: 1.68-4.99; P<0.0001) and tobacco-betel quid chewers (OR=3.0, 95% CI: 1.8-5.03; P<0.0001) had significantly increased risk of HNC. Similarly, heavy alcohol drinkers had nearly 2 fold (95% CI: 1.07-3.15; P=0.025) increased risk of HNC. The risk of HNC increased 5 fold (95% CI: 2.53-10.0; P<0.0001) for smokers with tobacco-betel quid chewing. Similarly, significantly increased risks of HNC were observed for alcohol drinkers with smoking habit (OR=3.32, 95% CI: 1.77-6.24; P<0.0001) or with tobacco betel quid chewing (OR=4.18, 95% CI: 2.09-8.33; P<0.0001). However, highest risk of HNC (OR=6.09, 95% CI: 2.35-15.73; P<0.0001) was observed in individuals with combine habits of smoking, alcohol drinking and tobacco-betel quid chewing (Table 1).

Polymorphism in CYP1A1 gene and association with HNC risk

The genotypes of *CYP1A1* T3801C were determined by detecting the PCR-RFLP band pattern on 1.5% agarose gel. The PCR-RFLP result was confirmed by randomly sequencing 10% of the samples (Figure 1). The three genotypes of *CYP1A1* T3801C viz. TT, TC and CC had frequency distributions of 45.3%, 41.2%, 13.5% and 54.3%, 36.1%, 9.6% in cases and controls, respectively. Logistic regression analysis show that *CYP1A1* CC (OR=2.06, 95% CI: 1.01-4.17; P=0.045) genotype had significant risk association with HNC in the study population when compare to the TT genotype (Table 2). Combine TC and CC (TT + CC) genotypes also showed a risk (OR=1.54, 95% CI: 1.02-2.39, P=0.039) of HNC. The risk associated with each allele was also investigated. We observed significant risk of HNC in those individual

Table 1. Environmental Risk Factors and Risk of Head And Neck Squamous Cell Carcinoma

Risk factors	Cases n=170 (%)	Controls n=230 (%)	OR* (95% CI)	P-value
Smoking				
Never	65 (38.2)	131 (57)	1 (ref)	
Light	45 (26.5)	58 (25.2)	1.58 (0.92 - 2.7)	0.094
Heavy	60 (35.3)	41 (17.8)	2.9 (1.68 - 4.99)	<0.0001
Tobacco-betel quid chewing				
Never	47 (27.6)	102 (44.3)	1 (ref)	
Light	51 (30)	75 (32.6)	1.49 (0.89 - 2.49)	0.126
Heavy	72 (42.4)	53 (23)	3.0 (1.8 - 5.03)	<0.0001
Alcohol drinking				
Never	74 (43.5)	125 (54.3)	1 (ref)	
Light	52 (30.6)	60 (26.1)	1.6 (0.97 - 2.64)	0.064
Heavy	44 (25.9)	45 (19.6)	1.84 (1.07 - 3.14)	0.025
Smoking + Tobacco-betel quid chewing				
Never	17 (10)	56 (24.3)	1 (ref)	
Both	77 (45.3)	52 (22.6)	5.04 (2.53 - 10.02)	<0.0001
Single	76 (44.7)	122 (53)	2.09 (1.11 - 3.94)	0.022
Alcohol drinking + Smoking				
Never	27 (15.9)	70 (30.4)	1 (ref)	
Both	55 (32.4)	44 (19.1)	3.32 (1.77 - 6.24)	<0.0001
Single	88 (51.8)	116 (50.4)	1.79 (1.04 - 3.09)	0.034
Alcohol drinking + Tobacco-betel quid chewing				
Never	15 (8.8)	57 (24.8)	1 (ref)	
Both	65 (38.2)	54 (23.5)	4.18 (2.09 - 8.33)	<0.0001
Single	90 (52.9)	119 (51.7)	2.69 (1.4 - 5.15)	0.003
Smoking + Tobacco-betel quid chewing + Alcohol drinking				
Never	9 (5.3)	30 (13)	1 (ref)	
Both	39 (22.9)	21 (9.1)	6.09 (2.35 - 15.73)	<0.0001
Multiple	122 (71.8)	179 (77.8)	2.26 (1.01 - 5.05)	0.047

*Odds ratio (OR) are adjusted for age, gender, smoking, and alcohol drinking and tobacco-betel quid chewing as appropriate; P < 0.05 considered as statistically significance; Bold values indicate statistical significance (P < 0.05)

Table 2. Distribution of CYP1A1 (T3801C) Genotype in Different Types of Head and Neck Cancer

Genotype	Overall		Oral		Nasopharyngeal		Larynx		Pharynx and others	
	Ca/Co;	OR* (95% CI)	N=63	OR* (95% CI)	N=60	OR* (95% CI)	N=24	OR* (95% CI)	N=23	OR* (95% CI)
	170/230	P value		P value		P value		P value		P value
TT	77/125	1(ref)	26	1(ref)	26	1(ref)	9	1(ref)	12	1(ref)
TC	70/83	1.45 (0.92-2.27)	25	1.5 (0.79-2.87)	26	1.63 (0.84-3.15)	12	1.9 (0.72-5.05)	8	0.99 (0.37-2.62)
		0.108		0.213		0.145		0.194		0.991
CC	23/22	2.06 (1.01-4.17)	12	2.99 (1.25-7.16)	8	2.23 (0.77-6.48)	3	2.36 (0.54-10.25)	3	1.79 (0.43-7.36)
		0.045		0.014		0.138		0.251		0.415
TC+CC	93/105	1.54 (1.02-2.39)	37	1.8 (0.99-3.26)	34	1.73 (0.92-3.23)	15	1.99 (0.79-5.0)	11	1.14 (0.47-2.76)
		0.039		0.049		0.085		0.141		0.768
T-allele	224/333	1(ref)	77	1(ref)	78	1(ref)	30	1(ref)	32	1(ref)
C-allele^^	116/127	1.37 (1.01-1.85)	49	1.67 (1.11-2.52)	42	1.41 (0.92-2.16)	18	1.57 (0.85-2.92)	14	1.15 (0.59-2.22)
		0.044		0.016		0.116		0.178		0.731

*Odds ratio (OR) are adjusted for age, gender, smoking, and alcohol intake and tobacco-betel quid chewing as appropriate; P<0.05 considered as statistically significance; ^^Crude odds ratio (OR); Ca cases, Co controls; Bold values indicate statistical significance (P<0.05)

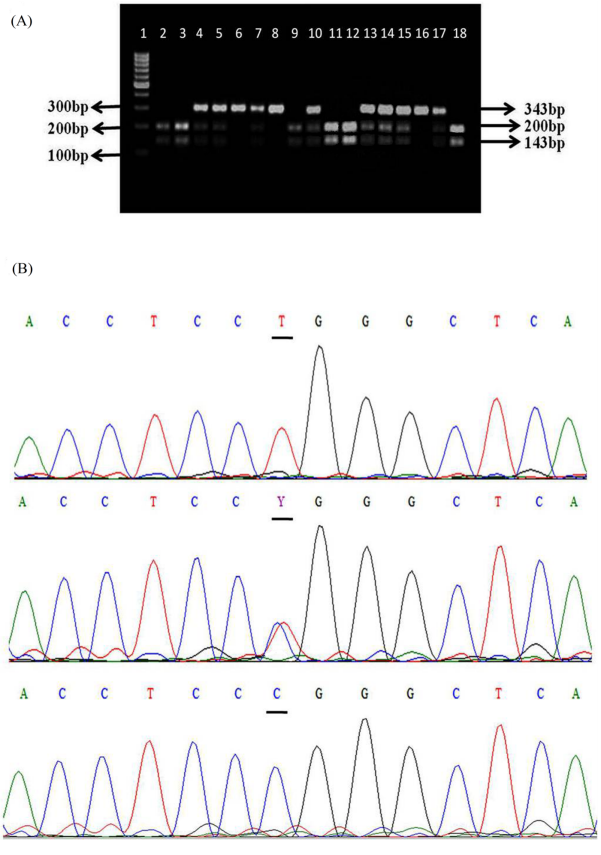

Figure 1. Polymorphism of CYP1A1 T3801C Metabolic gene. (A) Ethidium bromide stained gel showing CYP1A1 T3801C polymorphism: 100-bp ladder (lane 1); CYP1A1 TT wild genotype (lanes 6, 8 and 16); CYP1A1 TC heterozygous genotype (lanes 4, 5, 7, 10, 13, 14, 15, and 17); CYP1A1 CC mutant genotype (lanes 2, 3, 9, 11, 12, and 18); (B) DNA sequencing results showing nucleotide changes (mark by black line). Nucleotide change marked as Y indicates the heterozygous genotype, where red peak denotes Thymine (T) while blue peak denotes Cytosine (C)

carrying the C-allele as compared to T- allele (P=0.04). Further, the association between *CYP1A1* T3801C genotype and different types of HNC was analysed. It was observed that CC (P=0.014) and combined TC+CC (P=0.049) genotypes were significantly associated with the risk of oral cancer (Table 2). However, *CYP1A1* polymorphism was not associated with the risk of other types HNC in our study.

Interaction of tobacco habits and alcohol drinking and CYP1A1 polymorphism in HNC

The potential interactions of smoking, tobacco-betel quid chewing, and alcohol drinking and *CYP1A1* T3801C polymorphism in HNC risk were investigated (Table 3). Heavy smokers carrying the *CYP1A1* TT and TC genotypes had 2.55 fold (95% CI: 1.27-5.13; P=0.012) and 3.42 fold (95% CI: 1.65-7.11; P=0.002) increased risk of HNC. However, highest risk was observed among heavy smokers carrying CC genotype (OR=7.55, 95% CI: 1.62-35.1; P=0.009). We examined interaction of tobacco-betel quid chewing with *CYP1A1* genotypes in HNC risk. Heavy tobacco-betel quid chewers carrying the TC genotype had 4.91 fold risk (95% CI: 2.16-11.1; P<0.0001) of HNC, whereas a 10 fold (95% CI: 2.09-37.6; P<0.0001) elevated risk was observed among chewers carrying the CC genotype. Tobacco-betel quid chewing also show significantly interaction with the TT genotype (P<0.05). Among heavy alcohol drinkers, individuals carrying the TC genotype had significantly increased (OR=3.26, 95% CI: 1.43-7.43; P=0.006) risk of HNC, whereas individual with CC genotype had 7.21 fold (95% CI: 1.55-33.5; P=0.011) elevated risk.

The combinations of smoking, alcohol drinking and tobacco-betel quid chewing habits and their interactions with *CYP1A1* T3801C polymorphism in HNC risk were also considered (Table 3). A significantly elevated risk of

Table 3. Interaction of smoking, alcohol intake and tobacco-betel quid chewing and CYP1A1 polymorphism in HNC

CYP1A1 (T3801C, rs4646903) polymorphism						
	TT genotype		TC genotype		CC genotype	
	Ca/Co	OR (95% CI) **P value	Ca/Co	OR (95% CI) **P value	Ca/Co	OR (95% CI) **P value
Smoking						
Never	32/69	1 (reference)	25/48	1.12 (0.59-2.12) 0.746	8/14	1.23 (0.48-3.17) 0.802
Light	19/34	1.20 (0.60-2.41) 0.719	18/18	2.16 (1.0-4.65) 0.069	8/6	2.88 (0.96-8.65) 0.076
Heavy	26/22	2.55 (1.27-5.13) 0.012	27/17	3.42 (1.65-7.11) 0.002	7/2	7.55 (1.62-35.1) 0.009
Tobacco-betel quid chewing						
Never	14/55	1 (reference)	27/35	3.03 (1.41-6.52) 0.005	6/12	1.96 (0.64-6.0) 0.344
Light	27/41	2.59 (1.21-5.51) 0.016	18/28	2.53 (1.11-5.77) 0.034	6/6	3.93 (1.15-13.4) 0.063
Heavy	36/29	4.88 (2.29-10.4) <0.0001	25/20	4.91 (2.16-11.1) <0.0001	11/4	10.80 (2.09-37.6) <0.0001
Alcohol drinking						
Never	33/68	1 (reference)	30/43	1.44 (0.77-2.68) 0.267	11/4	1.62 (0.67-3.89) 0.35
Light	26/26	2.06 (1.05-4.06) 0.053	21/28	1.55 (0.77-3.10) 0.277	5/6	1.72 (0.51-5.73) 0.505
Heavy	18/31	1.20 (0.59-2.43) 0.714	19/12	3.26 (1.43-7.43) 0.006	7/2	7.21 (1.55-33.5) 0.011
Smoking + Tobacco-betel quid chewing						
Never	8/27	1 (reference)	6/20	1.01 (0.31-3.31) 1	3/9	1.13 (0.26-4.91) 1
Single	30/70	1.45 (0.60-3.51) 0.515	39/44	2.99 (1.23-7.27) 0.023	8/8	3.38 (0.99-11.5) 0.102
Both	39/28	4.70 (1.88-11.7) 0.001	25/19	4.44 (1.67-11.7) 0.003	12/5	8.10 (2.26-29) 0.002
Alcohol drinking + Smoking						
Never	11/39	1 (reference)	13/22	2.10 (0.81-5.40) 0.148	3/9	1.18 (0.29-4.87) 1
Single	41/59	2.46 (1.14-5.33) 0.029	33/47	2.49 (1.12-5.52) 0.035	14/10	4.96 (1.76-13.9) 0.003
Both	25/27	3.28 (1.4-7.71) 0.007	24/14	6.08 (2.4-15.3) <0.0001	6/3	7.09 (1.64-30.6) 0.013
Alcohol drinking + Tobacco-betel quid chewing						
Never	5/28	1 (reference)	24/14	2.8 (0.80-9.80) 0.124	2/10	1.12 (0.2-6.31) 1
Single	37/68	3.05 (1.1-8.44) 0.031	40/45	4.98 (1.78-13.9) 0.001	13/6	9.60 (2.91-35.3) 0.001
Both	35/29	6.76 (2.35-19.4) <0.0001	22/22	5.6 (1.86-16.8) 0.022	8/6	7.47 (1.87-29.8) 0.01

Bold values indicate statistical significance (P < 0.05); Ca cases, Co controls; ** Fisher's exact test used to calculate P value and P < 0.05 considered as statistically significance

Table 4. Summary of Multifactorial Dimensionality Reduction Analysis (MDR) for HNC Risk Prediction

Model	TrBA	TBA	CVC	P value
TBS	0.593	0.593	100/100	<0.001
TBS, CYP1A1	0.592	0.57	94/100	<0.001
TBS, TBC, CYP1A1	0.611	0.605	99/100	<0.0001
TBS, TBC, ALC, CYP1A1	0.59	0.516	100/100	<0.0001

TBA, testing balance accuracy; TrBA, training balance accuracy; CVC, cross-validation consistency; TBS, tobacco smoking; TBC, tobacco chewing, ALC; Alcohol consumption; Bold values indicate best model prediction for NPC risk with highest TrBA, TBA and maximum CVC

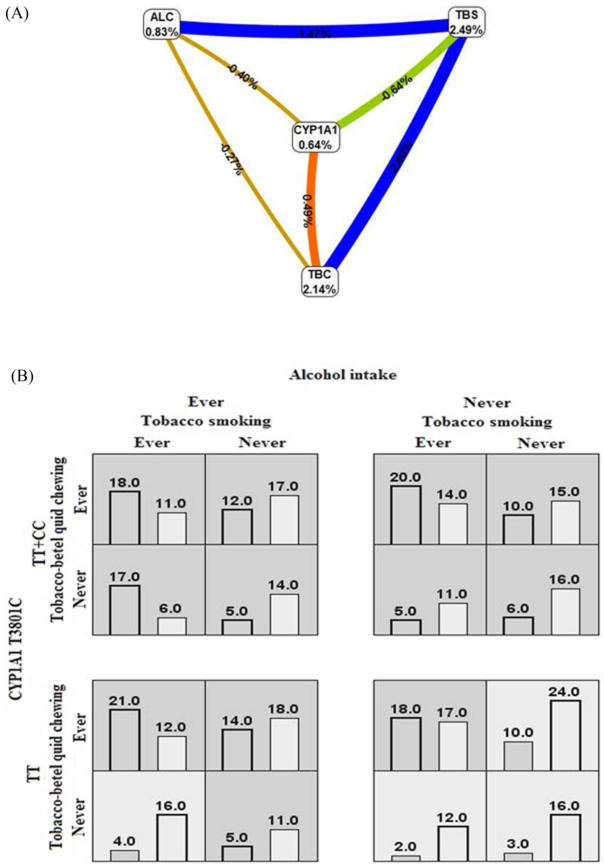

Figure 2. MDR analysis of genetic and Environmental Factors. (A) Interaction entropy graph for gene-environment interaction and HNC risk. This graphical model explains the percent of the entropy in case-control removed by each factor (independent effect) and by each pair-wise combination of attributes (interaction effect). Positive percentage of entropy indicating synergistic interaction and negative values of entropy represent redundancy. The red colour indicating a high degree of synergistic interaction, orange a lesser degree whereas; gold represent midpoint; blue represents the highest level of redundancy followed by green. TBC-tobacco-betel quid chewing, TBS-tobacco smoking, and ALC-alcohol consumption; (B) Summary of the four-factor model (CYP1A1, alcohol, smoking and tobacco-betel quid chewing) in MDR analysis. The distribution of high risk (dark shading) and low risk (light shading) combinations associated with HNC risk. The percentage of patients having HNC was represented by left column in each box, whereas right column in each box indicated percentage of controls

HNC was observed for alcohol drinkers with smoking (OR=7.09, 95% CI: 1.64-30.2; P=0.013) or with tobacco-betel quid chewing (OR=7.47, 95% CI: 1.87-29.8; P=0.010) and carrying CC genotype. However, highest risk of HNC (OR=8.10, 95% CI: 2.26-29.03; P=0.002) was observed for smokers with tobacco-betel quid chewing and carrying *CYP1A1* CC genotype.

MDR analysis

MDR analysis was used to determine the best-model gene-environment interaction for HNC risk (Table 4). The analysis suggest that smoking was the best one-factor model with CVC 100/100 (TBA=0.59; P<0.001). While smoking and *CYP1A1* variant genotype was the best two factors model with CVC of 94/100 (TBA=0.57; P<0.001). The best four-factors model was the combinations of smoking, tobacco-betel quid chewing, alcohol drinking and *CYP1A1* variant genotype with CVC of 100/100 and TBA (0.51) and P<0.0001. However, the best model of all the predictive models was the three-factor model combination of smoking, tobacco-betel quid chewing, and *CYP1A1* variant genotype having CVC of 99/100 and highest TBA of 0.60 (P<0.0001).

Interaction entropy graphs

Interaction entropy graph was constructed using MDR results to determine synergistic or antagonistic interactions between the genetic and environmental factors in HNC risk (Figure 2). Entropy graph, revealed smoking, and tobacco-betel quid chewing had highest independent effect with percentage entropy of 2.49% and 2.14%, respectively. Tobacco-betel quid chewing had a synergistic interaction with *CYP1A1* variant (0.64%) by removing 0.49% of entropy. Alcohol consumption (0.83%) might also explain considerable entropy independently.

Discussion

In this study, we investigated the role of *CYP1A1* T3801C polymorphism in modulating the relationship between smoking, alcohol drinking and tobacco-betel quid chewing and HNC risk among the northeast Indian population. Our findings suggested that the variant *CYP1A1* T3801C genotype, by increasing the *CYP1A1* activity, modified HNC risk by interacting with tobacco and alcohol.

Northeast India is reported to have a very high morbidity and mortality rates of HNC (Bhattacharjee et al., 2006). Epidemiological studies from the northeast India reported tobacco smoking as one of the strongest risk factor for HNC (Yadav et al., 2010; Talukdar et al., 2013; Choudhury and Ghosh, 2014; Ghosh et al., 2014a) . Moreover, it was seen that tobacco-betel quid chewing also had a very high impact on HNC risk (Sharan et al., 2012; Mondal et al., 2013; Choudhury et al., 2014) . Tobacco in any form generates free radicals that deplete antioxidants and cause oxidative damage to DNA, proteins and lipids resulting in cancers. Similarly; alcohol consumption is linked to an increase in risk of HNC (Bektas-Kayhan et al., 2014; Ghosh et al., 2014a; Krishna et al., 2014). Recent studies have revealed mutagenic effects of alcohol on human chromosomes *in vitro* (Hsu et al., 1991). Our results also suggest for a strong risk of HNC with smoking,

alcohol drinking and tobacco-betel quid chewing (Table 1).

In our study, significant associations between HNC risk and *CYP1A1* T3801C variant genotypes were observed (Table 2). Our study suggests an increase risk of HNC in the *CYP1A1* CC genotypes (2 fold) that could reflect the higher enzymatic activity. *CYP1A1* belongs to a superfamily of phase I xenobiotics metabolizing enzymes. These enzymes play a vital role in resisting a large variety of chemical carcinogens and environmental toxicants that are probably associated with cancer risk. Altered forms of enzymes are known to enhance enzyme activities and therefore, have been linked with increasing incidence of cancers (Sharma et al., 2013; Shukla et al., 2013), most likely due to increased susceptibilities to environmental toxins and carcinogens. Moreover, they are involved in the induction of various enzymes and proteins important in cellular function, e.g., the modulation of DNA repair genes that in turn determines individual susceptibility to cancer risks (Guo et al., 2008).

In the present study, the confounding effect of smoking on the HNC association with *CYP1A1* T3801C polymorphism shows that *CYP1A1* CC genotypes were strongly associated with risk of HNC in smokers (Table 3). Recent studies also observed that the risk of cancers in smokers was modulated by *CYP1A1* genetic variations (Guo et al., 2008; Sam et al., 2008; Liu et al., 2013; Jiang et al., 2014). However, these studies were conducted in a bi-modal manner (presence/absence) whereas our study was conducted on a dose-dependent manner. Tobacco smoke is a complex mixture of over 4,000 compounds; more than 60 of the compounds are potent carcinogens, including polycyclic aromatic hydrocarbons (PAHs), aromatic amines, N-nitroso compounds. *CYP1A1* is known to metabolize PAHs generated from tobacco smoke such as benzo [α] purene in addition to a various other carcinogenic compounds (Guengerich and Shimada, 1998; Bartsch et al., 2000). The carcinogenic substrates on exposure to *CYP1A1* may therefore increase the potential for genomic damage (Sam et al., 2008). Studies also revealed the expression of *CYP1A1* in the buccal mucosa, suggesting the in situ activation of tobacco carcinogens (Vondracek et al., 2001). Moreover, cigarette smoke has also been shown to up regulate *CYP1A1* under *in vitro* conditions as well as in smokers (Nagaraj et al., 2006; Chi et al., 2009). Previous studies reported genetic variability altered enzyme activity and subsequently affect carcinogens detoxification in tobacco smoke, thereby increasing susceptibility to HNC risk (Soya et al., 2007; Sabitha et al., 2010; Sam et al., 2010; Tai et al., 2010; Khlifi et al., 2014). However, few studies did not find a relationship between smoking and risk of HNC among cases with the *CYP1A1* polymorphisms (Matthias et al., 1998; Oude Ophuis et al., 1998; Varela-Lema et al., 2008). In addition, *CYP1A1* TC and CC genotypes appeared to be strongly associated with risk of HNC in tobacco-betel quid chewers. Betel-quid chewing along with tobacco results in the exposure to nitrosamines derived from tobacco and areca or betel nut alkaloids. Betel quid chewing generate a high amount of ROS, which has been implicated in multistage carcinogenesis (Soya et al., 2007). Recent studies reported a significant association between *CYP1A1* polymorphism and HNC among tobacco-betel quid chewers (Anantharaman et al., 2007; Hernando-Rodriguez et al., 2012; Chaudhuri et al., 2013), whereas few studies did not found any relationship between *CYP1A1* and chewing (Khlifi et al., 2014).

Furthermore, a strong risk modulation of HNC was observed among individual carrying the *CYP1A1* CC genotype and alcohol drinking (Table 3). This is the first study to report an interaction of *CYP1A1* T3801C polymorphism and environmental factors in the development of HNC in northeast Indian population. Similar type of result, have been reported in the previous other study in oral cancers (Chatterjee et al., 2009; Chatterjee et al., 2010). On the other hand, individual carrying the *CYP1A1* TC and CC genotypes and taking alcohol either with smoking or tobacco-betel quid chewing has an overall higher risk for HNC risk. Alcohol in general is not a genotoxic substance; however, its degradation results in the formation of acetaldehyde, which is carcinogenic in nature. Besides, alcohol drinking makes suppresses the removal of nitrosamine molecules of low molecular weight released by tobacco in the liver and inhibiting several isoforms of the cytochrome P450 superfamily (Cury et al., 2012). Furthermore, there was an increase of nitrosamines to post-hepatic tissues and an increase in DNA adducts formation, which can lead to cancer development (Warnakulasuriya et al., 2005; Hashibe et al., 2007).

We include MDR analysis to study the higher order gene-environmental interaction. MDR approach identifies the combination of tobacco habits and *CYP1A1* T3801C polymorphism as the best model for HNC risk in our study population (Table 4). Interaction entropy graph was drawn to determine synergistic or antagonistic interactions using the MDR results (Figure 2). Entropy graph, revealed tobacco habits had highest independent effect and had a synergistic interaction with *CYP1A1* variant genotypes. These results indicated that besides tobacco exposure, individual detoxification capacity also plays an important role in the development of HNC. Our study might have certain drawback for predicting high-order interactions due to the relatively small sample size; however, MDR approach overcomes the limitation for low sample sizes by using cross validation and permutation testing strategy.

Our study indicates that smoking, alcohol drinking and tobacco-betel quid chewing are risk factors associated with HNC in the northeast Indian population. In addition, it is suggested that *CYP1A1* T3801C polymorphism is a predisposing risk factor for HNC. The result also indicates that the risk of HNC associated with tobacco and alcohol is modulated by *CYP1A1* T3801C polymorphism. Highest risks of HNC were observed among individual consuming tobacco and alcohol and carrying the *CYP1A1* CC genotypes. These findings demonstrated importance of gene-environment interaction in prediction the susceptibility of HNC.

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RESEARCH ARTICLE

Tobacco carcinogen-metabolizing genes CYP1A1, GSTM1, and GSTT1 polymorphisms and their interaction with tobacco exposure influence the risk of head and neck cancer in Northeast Indian population

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Abstract Genetic polymorphisms in tobacco-metabolizing genes may modulate the risk of head and neck cancer (HNC). In Northeast India, head and neck cancers and tobacco consumption remains most prevalent. The aim of the study was to investigate the combined effect of cytochrome P450 1A1 (*CYP1A1*) T3801C, glutathione S-transferases (*GSTs*) genes polymorphisms and smoking and tobacco–betel quid chewing in the risk of HNC. The study included 420 subjects (180 cases and 240 controls) from Northeast Indian population. Polymorphisms of *CYP1A1* T3801C and *GST* (*M1 & T1*) were studied by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and multiplex PCR, respectively. Logistic regression (LR) and multifactor dimensionality reduction (MDR) approach were applied for statistical analysis. LR analysis revealed that subjects carrying *CYP1A1* TC/CC+*GSTM1* null genotypes had 3.52-fold ($P<0.001$) increase the risk of head and neck squamous cell carcinoma (HNSCC). Smokers carrying *CYP1A1* TC/CC+*GSTM1* null and *CYP1A1* TC/CC+*GSTT1* null genotypes showed significant association with HNC risk (odds ratio [OR]=6.42; $P<0.001$ and 3.86; $P=0.005$, respectively). Similarly, tobacco–betel quid chewers carrying *CYP1A1* TC/CC+*GSTM1* null genotypes also had several fold increased risk of HNC ($P<0.001$). In MDR analysis, the best model for HNSCC risk was the four-factor model of tobacco–betel quid chewing, smoking, *CYP1A1* TC/CC, and *GSTM1* null genotypes (testing balance accuracy [TBA]=0.6292; cross-validation consistency [CVC]=9/10 and $P<0.0001$). These findings suggest that interaction of combined genotypes of carcinogen-metabolizing genes with environmental factors might modulate susceptibility of HNC in Northeast Indian population.

Keywords Head and neck cancer · CYP1A1 · GSTM1 · GSTT1 · Tobacco · MDR analysis

Introduction

Head and neck cancer (HNC) is one of the most common malignancy worldwide, and it refers to a group of biologically similar cancers arising from the mucous membranes of the oral cavity, nasal cavity, paranasal sinuses, pharynx, and larynx [1–3]. In India, it acquires 30–40 % cancers at all sites and is the sixth most common cause of death in males and seventh in females [4]. Tobacco chewing and smoking are given special attention in relation to HNC as they contain various types of carcinogens, including polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines, and N-nitrosamine. Xenobiotics metabolizing enzymes are responsible for metabolism of many exogenous chemicals that are toxic, mutagenic, or carcinogenic [5]. Carcinogen-metabolizing enzymes include the phase I enzymes involved in the detoxification of

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carcinogens and either neutralize them or change them into electrophilic compounds that are detoxified by the phase II enzymes. The human cytochrome P450 family-1 member-A1 is encoded by the cytochrome P450 1A1 (*CYP1A1*) gene, is a member of the CYP superfamily and located in chromosome 15q22 to q24 and encodes aryl hydrocarbon hydroxylase (AHH), a phase I enzyme [6, 7]. The CYP1A1 enzymes activate these environmental carcinogens to get converted in to epoxide intermediates. Subsequently, the phase II detoxification enzymes, such as glutathione *S*-transferases (GSTs) make these intermediates into the water-soluble form by conjugation reactions, so that carcinogenic substances easily excreted out from the human body and protect the body from toxic effects of carcinogens [8–10].

The most widely studied polymorphism of *CYP1A1* is *m1* (T3801C or rs4646903), which is characterized by T→C transition at 3801 nucleotide position in the 3′ non-coding region and that creates a *Msp*I restriction enzyme cleavage site [11, 12]. This variation in nucleotide may affect the level of gene expression or messenger RNA (mRNA) stability and alter the enzyme activity [13]. Thus, polymorphisms of *CYP1A1* gene, which may cause enhanced enzymatic activity, appear to play a crucial role in susceptibility to DNA adduct formation and increased cancer risk. Among the glutathione *S*-transferases (GSTs) superfamily, *GSTM1* and *GSTT1* genes were found to be associated with higher susceptibility to various cancers [14]. The *GSTM1* and *GSTT1* genes have been localized to chromosome 1p13.3 and 22q11.2, respectively. Both *GSTM1* and *GSTT1* genes are polymorphic in nature, and their frequent homozygous deletions result in a loss of functional activity of corresponding enzymes and thus decreased detoxification capacity [15]. The polymorphisms of *CYP1A1*, *GSTM1*, and *GSTT1* genes have been associated with increased risk for various tobacco-related cancers in various populations. Singh et al. [16] reported that *GSTM1* and *GSTT1* gene polymorphism modulate head and neck cancer risk in North Indian population. Soya et al. [10] also reported that *GSTT1* null genotype is strongly associated with upper aerodigestive tract cancer risk in the South Indian population. Similarly, Anantharaman et al. [17] demonstrated that *GSTM1* null is a risk factor to oral cancer among Indian tobacco users, although *GSTT1* null genotype showed a protective role. A case–control study was undertaken in North Indian population, demonstrating the role of CYP1A1 polymorphism in the development of head and neck squamous cell carcinoma (HNSCC) to investigate the association of polymorphisms in cytochrome P450 1A1 (CYP1A1) with HNSCC [18]. A meta-analysis also indicated that GSTM1 deletion polymorphism had a significant effect on the susceptibility of oral cancer in the Indian population [19]. A review study conducted by Masood et al. [20] elucidated that Asians are more prone to head and neck cancers with GSTM1 and GSTT1 null genotypes as compared to Europeans and Americans. In our previous study, we also found individual effect of *GSTM1* and *GSTT1* null genotypes to the development of HNSCC [21]. However, there is no previous report that explains combined effect of either *GSTM1* or *GSTT1* null genotype with *CYP1A1* variant genotype to the risk of head and neck cancer in Northeast Indian population.

Progression of the cancer is not only due to environmental carcinogens, but also their interactions with metabolizing genes that are involved in the detoxification of these carcinogens. Identification of genetic susceptibility along with tobacco consumption status of population will guide toward better understanding of HNC etiology. The incidence of tobacco-related HNC increased significantly in northeast India [4] and to date, no report has been published so far on interaction between combined genotypes of phase I (*CYP1A1*) and phase II (*GSTM1* and *GSTT1*) carcinogen-metabolizing genes and tobacco (smoking and chewing) that modify the susceptibility toward HNC. We, therefore, performed a case–control study in Northeast (NE) Indian population, to test the hypothesis that combined effects of tobacco carcinogen-metabolizing genes (*CYP1A1*, *GSTM1*, and *GSTT1*) and their interaction with tobacco smoking and chewing might be associated with increased risk of head and neck cancer. Moreover, we also included the multifactor dimensionality reduction (MDR) approach to predict high-risk and low-risk gene–gene and gene–environment interaction models that confer HNC risk.

Materials and methods

Study subjects

The present study consisted of a total of 420 subjects, which includes 180 patients with pathologically confirmed HNC and 240 healthy individuals as controls. The information concerning age, gender, family history of cancer, and tobacco and food habits as well as their frequency, were obtained by using a standard questionnaire. Various tobacco habits included in our study were smoking (*cigarettes* and *beedi*), tobacco chewing (*khaini*, *zarda*, *gutkha*), and betel quid chewing (betel leaf along with areca nut, lime with tobacco, or without tobacco). The study was approved by the Institutional Ethical Committee, Assam University, Silchar, Assam, India.

Sample collection and DNA isolation

Blood and oral swab samples were collected from patients and healthy individuals. Genomic DNA was extracted using a standard phenol–chloroform–isoamyl alcohol method from each sample [22], and then stored in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) buffer at −20 °C and

subsequently used for genotyping of *CYP1A1*, *GSTM1*, and *GSTT1*. To avoid any cross-contamination during collection and processing of the samples every possible precautionary measure was taken.

Determination of CYP1A1 genotypes

The polymorphism of *CYP1A1* T3801C was analyzed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. The polymorphic site of *CYP1A1* was amplified using forward and reverse primers 5′-TAGGAGTCTTGTCTCATGCCTT-3′ and 5′-CAGTGAAGAGGTGTAGCCGCT-3′, respectively [23] in 20-μl polymerase chain reaction (PCR) reactions. Each PCR reaction mixture contains 10–100 ng genomic DNA, 20 pmol of each primer, 10X reaction buffer, deoxyribonucleotide triphosphate (dNTP) mix (2.5 mM of each dNTPs), *Pfu* DNA polymerase, $MgCl_2$, and nuclease-free water (NFW). The reaction mixture was subjected to initial denaturation at 94 °C for 8 min, followed by 35 cycles at 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min. The final extension was done at 72 °C for 10 min. PCR products were then digested with restriction enzyme *Msp1* (New England BioLabs, USA) for 3–5 h at 37 °C, and electrophoresed on a 2.5 % agarose gel. DNA molecular weight marker was used to assess the size of the PCR-RFLP products. The PCR products of *CYP1A1* were sequenced to confirm the PCR-RFLP result by using 3500 Genetic Analyzer, Applied Biosystems (Molecular Medicine Lab, Department of Biotechnology, Assam University, Silchar, India).

Determination of GSTM1 and GSTT1 genotypes

Genotyping of the *GSTM1* and *GSTT1* was done by multiplex PCR using primer pairs of the respective genes (a) *GSTT1:* F5′-TTCCTTACTGGTCCTCACATTCTC-3′, R5′-TCAC GGGATCATGGCCAGCA-3′; (b) *GSTM1*: F5′-GAACTC CCTGAAAAGCTAAAGC-3′, R5′-GTTGGGCTCAAATA TACGGTGG3′; and (c) *CYP1A1*: F5′ ACTGCCACTTCA GCTGTCT, R5′-GCTGCATTTGGAAGTGCTC-3′ [24]. Here, primers of this *CYP1A1* gene act as an internal control. Extracted DNA of each sample was amplified in a total volume of 10-μl reaction mixture containing 2× BioMix (Bioline, UK) and 10 pmol of each of the forward (F) and reverse (R) primers. PCR conditions were set as initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 59 °C for 45 s and 72 °C for 1 min and the final extension at 72 °C for 10 min. To check the desired band, PCR products were electrophoresed in 1.5 % agarose gel stained with ethidium bromide.

Statistical analysis

In our study, we used chi-square (χ^2) tests to determine any difference between cases and controls groups according to age, sex, smoking, and tobacco–betel quid chewing. To analyze the gene–gene interactions and risk of HNC, we calculated odds ratio (OR) and 95 % confidence intervals (CI) using the logistic regression model. To strengthen the association between variant genotypes and HNC risk, odds ratio were calculated after adjusting confounding factors such as age, gender, smoking, and tobacco–betel quid chewing status. Interactions between combined genotypes of metabolizing genes and environmental factors were estimated using the unconditional logistic regression model. *P* values less than 0.05 are considered statistically significant. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software, version 16 for Windows.

Multifactor dimensionality reduction approach

The MDR is a novel, non-parametric, and model-free statistical method used to detect high-order gene–gene and gene–environment interaction associated with disease risk [25]. The advantage of using MDR analysis over conventional parametric statistical methods is to overcome sample size limitations often encountered by case–control study. In this study, we used MDR software package MDR 3.0.2 (www.multifactordimensionaliltyreduction.org) to create a multifactors model to predict HNC susceptibility. To reduce the chance of observing false results due to the divisions of data, we used 10 random seed numbers and 10-fold cross-validation procedure and 1000-fold permutation testing during MDR analysis. The best prediction model was selected on the basis of maximum testing balance accuracy (TBA), training balance accuracy (TrBA), and cross-validation consistency (CVC). MDR results were considered to be statistically significant at the *P* value of <0.05. The advantage of using the MDR approach is to overcome sample size limitations often encountered by conventional parametric statistical method [26, 27].

Interaction entropy graphs

Interaction entropy graphs were created using MDR results, for better verification and visualization of interactions between gene and environment factors. Entropy estimates are used to determine the information gain about a class variable from integrating two variables together. Interaction entropy graphs are consisting of a node for each variable with pairwise connections between them. The percentage of entropy removed (information gain) by each variable or by each pairwise connection of variables is visualized for each node or connection. To determine whether interactions are synergistic

or not, the independent effect of each variable can be therefore compared with the interaction effect of variables [28].

False positive report possibility

Results generated by multiple comparison analysis are often encountered by false positive discoveries, thus false positive report possibility (FPRP) is conducted to evaluate the robustness of the findings from studies of gene–environment interaction using Bayesian approach [29]. In view of a small sample size of our epidemiological data, we set prior probabilities ranging from 0.25 to 0.001 with statistical power to detect OR of 1.5 and 2.0 and α level equal to the observed *P* value. The FPRP cutoff point was kept at 0.5.

Results

Demographic characteristics of subjects

Table 1 summarized the demographic characteristics of cases and healthy controls. Among the patients, 68.9 % were male and 31.1 % female, whereas in the control group, 61.7 % were male and 38.3 % female. The mean age of patients and healthy controls were 55.42 and 52.15, respectively. In this case–control study, no statistically significant difference was observed in gender and age distribution (P=0.148 and 0.622, respectively). Smokers and tobacco–betel quid chewers were significantly higher in the case group compared to the control group (P<0.05). HNC patients having both smoking and chewing habits (duel habits) also showed significant risk (<0.001). Of the 180 head and neck cancer patients; 63 (35 %) had oral cancer, 24 (13.3 %) had laryngeal malignancy, 70 (38.9 %) had nasopharyngeal cancer, 10 (5.6) pharyngeal cancer and 13 (7.6 %) had other types of cancer in the head and neck region (Table 1).

Table 1 Demographic characteristics of cases and controls

Parameters	Cases *N* (%)	Controls *N* (%)	*P* value
Gender			
Male	124 (68.9)	148 (61.7)	
Female	56 (31.1)	92 (38.3)	0.148
Age			
Mean±SEM	55.42±0.92	52.15±0.88	
>50	98 (54.4)	124 (51.7)	
≤50	82 (45.6)	116 (48.3)	0.622
Smoking			
Never	73 (40.6)	132 (55)	
Ever	107 (59.4)	108 (45)	<0.05
Tobacco–betel quid chewing			
Never	49 (27.2)	107 (44.6)	
Ever	131 (72.8)	133 (55.4)	<0.05
Smoking+tobacco–betel quid chewing			
Never	18 (10)	56 (23.3)	
Duel habit	77 (42.8)	58 (24.2)	<0.0001
Single habit	85 (47.2)	126 (52.5)	0.013
Site of tumor			
Oral	63 (35)		
Nasopharyngeal	70 (38.9)		
Larynx	24 (13.3)	–	–
Pharynx	10 (5.6)		
Other	13 (7.2)		

Interaction between genotypes of CYP1A1 T3801C, GSTM1 and GSTT1 in the risk of HNC

The allele types of *CYP1A1* gene were determined by three distinct banding patterns such as: a single 343-bp fragment indicates the wild-type TT allele, three fragments of 343, 200, and 143 bp for the TC heterozygous allele and two fragments of 200 and 143 bp indicate variant CC allele of CYP1A1 T3801C (Fig. 1a). The genotypes of *GSTT1* and *GSTM1* were also detected by the observing present/absent of the desired band in 1.5 % agarose gel (Fig. 1b).

We investigated the gene–gene interaction of *CYP1A1* T3801C and *GSTs* genes and their association with HNC using the combined low-risk wild genotypes of *CYP1A1*, *GSTM1*, and *GSTT1* as a reference group (Table 2). Individuals carrying *CYP1A1* TC/CC+*GSTM1* null genotypes combination, showed the highest risk of HNC (OR=3.52; 95 % CI=1.90–6.51; P<0.001). However, *CYP1A1* TT+*GSTM1* null genotypes and *CYP1A1* TC/CC+*GSTM1* wild genotypes combination showed no significant association with risk of HNC (P=0.376 and 0.724, respectively). On the other hand, a significant increased risk of HNC was observed in individuals carrying *CYP1A1* TC/CC+*GSTT1* null combined genotypes, when compared with combined wild genotypes of *CYP1A1* and *GSTT1* (OR=1.91; 95 % CI=1.05–3.47; P=0.033). Similarly, *CYP1A1* TC/CC+*GSTT1* wild genotypes combination also showed an increased risk of HNC (OR=1.75; 95 % CI=1.06–2.89 and P=0.027) (Table 2).

Interaction of smoking and combined genotypes of CYP1A1 T3801C, GSTM1, and GSTT1 and HNC risk

In our present study, we also evaluated the interaction of smoking and the genotypes combinations of *CYP1A1* T3801C and *GSTM1* or *GSTT1* in HNC risk (Table 3). The results showed that smokers carrying both *CYP1A1* TC/CC and *GSTM1* null genotypes had the highest increase risk of developing HNC compared to never smokers with *CYP1A1* TT+*GSTM1* (wild) genotype (OR=6.42; 95 % CI=2.70 to 15.26; P<0.001). About 3.86-fold (95 % CI=1.66 to 8.96;

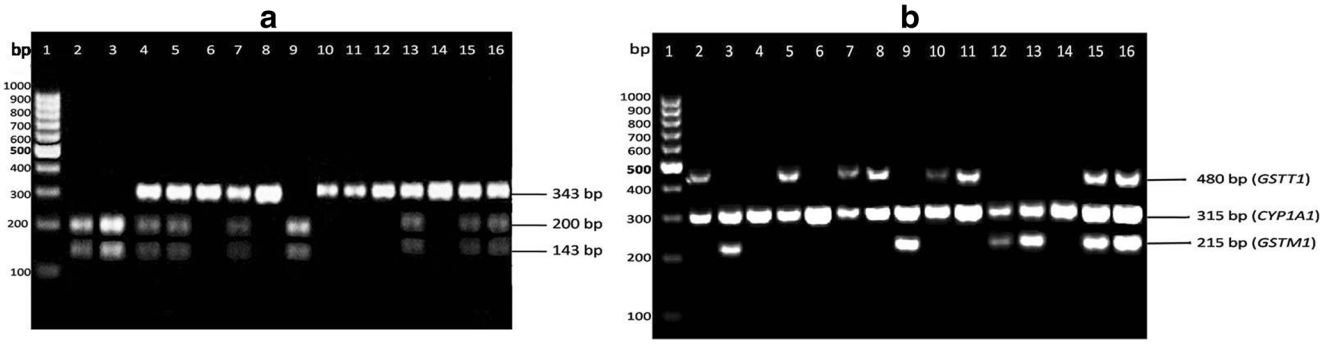

Fig. 1 Polymorphisms of *CYP1A1* T3801C, *GSTM1*, and *GSTT1* genes. **a** Ethidium bromide-stained gel showing *CYP1A1* T3801C polymorphism: 100 bp ladder (*lane 1*); *CYP1A1* TT wild type (*lanes 6,8,10,11,12,* and *14*); TC heterozygous genotype (*lanes 4, 5, 7, 13, 15,* and *16*) and CC genotype (*lanes 2, 3* and *9*). **b** Ethidium bromide-stained gel *GSTM1* null genotype (*lanes 2, 5, 7, 8, 10,* and *11*); *GSTT1* null genotype (*lanes 3, 9, 12,* and *13*); both *GSTM1* and *GSTT1* null genotype (*lanes 4, 6,* and *14*) and *GSTM1* and *GSTT1* wild genotype (*lanes 15* and *16*)

P=0.003) increased the risk of HNC in those who were smokers and also carrying *CYP1A1* TC/CC+*GSTT1* null genotype. Again, smokers having *CYP1A1* TC/CC+*GSTT1* wild genotype also showed a significant increased risk of HNC (OR=3.58; 95 % CI=1.75–7.34; P=0.001).

Interaction of tobacco–betel quid and combined genotypes of CYP1A1 T3801C, GSTM1, and GSTT1 and HNC risk

Our study evaluated the interaction of tobacco–betel quid chewing and *CYP1A1* T3801C and *GSTM1* or *GSTT1* gene polymorphisms in HNC risk (Table 4). The results showed that tobacco–betel quid chewers carrying both *CYP1A1* TC/CC and *GSTM1* null genotypes had a higher risk of developing HNC compared to never chewers with *CYP1A1* TT+*GSTM1* wild genotypes (OR=9.31; 95 % CI=3.33–25.98; P<0.001) increase risk (Fig. 2). Again, increased risk of HNC was about 3.98-fold (P=0.005) in those who were chewers and also carrying *CYP1A1* TT+*GSTM1* null genotypes. Individuals who were tobacco–betel quid chewers and carrying *CYP1A1* TT+*GSTT1* null genotype and *CYP1A1* TT/CC+*GSTT1* null genotypes also showed 4.77-fold (95 % CI=1.95–11.65; P=0.001) and 3.82 (95 % CI=1.57–9.30; P=0.005) increased risk of HNC, respectively.

Multifactor dimensionality reduction

Interactions between genetic and environmental factors for HNC risk were evaluated by MDR analysis and the best models to detect high-order interactions were summarized in Table 5. The MDR results showed that tobacco–betel quid chewing was the best one-factor model with TBA=0.55, CVC of 9/10 and P<0.0003. The combination of tobacco–betel quid chewing and *GSTM1* null was predicted as two factors model with 100 % CVC (TBA=0.6118 and P<0.0001). However, the four-factor model consists of tobacco–betel quid chewing, smoking, *CYP1A1* TC/CC and *GSTM1* null genotypes with CVC of 9/10 and highest TBA of 0.6292 and P<0.0001 was considered as the best model predicted among all the models (Table 5).

Table 2 Combined genotypes of *CYP1A1* T3801C, *GSTM1*, and *GSTT1* genes and HNC risk

Combined genotypes	Cases *N* (%)	Controls *N* (%)	OR[a] (95 % CI)	*P* value
CYP1A1 T 3801C+*GSTM1* (wild/null)				
TT+wild type	37 (20.5)	66 (27.5)	1 (reference)	
TT+null type	43 (23.9)	64 (26.7)	1.29 (0.72 to 2.31)	0.376
TC/CC+wild type	43 (23.9)	75 (31.2)	1.10 (0.62 to 1.96)	0.724
TC/CC+null type	57 (31.7)	35 (14.6)	3.52 (1.90 to 6.51)	**<0.001**
CYP1A1 T 3801C+*GSTT1* (wild/null)				
TT+wild type	50 (27.8)	90 (37.5)	1 (reference)	
TT+null type	30 (16.7)	40 (16.7)	1.42 (0.78 to 2.62)	0.249
TC/CC+wild type	65 (36.1)	72 (30)	1.75 (1.06 to 2.89)	**0.027**
TC/CC+null type	35 (19.4)	38 (15.8)	1.91 (1.05 to 3.47)	**0.033**

Bold values designate statistical significance (P<0.05)

[a] OR (odds ratio) adjusted for age, gender, tobacco–betel quid chewing and smoking

Table 3 Interaction of smoking and combined genotypes of *CYP1A1* T3801C, *GSTM1*, and *GSTT1* in the risk of HNC

	Smoking					
	Non smokers			Smokers		
	Cases/controls	OR (95 % CI)	*P* value	Cases/control	OR (95 % CI)	*P* value[a]
CYP1A1 T3801C+*GSTM1* (wild/null)						
TT+wild	14/37	1 (reference)		23/29	2.10 (0.93 to 4.74)	0.101
TC/CC+wild	17/41	1.10 (0.48 to 2.51)	1.000	26/34	2.02 (0.92 to 4.46)	0.112
TT+null	19/33	1.52 (0.67 to 3.48)	0.400	24/31	2.05 (0.91 to 4.58)	0.106
TC/CC+null	23/21	2.89 (1.24 to 6.73)	0.020	34/14	6.42 (2.70 to 15.26)	<0.001
CYP1A1 T3801C+*GSTT1* (wild/null)						
TT+wild	17/50	1 (reference)		33/40	2.43 (1.19 to 4.95)	0.021
TC/CC+wild	25/40	1.84 (0.88 to 3.84)	0.135	39/32	3.58 (1.75 to 7.34)	0.001
TT+null	16/20	2.35 (1.01 to 5.49)	0.075	14/20	2.06 (0.87 to 4.90)	0.116
TC/CC+null	15/22	2.01 (0.86 to 4.68)	0.125	21/16	3.86 (1.66 to 8.96)	0.003

[a] Fisher's exact test used to calculate *P* value and $P<0.05$ considered as statistically significant

Interaction entropy graphs

Using MDR results, we constructed interaction entropy graphs for HNC risk to determine synergistic or not-synergistic interactions (Fig. 2a, b). In interaction entropy graph, tobacco chewing showed the highest independent effect (2.32 %) and also had synergistic interaction with *GSTM1* null genotype (1.45 %) by removing 0.56 % of entropy. On the other hand, *GSTM1* null genotype and *CYP1A1* TC/CC showed highest synergistic interaction removing 0.87 % of entropy. Smoking (1.48 %) and *CYP1A1* TC/CC (0.67 %) might also explain considerable entropy independently.

False positive report possibility

The FPRPs of logistic regression (LR) and MDR analysis are summarized in Table 6. Results of interaction between smokers and tobacco–betel quid chewers with CYP1A1 (TT/CC)+GSTM1 null genotypes and higher order predictor models obtained from the multiple comparison analysis showed excellent reliability even when assuming very low prior probabilities (up to 0.1 to 0.001) for detecting ORs of 1.5 and 2.0.

Discussion

In this study, we investigated the interaction of combined genotypes of tobacco carcinogen-metabolizing genes (*CYP1A1*, *GSTM1*, and *GSTT1*) and tobacco exposure (smoking and chewing) toward the susceptibility of head and neck cancer (HNC). To the best of our knowledge, this is the first report on an interaction effect of smoking, tobacco–betel quid chewing, and combined metabolizing genes polymorphisms to the risk

Table 4 Interaction of tobacco–betel quid chewing and combined genotypes of *CYP1A1* T3801C, *GSTM1* and *GSTT1* in the risk of HNC

	Tobacco–betel quid					
	Non chewers			Chewers		
	Cases/controls	OR (95 % CI)	*P* value	Cases/controls	OR (95 % CI)	*P* value[a]
CYP1A1 T3801C+*GSTM1* (wild/null)						
TT+wild	7/24	1 (reference)		30/42	2.45 (0.95 to 6.33)	0.076
TC/CC+wild	16/29	1.89 (0.68 to 5.27)	0.311	27/46	2.01 (0.78 to 5.22)	0.176
TT+null	7/33	0.73 (0.23 to 2.31)	0.765	36/31	3.98 (1.53 to 10.35)	0.005
TC/CC+null	19/21	3.10 (1.11 to 8.69)	0.046	38/14	9.31 (3.33 to 25.98)	<0.001
CYP1A1 T3801C+*GSTT1* (wild/null)						
TT+wild	11/35	1 (reference)		39/55	2.26 (1.03 to 4.94)	0.060
TC/CC+wild	23/32	2.29 (0.97 to 5.38)	0.090	41/40	3.26 (1.47 to 7.24)	0.005
TT+null	3/22	0.43 (0.11 to 1.69)	0.351	27/18	4.77 (1.95 to 11.65)	0.001
TC/CC+Null	12/18	3.89 (1.57 to 9.66)	0.004	24/20	3.82 (1.57 to 9.30)	0.005

[a] Fisher's exact test used to calculate *P* value and $P<0.05$ considered as statistically significant

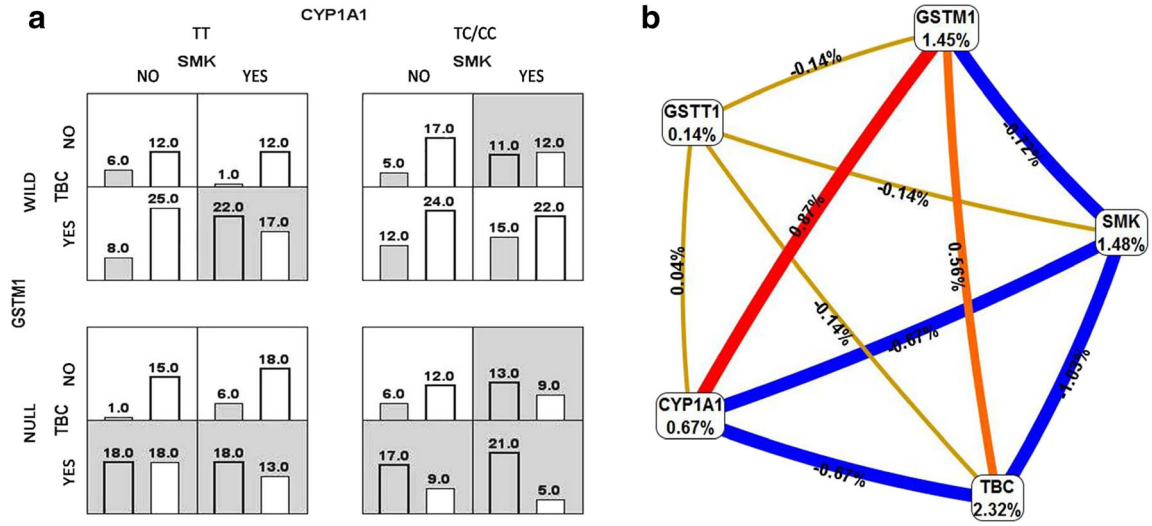

Fig. 2 MDR analysis. **a** Summary of the four-factor model (*CYP1A1*, *GSTM1*, tobacco–betel quid chewing, and smoking) in MDR analysis. The distribution of high-risk (*dark shading*) and low-risk (*light shading*) combinations associated with HNC risk. The percentage of patients having HNC is represented by the *left column* in each box, whereas the *right column* in each box indicated percentage of controls. **b** Interaction entropy graph for gene–environment interaction and HNC risk. This graphical model explains the percent of the entropy in case–control removed by each factor (independent effect) and by each pair-wise combination of attributes (interaction effect). Positive percentage of entropy indicating synergistic interaction and negative values of entropy represent redundancy. The *red color* indicating a high degree of synergistic interaction, *orange* a lesser degree whereas; gold represent midpoint, *blue* represents the highest level of redundancy followed by *green*. *TBC* tobacco–betel quid chewing and *SMK* smoking

of HNC in Northeast (NE) Indian population. Both genetic polymorphisms in metabolic genes and tobacco use play significant roles in different inter-individual susceptibility to various cancers [21, 30]. Many studies also investigated the possible role of *CYP1A1*, *GSTM1*, and *GSTT1* polymorphisms and environmental factors in HNC development, though inconsistent results have been reported [31–34].

In NE India, the incidence ratio of HNC is very high with a serious increase of morbidity and mortality rates [4]. Smoking, betel–quid chewing with or without tobacco, and other forms of tobacco consumption are major risk factors for head and neck cancer development [22]; though these factors cannot alone explain the high cancer incidence and prevalence in NE India. Previous studies have reported the role of *CYP1A1* polymorphisms and the risk of various cancers [35, 18, 36]. *GSTM1* and *GSTT1* are phase II metabolism pathway genes and play a crucial role in detoxification of tobacco carcinogens through conjugation reaction. Deletion or null genotype of *GSTM1* and *GSTT1* would decrease the capacity of the detoxification process. Thus, polymorphisms of these metabolism pathway genes may contribute toward the variable cancer susceptibility. The interaction of phase I and II tobacco carcinogen-metabolizing genes may explain the accumulation of the larger amount of toxic substances inside the body that might play the major role during the development of tobacco-related head and neck cancer.

Many epidemiologic studies in worldwide populations showed an association of *CYP1A1*, *GSTM1*, and *GSTT1* polymorphisms with susceptibility to HNC [37, 38, 17, 39, 40]. However, other studies have shown no association between *CYP1A1* or *GSTM1* or *GSTT1* polymorphism and HNC [17, 41]. Previous studies in different Indian populations also have shown an association between *CYP1A1* gene polymorphisms and HNC. A strong correlation between HNC and polymorphism of *CYP1A1* and *GSTM1* was found in North Indian population [42]. Similar result has been reported in Tamilians

Table 5 Summary of MDR analysis for HNC risk prediction

No. of locus	Best model	TBA	TrBA	CVC	*P* value
1st order	TBC	0.5500	0.5877	8/10	0.0003
2nd order	TBC, GSTM1	0.6118	0.6118	10/10	<0.0001
3rd order	SMK, TBC, GSTM1	0.5847	0.6359	6/10	<0.0001
4th order**	SMK, TBC, CYP1A1, GSTM1	**0.6292**	**0.6620**	**9/10**	**<0.0001**

CVC cross-validation consistency, *TBC* tobacco–betel quid chewing; *SMK* smoking; *TBA* testing balance accuracy; *TrBA* training balance accuracy

**Best model predicted for HNSCC risk with highest test balance and training balance accuracy and maximum CVC

Table 6 False positive reports probability (FPRP) for odd ratios of the logistic regression (LR) and multifactor dimensionality reduction (MDR) analysis

	OR (95 % CI) *P* value	FPRP for OR=1.5 (prior probability)				FPRP for OR=2.0 (prior probability)			
		0.25	0.1	0.01	0.001	0.25	0.1	0.01	0.001
LR analysis									
SMK+*CYP1A1* (TT/CC)+*GSTM1* (null)	6.42 (2.70–15.26) <0.001	**0.134**	**0.316**	0.836	0.981	**0.018**	**0.053**	**0.380**	0.861
SMK+*CYP1A1* (TT/CC)+*GSTT1* (null)	3.86 (1.66–8.96) 0.003	**0.265**	0.519	0.922	0.992	**0.074**	**0.193**	0.724	0.964
TBC+*CYP1A1*(TT/CC)+*GSTM1* (null)	9.31 (3.33–25.98) <0.001	**0.200**	**0.428**	0.892	0.988	**0.036**	**0.100**	0.549	0.925
TBC+*CYP1A1* (TT/CC)+*GSTT1* (null)	3.82 (1.57–9.30) 0.005	**0.324**	0.590	0.941	0.994	**0.109**	**0.269**	0.802	0.976
MDR analysis									
TBC	2.15 (1.41–3.26) 0.0003	**0.020**	**0.059**	**0.408**	0.874	**0.003**	**0.008**	**0.078**	**0.460**
TBC, *GSTM1*	3.02 (1.94–4.69) <0.0001	**0.003**	**0.008**	**0.085**	**0.484**	**<0.001**	**<0.001**	**0.003**	**0.025**
SMK, TBC, *GSTM1*	2.98 (2.01–4.46) <0.0001	**0.001**	**0.002**	**0.025**	**0.207**	**<0.001**	**<0.001**	**<0.001**	**0.004**
SMK, TBC, *CYP1A1*, *GSTM1*	3.78 (2.51–5.69) <0.0001	**<0.001**	**<0.001**	**0.004**	**0.038**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Prior probability range=0.25–0.001 to detect OR=1.5 or 2.0; α level=observed *P* value; Values in bold=noteworthy association at 0.5 FPRP

(south Indian) population [43] showing association between polymorphisms of these genes and upper aerodigestive tract (UADT) cancers [10]. We also previously reported the association of *GSTM1* and *GSTT1* null genotypes (OR=2.18 and 1.61, respectively) with head and neck squamous cell carcinoma risk [21]. One previous study also showed association of *CYP1A1* and *GST*s polymorphisms and oral cancer susceptibility in Northeast Indian population [44]. However, only few studies have explained combined genotypes of *CYP1A1* and *GST* (M1 and T1) genotypes in cancer. To date, there is no report so far that reveal combined genotypes of phase I (*CYP1A1* T3801C) and phase II (*GSTM1* and *T1*) genes and HNC risk in Northeast Indian population. In our present study, we found that, individuals carrying combined variant genotypes of *CYP1A1* T3801C and *GSTM1* null genotypes had a 3.52–fold increased risk of HNC. This increase risk was higher than the risk reported in our previous study that considered an individual genotype at a time. The results of our study were in accordance with a study conducted in Southern India (Tamilian population) that reported a significant risk for UADT cancer among tobacco smokers and chewers and carrying both *CYP1A1* variant and *GSTM1/GSTT1* null genotype [45]. Sam et al. [45] found 2.68-fold increased risk of UADT cancer in individuals carrying *CYP1A1* variant and *GSTM1* null genotypes. The study conducted by Anantharaman et al. [17] in West Indian population found that combined effect of either *GSTM1* or *GSTT1* null genotype with *CYP1A1* variant genotypes to the risk of oral cancer is similar to individual genes effect. Our finding also revealed that the combination of *CYP1A1* TC/CC and *GSTT1* null genotypes increased the susceptibility of HNC by 1.92-fold. These findings were also supported by Sam et al. [45] study, although the risk reported was much higher (combination effect of *CYP1A1* TC/CC and *GSTT1* null genotypes increased risk by 4.88-fold) compared to our study. The difference in the results may be elucidated by the distinction subjects' ethnicities or sample size. Thus, our findings suggest that cross talk between these phase I and II tobacco carcinogen-metabolizing genes might modulate susceptibility of HNC.

In the present study, we also examined gene–environment interaction taking into account the impact of habit-related factors like smoking and tobacco–betel quid chewing on HNC progression. The results of this interaction study showed a very high occurrence of HNC in tobacco–betel quid chewers and smokers in comparison to non smokers and non chewers (Table 1). The detoxification processes present in cellular system protects the cells from DNA damage caused by various carcinogens present. *CYP1A1* plays an important role in the bioactivation of pro-carcinogens and *GSTs* genes take part in detoxification of activated carcinogens present in tobacco products. Thus it is rational to study the combined effect of phase I (*CYP1A1*) and phase II (*GSTM1* and *GSTT1*) metabolizing genes and tobacco with the susceptibility to tobacco-related cancers. Many previous studies investigated the combined association of tobacco and *CYP1A1*, *GSTM1* and *GSTT1* polymorphisms [45, 42, 46, 37], but in a too simplest way. Also, no previous studies have found that investigate the association between polymorphisms of *CYP1A1* T3801C, *GSTM1* and *GSTT1* tobacco carcinogen-metabolizing genes and various tobacco habits with HNC risk using MDR

analysis in NE Indian population. Therefore, we investigated the association of smoking and tobacco–betel quid chewing and metabolism genes polymorphisms and HNC risk in a combined way (combined genotypes × tobacco uses). Our study showed that, combination of smoking and tobacco chewing and variant genotype of *CYP1A1*, *GSTM1* and *GSTT1* genes increased the risk of HNC. Smokers carrying *CYP1A1* TC/CC and *GSTM1* null genotype had been significantly associated with increased the risk of HNC compared to non smokers with wild-type *CYP1A1* and *GSTM1* genotypes (Table 3). However, tobacco–betel quid chewers carrying both *CYP1A1* TC/CC and *GSTM1* null genotype showed the highest risk of HNC compared to non chewers (Table 4). Our reports on gene–environment interaction were supported by the study conducted by Sam et al. [45] in Southern India, that showed higher elevated UADT cancer risk among tobacco users carrying combined genotypes of *CYP1A1* and *GST* (M1 and T1) on UADT cancers. Our findings were also supported by the Brazilian study, that found an increased risk for head and neck cancers associated with polymorphic *CYP1A1* MspI and *GSTM1* null genotypes among the tobacco users [32]. A study in the German population showed the simultaneous deletion of *GSTM1* and *GSTT1* genes increased the HNSCC risk among tobacco and alcohol consumers [47]. The MDR analyses also explained the gene–environment interaction (Fig. 2); and identify the combination of tobacco–betel quid chewing, smoking, *CYP1A1* TC/CC and *GSTM1* null genotype as the best model for HNC risk (Table 5). Interaction entropy graphs were drawn for visualization and interpretation of MDR interactions. Tobacco–betel quid chewing and *CYP1A1* and *GSTM1* genes polymorphism showed significant individual effects as well as strongest synergistic effects among each other in HNC risk and validated the results of gene–environment interaction. These results indicated that besides tobacco exposure, individual detoxification capacity also plays a crucial role in the development of HNC.

The relatively small sample size in our study might be a drawback for predicting high-order interactions and multiple comparison analysis; however, MDR approach improves statistical power to overcome small sample size limitation by using cross-validation and permutation testing strategy. Moreover, we further strengthened the data by testing the robustness and consistency of results of gene–environment interaction obtained from LR and MDR analysis using false positive report probability (FPRP).

Conclusion

To the best of our knowledge, we are the first to report that the combined genotypes of *CYP1A1*, *GSTM1*, and *GSTT1* and their interaction with tobacco smoking and chewing modified susceptibility toward HNC in Northeast (NE) Indian population. Our findings suggest that inter-individual susceptibility of tobacco-related HNC could be explained better by the genotype combinations of *CYP1A1* and *GST*s genes, than by the attribution of a single-carcinogen metabolism gene. Further, our study emphasizes the importance of the interaction study to predict susceptibility for tobacco-related cancers.

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Conflicts of interest None

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Association of Epstein Barr virus and lifestyle on nasopharyngeal cancer risk among the ethnic population of northeast India

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Abstract

Nasopharyngeal Cancer (NPC) is a multifactorial disease and is common in the Northeast States of India. Both Epstein Barr virus (EBV) infection and environmental factors are known to be associated with NPC. However, it is unclear whether EBV influences NPC risk is associated with environmental risk factors. To investigate this, we performed a case-control study among the ethnic population of northeast India with 70 NPC cases and 100 matched controls. Lifestyle information was ascertained through personal interview, and blood was collected to assess the presence of EBV. Conditional logistic regression models were applied for statistical analysis. EBV was associated with NPC risk (OR=2.6, P=0.007). Smoked meat (OR=2.39, P=0.016), fermented fish (OR=2.07, P=0.044), tobacco smoking (OR=4.57, P<0.0001) and herbal medicine (OR=3.18, P=0.001) were the major risk factors associated with NPC in this region. NPC risk was much higher among EBV positive subjects with tobacco smoking (OR=11.44, P<0.0001) herbal medicine use (OR=9.15, P<0.0001), intake of smoked meat (OR=8, P=0.001) and fermented fish (OR=4.67, P=0.002) respectively. EBV infection and smoked meat intake remain NPC risk factors in our study population. Fermented fish intake, tobacco smoking and herbal medicine use are additional risk factors for NPC. We also report for a potential association between major lifestyle factors and EBV infection towards NPC development.

Keywords: Nasopharyngeal carcinoma, Epstein Barr virus, lifestyle, smoking, northeast India.

Introduction

Nasopharyngeal Carcinoma (NPC) is a rare malignancy in most parts around the world, with an incidence well under 1 per 100 000 population per annum. Exceptions are the individuals from China and Southeast Asia, the natives in the Arctic region, and the Arabs in North Africa and parts of the Middle East [1]. In the Indian subcontinent, NPC has a low incidence which is comparable to other parts around the world except in some ethnic groups across the Northeast region [2]. These racial and geographic distributions of NPC suggest the involvement of both environmental/lifestyle and genetic factors in its development. Epstein-Barr virus (EBV) infection has been reported as an important contributing etiological factor in NPC development [3]. Studies have demonstrated the expression of EBV latent genes – Epstein–Barr virus nuclear antigen (*EBNA*), latent membrane protein-1 (*LMP-1*), *LMP-2*, and EBV encoded small RNAs (*EBER*) - in NPC cells confirming the infection of tumor cells by EBV. However, given the universality of EBV infection but the unique geographic distribution of NPC, factors other than EBV are also believed to be important determinants for NPC risks. Studies in endemic areas have indicated Cantonese salted fish [4,5], other preserved foods [6,7], uses of herbal medicine [8,9], long term cigarette smoking [10,11], and/or alcohol consumption [12,13] as a possible etiological factor in the development of NPC.

Different indigenous, tribal and non-tribal groups inhabit the northeast Indian states. Consumption of traditional foods (smoked meat, fermented fish and fermented soybean) and use of herbal medicine for various ailments has always been a custom to this indigenous population. Moreover, tobacco smoking is also a common practice among this population. It is well established that the Epstein Barr virus (EBV). infection and environmental factors are associated with NPC. However, it is unclear whether EBV influences NPC risk is associated with environmental risk factors. We investigated whether EBV is a confounder or an effect modifier of the relationship between lifestyle factors and risk of NPC. We further compared NPC risk and lifestyle factors with and without EBV infection after adjustment for potential confounding factors.

Materials and methods

Study Population

We conducted a population based case-control study among the ethnic population Northeastern States of India *viz.*, Manipur, Nagaland and Mizoram. Cases were histologically confirmed NPC newly diagnosed between May 2012 and June 2013 and were permanently residing in this region. Cases were identified through the population based cancer registries (PBCRs) and hospital based cancer registries (HBCRs) located in these regions. In total, 95 incident NPC cases were identified. Four (4.2%) died before we could contact them and were not interviewed. Permissions to contact 3 (3.1%) patients were refused by their physicians. Eighteen (19%) patients were not recruited as they had started diagnosis or refused to participate in the study. Seventy (73.7%) NPC patients were interviewed who do not have previous diagnosis or treatment of NPC. There were no age or race restrictions and subjects ranged from age 20 to 90 years of age. Controls were randomly selected and were individually matched to the case by age, gender, ethnicity (*Manipuri*, *Naga*, or *Mizo*), and neighborhood of residence. 70 patients and 100 eligible control subjects were identified and interviewed. The Institutional Review Boards of all the participating institutions approved the study protocol and the individual subjects signed informed consent forms.

Collection of data

A personal interview was administered to the subjects, and a standard pre-designed questionnaire was used to collect general and exposure information on the patients and matched controls. Each subject was requested to report information on demographic characteristics (age, gender, education and ethnicity, etc.), consumption of traditional foods (includes smoked meat and fermented fish), tobacco smoking, herbal medicine use, prior medical conditions, and use of medications. Subjects were categorized as *ever* for those who consumed the traditional food items >1 times per week and *never* for those who do not consume. *Ever* smokers were defined as subjects who smoked at least 100 cigarettes in their lifetime and the rest as *never* smokers. *Ever* use of herbal medicine was assessed, and for those who reported use, the names of the medicines used were assessed. In addition, 2ml venous blood was collected for determining the presence of gene coding for EBV latent membrane protein 1 (*LMP1*) in their genome. Further, subjects were categorized as *positive* for those who showed the presence of EBV and the rest as *negative*. Precaution was taken to avoid any cross-contamination while collecting and processing the samples.

PCR detection of EBV

Each blood sample was digested in TES buffer and incubated overnight at 55°C to digest the tissue. DNA was subsequently isolated by phenol/ chloroform/ isoamylalcohol method [14] followed by ethanol precipitation and re-suspended in TE buffer and stored at -20°C. EBV infection was detected through PCR amplification of the *LMP1* gene using the primers 5′-GGAGCCCTTTGTCTACTCCTA CTG-3′ and 5′-TGCCTGTCCGTGAAATTC-3′. The PCR programme used for amplification was: initial denaturation step at 94°C for 5mins; 40 cycles of denaturation at 94°C for 30s; annealing at 62°C for 30s, extension at 72°C for 45s and final extension at 72°C for 5mins. A 150 bp product in 2% agarose gel determines the presence of the EBV-*LMP1* gene.

Statistical analysis

Statistically significant differences between cases and controls for demographic and socioeconomic characteristics were assessed by Chi-square (χ^2) test. Conditional logistic regression models were used to estimate the odds ratios (ORs), their 95% confidence intervals (95% CI), and corresponding p-values using the Statistical Package for the Social Sciences (SPSS) program software. A p-value of less than 0.05 was considered to be statistically significant.

Results

The demographic characteristics of the 70 NPC cases and 100 controls enrolled in this study are shown in Table-1. There was no statistically significant difference between the cases and controls in terms of sex, age group, BMI, ethnicity, profession, which indicated that the frequency matching was sufficient. Statistically significant variation was observed for consumption of smoked meat (P=0.02), fermented fish (P=0.05), herbal medicine use (P<0.0001) and tobacco smoking (P<0.0001). EBV status (P=0.011) also showed significant variation among the study population.

Table-2 presents the association between lifestyle factors as well as EBV infection and NPC risk in the case-control dataset. 72.8% cases and 54% controls were positive for EBV infection (Fig. 1a). When

Table-1: Demographic characteristics and socioeconomic status of the study population

Variables	Case, *n* (%)	Control, *n* (%)	χ^2 test	*P*-value^^
Sex :				
Male	54 (77.1)	79 (79)		
Female	16 (22.9)	21 (21)	0.08	0.77
Age Group:				
≤50	26 (37.1)	39 (39)		
>50	44 (62.9)	61 (61)	0.06	0.8
BMI Kg/m^2:				
Underweight (< 18.5)	16 (22.8)	10 (10)		
Normal Weight (18.5-22.99)	30 (42.8)	52 (52)	5.7	0.12
Overweight (23.0-27.49)	18 (25.7)	31 (31)		
Obesity (27.5-more)	6 (8.6)	7 (7)		
Ethnicity:				
Manipuri	30 (42.8)	45 (45)		
Naga	29 (41.5)	39 (39)	0.11	0.94
Mizo	11 (15.7)	16 (16)		
Profession:				
Famer	39 (55.7)	40 (40)		
Service	17 (24.3)	35 (35)	4.18	0.13
Others	14 (20)	25 (25)		
Smoked Meat:				
Never	18 (25.7)	44 (44)		**0.02**
Ever	52 (74.3)	56 (56)	5.18	
Fermented Fish:				
Never	41 (58.6)	74 (74)		
Ever	29 (41.4)	26 (26)	3.8	**0.05**
Tobacco Smoking:				
Never	27 (38.6)	73 (73)		
Ever	43 (61.4)	27 (27)	18.75	**<0.0001**
Herbal Medicine:				
Never	31 (44.3)	71 (71)		
Ever	39 (55.7)	29 (29)	15.07	**<0.0001**
EBV-*LMP1*				
Negative	19 (27.2)	46 (46)		
Positive	51 (72.8)	54 (54)	5.43	**0.0198**

*Distribution in frequencies was tested by chi-square test and $P<0.05$ is considered statistically significant value
Bold values indicate statistical significance ($P<0.05$)

compared to the reference group, there was a 2.6 (95% CI: 1.3-5.2; $P=0.007$) fold risk of NPC development among EBV positive subjects. Certain traditional foods were also analyzed for their risk towards NPC development in our study population. These include smoked meat and fermented fish where the intake frequencies were 74.3% and 41.4% in cases whereas 56% and 26% in controls. After adjustment for possible confounders, smoked meat and fermented fish intake showed 2.39 (95% CI: 1.18-4.85; $P=0.016$) and 2.07 (95% CI: 1.02-4.22; $P=0.044$) fold NPC risk when compare to the reference group. Others factors like smoking and herbal medicine use also seem to induce the risks for NPC development. 61.4% and 55.7 % cases were smokers and use herbal medicine, which were much higher compared with the controls with

Table-2: OR and 95% CI for association of NPC with EBV and lifestyle related habits

Variables	Case n=70 (%)	Control n=100 (%)	Adjusted ORs (95% CI)^^	*P*-value*
EBV-*LMP1*				
Negative	19 (27.2)	46 (46)	1	Ref.
Positive	51 (72.8)	54 (54)	2.6 (1.3-5.2)	**0.007**
Smoked Meat:				
Never	18 (25.7)	44 (44)	1	Ref.
Ever	52 (74.3)	56 (56)	2.39 (1.18-4.85)	**0.016**
Fermented Fish:				
Never	41 (58.6)	74 (74)	1	Ref.
Ever	29 (41.4)	26 (26)	2.07 (1.02-4.22)	**0.044**
Tobacco Smoking:				
Never	27 (38.6)	73(73)	1	Ref.
Ever	43 (61.4)	27 (27)	4.57 (2.28-9.18)	**<0.0001**
Herbal Medicine:				
Never	31 (44.3)	71 (71)	1	Ref.
Ever	39 (55.7)	29 (29)	3.18 (1.62-6.24)	**0.001**

^^ Odds adjusted for sex, age, BMI, ethnicity and profession as appropriate
* Fisher's exact test used to calculate *P* value and $P<0.05$ considered as statistically significance
Bold values indicate statistical significance values after adjustment for confounders ($P<0.05$)

only 27% and 29%. Smoking and herbal medicine use showed 4.57 (95% CI: 2.28-9.18; $P<0.0001$) and 3.18 (95% CI: 1.62-6.24; $P=0.001$) fold risk for NPC development (Fig. 1b).

We also analysed whether the relationship between these major lifestyle factors and the risk of NPC differs by EBV infection status (Table-3). Risk associated with NPC increases among EBV positive subjects with intake of smoked meat (OR=8.0, 95% CI: 2.22-28.79; $P=0.001$) and fermented fish (OR=4.67, 95% CI: 1.79-12.17; p=0.002) respectively and were statistically significant. However, higher risk

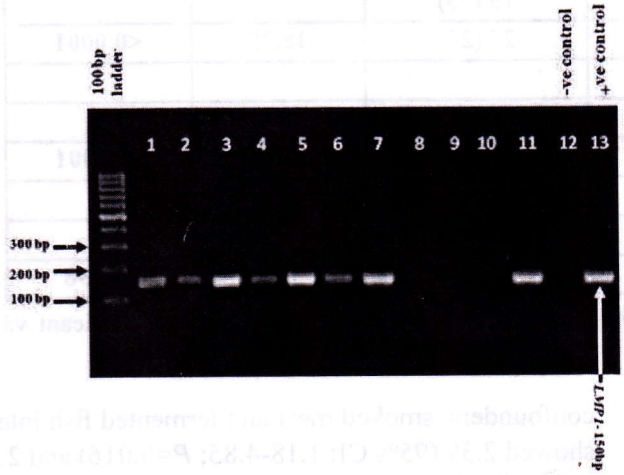

Fig. 1a: PCR based detection of *EBV-LMP1* in NPC; representative agarose gel stained with ethidium bromide for PCR detection of Epstein Barr Virus (*EBV-LMP1* gene at expected size of 150 base pairs) in the study population. Lane 1-7 & 11 represent samples positive for EBV, lane 8-10 represents samples negative for EBV along with –ve control in lane 12 and +ve control in lane 13.

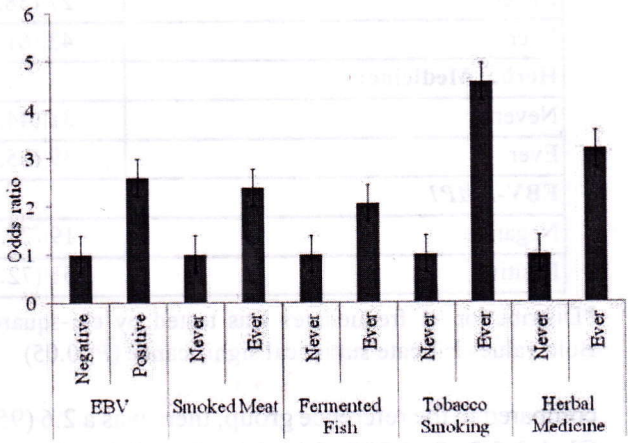

Fig.1b: Odds ratio for association of NPC with EBV, smoked meat and fermented fish, tobacco smoking, and herbal medicine. A significant elevated risk of NPC was observed in individuals with EBV infection (OR=2.6). Smoked meat (OR=2.39) and fermented fish (OR=2.07) intake also show increased risk of NPC. Highest risk of NPC were observed with tobacco smoking (OR=4.57) and herbal medicine use (OR=3.18)

of NPC were observed with tobacco smoking (OR=11.44, 95% CI: 4.03-32.48; $P<0.0001$) and herbal medicine use (OR=9.15, 95% CI: 3.29-27.13; $P<0.0001$).

Table-3: Risk of NPC by intake of smoked meat, fermented fish, tobacco smoking, herbal medicine use and EBV infection

Variables	EBV-*LMP1* Negative			EBV-*LMP1* Positive		
	Ca /Co	ORs (95% CI)^^	*P*-value*	Ca/Co	ORs (95% CI)^^	*P*-value*
Smoked Meat						
Never	Mar-20	1	Ref.	15/24	4.17 (1.08-16.02)	**0.044**
Ever	16/26	4.10 (1.08-15.62)	**0.046**	36/30	8.0 (2.22-28.79)	**0.001**
Fermented Fish						
Never	Oct-35	1	Ref.	31/39	2.78 (1.20-6.43)	**0.018**
Ever	9-Nov	2.86 (0.95-8.64)	0.08	20/15	4.67 (1.79-12.17)	**0.002**
Tobacco smoking						
Never	Jul-31	1	Ref.	20/42	2.11 (0.80-5.54)	0.116
Ever	Dec-15	3.54 (1.18-10.64)	**0.029**	31-Dec	11.44 (4.03-32.48)	**<0.0001**
Herbal Medicine						
Never	Aug-27	1	Ref.	23/44	1.76 (0.70-4.45)	0.264
Ever	Nov-19	1.95 (0.67-5.68)	0.279	28-Oct	9.15 (3.29-27.13)	**<0.0001**

Ca/Co: Case/Control Ca *cases*, Co *controls*
* Fisher's exact test used to calculate *P* value and $P<0.05$ considered as statistically significance
Bold values indicate statistical significance ($P<0.05$)

Discussion

In the present study, we report the risk associated with lifestyle factors as well as EBV infection and NPC among the ethnic population of Northeast India. To the best of our knowledge, this is the first case-control design, to discuss, whether the relationship between major lifestyle factors and the risk of NPC differs by EBV infection status.

The presence of EBV in NPC was firmly established as early as 1973 [15]. Due to its association with NPC, it has been classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) [16]. EBV derived latent membrane protein-1 (*LMP1*) gene was used to detect the presence of EBV infection in our study. EBV *LMP-1* gene can transform normal cell to cancerous form and support to the concept that EBV is involved in the pathogenesis of NPC [17]. Our finding also supports the role of EBV in NPC development which is consistent with other studies [18,19].

Our study suggests an intake of smoked meat as major risk factors associated with NPC. Smoked meat remains a stable contributor to NPC risk in the ethnic population of northeast India. Our result is consistent with previous studies conducted in this region [8,20]. Smoked meat contained nitrosodimethylamine (NDMA), nitrosodiethylamine (NDEA) and nitrosopyrrolidine (NPYR) which are known mutagen and may have contributed to NPC development [21]. Exposure to other preserved food, such as fermented fish, was also found to be associated with an increased risk of NPC. This is consistent with previous publications [5,22,23]. It has been reported that preserved foods including mouldy bean curd, salted shrimp paste, salted eggs and various vegetables were associated with an increased risk of NPC. Intake of preserved foods also has been shown to be associated with NPC risk among Arabs of North Africa, another high risk population of NPC [24]. However, we should interpret our result with care, since it may cast doubt on the validity of the information obtained using a food-frequency questionnaire.

We further analysed if the risk associated with these major lifestyle factors were modified by EBV infection. No case-control study has been conducted to determine an association of lifestyle factors and EBV infection in NPC carcinogenesis. Statistically significant increased risks of NPC were observed in individuals with EBV infection and consuming smoked meat and fermented fish (OR=8.0 and 4.67). Our results may be supported by experimental evidence that determine the presence of possible EBV

inducers, activators, mutagens and volatile nitrosamines in preserved food samples from high-risk areas for nasopharyngeal carcinoma [25,26]. Results from the present study indicate that cigarette smoking is also associated with risk of NPC. An etiological link between cigarette smoking and NPC risk is biologically plausible since the nasopharynx is a site directly exposed to smoke during cigarette smoking. Tobacco smoke also contains over 4,000 compounds; such as polycyclic aromatic hydrocarbons, aromatic amines, and N-nitrosamines, which are carcinogens [27] and it is possible that carcinogenic products in cigarettes could cause genetic mutations and methylation [28] thereby resulting in the transformation of epithelial cells in the nasopharynx. Several studies in endemic areas have also observed a positive association between cigarette smoking [10,29] and NPC although some studies failed to detect such an association [30,31]. However, in NPC carcinogenesis, cigarette smoking may play an alternative role by acting as a cofactor to EBV infection or via inducting EBV reactivation [18,32]. We observed consistent evidence that a strong association exists between EBV infection and tobacco smoking among our study population. We verified that risk associated with NPC was much higher among tobacco smokers and EBV infected subjects. Though not statistically significant a very high interaction of EBV infection and tobacco smoking was observed in previous studies [29,33]. In our study population EBV infected individuals with tobacco smoking were associated with 11.44 fold risk for NPC development and were statistically significant.

Plants commonly of the *Euphorbiaceae* and *Thymelaeceae* families are associated with NPC risk in endemic areas. It has been found that these plants contain phorbol esters, which is thought to be linked to nasopharyngeal carcinoma, either through its ability to reactivate the Epstein-Barr virus (EBV) infection [34] or through a direct promoting effect on cells transformed by the EBV [35]. In our study, it was observed that the consumption of herbal medicine is associated with 3.18 fold risk of developing NPC, similar to other endemic areas [8,9] though another study denied this association [36]. Of particular note is our finding of an apparent association between EBV and herbal medicines. Herbal medicine was found to act jointly with EBV infection. The risk associated with NPC increases to 9.15 fold among EBV positive subjects with herbal medicine use. We also assessed the identity of the herbal medicine used in our study areas. We could identify only a few herbal medicines use in common ailments, such as *Alnus nepalensis* D. Don, *Ananas comosus* (L.) Merr., *Asparagus racemosus* Wilt., *Callicarpa arborea* Roxb., *Curcuma caesia* Roxb., *Drymaria cordata* Willd., *Fagopyrum esculentum* Moench., *Phyllanthus fraternus* Web., *Ricinus communis* Linn., *Sonchus wightianus* Linn., *Adhatoda vasica* Nees., *Bombax ceiba* Linn., *Cassia alata* Linn, *Phlogacanthus thyrsiflorus*, *Croton caudatus* (variety not specify). Interestingly, use of plants of the *Euphorbiaceae* family was common, which have been observed to be linked to nasopharyngeal carcinoma [34,35]. This could explain the increased risk of NPC among EBV infected individual with herbal medicine use in our study population.

In conclusion, intake of smoked meat remains a risk factor for NPC in northeast India. Moreover, fermented fish acts as an additional risk factor. Tobacco smoking and used of herbal medicine also plays a key role as NPC risk factors in this region. To our knowledge, this is the first epidemiological study for any potential association between lifestyle and EBV infection towards the risk of NPC development among the ethnic population of northeast India. Taken together, our study reflect that frequent intake of smoked and preserved food items, tobacco smoking and herbal medicine or the chemicals present in them may interact with or reactivate or induce EBV or may have a synergistic effect on EBV reactivation, which may lead to genomic instability of the host cells and thereby causing carcinogens. This interaction is also biologically possible, but need further validation by correlating the EBV load at different stages of the cancer, by increasing the sample size during the study and inclusion of both in vitro and in vivo studies.

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Conflict of interest

The authors declare no conflict of interest.

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Dysfunction of mitochondria due to environmental carcinogens in nasopharyngeal carcinoma in the ethnic group of Northeast Indian population

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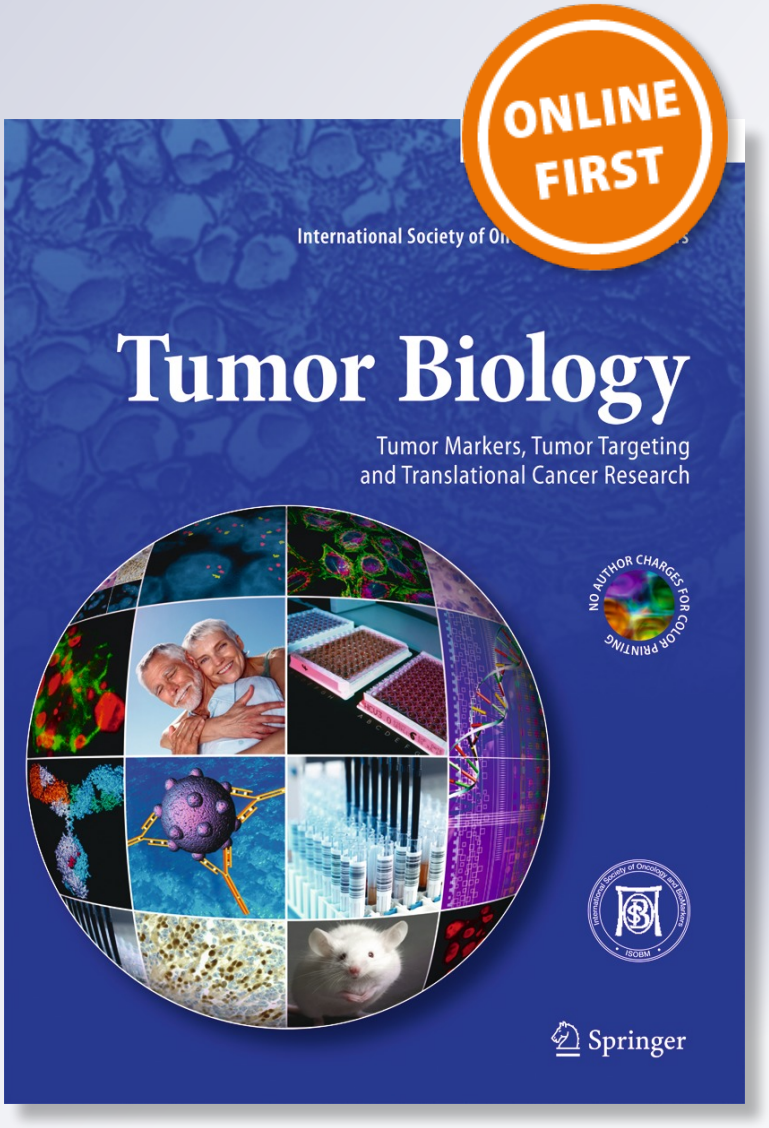

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RESEARCH ARTICLE

Dysfunction of mitochondria due to environmental carcinogens in nasopharyngeal carcinoma in the ethnic group of Northeast Indian population

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Abstract Nasopharyngeal carcinoma (NPC) is a rare cancer worldwide, but in India, NPC is uncommon in its subcontinent except in the north-eastern part of the country. NPC is thought to be caused by the combined effects of environmental carcinogens, genetic susceptibility and Epstein-Barr virus (EBV). This is the first study that aimed to examine the selected risk factors, mostly dietary, viral environmental, metabolic gene polymorphisms, mitochondrial DNA (mtDNA) copy number variation and their risk, in subjects who are highly prone to NPC in the ethnic groups of Northeast India, which has included cases, first-degree relatives and controls. The cases and controls were selected from three ethnic groups (Manipuri, Naga and Mizo) of Northeast India with high prevalence of NPC. This case–control family study includes 64 NPC patients, 88 first-degree relatives and 100 controls having no history of cancer. PCR-based detection was done for EBV–latent membrane protein 1 (*LMP1*) gene and glutathione S-transferase Mu 1 (*GSTM1*)–glutathione S-transferase theta 1 (*GSTT1*) polymorphism. A comparative ΔCt method was used for the determination of mtDNA content. An increased risk of 2.00–6.06-folds to NPC was observed with those who intake smoked meat and fish, salted fish and fermented fish; betel nut chewers; tobacco smokers; alcohol drinkers; and those who have kitchen inside the living room, glutathione S-transferase null genotype and EBV infection. The risk of NPC increased in cases with decreased mtDNA copy number (P_{trend}=0.007). A significant difference between *GST* null genotypes and EBV infection with mtDNA content was found in the cases (P<0.0001). The understandings of environment–genetic risk factors and their role in the etiology of NPC are helpful as preventive measures and screening.

Keywords EBV · Mitochondrial DNA copy number variation · Carcinogen metabolism genes (*GSTs*) · Dietary habits · Environmental factors · Ethnic groups

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Introduction

Nasopharyngeal carcinoma (NPC) represents about 0.7 % of the global cancer burden, and the disease may be considered one of the rarer cancers found globally, ranking as the 24th most frequently diagnosed cancer form worldwide and 22nd within the developing world. The global statistics by world region reveal the distinct features of its descriptive epidemiology. However, there is contrasting geographical and ethnic variations in the distribution of its incidence worldwide. NPC is a rare cancer worldwide except in Southeast Asia, Southern China and North Africa [1, 2]. India has a high incidence of oral cavity cancer, but NPC is uncommon in Indian

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subcontinent except in the north-eastern part of the country. The Mongoloid race in this region has shown an increase in NPC incidence. According to the National Cancer Registry programme (NCRP), 2006–2008 has been reported to have the highest age-adjusted incidence rates (AARs) of NPC in Mizoram (6/100,000) followed by Manipur State (5/100,000) and Nagaland State in Northeastern India belonging to the ethnic groups of Mizo, Manipuri and Naga [3]. The significant difference in geographical, ethnicity and dietary habits could predispose people of Northeastern India for high incidence of NPC.

NPC is thought to be caused by the combined effects of environmental carcinogens, viz. cigarette smoke, infectious agents, chemical agents, etc. [4]; genetic susceptibility, e.g. mutations in one or more genes, family history of NPC, genetic polymorphism, etc. [5]; and Epstein-Barr virus (EBV) [6, 7]. In addition, a family history of NPC, cigarette smoking, alcohol consumption and eating a diet high in salt-cured fish meat and other preserved foods are probably important etiological factors increasing the risk of developing NPC [8]. Moreover, environmental chemical pollutions, widely spread carcinogens, are difficult to be degraded in the environment and thus may have a long-term effect on human health. Recent evidence indicates that carcinogen-metabolizing genes and DNA-repair genes may play critical roles in determining individual susceptibility to cancers [9, 10]. Polymorphisms in the genes encoding both phase I and phase II enzymes, possibly by altering their expression and function, may increase or decrease carcinogen activation/detoxification and modulate DNA repair which, in turn, determines individual susceptibility to cancer risk.[11].

Many reports have indicated that mitochondria is involved in apoptosis [12–14] and probably also in tumourigenesis [15], which has led researchers to examine the potential role of mitochondrial DNA (mtDNA) alterations in the development and maintenance of cancers. Tobacco smoke is a complex mixture of carcinogenic compounds; smokeless tobacco and salted fish, meat and other preserved foods are rich in nitrosamines and generate reactive oxygen species (ROS) [16]. The mitochondrial genome is susceptible to ROS and other types of genotoxic damage due to lack of protective histones and its limited mtDNA repair capabilities [17]. The mtDNA copy number per cell is maintained within a constant range to meet the energy requirement of the cell to sustain normal physiological functions. It is likely that the variations in the copy number of mitochondria reflect the net results of gene–environmental interactions between unknown hereditary factors and the levels of oxidative stress (an imbalance between ROS production and the antioxidant capacity), caused by a variety of endogenous and exogenous factors, such as hormones, age, dietary and environmental oxidants/antioxidants and reaction to oxidative damage, all of which are thought to be the risk factors for various types of cancer development [18, 19]. We aimed to examine the selected risk factors which include mostly diet and lifestyle, EBV infection, carcinogen metabolism genes (glutathione S-transferases, *GSTs*), mtDNA copy number variation and their contribution in the development NPC. This is a first kind of study on ethnic groups from Northeast India prone to NPC that has included cases, first-degree relatives and controls to examine the prospects of genetic susceptibility to cancer.

Materials and methods

Demographic data collection

The present study was conducted on three ethnic groups of Northeast India, viz. Manipuri, Naga and Mizo, which are highly prone to NPC. All the cases and controls were ethnically matched. The lifestyle which included their housing patterns, ventilation and dietary habit was recorded. Data regarding age, gender, occupation and nature of consuming tobacco–betel quid habit (smoking or smokeless) and alcohol intake from NPC subjects was abstracted from hospital records and on personal interviews.

Subjects and sample collection

The oral swab/peripheral blood/formalin-fixed paraffin-embedded (FFPE) tissues of 64 NPC cases, 88 first-degree relatives of NPC cases and 100 healthy controls (without family history of cancer) were collected from hospitals with written informed consent, and the study was approved by the Institutional Review Board (IRB), Assam University, Silchar. All possible precautions were taken to avoid any cross-contamination while collecting as well as processing the samples.

DNA isolation

DNA was isolated from preselected regions of peripheral blood and oral swab. The tissues were digested in Tris–HCl/EDTA/sodium chloride (TES) buffer, and the tissue digests were incubated overnight at 55 °C. The DNA was subsequently isolated by phenol/chloroform/isoamyl alcohol method followed by ethanol precipitation and resuspended in Tris–HCl/EDTA (TE) buffer and stored at −20 °C [20]. Genomic DNA isolation kit (Bioline, UK) was used from FFPE tissues following the manufacturer's instructions.

Detection of EBV latent membrane protein 1 gene using PCR

PCR-based detection of EBV–latent membrane protein 1 (*LMP1*) gene was carried out using forward (F) 5′-GGAG CCCTTTGTCTACTCCTACTG-3′ and reverse (R) 5′-

TGCCTGTCCGTGCAAATTC-3′ primers. The PCR programme used for amplification was as follows: initial denaturation step was done at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 30s, annealing at 62 °C for 45 s and elongation at 72 °C for 90 s.

Multiplex PCR for *GSTM1* and *GSTT1*

Analysis for *GSTM1–GSTT1* gene polymorphism using *CYP1A1* gene as an internal control was done by multiplex PCR. The forward (F) and reverse (R) primers used for amplification were as follows: for *GSTT1*, F5′-TTCCTTACTG GTCCTCACATTCTC-3′ and R5′-TCACGGGATCATGG CCAGCA-3′; for *GSTM1*, F5′-GAACTCCCTGAAAAGC TAAAGC-3′ and R5′-GTTGGGCTCAAATATACGGTGG-3′; and for *CYP1A1*, F5′-GAACTGCCACTTCAGCTGTCT and R5′-GCTGCATTTGGAAGTGCTC [21]. The PCR programme used for amplification was as follows: initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 45 s and elongation at 72 °C for 90 s. The amplified product was observed in 1.5 % agarose gel.

Quantitative real-time PCR

The StepOne™ Real-Time PCR System (Applied Biosystems) was used to perform PCR amplification for mtDNA D-loop (C-tract) region. *GAPDH* was used as a 'housekeeping gene' to normalize all the threshold cycle (Ct) values. The forward (F) and reverse (R) primers used for the amplification of C-tract region were F5′-CAGGGTCA TAAAGCCTAAATAG-3′ and R5′-GAGGTAAGCTACAT AAACTGTG-3′ (109 bp) and, for *GAPDH*, were F5′-GAAATCCCATCACCATCTTCC-3′ and R5′-GAGCCCCA GCCTTCTCCATG-3′ (125 bp), respectively. For each 10-μl reaction, 1 μl of unknown DNA was amplified containing 0.5 μl of each primer (20 pmol/μl), 5 μl of 2× SYBR Green Master Mix (Applied Biosystems) and 3 μl of nuclease-free water. The real-time PCR conditions consisted of initial denaturation and *Taq* polymerase activation at 95 °C for 10 min followed by 40 cycles of 95 °C for 45 s, 54 °C for 45 s and 72 °C for 1 min and followed by a melting curve analysis. Each measurement was repeated in triplicate, and a non-template control was included in each experiment.

To determine the quantities of mtDNA and nuclear DNA (nDNA) present in the samples, the average threshold cycle (Ct) number values of the nDNA and mtDNA were obtained from each case. The level of mtDNA was calculated using the delta Ct (ΔCt) of average Ct of mtDNA and nDNA (ΔCt= CtmtDNA−CtnDNA) same as with that having an exponent of 2 ($2^{-\Delta Ct}$) [18].

Statistical analysis

Medians and frequencies of selected characteristics of cases and controls were compared using the Mann–Whitney *U* test for continuous and the Pearson chi-square for all other categorical variables. mtDNA copy number was categorized into quartiles based on the distribution among controls. Odds ratios (ORs) and 95 % confidence intervals (CIs) were estimated using logistic regression models. A test for trend was calculated using the mtDNA copy number as a continuous variable. Non-parametric Mann–Whitney test was used to test if mtDNA content alteration in tumour is different in NPC cases, first-degree relatives (FDRs) and controls with or without *GSTM1* and *GSTT1* null genotypes. *P* values less than 0.05 are considered statistically significant.

Results

The characteristics of the 64 NPC cases, 88 FDRs and 100 controls enrolled in this study along with the parameters which are considered to cause NPC are shown in Table 1. There was no statistically significant difference between the cases, FDRs and controls in terms of age; sex; ethnicity; dietary habits like intake of meat, fish, smoked fish, fermented fish and green vegetable/salads; alcohol consumption; house types; kitchen (outside/inside); and ventilation. The individual risk factors associated with NPC were examined for the cases, FDRs and controls and depicted in Table 2. The results were divided into three parts for better understanding as follows:

1. Risk factors associated with nasopharyngeal carcinoma in cases

In cases, it was observed that there is an increased risk for NPC with intake of smoked meat (OR=2.00; 95 % CI, 1.02–3.93; *P*=0.04), smoked fish (OR=2.21; 95 % CI, 1.15–4.26; *P*=0.01), salted fish (OR=2.61; 95 % CI, 1.17–5.81; *P*= 0.01), fermented fish (OR=2.17; 95 % CI, 1.04–4.51; *P*= 0.02) when their dietary practices were examined. Among the other common practices like betel nut chewing, tobacco smoking and alcohol consumption, which were categorized with respect to their intake status, viz. yes and no, were examined. It was observed that there was an increased risk of 2.77-fold (95 % CI, 1.33–5.91; *P*=0.003) for NPC in betel nut chewers compared to non-chewers. Similarly, a 5.54-fold (95 % CI, 2.25–11.63; *P*<0.0001) and 2.13-fold (95 % CI, 1.06-4.34; *P*=0.02) increase risk in tobacco smokers and alcohol consumers for NPC was observed compared non-smokers and alcohol consumers, respectively. Some variables other than the dietary practices were examined like kitchen inside or outside of the living room which was found to be 2.2-fold (95 % CI, 1.00–4.90; *P*=0.04) for NPC. The *GST* null

Table 1 Selected characteristics of NPC cases, their FDRs and controls

Characteristics	Case (n=64)	FDR (n=88)	Control (n=100)	P value
Sex				
Male	49	69	79	0.93
Female	15	19	21	
Age group				
<50	23	32	39	0.90
≥50	41	56	61	
Ethnicity				
Manipuri	28	42	45	0.96
Naga	27	32	39	
Mizo	9	14	16	
Dietary habits				
Meat intake				
Daily	6	13	15	0.80
≤3 times a week	15	22	26	
>3 times a week	43	53	59	
Smoked meat intake				
Yes	46	63	56	0.03
No	18	25	44	
Fish intake				
Daily	2	7	13	0.18
≤3 times a week	28	35	29	
>3 times a week	34	46	58	
Smoked fish intake				
Yes	43	56	52	0.10
No	21	32	48	
Salted fish intake				
Yes	18	23	13	0.02
No	46	65	87	
Fermented fish intake				
Yes	26	27	24	0.07
No	38	61	76	
Fresh fruit				
Never	2	2	3	0.01
Daily	11	9	19	
≤3 times a week	31	28	22	
>3 times a week	20	49	56	
Green salad/cooked vegetables intake				
Never	4	2	3	0.42
Daily	14	24	34	
≤3 times a week	31	45	49	
>3 times a week	15	17	14	
Betel nut chewing				
Current	25	28	24	0.0002
Former	18	6	9	
Never	16	41	49	
Occasionally	5	13	18	
Tobacco smoking				
Current	9	7	6	<0.0001
Former	26	11	12	
Never	21	64	73	
Occasionally	8	6	9	
Alcohol consumption				
Current	25	21	23	0.09
Former	12	21	14	
Never	20	34	51	
Occasionally	7	12	12	
Type of house				
Kachha-bamboo/mud/wood	38	48	59	0.77
Pucca-RCC	26	40	41	
Ventilation per room				
No window	2	7	4	0.24
Single window	39	42	62	
≥2 windows	23	39	34	
Kitchen				
Outside	47	69	86	0.12
Inside	17	19	14	
Cooking fuel used				
Gas	27	38	57	0.04
Wood fire	31	32	29	
Both	6	18	14	

genotypes have been linked with a number of cancers likely due to an increased susceptibility to environmental toxins and carcinogens, and here in this study, we found that *GSTM1* null genotype has increased NPC risk by 2.15-fold (95 % CI, 1.13–4.08; P=0.01) whereas no risk association of *GSTT1* null genotype (OR=1.32; 95 % CI, 0.66–2.61; P=0.42) with NPC was observed. However, the risk further increases by 6.06-fold (95 % CI, 1.15–31.82; P=0.02) with both *GSTM1* and *GSTT* null genotypes (Fig. 1). Moreover, the EBV (*LMP1* gene) infection was examined and a 2.17-fold (95 % CI, 1.11–4.26; P=0.02) of increased risk for NPC was found (Fig. 1).

2. Risk factors associated with nasopharyngeal carcinoma in FDRs

In FDRs, when their dietary practices were examined with respect to those of controls, it was observed that there is an increased risk for NPC with intake of smoked meat (OR=1.98; 95 % CI, 1.07–3.63; P=0.02), smoked fish (OR=1.89; 95 % CI, 1.05–3.40; P=0.03), salted fish (OR=2.36; 95 % CI, 1.11–5.02; P=0.02) and fermented fish (OR=1.99; 95 % CI, 1.06–3.73; P=0.03). Similarly, in FDRs, betel nut chewing, tobacco smoking and alcohol consumption were examined and no significant association of NPC was found in FDRs with the practice of betel nut chewing, tobacco smoking and alcohol consumption. FDRs with *GSTM1* null genotype have increased NPC risk by 2.80-fold (95 % CI, 1.54–5.09; P=0.0005), whereas those without *GSTT1* null genotype have no

Table 2 Risk factors associated with nasopharyngeal carcinoma in Northeast

Variable	Cases (n=64)	Controls (n=100)	Odds ratio (95 % CI)	P value	FDR (n=88)	Odds ratio (95 % CI)	P value
Smoked meat intake							
No	18	44	1 [ref]		25	1 [ref]	0.02
Yes	46	56	2.00 [1.02–3.93]	0.04	63	1.98 [1.07–3.63]	
Smoked fish intake							
No	21	52	1 [ref]		32	1 [ref]	0.03
Yes	43	48	2.21 [1.15–4.26]	0.01	56	1.89 [1.05–3.40]	
Salted fish intake							
No	46	87	1 [ref]		65	1 [ref]	0.02
Yes	18	13	2.61 [1.17–5.81]	0.01	23	2.36 [1.11–5.02]	
Fermented fish intake							
No	38	76	1 [ref]		61	1 [ref]	0.03
Yes	26	24	2.17 [1.04–4.51]	0.02	27	1.99 [1.06–3.73]	
Betel nut with betel leaf							
Non-chewers	16	48	1 [ref]		41	1 [ref]	0.88
Chewers	48	52	2.77 [1.33–5.91]	0.003	47	1.06 [0.57–1.96]	
Tobacco							
Non-smokers	21	73	1 [ref]		64	1 [ref]	
Smokers	43	27	5.54 [2.25–11.63]	<0.0001	24	1.01 [0.50–2.03]	0.92
Alcohol consumption							
No	21	51	1 [ref]		34	1 [ref]	0.08
Yes	43	49	2.13 [1.06–4.34]	0.02	54	1.65 [0.89–3.08]	
Kitchen							
Outside	47	86	1 [ref]		69	1 [ref]	
Inside	17	14	2.22 [1.00–4.90]	0.04	19	1.56 [0.73–3.29]	0.24
GSTM1							
Present	25	58	1 [ref]		29	1 [ref]	0.0005
Null	39	42	2.15 [1.13–4.08]	0.01	59	2.80 [1.54–5.09]	
GSTT1							
Present	43	73	1 [ref]		59	1 [ref]	0.37
Null	21	27	1.32 [0.66–2.61]	0.42	29	1.32 [0.71–2.48]	
Both *GSTM1* and *GSTT1*							
Present	15	13	1 [ref]		20	1 [ref]	
Null	14	2	6.06 [1.15–31.82]	0.02	23	7.86 [1.57–39.28]	0.005
EBV–LMP1 gene							
Uninfected	18	46	1 [ref]		39	1 [ref]	0.82
Infected	46	54	2.17 [1.11–4.26]	0.02	49	1.07 [0.60–1.90]	

association (OR=1.32; 95 % CI, 0.71–2.48; P=0.37). Furthermore, with both *GSTM1* and *GSTT1* null genotypes, the risk increases to 7.86-fold (95 % CI, 1.57–39.28; P=0.005) for NPC.However, no association of EBV infection with NPC was observed.

3. Association of mtDNA copy number variation with NPC

Using quantitative PCR techniques, we determined the relative content of mtDNA with respect to the *GAPDH* gene in NPC cases, controls and FDRs (Fig. 2). Overall, the relative median of the mtDNA content is lower in cases (1.98 relative copies) than that in controls (4.11 relative copies) and FDRs (4.69 relative copies). NPC cases in the lowest quartile of the mtDNA copy number experienced a significantly increased risk of 2.90-fold for NPC (95 % CI, 1.06–7.91) compared with those in the highest quartile (Table 3). We observed that risk of NPC increased with the decrease in mtDNA copy number (P_{trend}=0.007) compared to FDRs where in the lowest quartile, no risk (OR=0.40; 95 % CI, 0.15–1.03) was observed and P_{trend}=0.05 (Table 3). Furthermore, we found a significant difference between *GSTM1* and *GSTT1* null genotypes with mtDNA content in controls and FDRs (P<0.0001 and P<0.0001, respectively).

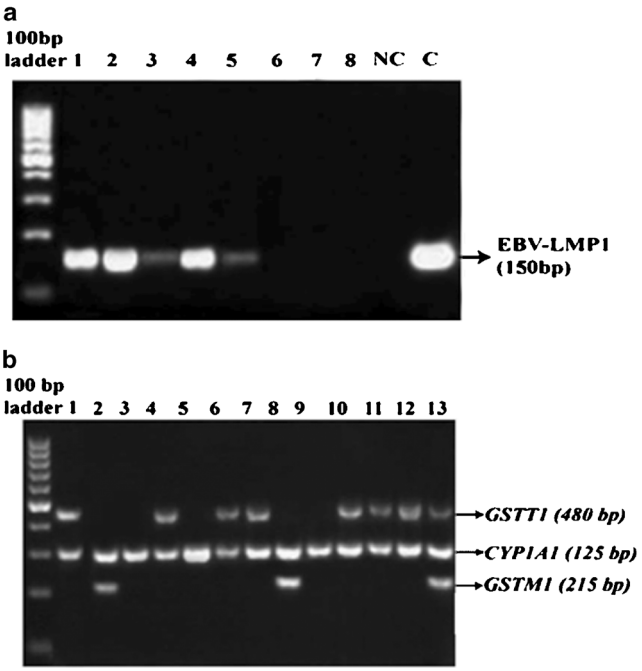

Fig. 1 Environment-genetic risk factors and their epidemiology. EBV prevalence in NPC: **a** Agarose gel electrophoresis showing PCR amplification of EBV (*LMP1*gene) in *lane nos. 1*, *2*, *3*, *4* and *5*. *Lane nos. 6*, *7* and *8* show absence of EBV infection and lane *NC* is representing negative control and *lane C* as positive control. GSTs null genotyping: **b** Agarose gel electrophoresis showing multiplex PCR of the *GSTM1* (215 bp), *GSTT1* (480 bp) and *CYP1A1* at 315 bp as an internal control. The *GSTM1* genotype was presented by *lane nos. 2* and *8* and *GSTT1* genotype in *lane nos. 1*, *4*, *6*, *7*, *10*, *11* and *12*. Both *GSTM1* and *GSTT1* null genotypes were showed in *lane nos. 3*, *5* and *9*

Discussion

This is the first study that consisted of NPC cases, their relatives (FDRs) and controls from the three highly NPC-prone ethnic groups in Northeast India, i.e. Manipuri, Naga and Mizo. The lifestyle and environment have a huge impact on human health. This is what we have observed in this study. The first and foremost thing to be observed was the housing pattern with poor or no ventilation, especially in the economically poor people and those who are obviously more susceptible to NPC. These houses even do not have a separate kitchen; as a consequence, the smoke generated from firewood, charcoal, etc. is inhaled by the members in the house which can be one of the contributing factors to NPC. The intake of smoked meat and fish, salted preserved and fermented fish is a traditional staple food in several regions of Northeast India, especially NPC-endemic areas. Increased risks of 1.8–2.6-folds to NPC were observed in both patients and their FDRs with the intake of smoked, fermented and salt-preserved meat and fish. The process of cooking and preserving fish and meat results in the accumulation of significant levels of nitrosamines, which are known carcinogens in human health. Smoked meat consumption is very common in Nagaland, which is linked with high prevalence of NPC, and in our study, we observed a twofold increased risk to NPC in smoked meat consumers. Smoking process contaminates the meat with polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs), which are known carcinogens, which affects human health and has proven to be risk factors for NPC [22, 23]. These compounds not only cause single-strand DNA breaks but also result in the oxidation of protein thiols and lipid peroxidation, thereby triggering damage to mtDNA. Moreover, mitochondrial dysfunction has been reported in various cancers like oral cancer in our previous studies [18, 21] as well as in NPC [24, 25]. Generally, it is believed that ROS is a relevant class of carcinogens (Park et al. 2009) and it was proven that ROS can stimulate cancer development at all three stages: initiation (the induction of DNA mutations in a somatic cell), promotion (the stimulation of tumourigenic expansion of the cell clone) and progression (the malignant conversion of the tumour to cancer). Interestingly, in some systems, ROS mediated both pro- and anti-apoptosis effects, depending on the ROS concentration [15, 26]. Similarly, many studies have shown an association of salted and fermented fish to NPC. These foods accumulate significant levels of nitrosamines, which are known carcinogens in animals [27]. Salt-preserved fish also contains bacterial mutagens and EBV-reactivating substances, some or all of which could also contribute to NPC [23].

The involvement of EBV in NPC has been postulated since 1966 [28]. Some hypothesis proposed that EBV played a critical role in transforming nasopharyngeal epithelial cells into invasive cancer [29]. In the present study, we have analyzed the EBV *LMP1* gene in cases, controls and FDRs and found to be a risk factor in the NPC cases with EBV infection. *LMP1* is of special interest since it is generally considered to be the main EBV oncogene that is believed to be important in the pathogenesis nasopharyngeal carcinoma [30, 31]. EBV, in conjunction with environmental and genetic factors, plays a role in the development of NPC. *LMP-1* localizes to cellular membranes and functions as a constitutively active tumour necrosis factor receptor homologue that propagates intracellular signaling, including the NF-κB, cJun N-terminal protein kinase/AP-1 and Janus kinase/STAT pathways [32, 33]. Through these signaling pathways, *LMP-1* generates innumerable effects on host cell growth, differentiation and apoptosis, including growth promotion and survival in epithelial cells [34, 35]. Although the mechanisms by which *LMP-1* influences cell biology have been intensively studied, little is known about the mechanisms that regulate *LMP-1* oncogenic activity [36]. In the present study, we found a significant difference with mtDNA content in cases and controls with or without EBV infection (P=0.002). However, there are no reports of association of EBV infection with the mtDNA copy number. The EBV codes for BamHI fragment H rightward open reading frame-1 (BHRF1) an early protein, which localizes to the mitochondrial outer membrane and co-localizes

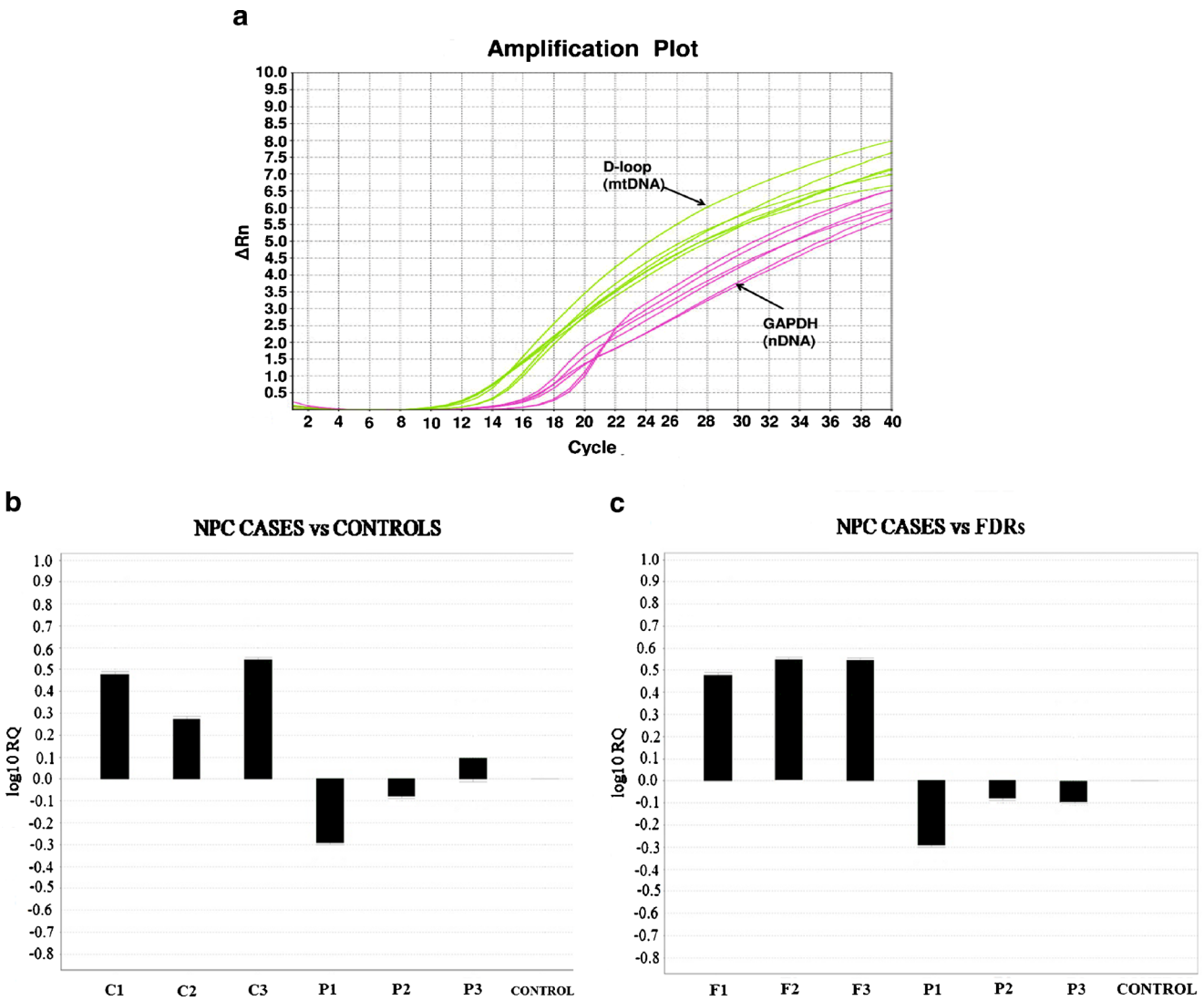

Fig. 2 Quantitative PCR for mitochondrial DNA copy number determination. **a** Quantitative PCR of D-loop region and GAPDH gene (representative curve). D-loop region and GAPDH are ubiquitous genes found in the mitochondrial and nuclear genomes, respectively. Using quantitative PCR in samples from the NPC cases, FDRs and control, the relative mitochondrial content was calculated. **b** Mitochondrial content decreases in NPC cases as compared to that in controls, and **c** mitochondrial content decreases in NPC cases in comparison to the FDRs with respect to $\log_{10}$RQ (log fold change).

with Bcl-2 [37, 38]. The BHRF1 interacts with the cellular protein VRK2 [39] and enhances the cell survival. Several EBV-associated proteins, such as BALF1, BHRF1, EBNA and LMP1, have been shown to interfere with fatty acid metabolism, mitochondrial function and apoptosis pathways [40, 41]. It may be possible that by inhibition of apoptosis, thereby facilitating tumour development with corresponding variation to mtDNA content, for which the exact mechanism is unclear. We are reporting for the first time the association of EBV infection with mtDNA content variation.

The GST genes *GSTM1* and *GSTT1* are involved in the detoxification of a broad range of toxic substances. Genetic polymorphisms in these genes have been studied intensively for their potential role in cancer susceptibility. The presence of both *GSTM1* and *GSTT1* are essential for the detoxication of carcinogenic compound. In the present study, multiplex PCR was done for the detection of *GSTM1* and *GSTT1* null genotype using *CYP1A1* as an internal control. In both cases and FDRs, we found a significant risk of *GSTM1* null genotype associated with NPC whereas no association was found with *GSTT1* null genotype. The absence of a homozygous allele of *GSTM1* gene yields a complete loss of enzyme activity [42]. Previous studies have demonstrated that the *GSTM1* polymorphism is associated with susceptibility to a number of malignant cancers [43, 44]. Many case–control studies were published to assess the association between the polymorphism of *GSTM1*, located on chromosome 1p13.3, and NPC risk, but the existing evidence was still weak due to limited sample size, ethnic difference or disagreements among the published studies [11, 45]. However, certain meta-analytic results have

Table 3 Odds ratios (ORs) and 95 % CI for relative mtDNA copy number and risk of NPC

mtDNA copy number quartile	Cases (n=64)	Controls (n=100)	OR (95 %CI)	FDRs (n=88)	OR (95 %CI)
Quartile 1 (≤0.2)	31	29	2.90 [1.06–7.91]	11	0.40 [0.15–1.03]
Quartile 2 (>0.2–2)	15	27	1.51 [0.51–4.40]	28	1.09 [0.47–2.51]
Quartile 3 (>2–12)	11	25	1.19 [0.38–3.65]	31	1.30 [0.56–3.00]
Quartile 4 (>12)	7	19	1 [ref]	18	1 [ref]
P_{trend}	0.007			0.05	

demonstrated a significant association of *GSTM1* polymorphism and NPC risk [46, 47]. Moreover, the null genotypes of both *GSTM1* and *GSTT1* further increase the risk of NPC as observed in this study both in cases and FDRs as it is completely incapable of detoxification of the carcinogens generated from the different smoked foods, tobacco and betel quid consumption. A significant difference between *GSTM1* and *GSTT1* null genotypes with mtDNA content in cases, FDRs and controls (P<0.0001 and P<0.0001) was observed. The increased risk factor of null GSTs with the accumulation of mtDNA mutations possibly plays inside the mitochondrial matrix as an mtDNA protection factor regarding damage caused by reactive oxygen species which in turn affect the mtDNA content and may lead to causation of NPC as well [18, 21]. The associations of GST null genotypes and mtDNA content have not yet been reported.

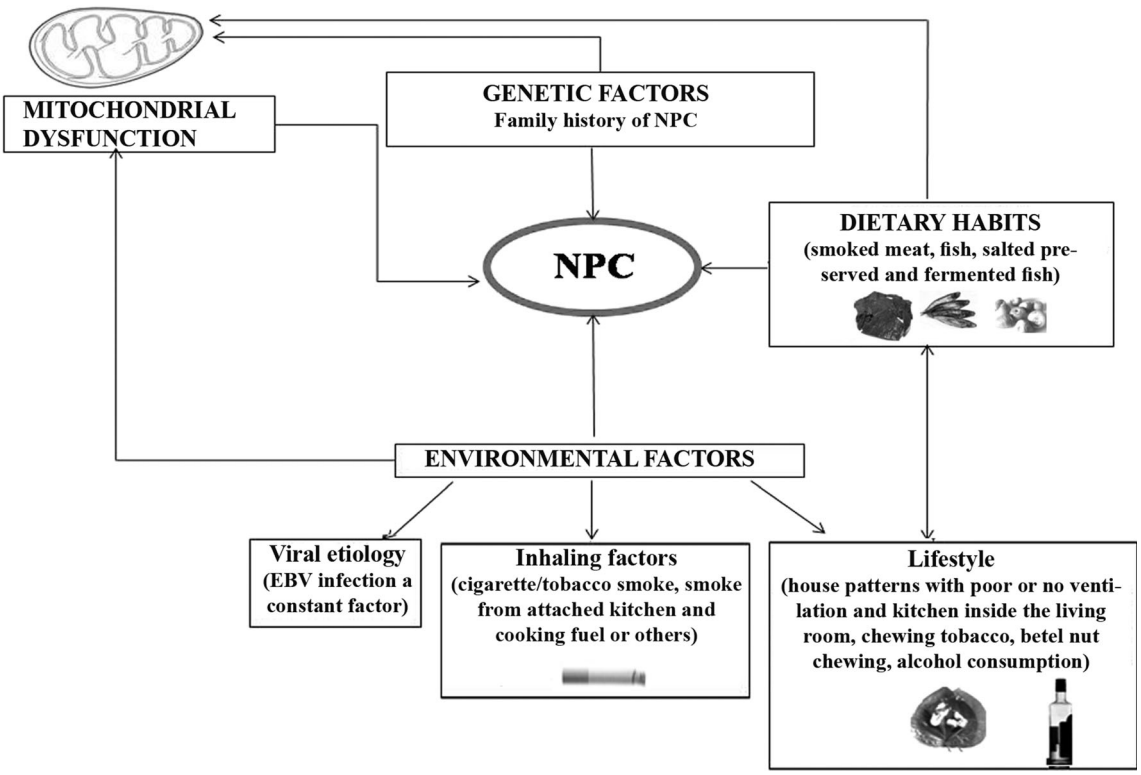

Fig. 3 Schematic representation of the etiology of NPC and mitochondrial dysfunction. The combined effects of environmental factors and genetic factors led to the development of NPC directly as well as caused mitochondrial dysfunction which is related to NPC in the ethnic group of Northeast India. The environmental factors include viral etiology, i.e. EBV infection remains a constant factor for the development of NPC. The EBV-associated proteins like LMP1, BALF1, BHRF1 and EBNA interfere with fatty acid metabolism, mitochondrial function and more importantly apoptosis pathways, thereby facilitating tumour development. The dietary factors, inhaling factors and lifestyle were few environmental factors which contain significant levels of nitrosamines, HCAs and PAHs which are involved in the development of NPC and responsible for mitochondrial dysfunction by production of ROS, which also cause oxidation of protein thiols and lipid peroxidation triggering mtDNA damage. Genetic factors—The family history of NPC has consistently been associated with an increased risk of NPC; it may be because of inherited genes, shared environmental factors (such as the same diet or living quarters) or combination of these. The polymorphism of carcinogen-metabolizing genes is another genetic factor, which has a potential role in cancer susceptibility. The presence of *GSTM1* and *GSTT1* is essential for the detoxication of carcinogenic compounds. The null GSTs increase the risk for NPC as incompetent to detoxify the carcinogens generated from different smoked foods, tobacco and betel quid consumption and also lead to the accumulation of mtDNA mutations as they play inside the mitochondrial matrix as mitochondrial protection factor from the damage caused by ROS

In our study, low level of the mtDNA copy number in cases was found to be associated with high risk of NPC, which is in contrary to the findings in the FDRs. It may be probably due to the release of substantial amounts of ROS generated from smoke inhalation from cooking fuel in the houses with attached kitchen and improper ventilation and of course from the smoked, salt preservative and fermented food which in turn increase mtDNA mutation. Similar results were reported in OSCC but not in tobacco chewers [18]. The accumulation of mtDNA deletions and subsequent cytoplasmic segregation of these mutations during cell division could be important contributors to the early phase of NPC [18, 48]. We evaluated patients treated with radiation therapy and without therapy and found that those who underwent radiation therapy have a low copy number than the non-radiated ones. This decrease of mtDNA after radiotherapy may reflect an effect of external beam radiation that influences the mitochondrial number in cells, effectively reducing mtDNA [49]. Alternatively, the depletion in mtDNA may be the result of the repression of mitochondrial biogenesis. The mtDNA copy number in cancer probably depends on several factors, including the site of mutation in the mitochondrial genome as demonstrated in D-loop region, a highly susceptible site for oxidative damage compared with the other regions of mtDNA [50]. For better understanding, the overall probable risk factors associated with the development of NPC and prevalence in the ethnic groups of Northeast Indian populations are presented by a schematic diagram in Fig. 3.

Conclusion

The environment–genetic risk factors which were examined in this study with their role in the etiology of NPC were discussed above and reached to a conclusion that the understandings of the risk factors are helpful as preventive measures and screening. The association of GST null polymorphism with the mtDNA copy number can be used as a preventive measure for the patient either at risk of NPC due to lifestyle or those who have heredity of oral cancer in their family. Furthermore, the understanding that the environment–genetic interactions related to NPC pathogenesis where EBV remains a constant factor using next-generation sequencing technology will be of great potential along with in-depth study of EBV interaction with mitochondria in inhibiting apoptosis can be an addition, which may give a new direction in treatment of enigmatic NPC.

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Conflicts of interest None

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RESEARCH ARTICLE

Association of HPV with genetic and epigenetic alterations in colorectal adenocarcinoma from Indian population

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Abstract Several studies from developing countries have shown human papillomavirus to be associated with colorectal cancers, but the molecular characteristics of such cancers are poorly known. We studied the various genetic variations like microsatellite instability (MSI), oncogenic mutations and epigenetic deregulations like CpG island methylation in HPV associated and nonassociated colorectal cancer patients from Indian population. HPV DNA was detected by PCR using My09/My11 and Gp5+/Gp6+ consensus primers and typed using HPV16 and HPV18 specific primers. MSI was detected using BAT 25 and BAT 26 markers, and mutation of *KRAS*, *TP53* and *BRAF* V600E were detected by direct sequencing. Methyl specific polymerase chain reaction (MSP) was used to determine promoter methylation of the classical CIMP panel markers (*P16*, *hMLH1*, *MINT1*, *MINT2* and *MINT31*) and other tumour-related genes (*DAPK*, *RASSF1*, *BRCA1* and *GSTP1*). HPV DNA was detected in 34/93 (36.5 %) colorectal tumour tissues, HPV 18 being the predominant high-risk type. MSI was detected in 7.5 % cases; KRAS codon 12, 13, *BRAF* V600E and *TP53* mutations were detected in 36.5, 3.2 and 37.6 % of the cases, respectively. CIMP-high was observed in 44.08 % cases. HPV presence was not associated with age, stage or grade of tumours, MSI or mutations in *KRAS*, *TP53* or *BRAF* genes. Higher methylation frequencies of all genes/loci under study except *RASSF1*, as well as significantly higher CIMP-high characteristics were observed in HPV positive tumours as compared to negative cases. HPV in association with genetic and epigenetic features might be a potent risk factor for colorectal cancer in Indian population.

Keywords Colorectal cancer · HPV · Genetic alterations · Epigenetics · CIMP · *KRAS* · *BRAF* · *P53* · MSI · Northeast India

Introduction

Colorectal cancer (CRC) is the fourth leading cancer worldwide with approximately 1,360,602 new cases annually [1]. The genesis of CRC is thought to be a multistep process involving complex interaction of genetic, epigenetic and environmental factors or viral components in distinct geographical regions [2–5]. Although, epidemiological research have established several environmental risk factors like smoking, alcohol consumption [6] etc.; evidences of probable human papillomavirus (HPV) infection in CRC have accrued over quite some time [7–9]. However, the detection rates exhibited significant geographic variations, some studies even failing to detect HPV altogether [10–12]. This discrepancy was addressed in two comprehensive meta-analyses conducted by Damin et al. (2013) [13] and Baandrup et al. (2014) [5], where they identified two main grounds of disparity: distinct geographical variances in HPV prevalence and differences in detection techniques used. Overall, HPV presence was found to confer 6-10 folds higher risk of CRC compared to controls (Table 1). Following the well-recognised role of HPV in cervical and other anogenital carcinoma, this finding is indeed intriguing and indicative of probable HPV mediated CRC pathogenesis.

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Table 1 Prevalence of HPV in colorectal cancers of different regions across the world, as described in two meta-analysis by Damin et al. (2013) [13] and Baandrup et al. (2014) [5]

Study	HPV detection technique	CRC cases				Normal controls			Odds ratio [95% CI]
		Region	Number of studies included	Number of patients	Pooled HPV prevalence (%)	Number of studies included	Number of subjects	Pooled HPV prevalence (%)	
Damin et al. (2013) [13]	PCR-based techniques	Total	18	1436	31.9	5	104	3.6	10.04 [3.67–27.46]
		USA	4	185	18				
		Europe	4	201	7.7				
		Asia	7	461	53.6				
		South America	3	586	60.8				
Baandrup et al. (2014) [5]	Non-PCR based + PCR based	Total	34	2630	11.2	8	419	1.6	6.0 [2.0–17.9]
		USA	10	918	3.2				
		Europe	10	801	1.9				
		Asia	5	286	39.2				
		South America	4	208	45.1				
		Middle East	5	397	32.2				
		Australia	2	20	0				

The earliest known model of sporadic CRC includes the adenoma-carcinoma sequence characterised by chromosomal instability and alterations in specific oncogenes (e.g., *KRAS*) or tumour suppressor genes (e.g. *TP53*, *APC* etc.) [14]. A second pathway, known as the microsatellite instability (MSI) pathway accounts for around 10–15 % CRC, including both familial (pure MSI) and sporadic cancers [15]. More recently, alternative molecular pathways characterised by multiple CpG Island Methylation (termed as CIMP) associated with distinct genetic alterations in *BRAF* and *KRAS* oncogenes are also identified [16].

Apart from P53 degradation and Rb inactivation, HPV oncoproteins are now known to induce many other oncogenic events like genomic instability, DNA damage, accumulation of mutations, evasion of apoptosis etc., the synergistic effect of which produces malignant transformation [17]. In addition to genetic events, the ability of HPV to modulate aberrant DNA methylation of the host genome is evident to both cell culture as well as clinical studies [18, 19]. HPV positive and negative host genome exhibits large differences in their methylation patterns, resulting in deregulation of crucial cellular pathways.

Although, alteration in crucial genetic and epigenetic events like MSI, oncogenic mutations of *KRAS*, *BRAF*, *TP53* genes and CpG island methylation are extensively studied in colorectal cancers, but there are no reports in existing literature investigating these alterations in HPV associated colorectal cancers. The present study was undertaken to investigate the association of various genetic, epigenetic and viral risk factors in colorectal tumours in a unique population of India, characterised by rectal predominance and high proportion of young patients.

Materials and methods

Patients and clinicopathological data

A total of 93 colorectal adenocarcinoma samples, 30 adjacent normal tissues and 40 matched peripheral blood samples were collected from Cachar Cancer Hospital and Research Centre, Assam; Silchar Medical College and Hospital, Assam, and Agartala Government Medical College, Tripura, India, from September 2010 to September 2013. Demographic and clinicopathological data like age, gender, family history of CRC, location (colonic/rectal tumours), stage, differentiation and metastasis were collected prospectively. The study was approved by the ethics committee of Cachar Cancer Hospital and Research Centre, Assam, India (Approval No: IRB/CCHRC/01/2010). Written consent was obtained from the patients, and the procedure was approved by the Institutional Review Board (IRB).

DNA extraction

Genomic DNA was isolated from cancerous biopsy samples, surgically excised cancer tissues and peripheral blood samples by standard phenol/chloroform protocol. The isolated DNA was then dissolved in Tris-EDTA buffer, and stored at −80 °C for further analysis [20].

Detection and typing of HPV

PCR detection and typing of HPV DNA were carried out using consensus and type specific primers (Online Resource 1) in duplicate for each sample and at specific time intervals. Prior to HPV detection, DNA from all the samples were subjected to spectrophotometric analysis for quality and quantity assessment, as well as PCR amplified using GAPDH gene primers. DNA obtained from all the tissue samples as well as peripheral blood was subjected to PCR using two sets of consensus primers Gp5+/Gp6+ and My09/My11 for amplifying HPV L1 gene fragments. Amplification was performed on 20 μl of reaction mixtures containing 2× PCR master mix (Bioline, USA), forward and reverse primers, 2 μl of sample and nuclease-free water with the following cycling profile: initial denaturation at 95 °C for 5 min followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 1 min elongation at 72 °C followed by a final extension step of 8 min at 72 °C. Positive samples were tested using two different sets of specific primers each for HPV 16 and HPV18 as previously described [21]. DNA from cervical cancer cell lines infected with known HPV types was used as positive controls and PCR mixtures with nuclease-free water was used as negative controls. PCR products were analysed by electrophoresis on 2.5 % agarose gels stained with ethidium bromide. All possible precautions like sample processing and PCR reaction preparation in separate biosafety cabinet, sterile gloves changing after each batch of reactions, inclusion of negative controls in all PCR reactions, etc. were maintained to minimise contamination.

Microsatellite instability detection

MSI was detected using two mononucleotide markers BAT25 and BAT26 by PCR using specific primers (Online Resource 1) and separation in 7 M of urea denaturation PAGE, reconfirmed by PCR-SSCP. Gel visualisation was performed by a fast silver staining procedure as described previously [22].

KRAS, *TP53* and *BRAF* mutation detection by automated DNA sequencing

The primer sets to detect mutations of *KRAS* exon 2, *TP53* exon 7–8 and *BRAF* exon 15 mutations are tabulated in Online Resource 1. For each sample, 50 μL of reaction mixture was made up and PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52–59 °C for 45 s (according to each primer set) and 72 °C for 1 min, with a final extension for 6 min. The PCR products were purified using QIAquick® PCR Purification Kit (Qiagen) and subjected to automated DNA sequencing. The sequencing was done using purified products and the same sets of primers in a capillary automatic sequencer (ABI 3500 Genetic Analyser, Applied Biosystems).

Promoter hypermethylation analysis and evaluation of CIMP status

Bisulfite modification of genomic DNA was done by using Imprint® DNA Modification kit (Sigma-Aldrich, MOD50) following manufacturer's instructions. Promoter methylation statuses of five important tumour-related genes namely, *RASSF1*, *DAPK*, *ECAD*, *BRCA1* and *GSTP1*, in addition to the five classical CIMP panel markers (*P16*, *HMLH1*, *MINT1*, *MINT2* and *MINT31*) were analysed by Methylation Specific PCR (MSP) using primers and methods as described earlier [23–25]. DNA from peripheral blood lymphocytes treated with *Sss1 methyltransferase* was used as positive control for methylated primers, DNA from untreated normal lymphocytes were used as positive control for unmethylated primers and PCR mix with nuclease-free water was used as negative control and viewed in 3 % agarose gel or 6 % PAGE as applicable. CIMP was stratified into CIMP-high (CIMP-H, ≥5/10 markers methylated), CIMP-low (CIMP-L, <5/10 makers methylated) and CIMP-negative (CIMP-N, 0/10 markers methylated). Methylation index (MI) is defined as the number of methylated gene divided by the total number of gene studied in a sample.

Statistical analysis

Association between HPV data, clinicopathological, genetic and epigenetic data were carried out by Fisher's exact test. Whereas, all continuous data were analysed using Student's *t* test. All statistical analyses were carried out by JMP software package of SAS [26] and SPSS software and two-sided *P* value≤0.05 were considered statistically significant.

Results

Patient characteristics

Details of demographic, clinicopathological and molecular characteristics of the 93 colorectal adenocarcinoma patients are presented in Table 2. The mean age at presentation was 45.8 years, with significant difference between colonic and rectal cancer patients (53 vs. 43.7 years, $P=0.01$). As such, a lofty proportion of young age rectal cancers (below 40 years= 45.83 %) were observed in our series, the younger patients had significantly higher percentage of low rectal, advanced stage and aggressive tumours (stage III/IV/poorly differentiated/mucinous) as compared to the older cases ($P<0.01$). The major clinical presentations were bleeding per rectum, weight loss and anaemia, altered bowel habits, large gut obstruction

Table 2 Clinicopathological and molecular details of 93 CRC patients under study

Characteristics	Colon *N* (%)	Rectum *N* (%)	*P* value
Total patients (*N*=93)	21 (22.58)	72 (77.42)	-
Age			
Mean age at diagnosis (±SD)	53.0 (±14.79) years	43.74 (±15.73) years	*0.01*
≤40 years	5 (23.80)	33 (45.83)	0.08
>40 years	16 (76.19)	39 (54.16)	
Gender			
Male	9 (42.85)	40 (55.55)	0.33
Female	12 (57.14)	32 (44.44)	
Family history of CRC in $1^0/2^0$ relatives	3 (14.28)	2 (2.77)	0.07
Tumour stage			
I/II	10 (47.61)	31 (43.05)	0.80
III/IV	11 (52.38)	41 (56.94)	
Tumour differentiation			
Well differentiated	15 (71.42)	37 (51.38)	0.26
Moderately differentiated	2 (9.52)	10 (13.88)	
Poorly differentiated/ mucinous	4 (19.04)	25 (34.72)	
Metastasis			
Present	4 (19.04)	25 (34.72)	0.19
Absent	17 (80.95)	47 (65.28)	
Clinical presentation			
Abdominal pain	11 (52.38)	33 (45.83)	0.91
Altered bowel habits	9 (42.86)	32 (44.44)	
Bleeding per rectum	12 (57.14)	44 (61.11)	
Large gut obstruction	7 (33.33)	21 (29.17)	
Weight loss and anaemia	12 (57.14)	43 (59.72)	
HPV			
All types	9/21 (42.85)	25/72 (34.72)	0.81
HPV 16	2/21 (9.52)	4/72 (5.55)	
HPV 18	6/21 (28.57)	21/72 (29.16)	
Others	1/21 (4.76)	2/72 (2.77)	
KRAS			
Wild type	10 (47.61)	49 (68.05)	0.12
Codon 12/13 mutated	11 (52.38)	23 (31.94)	
BRAF			
Wild type	19 (90.47)	71 (98.61)	0.12
Mutated	2 (9.52)	1 (1.38)	
TP53			
Wild type	17 (80.95)	41 (56.94)	0.07
Mutated	4 (19.04)	31 (43.05)	
Microsatellite status			
MSI	4 (19.04)	3 (4.16)	*0.04*
MSS	17 (80.95)	69 (95.83)	
CIMP status			
CIMP-high	11 (52.38)	30 (41.66)	0.45
CIMP-low	10 (47.61)	42 (58.33)	

N Number of patients; values in italics represent gene [KRAS, BRAF & TP53] symbols

and abdominal lump. Almost 55 % of all cases presented with locally advanced or metastatic disease, with little site specific differences. Rectal cancer cases exhibited higher frequency of poorly differentiated histology and lower family history of CRC as compared to their colonic counterparts, although the difference was not statistically significant.

HPV detection and genotyping

HPV DNA was detected in 34/93 (36.5 %) colorectal tumour tissues and 6/30 (20 %) paired normal tissue from surgical cancer free margins. Five out of 6 (83.33 %) had HPV DNA in both cancer tissue as well as normal margin, whereas one (16.66 %) sample had HPV DNA only in the matched normal tissue. We further tested matched peripheral blood DNA form 40 patients (including all cases with matched normal tissues) and detected HPV DNA in 5/40 (12.5 %). HPV 18 was the most predominant type detected in tumour tissues, paired normal or peripheral blood; accounting for almost 80 % (27/34) of all positive cases (Fig. 1). Six out of 34 tumour tissues had HPV 16 DNA, whereas HPV 16–18 coinfection was observed in 2/34 cases. Further, 3/34 (8.82 %) samples harboured HPV types other than 18 or 16. Although the proportion of HPV 16 DNA in tumour and paired normal tissues were similar, we failed to detect HPV 16 DNA in peripheral blood of any patient having HPV 16 positive tumour tissue. HPV detection rates were not significantly different among colon or rectal cancer patients (Table 2).

Detection of MSI, *KRAS*, *TP53*, *BRAF* mutation

Microsatellite status, mutation status of *KRAS*, *TP53* and *BRAF* genes are illustrated in Table 2. MSI was detected in 7/93 (7.5 %) patients, *BRAF* V300E and *TP53* mutations were detected in 3/93 (3.2 %) and 34/93 (37.6 %) patients, respectively. Rectal tumours harboured lower microsatellite instability (4.16 vs 19.04 %) and higher *TP53* mutations (43.04 vs. 19.05 %) as compared to the colonic tumours. *KRAS* codon 12, 13 mutations were detected in 34/93 (36.5 %) patients with G12R, G12D and G13D, G13S being the predominant mutations among the codon 12 and 13 mutations, respectively (Fig. 2). Apart from overall higher frequency of *KRAS* codon12, 13 mutants in older patients, the codon12 mutants had significantly higher age than codon13 mutants (mean age=52.27 vs 60.50, P=0.03).

Detection of CIMP in CRC patients

A representative gel with amplified Methylation Specific PCR (MSP) products of both methylated (M) and unmethylated (U) forms of 5 CpG island loci for each gene under study is shown in Fig. 3. From a pairwise analysis of 30 tumour and surgically excised matched normal margin tissues, we identified significant difference

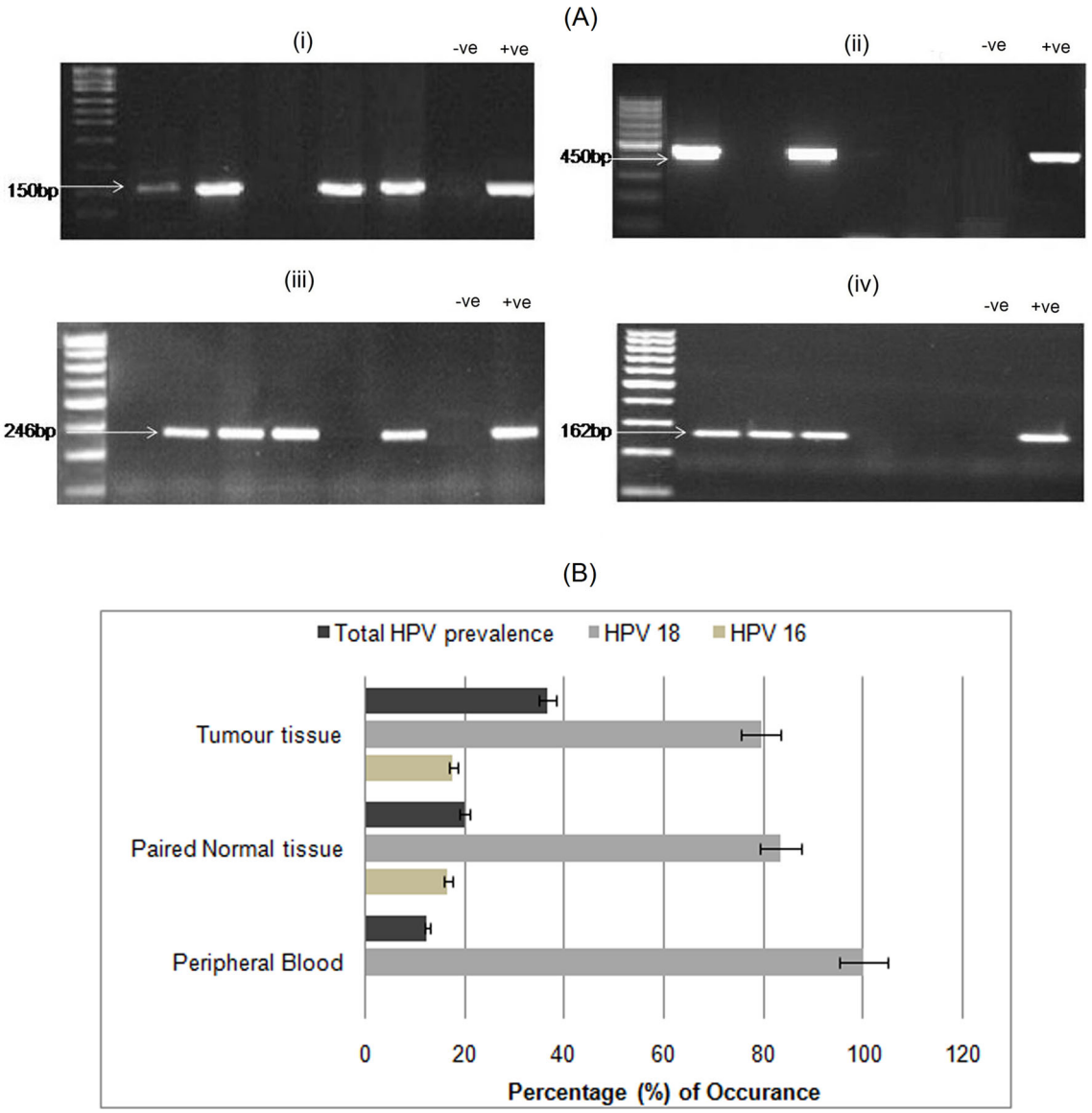

Fig. 1 HPV detection. **a** Representative agarose gel with amplified bands of HPV specific DNA sequences such as **i** amplified by Gp5 and Gp6 common primer **ii** amplified by MY9 and MY11 common primer **iii** amplified by HPV-16 specific primer and **iv** amplified by HPV-18 specific primer. **b** Frequency of total HPV, HPV-18 and HPV-16 among the CRC patients

($P<0.0001$) among the methylation signatures of tumour and corresponding normal tissues (Fig. 3). The methylation profile of 10 cancer-related genes/loci and corresponding data, MSI and mutation pattern of 93 CRC patients are depicted in Fig. 4. Highest methylation frequencies were observed in the three MINT loci markers MINT1 (56.98 %), MINT2 (64.51 %) and MINT31 (55.91 %) followed by *P16* (45.16 %), *RASSF1* (43.01 %), *DAPK1* (35.48 %), *GSTP1* (35.48 %), *ECAD* (30.1 %), *BRCA1* (10.75 %) and lowest in *MLH1* (6.45 %) genes. Among the 93 patients, 41 (44.08 %) exhibited CIMP-high with a mean methylation index of 0.62, whereas 52 patients were classified as CIMP-low and had a mean methylation index of 0.19.

Comparison of CIMP, MSI, *KRAS*, *BRAF* and *TP53* mutations in HPV associated and nonassociated CRCs

Clinicopathological and molecular features associated with HPV positive and HPV negative cases are shown in Table 3. HPV associated cases harboured higher *KRAS* codon 12 13 mutations (47.05 vs. 30.5 %) and lesser *TP53* mutations (29.4 vs. 42.3 %) than HPV negative cases, but the difference was not statistically significant. Methylation frequencies of all the genes/loci under study except *RASSF1* were higher in HPV positive cases as compared to the negative cases, the difference being significant for MINT1 loci ($P=0.004$) only (Fig. 3). As such, significantly higher frequency of CIMP-high features ($P=0.03$) was observed in HPV positive cases.

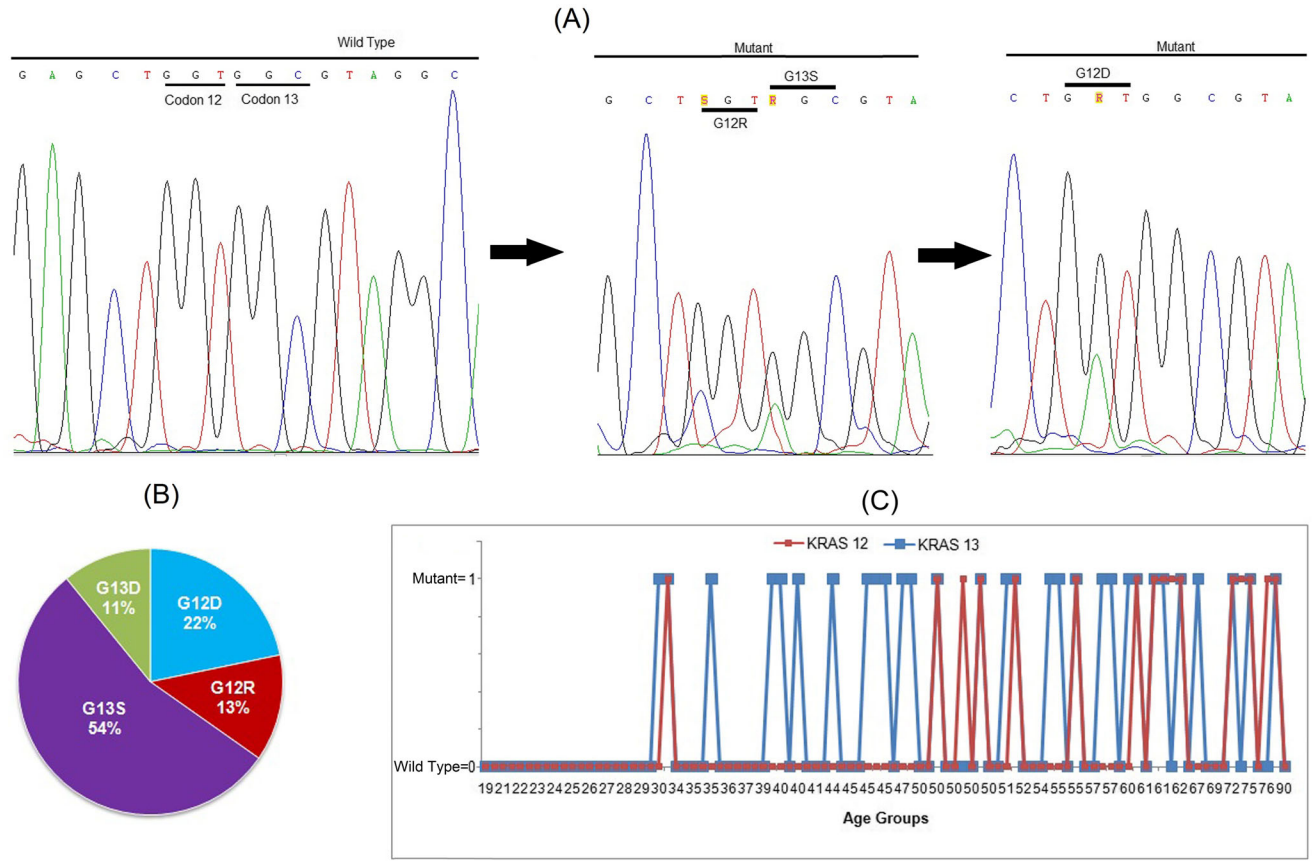

Fig. 2 *KRAS* mutation detection. **a** Comparison of the sequence chromatogram between wild type and mutant *KRAS* exon1, showing *KRAS* codon G12R, G12D and G13S mutations. **b** Frequency distribution of *KRAS* codon 12, 13 mutations. **c** Age distribution of *KRAS* codon 12, 13 mutations

The mean methylation indices of HPV positive cases were also higher than negative cases (0.42 vs. 0.35, $P=18$).

Discussion

This is an original report on HPV prevalence in Indian CRC patients, and also the first clinical study on frequently perturbed molecular features in colorectal cancer like MSI, *KRAS* codon 12, 13, *BRAF*, *TP53* mutations and CIMP in HPV-associated colorectal cancers. We report HPV prevalence of 35 % in CRC tissues, HPV 18 being the most prevalent strain. Comprehensive meta-analyses conducted by Damin et al. [13] and Baandrup et al. [5] revealed similar pooled percentage of HPV in CRC samples from Asia and found HPV18 as the major high-risk strain. HPV prevalence in CRC exhibited distinct geographical variations, the developing countries of South America, Asia and Middle East being the highest prevalence regions. The meta-analysis could not attribute detection techniques used or tissue types analysed to the observed variation. This can be rather due to geographical differences in HPV prevalence, distribution of HPV variants, susceptibility towards HPV infection or lifestyle and sexual behaviour as well [5]. Modes of transmission of HPV in colorectal cancer have been contentious; however, anal transmission by sexual behaviour, retrograde viral transmission from the anogenital area [27] or vertical transfer from HPV infected mother [28] has been anticipated. However, similar colonal and rectal viral detection rates in the current as well as previous studies suggest that HPV might not be a result of retrograde viral transmission from the anogenital areas [8, 9]. Lately, HPV transmission in epithelial cells of other organs through peripheral blood has also been proposed [29]. Although, HPV DNA was earlier detected in peripheral blood of cervical cancer patients, but was attributed to metastatic conditions rather than transmission [30, 31]. Inversely, few studies could find HPV DNA in cancer free healthy blood donors [32]. We could detect HPV DNA in almost 12 % of cases, but all harboured HPV DNA in tumour tissues as well. However, an association with metastasis was not documented either. Sexual mode of transmission could not be evaluated due to social and religious taboos associated. Largely, the route of viral transmission to the colon remains debatable and needs to be determined.

We failed to find any significant association of HPV with clinicopathological features of patients, similar to previous

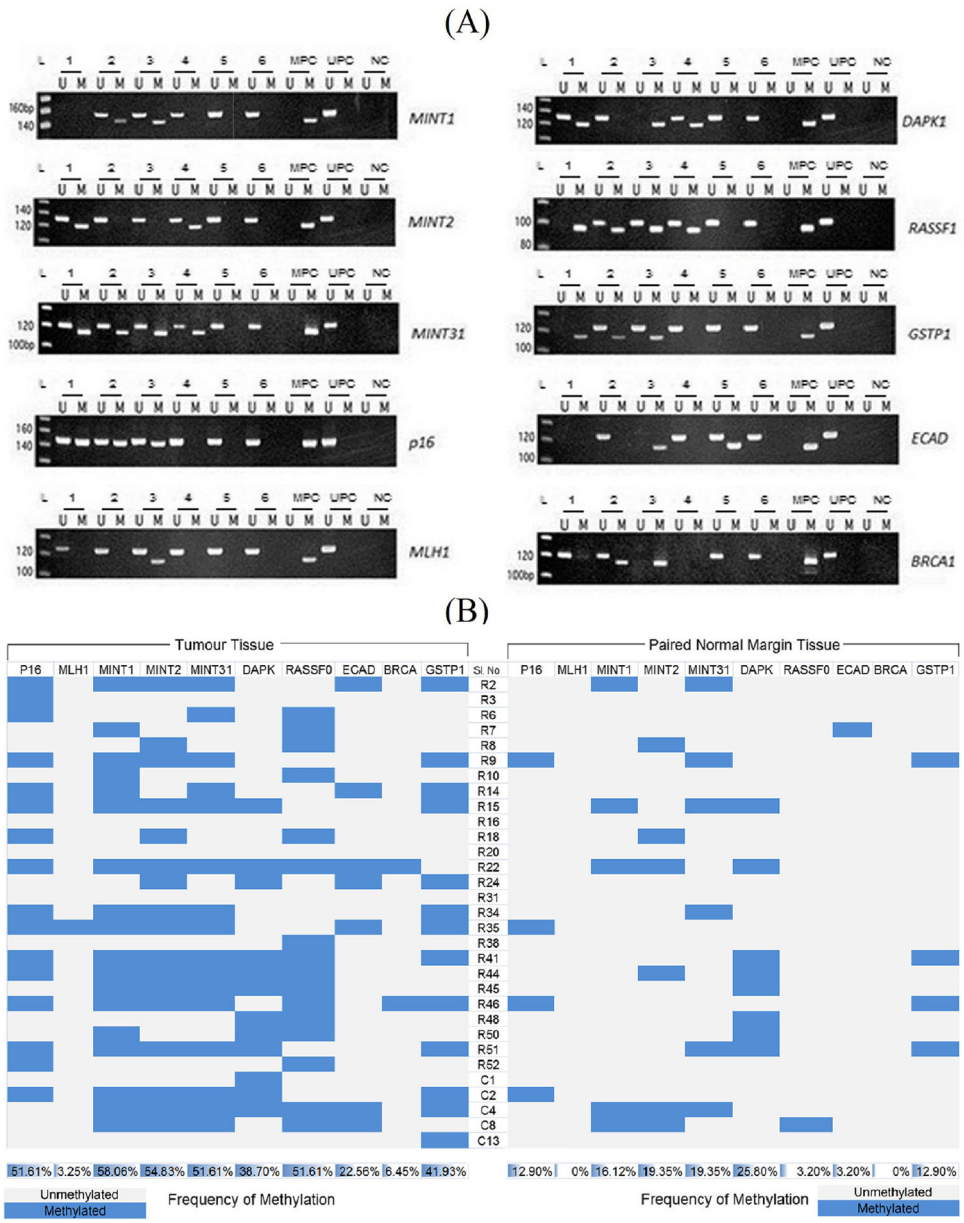

Fig. 3 Promoter methylation detection. **a** Electrophoresis pattern of promoter methylation of 10 genes/loci using methylation-specific PCR (MSP). L-DNA marker; 1,2,3,4,5,6-colorectal samples; M-methylated; U-unmethylated; MPC-methylated positive control; UPC-unmethylated positive control; NC-negative controls. Each sample as well as controls were subjected to MSP using both methylated and unmethylated primers. **b** Pairwise comparison of methylation pattern and frequencies of each gene/loci from the tumour and normal tissues of 30 CRC patients analysed in the study

studies [8, 9]. An earlier study on the role of HPV in CRC could demonstrate an association of HPV with early age at onset [9]. Inspite of a large proportion of young age patients in our study group as well in the region [33], we failed to establish any such association. However, a nonsignificant high proportion of HPV positive cases in early stage tumours might indicate a probable role of HPV in tumour initiation.

CRC is a heterogeneous complex disease having distinctive genetic-epigenetic milieu. The overall frequency of MSI in BAT 25 and BAT 26 markers were 7.5 %, with significant site specific variances (19 % in colon vs. 4 % in rectum). This frequency was lower than that of the Western population where MSI constitutes about 15 % of tumours [15]. Further, we found significantly lower frequency of *KRAS* codon 12, 13 mutations in younger patients. Our results were supported by previous findings of low *KRAS* mutations in young Indian and Bangladeshi population [34, 35]. The role of promoter hypermethylation of multiple tumour-related genes is recognised as one of the key events in initiation and progression of colorectal cancer. Here, we report high methylation frequencies of *P16* (45.16 %) and *RASSF1* (43.01 %) genes similar to earlier findings from Indian population (*P16 and RASSF1* promoter methylation in 42.1 % and 47 % of the cases respectively) [36, 37]. We found high CpG island methylation in 44 % patients with nonsignificant colonic predominance. Although, very few studies were conducted on CIMP based classification of Indian CRC patients, but both marker based as well as methylome-based studies on other population have reported

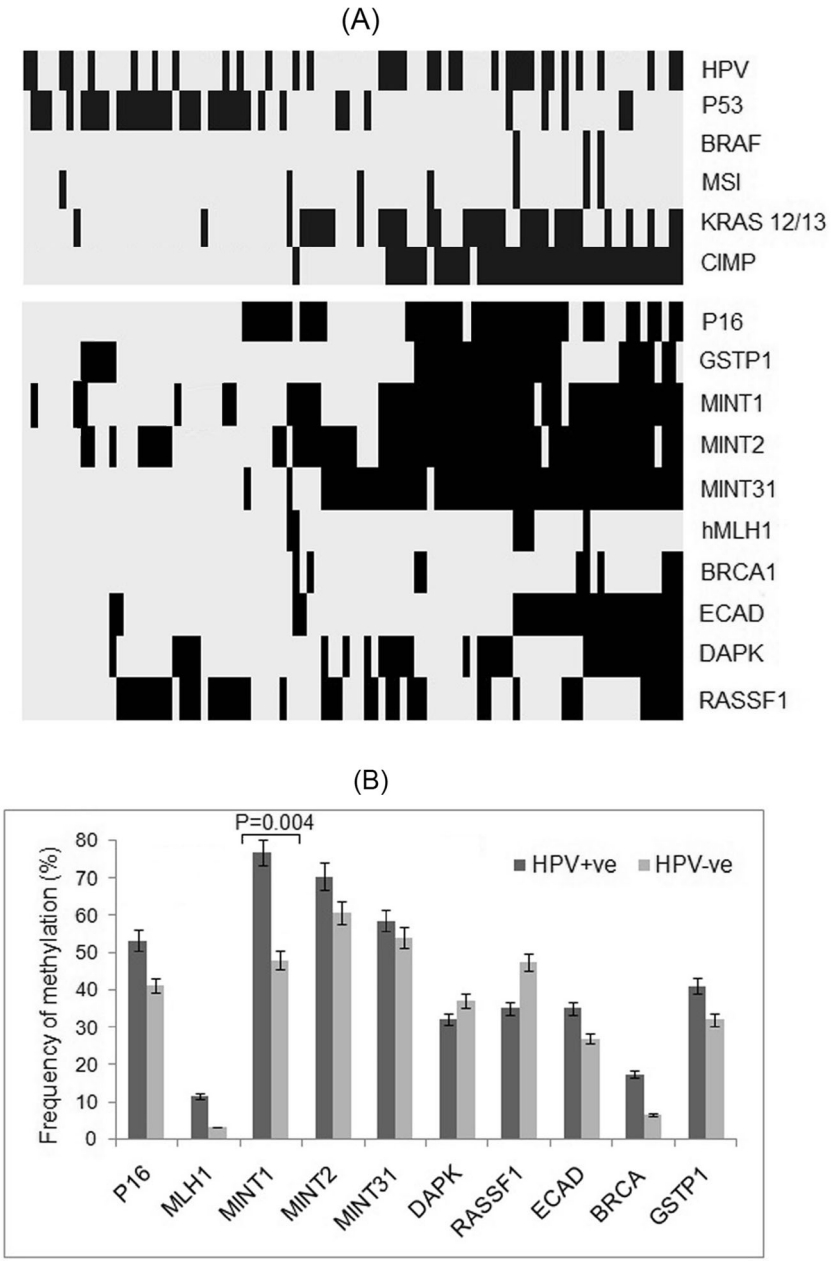

Fig. 4 HPV and promoter hypermethylation. **a** Heat-map representing 93 colorectal cancer cases showing the statuses of HPV presence, genetic (MSI, *KRAS*, *TP53* and *BRAF* mutation) and epigenetic (methylation pattern of 10 tumour-related genes/loci and CIMP). Each *rectangle* indicates a case, and each *row* indicates a marker; *grey colour* denotes absence and *black* demotes presence of a particular attribute. **b** Comparison of methylation frequencies of the 10 genes/loci between HPV positive and HPV negative cases

two mutually exclusive subclasses of *BRAF*-associated and *KRAS*-associated CIMP in 45–50 % CRC cases; the former being predominated by hypermutated proximal colonic tumours [38].

Although, literature corresponding to HPV infection in CRC as well as molecular characteristics of the disease has grown immensely, but how HPV relates to the key molecular events in CRC pathogenesis is very poorly understood. While, the genetic alterations did not differ significantly between HPV positive and negative groups, but a comparatively lower frequency of *TP53* mutants and higher *KRAS* mutations among HPV associated cases supports earlier observations of similar inverse association between HPV and P53 disruptive mutations in anal, cervical or head and neck cancers [39–41]; as well as HPV positive colon tumours having concurrent KRAS onco-mutations. It might be noted that, although HPV E6 and E7 oncoproteins can efficiently immortalise cells in culture, but are not sufficiently tumorigenic in mice models and require cofactors like, expression of additional oncogenes such as *RAS* to acquire tumorigenicity [17]. In vitro studies on E6–E7 immortalised primary human epidermal keratinocytes was also shown to become malignant after additional transfection with activated RAS oncogene [42, 43], while cooperation of HPV 18 E6 and RAS was required to overcome the anti-proliferative and anti-transformation effects of wild-type *P53* in NIH3T3 cells [44]. Therefore, the current findings

Table 3 Comparisons of clinicopathological data, genetic mutations and DNA methylation of HPV positive and HPV negative patients

	HPV negative (*N*=59)	HPV positive (*N*=34)	*P* value
Anatomic location			
Colon	12 (20.33)	9 (26.47)	0.80
Rectum	47 (79.66)	25 (73.52)	
Age			
Mean age (years)	44.33	48.41	
≤40 years	26 (44.06)	9 (26.47)	0.12
>40 years	33 (55.93)	25 (73.52)	
Gender			
Male	33 (55.93)	16 (47.05)	0.51
Female	26 (44.06)	18 (52.94)	
Stage			
I/II	22 (37.28)	19 (55.88)	0.08
III/IV	37 (62.71)	15 (44.11)	
Metastasis			
Present	17 (28.81)	12 (35.29)	0.64
Absent	42 (71.19)	22 (64.71)	
Differentiation			
Well differentiated	29 (49.15)	23 (67.64)	0.22
Moderately differentiated	9 (15.25)	3 (8.82)	
Poorly differentiated/mucinous	21 (35.59)	8 (23.52)	
KRAS Codon 12, 13			
Wild type	41 (69.49)	18 (52.94)	0.12
Mutated	18 (30.50)	16 (47.05)	
BRAF			
Wild type	58 (98.30)	32 (94.11)	0.55
Mutated	1 (1.69)	2 (5.88)	
P53			
Wild type	34 (57.62)	24 (70.58)	0.26
Mutated	25 (42.37)	10 (29.41)	
Microsatellite status			
MSS	56 (94.91)	30 (88.23)	0.25
MSI	3 (5.08)	4 (11.76)	
CIMP			
CIMP-High	21 (35.59)	20 (58.82)	0.03
CIMP-Low	38 (64.40)	14 (41.17)	

might indicate a probable role of HPV as an additional factor in CRC development and progression. Further, CIMP-high was associated with HPV positivity and methylation frequencies of most of the genes/loci were higher in HPV associated cases except *RASSF1*. This inverse association of HPV and *RASSF1* corroborates previous findings that HPV infection inversely correlates with *RASSF1A* methylation in human cervical squamous cell carcinomas [45]. The role of HPV in promoting aberrant host DNA methylation is evident in both clinical as well as cell culture studies. Transfection of high-risk HPV in primary human foreskin keratinocytes resulted in upregulation of cellular DNA methyl transferase enzymes DNMT1, DNMT3B expression, thereby changing the methylation status of crucial cellular genes involved in cancer [46]. Significantly higher methylation was observed in HPV associated tumours as compared to nonassociated tumours in many cancer types like head and neck squamous cell carcinoma (HNSCC) and anal cancers in both marker based as well as genome-wide studies [18, 19].

In conclusion, our study further establishes previous reports of high HPV prevalence in malignant colorectal tumours from developing countries. HPV in association with other genetic or epigenetic deregulations might be potent risk factor for CRC in Indian population. The increasing reports of association between HPV and several nongenital malignancies might build anticipation for extending preventive vaccination beyond cervical cancers to other HPV associated nongenital cancers as well. Nevertheless, our study is only a pilot association study and requires validation involving larger populations and in vitro models to establish a causal effect.

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Conflict of interest The authors declare no conflict of interest.

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Mitochondrial DNA Copy Number and Risk of Oral Cancer: A Report from Northeast India

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the sixth most common cancer globally. Tobacco consumption and HPV infection, both are the major risk factor for the development of oral cancer and causes mitochondrial dysfunction. Genetic polymorphisms in xenobiotic-metabolizing enzymes modify the effect of environmental exposures, thereby playing a significant role in gene–environment interactions and hence contributing to the individual susceptibility to cancer. Here, we have investigated the association of tobacco - betel quid chewing, HPV infection, *GSTM1-GSTT1* null genotypes, and tumour stages with mitochondrial DNA (mtDNA) content variation in oral cancer patients.

Methodology/Principal Findings: The study comprised of 124 cases of OSCC and 140 control subjects to PCR based detection was done for high-risk HPV using a consensus primer and multiplex PCR was done for detection of *GSTM1-GSTT1* polymorphism. A comparative ΔCt method was used for determination of mtDNA content. The risk of OSCC increased with the ceased mtDNA copy number ($P_{trend} = 0.003$). The association between mtDNA copy number and OSCC risk was evident among tobacco – betel quid chewers rather than tobacco – betel quid non chewers; the interaction between mtDNA copy number and tobacco – betel quid was significant ($P = 0.0005$). Significant difference was observed between *GSTM1 - GSTT1* null genotypes ($P = 0.04$, $P = 0.001$ respectively) and HPV infection ($P<0.001$) with mtDNA content variation in cases and controls. Positive correlation was found with decrease in mtDNA content with the increase in tumour stages ($P<0.001$). We are reporting for the first time the association of HPV infection and *GSTM1-GSTT1* null genotypes with mtDNA content in OSCC.

Conclusion: Our results indicate that the mtDNA content in tumour tissues changes with tumour stage and tobacco-betel quid chewing habits while low levels of mtDNA content suggests invasive thereby serving as a biomarker in detection of OSCC.

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Introduction

OSCC, the most frequent tumour of oral cavity, [1] and the sixth most common cancer globally that accounts for approximately 5 per cent of all malignant tumours worldwide [2,3]. The statistical analysis by the International Agency for Research on Cancer (IARC) indicated that the lip and oral cavity is the tenth most common tumour site in the human [4]. Smokeless tobacco products and betel quid with or without tobacco are the major risk factors for oral cavity cancer in Taiwan, India, and other neighboring countries [5–7]. In Northeast India, incidence of tobacco related oral cancers is about 33% [8]. Smoking, alcohol use, smokeless tobacco products, and HPV (Human papilloma virus) infections are the major risk factors for oral cavity cancer, with smoking and alcohol having synergistic effects [9,10].

The development of carcinogenesis due to environment-gene interaction has been well illustrated by phase I and phase II enzymes that are involved in the metabolism of carcinogens. The phase I enzymes are CYPs (Cytochrome P450) that are involved in activating the environmental procarcinogens adding or exposing their functional groups whereas phase II enzyme like GST (Glutathione S-transferase) are involved in detoxication of the activated metabolites of the carcinogens [11]. Tobacco smoke is a complex mixture of carcinogenic compounds, and smokeless tobacco is rich in nitrosamines. Furthermore, the concomitant use of betel quid leads to 50-fold increase in reactive oxygen species generation (ROS) [12,13]. A structural deletion in these genes represents a null genotype and has been associated with an increased risk to oral cancer [14].

Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer [15,16]. Mitochondrial respiratory activity is associated with the generation of ROS. The mitochondrial genome is susceptible to ROS and other types of genotoxic damage due to lack of

protective histones and its limited mtDNA repair capabilities. The mtDNA copy number per cell is maintained within a constant range to meet the energy requirement of the cell to sustain normal physiological functions. It varies significantly among the population from 1000 to 10,000 per cell [17] and also significantly varies by cell type. It is likely that the variations in the copy number of mitochondria reflect the net results of gene–environmental interactions between unknown hereditary factors and the levels of oxidative stress (an imbalance between ROS production and the antioxidant capacity), caused by a variety of endogenous and exogenous factors, such as, hormones, age, dietary and environmental oxidants/antioxidants, and reaction to oxidative damage, all of which are thought to be risk factors for various types of cancer development [18–20].

MtDNA content has been implicated as a potential biomarker for several cancer types [21,22]. Decreased mtDNA content had been reported for thyroid [21], renal [23,24], gastric [25], breast [26], previously-treated head and neck [27], ovarian [28] and hepatic cancer [29]. In contrast, several studies have revealed an increased mtDNA content in prostate [30], untreated head and neck [31], endometrial [32], lung [33], colorectal [34,35] and pancreatic cancer [36].

The aim of the present study was to investigate the association of tobacco - betel quid chewing, HPV infection, *GSTM1-GSTT1* null genotypes, with mtDNA content. We also evaluated the mtDNA content in the tumour and correlated with tumour stages. OSCC is a multifactorial and dynamic event in which numerous alterations contribute to disease development. Therefore, the risk of tobacco - betel quid chewing, *GSTM1-GSTT1* null genotypes, HPV infection and mtDNA content associated with OSCC was studied which may serve as a possible molecular biomarker for early detection of oral cancer, being the most prevalent cancer of Northeast region of India.

Materials and Methods

Subjects and Sample Collection

One hundred twenty four OSCC patient's post-treated tumour tissue/FFPE/oral swab and 140 non-OSCC (without cancer, having the habit of chewing tobacco-betelquid and also no family history of cancer) age and gender matched controls swab from inner cavity, was collected during July 2010 to August 2012 from hospitals as well as from home with written Informed consent and approved by IRB. The availability of such controls alone in the hospital was impossible as most of the patients come with their relatives does not fit within the criteria assigned for being controls in this present study. Data regarding age, gender, occupation and nature of consuming tobacco-betel quid habit (smoking or smokeless) and alcohol intake from OSCC subjects was abstracted from hospital records and on personal interviews. All possible precautions were taken to avoid any cross-contamination while collecting as well as processing of the samples.

Ethics statement. The present study was approved [No: IRB/CCHRC/01/2010] by Institutional Review Board (IRB), Cachar Cancer Hospital and Research Centre (CCHRC) (http://cacharcancerhospital.org), Meherpur, Assam, India.

DNA Isolation

DNA was isolated from preselected regions of tumour tissue, formalin fixed paraffin embedded tissue (FFPE) and oral swab. The tissues were digested in TES (50 mM Tris-HCl pH 7.4, 25 mM EDTA, 150 mM NaCl) buffer and incubated overnight at 55°C the tissue digests. The DNA was subsequently isolated by phenol/chloroform/isoamylalcohol method followed by ethanol precipitation and re-suspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) buffer and stored at −20°C [37]. Bioline Isolate Genomic DNA minikit (Bioline, UK) was used for isolation of genomic DNA from FFPE tissues following manufacturer's instructions.

Multiplex PCR for *GSTM1* and *GSTT1*

Analysis for *GSTM1 -GSTT1* gene polymorphism using *CYP1A1* gene as internal control was done by multiplex PCR. The forward (F) and reverse (R) primers used for the amplification *GSTT1* was F5'-TTCCTTACTGGTCCTCACATTCTC-3′ and R 5′-TCACGGGATCATGGCCAGCA-3′, *GSTM1* was F5'-GAACTCCCTGAAAAGCTAAAGC-3′ and R5'-GTTGGGCTCAAATATACGGTGG-3′, *CYP1A1* was F5'-ACTGCCACTTCAGCTGTCT and R5'-GCTGCATTTG-GAAGTGCTC respectively [38,39]. The PCR programme used for amplification was: initial denaturation step at 94°C for 2 mins; 30 cycles of denaturation at 94°C for 30s; annealing at 59°C for 45s and elongation at 72°C for 90s. The amplified product was observed in 1.5% agarose gel.

HPV Detection and Genotyping

PCR amplification for HPV detection were carried out with consensus primers GP5+/GP6+ followed by subtype detection of HPV 16 and 18 [40,41]. Reaction mixture without DNA template was used as a negative control and that with known DNA template was used as a positive control which yielded PCR products of expected results. PCR amplification was carried out with forty cycles. The PCR products were analyzed by electrophoresis on 2% agarose gel. A molecular weight marker of 50 bp was also run simultaneously to identify the molecular size of the PCR products.

Quantitative Real Time PCR

The StepOne^{TM} Real-Time PCR System (Applied Biosystems) was used to perform PCR amplification for mtDNA D-loop (C-tract) region. *GAPDH* was used as a 'housekeeping gene' to normalize all of the threshold cycle (Ct) values. The forward (F) and reverse (R) primers used for amplification of C-tract region was F5' CAGGGTCATAAAGCCTAAATAG 3′ and R5'GAGGTAAGCTACATAAACTGTG3' (109 bp) and GAPDH was F5'GAAATCCCATCACCATCTTCC 3′ and R5' GAGCCCCAGCCTTCTCCATG 3′ (125 bp) respectively. For each 10 µl reaction, 1 µl of unknown DNA was amplified containing 0.5 µl of each primer (20 pmol/µl), 5 µl of 2X SYBR Green Mastermix (Applied Biosystems), and 3 µl nuclease free water. The real-time PCR conditions consisted of initial denaturation and Taq polymerase activation at 95°C for 10 minutes followed by 40 cycles of 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 1 minute and followed by a melting curve analysis. Each measurement was repeated in triplicate and a non-template control was included in each experiment.

To determine the quantities of mtDNA and nDNA present in samples, the average threshold cycle number (Ct) values of the nDNA and mtDNA were obtained from each case. The level of mtDNA was calculated using the delta Ct (ΔCt) of average Ct of mtDNA and nDNA (ΔCt = CtmtDNA-CtnDNA) in the same well as an exponent of 2 ($2^{-\Delta Ct}$).

Statistical Analysis

Medians and frequencies of selected characteristics were compared between cases and controls using the Mann-Whitney U test for continuous and the Pearson chi-square for all other categorical variables. MtDNA copy number was categorized into

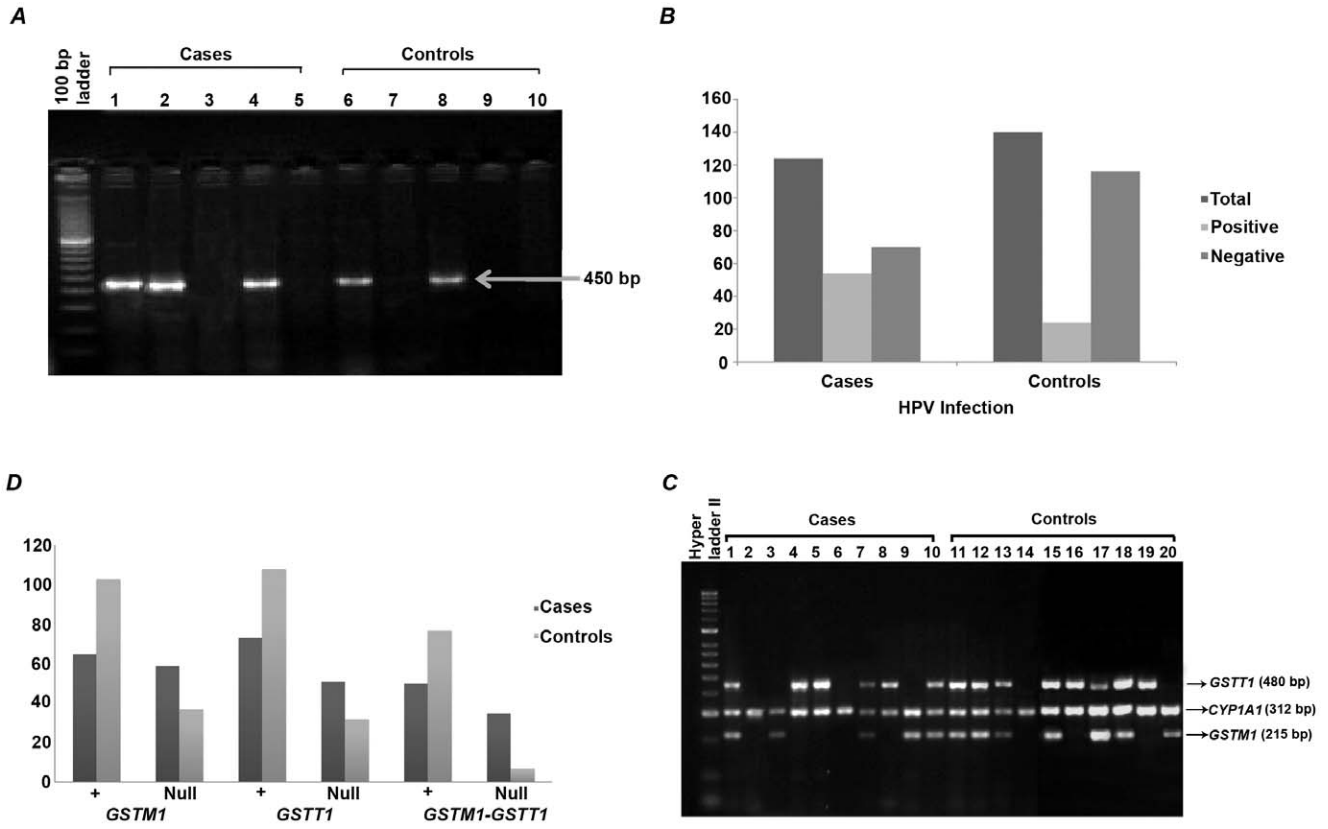

Figure 1. PCR based detection of HPV infection and *GSTs* polymorphism in oral cancer. (A) Representative agarose gel stained with ethidium bromide for fragment size determination (at expected size of ~450 base pairs) and polymerase chain reaction (PCR) amplification yield for common risk human papillomavirus (HPV) genomes from controls and oral cancer patients. (B) Bar graph showing the high incidence of HPV in OSCC patients than controls based on PCR detection of genomic DNA isolated from oral swab and tissues. (C) Multiplex PCR patterns for *GSTM1*, *GSTT1*, and *CYP1A* genes separated by agarose gel electrophoresis, corresponding to controls (lane 1–10) and oral cancer patients (lanes 11–20). The *CYP1A1* gene was used as an internal positive control. Lanes 1,4,5,7,10,11,12,13,15,11 and 18 represents the presence of both *GSTM1* and *GSTT1* genes, and lane 2, 6 and 14 represents null genotypes for both *GSTM1* and *GSTT1* genes. Lanes 3 and 20 represent presence of *GSTM1* gene and null genotypes for the *GSTT1*gene. Lanes 4, 58, 16 and 19 represent wild presence of *GSTT1* gene and null genotypes for the *GSTM1* gene. (D) Bar graph showing the distribution of *GSTM1* and *GSTT1* null genotypes among OSCC patients and controls based on multiplex PCR detection of genomic DNA isolated from oral swab and tissues.
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quartiles based on the distribution among controls. Odds ratios (OR) and 95% confidence intervals (CIs) were estimated using logistic regression models. A test for trend was calculated using the mtDNA copy number as a continuous variable. Non-parametric Mann-Whitney test was used to test if mtDNA content alteration in tumour is different in OSCC cases and controls with or without HPV infection, *GSTT1* and *GSTM1* null genotypes. *P*-values less than 0.05 are considered statistically significant.

Results

The characteristics of the OSCC subjects and controls as described in Table 1. There were no statistically significant differences between the cases and controls subjects in terms of age ($P = 0.82$), gender ($P = 0.2$), intake of current fruit ($P = 0.94$), salted dry fish ($P = 0.77$) and fermented fish ($P = 0.84$). However, significant differences were observed in daily tobacco-betel quid intake ($P = 0.01$) and vegetable ($P = 0.04$). The individual risk factors associated with oral cancer were examined. The increase risk to OSCC is 2.2- fold (95% CI, 1.31–3.68; $P = 0.002$) among the tobacco-betel quid chewers which is one of the major contributing factors for oral cancer and in Northeast India tobacco chewing is one of the customary practices. PCR of the oncogenic HPV genome was carried out using consensus primers GP5+/GP6+ and 450 bp band was observed (Figure 1A). The detection of common risk HPV in the individual was observed to be higher (Figure 1B) and was found 43.54% (54) in cases and 17.14% (24) in controls (Table 2). Upon genotyping HPV positive samples were found to be high risk subtype of HPV18. Infection with HPV has been implicated as one of the possible etiological factors for OSCC and in the present study, the risk of OSCC increased 3.72 -folds (95% CI, 2.11–6.56; $P<0.0001$) due to HPV infection.

Multiplex PCR was carried out among all the cases and the null genotype of *GSTT1* or *GSTM1* or both was detected by absence of the band when observed in 1.5% agarose gel (Figure 1C). We observed *GSTM1* null genotype in 47.58% (59) cases and 26.42% (37) controls, *GSTT1* null genotype in 41.12% (51) cases and 22.85% (32) controls, both *GSTT1* and *GSTM1* null genotypes were 28.22% (35) cases and 5% (7) controls respectively (Figure 1D). The *GSTM1* null genotype have increased oral cancer risk by 2.52 -fold (95% CI, 1.50–4.22; $P = 0.0003$) as null genotypes of this class gene have been linked with number of cancers, likely due to an increased susceptibility to environmental toxins and carcinogens, whereas the risk association of *GSTT1* null genotype with OSCC found to be statistically significant (OR,

Table 1. Selected characteristics of OSCC subjects and controls.

Characteristics	Subjects		*P* value
	Cases (n = 124) (%)	Control (n = 140) (%)	
Age (years)			
Median	58	56	0.82[a]
Gender			
Male	98(79)	101(72.1)	0.2[b]
Female	26(20.9)	39(27.8)	
Current vegetable intake			
<Once per week	23(18.5)	12(8.5)	0.04[b]
1–6 per week	61(49.1)	79(56.4)	
>1 per day	40(32.2)	53(37.8)	
Current fruit intake			
<Once per week	58(46.7)	63(45)	0.94[b]
1–6 per week	53(42.7)	61(43.5)	
>1 per day	13(10.4)	16(11.4)	
Non-veg intake(fish)			
Salted Dry fish			
<Once per week	12(9.7)	16(11.4)	0.77[b]
1–6 per week	63(50.8)	82(58.5)	
>1 per day	39(31.4)	42(30)	
Fermented fish			
<Once per week	18(14.5)	18(12.8)	0.84[b]
1–6 per week	71(57.2)	85(60.7)	
>1 per day	35(28.2)	37(26.4)	
Daily tobacco- betel quid intake			
No intake per day	35(28.2)	65(46.4)	0.01[b]
1–3 per day	50(40.3)	41(29.2)	
>3 per day	39(31.4)	34(24.2)	

[a]Mann - Whitney U was used to examine difference.
[b]Chi square was used to examine differences.
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2.35; 95% CI, 1.38–4.01; $P = 0.001$) (Table 2). Further the risk increases by 7.7-fold for OSCC with both *GSTM1-GSTT1* null genotypes (95% CI, 3.17–18.67; $P<0.0001$).

Using quantitative PCR techniques, we determined the relative content of mtDNA with respect to the *GAPDH* gene in 124 OSCC patients with different tumour stage and in the normal oral mucosal cells in 140 individuals without disease (Figure 2A). Overall, the relative median of the mtDNA content is significantly lower in cases (0.22 relative copies) than the controls (0.89 relative copies) ($P<0.009$). The distribution of mtDNA content in cases and controls was shown in Figure 2B. OSCC cases in the lowest quartile of the mtDNA copy number experienced a significantly increased risk of 2.92 fold to oral cancer (95% CI, 1.32–6.43) compared with those in the highest quartile (Table 3). We observed that risk of OSCC increased with the ceased mtDNA copy number ($P_{trend} = 0.003$).

The association between mtDNA copy number and OSCC risk was evident among tobacco – betel quid chewers rather than tobacco – betel quid non chewers (Table 4); the interaction between mtDNA copy number and tobacco – betel quid was significant ($P = 0.0005$). Similar results were observed when cases

Table 2. Risk of tobacco and betelquid chewing, HPV and *GSTT1- GSTM1* null genotypes associated with OSCC.

	Cases (n = 124)	Controls (n = 140)	OR [95%CI]	*P* value
Tobacco- betelquid				
Chewers	89	75	2.20 [1.31–3.68]	0.002
Nonchewers	35	65	1(ref)	
HPV				
Presence	54	24	3.72 [2.11–6.56]	<0.0001
Absence	70	116	1(ref)	
GSTM1				
+	65	103	1(ref)	0.0003
Null	59	37	2.52 [1.50–4.22]	
GSTT1				
+	73	108	1(ref)	0.001
Null	51	32	2.35 [1.38–4.01]	
GSTM1- GSTT1				
+	50	77	1(ref)	<0.0001
Null	35	7	7.7 [3.17–18.67]	

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and controls were classified as tobacco- betel quid chewers and non chewers based on low (≤1) and high (>1) mtDNA copy number, the tobacco–betel quid chewers with the low mtDNA copy number have 3.54 fold increased risk of OSCC (95% CI, 1.59–7.87). Furthermore, a significant difference was found with mtDNA content in cases and controls with or without HPV infection ($P<0.001$). Similarly, we found a significant difference between *GSTM1* and *GSTT1* null genotypes with mtDNA content in cases and controls ($P = 0.04$ and $P = 0.001$ respectively).

The mtDNA contents in tumour tissues was significantly higher in stage 0 and 1 tumours than in stage IV tumours, $P<0.001$. Out of 124 cases 18.5% (23) were stage 0, 25% (31) of tumours were stage I, 27.4% (34) stage III, 29% (36) were stage IV respectively. There were no samples available at tumour stage II. The mtDNA content correlated with tumour stage, where we observed that mtDNA content decreases with the increase in tumour stage ($P<0.001$) (Figure 2C).

Discussion

The habit of chewing tobacco and betelquid is an endemic habit throughout the Indian subcontinent. The betel quid is commonly referred to as 'paan' in South Asian countries. The main constituents of a betel quid are Piper betel leaves and areca nut (the seed of the Areca catechu plant). It is made by wrapping chopped areca nut in a Piper betel leaf, and some lime (calcium hydroxide) and tobacco leaves or zarda (flavoured tobacco) may be included to improve the taste; combinations of ingredients are altered according to individual preferences. Tobacco consumption by smoking or chewing is thought to be the major etiological risk factors for the development of oral cancer caused by irritation from direct contact with the mucous membranes of mouth.

The elevated number of tobacco-related OSCC cases is a major concern Northeast region of India. All forms of tobacco produce free radicals that deplete antioxidants and cause oxidative damage to DNA, proteins and lipids [42,43]. Antioxidant-rich foods such as green-leafy vegetables and fruits that may help reduce the

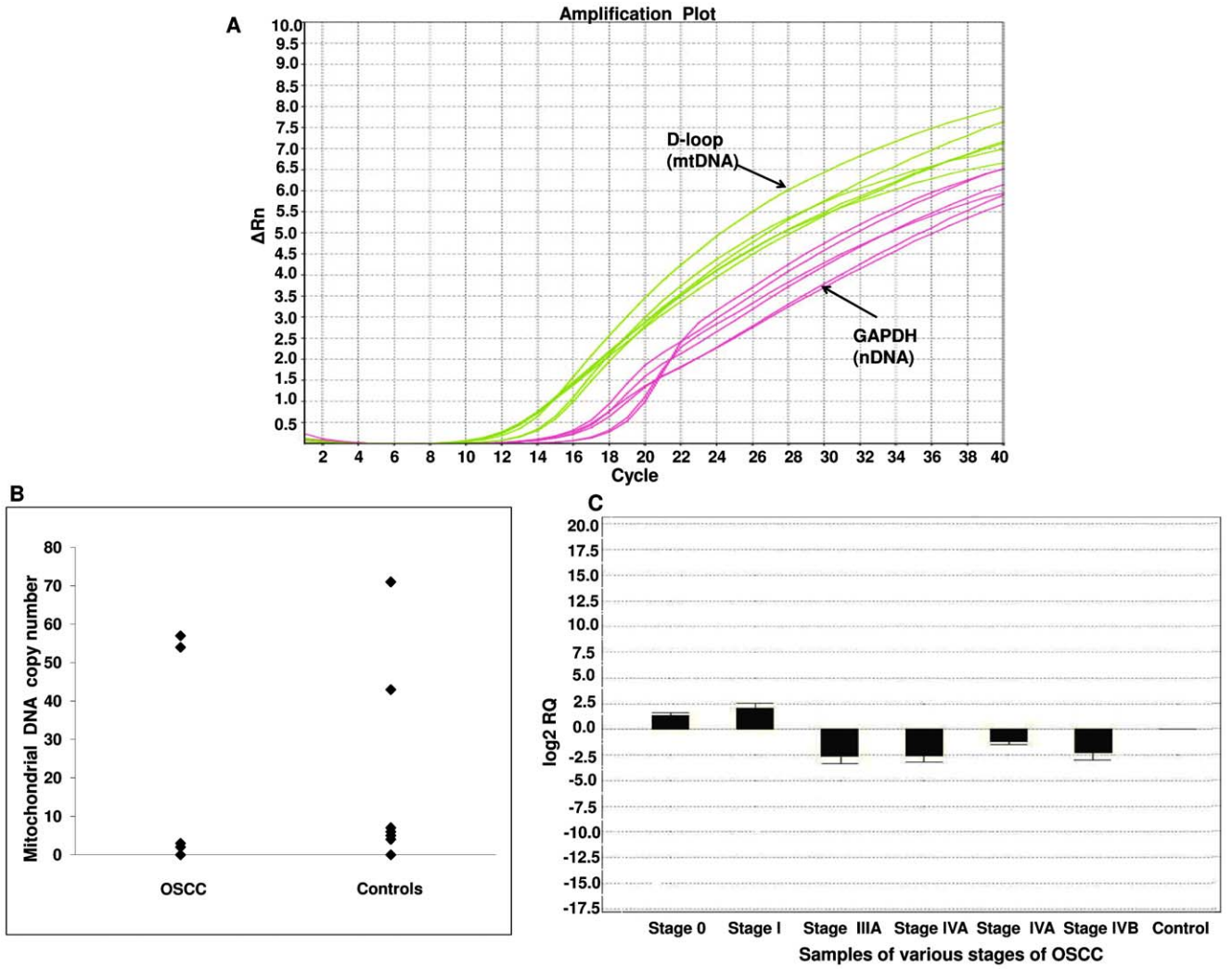

Figure 2. Real time PCR based mitochondrial DNA copy number determination in oral cancer. (A) Quantitative PCR of D-loop region and *GAPDH* gene (representative curve). D-loop region and *GAPDH* are ubiquitous genes found in the mitochondrial and nuclear genomes, respectively. Using quantitative PCR in samples from the patient and control, the relative mitochondrial content was calculated. (B) Distribution of mtDNA copy number in OSCC and controls. (C) Mitochondrial content decreases with increase in tumour stage: the various stages of OSCC samples from stage 0 to stage IVB with respect to log 2 RQ (log fold change).
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oxidative stress caused by tobacco [44,45] are usually lacking in the diet [46,47], the reasons may be the poor socio-economic condition and also customary practice of oral consumption.

In the present study, we examined the high risk HPV infection, a known independent causative agent for oral cancer in OSCC patients and a significant difference with mtDNA content in cases and controls with or without HPV infection ($P<0.001$) is obtained. However, no reports of association of HPV infection with the mtDNA copy number are there, although the correlation of HPV infection with mitochondrial mutation was reported in a study of cervical cancer [48]. Bak protein is pro-apoptotic member which localizes in mitochondria, and functions to induce apoptosis. The

Table 3. ORs and 95% CI for mtDNA copy number and risk of oral cancer.

mtDNA copy number quartile	Cases (n = 124)	Controls (n = 140)	OR (95%CI)
Quartile 1 (≤0.1)	58	43	2.92[1.32–6.43]
Quartile 2 (>0.1–1)	35	42	1.80[0.79–4.09]
Quartile 3 (>1–10)	19	29	1.41[0.57–3.47]
Quartile 4 (>10)	12	26	1 (ref)
P_{trend} 0.003			

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Table 4. mtDNA copy number and risk of oral cancer stratified by tobacco-betel quid chewing.

mtDNA content	Tobacco- betel quid Chewers		Tobacco- betel quid Non-chewers		*P*-interaction
	Cases/Controls	OR (95%CI)	Cases/Controls	OR (95%CI)	
low≤1	69/46	3.54 [1.59–7.87]	24/39	1.45 [0.60–3.46]	0.0005
high>1	20/29	1.63 [0.65–4.03]	11/26	1(ref)	

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elimination of Bak protein by HPV *E6* promotes survival of HPV infected cells by delaying apoptosis thereby facilitating tumour development with corresponding variation to mtDNA content, for which the exact mechanism is yet to be revealed. We are reporting for the first time the association of HPV infection with mtDNA content variation.

A significant difference between *GSTT1* and *GSTM1* null genotypes with mtDNA content in cases and controls ($P = 0.04$ and $P = 0.001$) was observed. The presence of both *GSTM1* and *GSTT1* are essential for detoxication of carcinogenic compound. The most important risk factor for oral cancer is smoking, tobacco chewing and betel quid. The concomitant use of betel quid leads to a 50-fold increase in reactive oxygen species generated [12]. The increased risk factor of null *GSTs* with accumulation of mtDNA mutations enzyme as because possibly plays inside the mitochondrial matrix as mtDNA protection factor regarding damage caused by reactive oxygen species which in turn affect the mtDNA content and may lead to causation of OSCC as well [39]. The associations of GST null genotypes and mtDNA content is not yet been reported.

Low levels of mtDNA copy number in tobacco- betel quid chewers found in our study are associated to high risk of OSCC due to release of substantial amounts of ROS. [49] which in turn increase mtDNA mutation in human oral tissues. The accumulation of mtDNA deletions and subsequent cytoplasmic segregation of these mutations during cell division could be important contributors to the early phase of OSCC [50,51]. The depletion in mtDNA may be result of the repression of mitochondrial biogenesis. The mtDNA copy number in cancer probably depends on several factors, including the site of mutation in the mitochondrial genome as demonstrated in D-loop region, a highly susceptible site for oxidative damage compared with the other regions of mtDNA [52]. The findings of the present study well demonstrate the risk of OSCC and mtDNA copy number to tobacco-betel quid chewers in this region. We did not evaluate the cancer tissue specimens for mtDNA determination before treatment due to its non availability from the biorepositary. Thus, we could not determine the mtDNA changes before chemotherapy. This might be a limitation in this type of study, although it would offer us additional information.

The inverse correlation of mtDNA content correlated with histopathological tumour stage and observed in our study were supported by similar finding in post-treatment salivary rinses in head and neck squamous cell carcinoma [27]. However, decrease of mtDNA copy number in tumour tissues have been reported in a variety of human cancers, including HCC [53,54], breast [55,56], gastric [57], osteosarcoma [58] and other cancers [59,60]. The underlying mechanism behind the low level of mtDNA content with increased tumour size is not clear. Furthermore, it was reported that decreased mtDNA content may result in decreased oxidative phosphorylation capacity that in turn may favor faster growth or increased invasiveness [61]. In general, decreased mitochondrial activity seems to be an adaptation to hypoxic environment of solid tumours during their development since low oxygen initiates lower oxidative stress under hypoxic conditions and hypoxia inducible factor (HIF) inhibits mitochondrial biogenesis [62] or disrupts mitochondria by mitophagy [63].When tumour is growing in size, cells are becoming more hypoxic, mitochondrial biogenesis is decreased [63]. Alternatively, the decrease of mtDNA posttreatment may reflect an effect of radiation that influences mtDNA content or mitochondrial number in cells, which may be responsible for reducing mtDNA.

The burden of oral diseases like oral cancer, periodontal disease, and tooth loss can be decreased by addressing common risk factors, which include avoiding smoking and consumption of tobacco related products and also intake of alcohol. Furthermore, practicing good oral hygiene like proper brush and floss daily along with routine cleaning and examination by the dentist can reduce the risk of oral diseases. The intake of fruits and vegetables can also protect against oral cancer as they are rich in antioxidants. HPV is one of the risk factor for oral cancer and the most reliable way to prevent infection with either high-risk or low-risk HPV is by avoiding any skin-to-skin oral, anal or genital contact with another person. Those who are sexually active, long term, term, mutually monogamous relationship with an uninfected partner is the strategy most likely to prevent HPV infection.

Conclusion

Our results indicate that the mtDNA content in tumour tissues changes with tumour stage and tobacco-betel quid chewing habits. Significant deviation from the medium range is associated with poor survival. High levels of mtDNA content may indicate that tumours may undergo rapid tumour growth while low levels of mtDNA content suggests invasive thereby serving as a biomarker in detection of OSCC.

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Author Contributions

Arranging the chemicals/PCR of the samples: BD. Collection of samples and isolation of DNA: MH BR PD SG MNC BC. PCR of HPV and GST gene polymorphism: RM RSL JC AS FRT. Conceived and designed the experiments: RM SKG. Performed the experiments: RM KS. Analyzed the data: RM SKG. Contributed reagents/materials/analysis tools: RM BD. Wrote the paper: RM SKG.

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