RESEARCH ARTICLE

MDM2 and *TP53* Polymorphisms as Predictive Markers for Head and Neck Cancer in Northeast Indian Population: Effect of Gene-Gene and Gene-Environment Interactions

Aditi Bhowmik[1], Sambuddha Das[1], Abhinandan Bhattacharjee[2], Biswadeep Choudhury[3], Momota Naiding[4], Sujata Deka[1], Sankar Kumar Ghosh[1], Yashmin Choudhury[1]*

Abstract

Background: Polymorphisms in the *MDM2* 309 (T>G) and *TP53* 72 (G>C) genes are reported to increase the susceptibility to head and neck cancer (HNC) in various populations. The risk for HNC is also strongly associated with etiologic habits such as smoking, alcohol consumption and/or chewing of betel quid (BQ). In a case-control study, we investigated the significance of the above polymorphisms alone, and upon interaction with one another as well as with various etiologic habits in determining HNC risk in a Northeast Indian population. Materials and Methods: Genotyping at 309 *MDM2* and 72 *TP53* in 122 HNC patients and 86 cancer free healthy controls was performed by PCR using allele specific primers, and the results were confirmed by DNA sequencing. Results: Individuals with the GG mutant allele of *MDM2* showed a higher risk for HNC in comparison to those with the TT wild type allele (OR=1.9, 95%CI: 1.1-3.3) (p=0.022). The risk was further increased in females by ~4-fold (OR=4.6, 95% CI: 1.1-19.4) (P=0.04). *TP53* polymorphism did not contribute to HNC risk alone; however, interaction between the *TP53* GC and *MDM2* GG genotypes resulted in significant risk (OR=4.9, 95% CI: 0.2-105.1) (p=0.04). Smokers, BQ- chewers and alcohol consumers showed statistically significant and dose-dependent increase in HNC risk, irrespective of the *MDM2* genotype. Conclusions: *MDM2* genotype could serve as an important predictive biomarker for HNC risk in the population of Northeast India.

Keywords: Head and neck cancer - 309 *MDM2* (T>G) - 72 *TP53* (G>C) - smoking- betel quid chewing- alcohol consumption

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Introduction

Head and neck cancers (HNC) constitute the sixth most common cancer world-wide (Macha et al., 2014) and occur widely in developing countries like India (Dikshit et al., 2012; Joshi et al., 2014). Myriad factors are known to contribute to a risk for head and neck cancer alone, or upon interaction with one another (Riaz et al., 2014). The most-well documented among these are genetic factors and exposure to various etiologic agents (Yu et al., 2011). These agents include smoking and alcohol consumption in Western countries and use of smokeless tobacco, areca nut and/or betel quid (BQ) in Southeast Asia. It has been estimated that consumption of tobacco could cause 8.4 million deaths in 70% of developing countries by 2020 (Joshi et al., 2014). Mutation of the *p53* tumor suppressor gene is the most common and among the earliest identified genetic alteration in head and neck squamous cell carcinoma (HNSCC), occurring in more than half of all cases (Yu et al., 2011; Rothenberg and Ellisen, 2012; Chakrobarty et al., 2014). The murine double minute 2 (MDM2) protein is one of the most extensively studied regulators of p53 activity. It regulates p53 activity in three ways. Firstly it binds to the p53 transactivation domain thereby inhibiting p53's transacriptional activity. Secondly *MDM2* acts as an E3 ubiquitin ligase to degrade p53. Finally *MDM2* helps p53 shuttle from the nucleus to the cytoplasm of the cell for degradation. Thus, the p53 and *MDM2* proteins constitute a negative feedback loop in which p53 transactivates *MDM2* and in return *MDM2* inhibits p53 activity (Yu et al., 2011; Nagata et al., 2014). The single nucleotide polymorphism (309*MDM2* T>G) in the Sp1 promoter region of the *MDM2* gene increases the affinity of the Sp1 transcriptional factor, therefore increasing *MDM2* mRNA and protein levels and thus attenuating the p53 pathway both *in vitro* and *in vivo* (Yu et al., 2011; Wang et al., 2014). On other hand, the single nucleotide polymorphism at codon 72 in exon 4 of

[1]*Department of Biotechnology, Assam University,* [2]*Department of ENT,* [3]*Department of Biochemistry,* [4]*Department of Pathology, Silchar Medical College and Hospital, Silchar, India *For correspondence: yashminchoudhury@gmail.com, yashminchoudhury@yahoo.co.in*

the p53 gene (codon 72; rs1042522; G>C) has functional significance in regulation of cancer progression and/or response to treatments, with the C-allele being associated with reduced apoptotic potential in comparison to the G-allele (Yu et al., 2011; Cai et al., 2014).

Xiao et al. (2010), Yu et al. (2011) and Wan et al. (2011), have previously reported that the *MDM2* GG genotype and 72*TP53* CC resulted in significant risk for nasopharyngeal carcinoma, HNSCC and carcinogenesis process in Chinese, Caucasians, and mixed ethnic populations, respectively.The highest incidence of nasopharyngeal cancer, tongue cancer and cancer of the lower pharynx in the world has been reported from Northeast India (Joshi et al., 2014). We therefore investigated the significance of 309 *MDM2* (T>G) and 72*TP53* (G>C) polymorphisms in determining the risk for HNC in the population of Northeast India. Furthermore we also investigated the effect of interaction of these genes with one another, and with the etiologic factors of smoking, betel quid and alcohol consumption on modulating HNC risk the population, through our case-control study.

Materials and Methods

Study subjects

Ethical clearance was obtained for this study from the Institutional Review Board (IRB) of Silchar Medical College and Hospital (No.SMC/11/7932-4, dated Silchar, 19/7/2011), Silchar, Assam, India and Institutional Ethics Committee (IEC/AUS/2013-008 dt-20/3/13), Assam University, Silchar, India. This study included 122 HNC patients and 86 healthy controls. Cases comprised male (n=105, Mean age ± SEM =55.9±1.2, median age =55) as well as female (n=17, Mean age±SEM =45.8±3.9, median age =50) patients with cancer at different sites of the head and neck region admitted at the Department of ENT, Silchar Medical College and Hospital, Silchar, Assam, India. Cancers were staged and graded at Department of Pathology, Silchar Medical College and Hospital, Silchar, Assam. HNC patients with stage III and IV cancer were included in the present study. Controls consisted of healthy male (n=52, Mean age±SEM =50.3±1.5, median age =50) and female (n=34, Mean age ± SEM =42.2±1.5, median age=42) volunteers without the disease. Venous blood samples were obtained from patients prior to chemotherapy or radiotherapy, as well as from age and gender matched controls, with informed consent during the time period of August 2012 to February 2014. The blood samples were collected in EDTA tubes and immediately refrigerated at 4ºC till further use.

Data pertaining to the habits of smoking, BQ chewing, and alcohol consumption among both patients and controls were collected with the help of a standard questionnaire. The study population was further divided into groups based upon the dosage of smoking (cigarettes/bidis per day), BQ chewing (units/day) and alcohol consumption (times of consumption/day). These groups were A (I) non-smokers (II) moderate smokers who smoked <20 cigarettes or bidis/day and (III) heavy smokers who smoked >20 cigarettes or bidis/day; B (I) BQ non- chewers (II) moderate BQ chewers who chewed <10 units of BQ per day and (III) heavy BQ chewers who chewed >10 units of BQ per day; (C) (I) Alcohol non-consumers (II) moderate consumers who consumed <5 times alcohol per day (III) heavy consumers who consumed >5 times alcohol per day.

Genetic polymorphism analysis

Genomic DNA was isolated from the blood samples of the study subjects using phenol:choloroform extraction method as described by (Talukdar et al., 2013) with some modifications. The purity and concentration of the isolated DNA was determined spectrophotometrically (BioRad SmartSpec, USA). Among the samples studied, 20 % were selected randomly and tested twice, and the results were found concordant.

Genotyping for *MDM2* SNP 309 was performed by polymerase chain reaction (PCR) using allele specific primers (Figure 1). Primers F1 (5´-GGATTTCGGACGGCTCTC-3´) and R1 (5´-TCCGGACCTCCCGCGCCGA-3´) were used to amplify the 121 bp wild type allele (T) and primers F2 (5´-GTTTTGTTGGACTGGGGCTA-3´) and R2 (5´-ATCCGGACCTCCCGCGCCGC-3´) were used to amplify the 168 bp mutant allele (G) as previously reported (Moradi et al., 2014). Two independent PCR reactions were performed for each allele and the amplification protocol consisted of 35 cycles of 94 ºC for 5 minutes, followed by 94 ºC for 30 seconds, 66ºC &

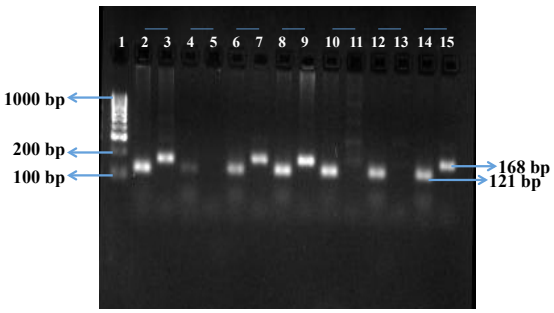

Figure 1. Representative Pattern for allele specific polymerase chain reaction amplification of MDM2 codon 309 polymorphism. Lane 1: 1000 bp DNA ladder. Lane 2, 3; 6, 7; 8, 9; 14, 15: TG heterzygotes. Lane4, 5; 10, 11; 12, 13: TT wild type homozygotes.

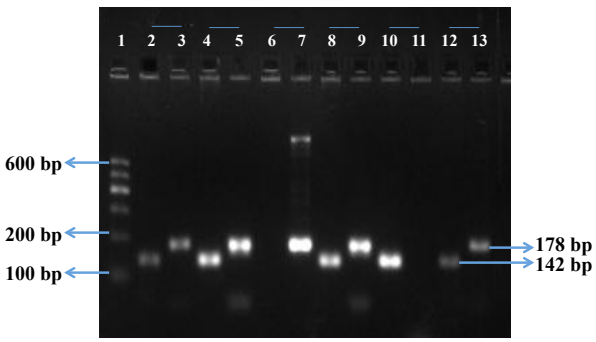

Figure 2. Representative Pattern for Allele Specific Polymerase Chain Reaction Amplification of *P53* Codon 72 Polymorphism. Lane 1: 600 bp DNA ladder. Lanes 2, 3; 4, 5; 8, 9; 12, 13: p53 GC heterozygotes. Lanes 6, 7: p53 CC homozygotes. Lane10, 11: p53 GG homozygotes

68°C for 30 seconds, 72°C for 30 seconds and the final elongation step of 30 seconds at 72°C.

Genotyping of polymorphism at codon 72 of the *p53* gene was also performed by allele specific PCR amplification (Figure 2). Primers *p53-p1* (GCCAGAGGCTGCTCCCC) and p53-p2 (CGTGCAAGTCACAGACTT) amplified the C allele for proline (178 bp) and p53-a1 (TCCCCCTTGCCGTCCCAA) and *p53-a2* (CTGGTGCAGGGGCCACG) amplified the G allele for arginine (142 bp) (Moradi et al., 2014). Amplification was performed using denaturation at 94 °C for 2 minutes, followed by 94 °C for 30 seconds, 62°C for 50 seconds, 72°C for 30 seconds for 35 cycles and the final elongation step at 72°C for 5 minutes.

More than 5 % of the samples were cross checked for the polymorphisms by direct DNA sequencing and the results obtained were found to be 100 % concordant.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences version 16.0 (SPSS Inc, Chicago, USA) and Graph Pad Prism 5.01 (Windows, GraphPad Software, San Diego California, USA). The differences in the distributions of categorical variables including demographic characteristics, smoking, BQ chewing and alcohol consumption as well as genotypes between the patients and controls were evaluated by two sided Chi-square test and Fisher's exact test where appropriate. The Hardy-Weinberg equilibrium (HWE) assumption was assessed by using Pearson goodness-of-fit statistic with the χ^2 distribution with 1 degree of freedom. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were obtained from unconditional logistic regression analyses to evaluate associations between SNPs and HNC risk in the case-control analysis. Distribution of genotypes and odds ratio of SNP for HNC patients and controls stratified on the basis of smoking habits, betel quid chewing and alcohol consumption and association between genotype frequencies of *MDM2* SNP 309 and risk of HNC development stratified for gender was also obtained from unconditional logistic regression analyses. Gene-gene and gene-environment interaction, allele frequencies between cases and controls were evaluated using two sided Fisher's exact test. Unpaired Student's t-test with Welch's correlation was used to analyse age group between controls and HNC patients with respect to gender. A p-value of <0.05 was considered to be statistically significant for all statistical analysis.

Multifactor dimensionality reduction (MDR)

MDR analysis is a popular non parametric, one dimensional, model free method for studying gene-gene and gene-environment SNP studies. Using this approach could help in overcoming sample size limitations which generally happens in conventional parametric statistical methods like logistic regression analysis. MDR software package (MDR 3.0.2) was used in this case control study to produce the best one dimensional multifactor model to predict HNC in North east Indian population. The best model was selected based on maximum cross-validation consistency (CVC) and testing balance accuracy. For that we adjusted the cross validation count up to 10 fold and 10 times random seed numbers to reduce the chance of false results. MDR results were considered as statistically significant at 0.05 levels (Choudhury and Ghosh, 2014).

Results

Demographics

A total of 86 healthy volunteers and 122 HNC patients were included in this case-control study. In comparison to controls, the patients were older with a higher proportion of males. Further statistical analysis of age with Welch's correlation analysis showed statistically significant difference in the age of males between controls and patients males (p=0.004) but not in females (p=0.4). Among the HNC patients, 50 (41%) were diagnosed with the cancer of larynx, 33 (27%) in oral cavity, 33(27%) in pharynx, 5(4%) in paranasal sinus and nasal cavity and 1% in salivary glands.

Association of etiologic habits with HNC risk

Dose-dependent increase in risk of HNC was observed in association with the habits of smoking, BQ use and alcohol consumption. The heavy smokers had 2.9 fold (95% CI=1.6-5.3, p=0.001) increased risk for HNC in comparison to non-smokers, heavy BQ chewers had a 3.4 fold (95%CI= 1.8-6.4, p=0.0002) increased risk in comparison to non-chewers, and heavy alcohol consumers had a 4.2 fold (95% CI= 1.4-12.7, p=0.008) increased risk in comparison to alcohol non-consumers. It was observed that cases who had multiple habits like smoking and BQ chewing or smoking and alcohol consumption showed 8.3 (95% CI= 3.4-20.2, p<0.0001) and 8.2 (95%CI= 2.3-29.5, p=0.003) fold higher risk respectively, in comparison to, those who reported no habit; while smoking, BQ chewing and alcohol consumption together contributed a 14.2 fold ((95% CI=2.97-67.5) higher risk than those who reported no habit. Odds ratio adjusted with age, gender, smoking, betel quid chewing and alcohol consumption were considered in the subsequent unconditional logistic regression analyses to evaluate associations between genetic polymorphisms and HNC risk in the case-control analysis (Table 1).

Genotype and allele distribution

The genotype frequencies of 309*MDM2* (T>G) for TT, TG and GG were 43, 50 and 7 % among controls, and 25.4, 51.6 and 23 % among HNC cases respectively. For *72TP53* (G>C) polymorphism the genotype frequencies for GG (Arg/Arg), GC (Arg/Pro) and CC (Pro/Pro) were 2.3, 94.2 and 3.5% respectively among controls and 0.0, 99.2 and 0.8% respectively among cases. The observed genotype frequencies of *MDM2* for both controls and cases did not deviate from those expected from HWE (χ^2=1.92 for controls, χ^2=0.12 for cases, χ^2=0.46 for entire cohort). Using the unconditional logistic regression adjusted for age, gender, etiological habits it was observed that the GG homozygous condition was overrepresented in HNC patients in comparison to controls (p=0.02) (OR=1.9, [95%CI=1.1-3.3]). Moreover the G allele was found to be associated with HNC risk rather than the T allele

Table 1. Genotype and Allele Distribution of *MDM2* and *TP53* Genes and Risk for HNC

309 MDM2 (T>G)	Controls	Cases	[a]OR (95% CI)	p-value
TT	37	31	1 (Ref.)	
TG	43	63	1.569 (0.792-3.106)	0.196
GG	6	28	1.906 (1.098-3.308)	0.022*
T allele	117	125	1 (Ref.)	
G allele	55	119	2.025 (1.348-3.043)	0.001*

72TP53 (G>C)	Control	Case	Adjusted OR^ (95% CI)	p-value
GG	2	0	1 (Ref.)	
GC	81	121	-	-
CC	3	1	1	1
G allele	85	121	1(Ref.)	
C allele	87	123	0.993 (0.672-1.467)	1

[a]Odd ratio adjusted with gender, age, smoking, betel quid chewing, and alcohol consumption at $\alpha<0.05$; * indicates statistical significance at statistical significance at $p<0.05$, -could not be calculated due to absence of particular genotype in the sample

Table 2. Association between Genotype Frequencies of *MDM2* SNP 309 and Risk for HNC in Females

309 MDM2 (T>G)	Controls	Cases	[a]OR (95% CI)	p-value
TT	19	4	1 (Ref.)	
TG	14	10	3.251(0.836-12.653)	0.089
GG	1	3	4.120(1.067-15.916)	0.04*

*a Odds ratio adjusted with age in female patients; * indicates statistical significance at $p<0.05$ *statistical significance ($p<0.05$)

(p=0.001) (OR=2.02, [95%CI=1.3-3.04]). In case of *TP53* polymorphism study we did not find statistically significant association between p53 polymorphism at codon 72 and HNC risk in this population. The population did not fall into HWE equation but overpowering heterozygous condition was observed among both controls and cases (Table 1).

Interaction between MDM2 309 T>G & 72TP53 G>C polymorphism

A significantly high risk for HNC was observed due to interaction of *MDM2* 309G/G-72*TP53* G/C (OR=21.9, [95% CI: 0.93-513.9]) (p=0.04) genotype. On other hand *MDM2* 309 T/T-72*TP53* G/C (OR=4.85, [95% CI: 0.2-105.1]) (p=0.4) and *MDM2* 309T/G-72*TP53* G/C (OR=7.2, [95% CI: 0.3-153.5]) (p=0.2) genotypic interactions did not increase the risk for HNC, significantly.

Association between genotype frequencies of MDM2 SNP 309 and HNC risk graded for gender

Gradation by gender highlighted a significant association between the *MDM2* GG genotype and the risk for HNC among females (Table 2), but not among males (not shown). As shown in Table 2, case/control analysis in the female subgroup showed that females with the GG genotype presented an ~4-fold higher risk (OR=4.6, [95% CI: 1.1-19.4]) (p=0.04) (adjusted for age, betel quid chewing habit) to develop HNC in comparison to females with the TT genotype.

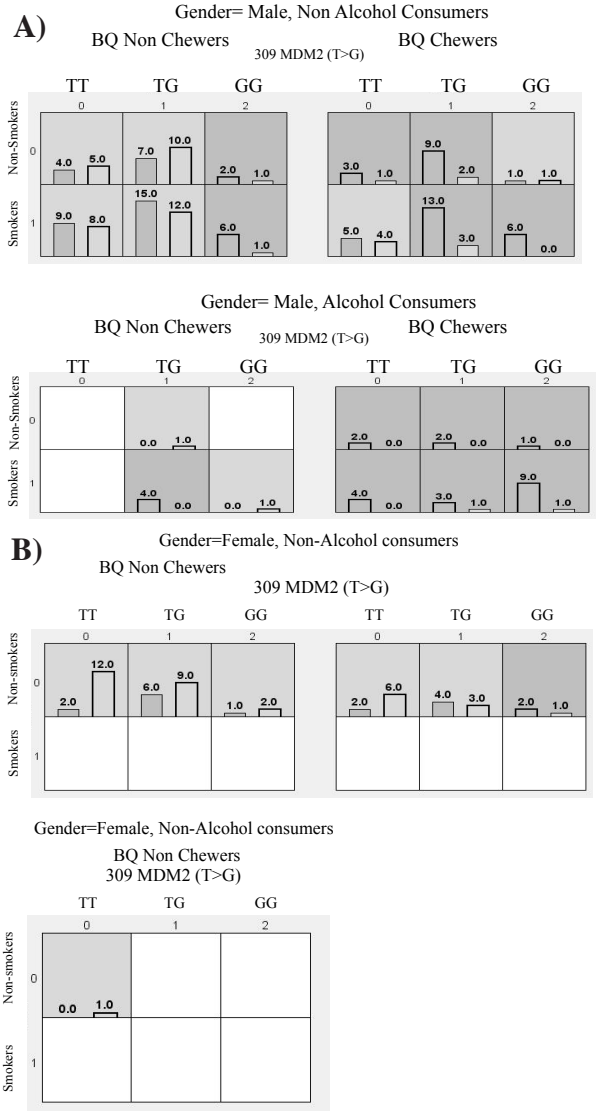

Figure 3. (MDR) Analysis of Genetic and Environmental Factors: Summary of the Five Factors Model, (MDM2, Smoking, BQ, Alcohol and Gender) in (A) Males, and (B) Females. The distribution of high-risk (dark shading) ,low-risk (light shading), and no-data observed (white shading) combinations associated with HNC risk. The percentage of patients having HNC was represented by left column (dark shaded) in each box, whereas right column (light shaded) in each box indicated percentage of controls

Effect of interaction between smoking, BQ chewing and/or alcohol consumption with MDM2 gene polymorphism on HNC risk

Interaction between smoking, BQ chewing and alcohol consumption and *MDM2* polymorphism was determined between cases with various genotypes reporting these habits at varied doses in comparison to non- smokers, BQ non-chewers and alcohol non-consumers with the wild type TT genotype. It was observed that heavy smokers with mutant GG genotype had 13.5 fold higher risk ($p<0.0001$), heavy smokers with TG genotype showed a 4.7 fold higher risk (p=0.003), for HNC in comparison to non smokers carrying wild type TT genotype (Table 3).

Analysis of interaction between BQ chewing and

Table 3. Interaction of Smoking, Betel-Quid Chewing and Alcohol Consumption with *MDM2* Gene Polymorphism in HNC

Smoking Habits	309 *MDM2* (T>G)	Controls	Cases	OR	95% CI	p-value
Non smokers	TT	25	13	1 (Ref.)		
	TG	27	28	1.99	0.8-4.7	0.12
	GG	3	7	4.5	0.99-20.3	0.07
Heavy smokers	TT	12	18	2.9	1.1-7.8	0.049
	TG	11	27	4.7	1.8-12.5	0.003
	GG	3	21	13.5	3.4-53.7	<0.0001
Moderate smokers	TT	0	0	----------	----------	----------
	TG	5	8	3.08	0.8-11.3	0.109
	GG	0	0	----------	----------	----------
BQ chewing Habits	309 *MDM2* (T>G)	Controls	Cases	OR	95% CI	p-value
BQ non chewers	TT	26	15	1(Ref.)		
	TG	34	32	1.6	0.7-3.6	0.2
	GG	3	9	5.2	1.2-22.2	0.03
BQ Heavy chewers	TT	8	13	2.8	0.95-8.3	0.07
	TG	7	23	5.7	1.97-16.4	0.002
	GG	3	18	10.4	2.6-41.3	0.0004
BQ Moderate chewers	TT	3	3	1.7	0.3-9.7	0.7
	TG	2	8	6.9	1.3-37.02	0.03
	GG	0	1	5.1	0.2-133.9	0.4
Alcohol consumption Habits	309 *MDM2* (T>G)	Controls	Cases	OR	95% CI	p-value
Non consumers	TT	36	26	1 (Ref.)		
	TG	41	54	1.8	0.95-3.5	0.08
	GG	4	17	5.9	1.8-19.6	0.002
Heavy consumers	TT	0	0	----------	----------	----------
	TG	2	9	6.2	1.2-31.3	0.021
	GG	2	11	7.6	1.6-37.3	0.006
Moderate consumers	TT	1	5	6.9	0.8-62.9	0.09
	TG	0	0	----------	----------	----------
	GG	0	0	----------	----------	----------

* indicates statistical significance at p<0.05; ----------could not be calculated due to absence of particular genotype in the sample.

MDM2 polymorphism suggested that Heavy chewers with TG and GG genotypes had 5.7 (p=0.002) and 10.4 (p=0.0004) fold higher risk respectively, while moderate chewers with TG genotype had a 6.93 fold increased risk (p=0.030) for HNC in comparison to non chewers with the TT genotype. Increased risk (p=0.03) at 5.2 fold for HNC was also observed in non chewers carrying mutant GG genotype in comparison to non chewers with the TT genotype (Table 3).

Heavy alcohol- consumers with TG and GG genotypes had 6.2 (p=0.021) and 7.6 (p=0.006) fold higher risk respectively for HNC than non consumers with wild type TT genotype (Table 3).

MDR analysis result

The best model chosen by MDR analysis was two factors (BQ, Gender) model with 90% CVC and 0.6419 testing balance accuracy at p<0.0001. On the other hand five factors model (MDM2, Smoking, BQ, Alcohol, and Gender) had maximum CVC of 10 in 10 and testing balance accuracy of 0.6043 at p<0.0001 (Figure 3A & B).

Discussion

A large number of case control studies as well as meta analysis studies have reported the significant role of *MDM2* 309 (T>G) polymorphism as a predictive biomarker for colorectal, breast, gastric, leukaemia and liver cancers (Ma et al., 2012; Gao et al., 2014; Song et al., 2014; Tang et al., 2014; Wang et al., 2014; Yan et al., 2014) in different populations. A meta-analysis on *MDM2* SNP309, gene-gene interaction, and tumor susceptibility showed that the GG genotype of *MDM2* 309 polymorphism is significantly associated with tumor risk in Asian population and possibly interacts with the p53 72 CC genotype in increasing the risk of carcinogenesis (Wan et al., 2011). Xiao et al. (2010) also reported that the *MDM2* 309 (T>G) polymorphism increases the risk for nasopharyngeal cancer in Chinese population (Xiao et al., 2010). In our study it was clearly visible that HNC risk associated with *MDM2* 309 GG polymorphism was 1.91 fold which indicates that 309*MDM2* could be used as a predictive biomarker for the Northeast Indian population (Table 1). The study also indicates that the 72 *TP53* polymorphism contributes to HNC risk in this population upon interaction with the *MDM2* GG genotype, but not alone. This is consistent with the findings of Suresh et al. (2010) in South Indian population (Suresh et al., 2010). We observed a gender-specific difference in HNC risk in association with *MDM2* polymorphism SNP309, with females carrying the GG genotype being at a greater risk than males (Table 2). Other studies have reported that head and neck squamous cell carcinoma tumors and cell lines expressed estrogen receptor and homozygous

condition of the G allele of the *MDM2* gene increases *MDM2* expression in an estrogen responsive manner, which further attenuates the p53 pathway, resulting in carcinogenesis (Yu et al., 2011). It may therefore be postulated that estrogen receptors may play a vital role in promoting HNC in females of the Northeast Indian population, in conjunction with *MDM2* polymorphism, though this hypothesis remains to be experimentally evaluated.

Majority of respondents in this study reported the habit of BQ chewing irrespective of sex and age, while only males reported the habits of smoking and very few reported alcohol consumption. The International Agency for Research on Cancer (IARC) has classified BQ with or without tobacco and areca nut as a *Group 1* carcinogen, that is, carcinogenic to humans (Cancer and Humans, 2004). It is clear that while a single etiological habit can result in a risk for HNC, the risk is compounded in individuals having multiple habits. However, among the three habits, BQ contributed the most significant risk for HNC to this population through logistic regression analysis which is consistent with the one factor model of MDR analysis with 90% CVC and 0.6241 test balance accuracy. The two factor model (BQ, Gender) with 90% CVC and 0.6419 test balance accuracy supported our finding of both genders involved in BQ mastication. The best five factors model (MDM2, Smoking, BQ, Alcohol and Gender) with 100% CVC and 0.6043 test balance accuracy supported our findings of different folds increased risk of HNC in males and females with respect to different etiological habits and *MDM2* genotypes.

In conclusion, the present case control study supported the hypothesis that *MDM2* 309 T>G polymorphism could serve as a predictive biomarker for HNC risk in Northeast Indian population, alone as well as upon interaction with the *TP53* 72 G>C polymorphism. Females with the *MDM2* GG genotype were more prone to HNC than males despite not smoking or consuming alcohol. Finally, BQ was found to be the major etiological risk factor for increasing the risk for HNC in this population, upon interaction with the *MDM2* GG genotype.

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