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


Accumulation of mutations over the complete mitochondrial genome in tobacco-related oral cancer from northeast India

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Association of mitochondrial D-loop mutations with GSTM1 and GSTTI polymorphisms in oral carcinoma: A case control study from Northeast India

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SUMMARY

Objectives: Mitochondrial dysfunction is a hallmark of cancer cells. Tobacco consumption in various forms is one of the major risk factors for the development of oral squamous cell carcinoma which makes the mitochondrial DNA susceptible to damage by reactive oxygen species. The GSTTI and GSTM1 members of the glutathione S-transferase multigene family are candidate carcinogen metabolizing genes. Here we determined the hot spot mutations in the D-loop region and revealing correlation if any, with clinical parameters, along with analysing the genetic polymorphism of GSTM1 and GSTTI and its susceptibility towards oral cancer.

Materials and methods: To determine the hot spot mutations 25 matched tissue samples of OSCC patients with 25 control subjects were used for PCR and direct sequencing. Analysis for GSTM1 and GSTTI gene polymorphism was done by multiplex PCR.

Results: Several mutations were found within the D-loop region among which mutations at nt46, nt52 and nt195 are found to be hot spot (P < 0.0001, P < 0.0001 and P < 0.001 respectively). A significant association was found between the numbers of D-loop mutation and GSTTI (OR = 2.03; 95% CI, 1.04-3.95, P = 0.003), GSTTI (OR = 1.73; 95% CI, 1.10-2.71, P = 0.0027) null genotypes respectively. We observed a significant correlation between the increased number of D-loop mutations with the advancement in tumour stage of the patients (P = 0.009, r = 0.48).

Conclusion: The association of null genotypes and mutations can be used as a possible biomarker for early detection and preventive measure of oral cancer for those habituated to tobacco consumption.
region lies in a conserved sequence block that is hypothesised to be involved in some aspect of mtDNA replication and transcription. The development of cancer is influenced by both genetic and environmental factors. This environment–gene interaction on carcinogenesis 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 the phase II enzyme like GST (Glutathione S-transferase) are involved in detoxication of the activated metabolites of the carcinogens. Polymorphisms in the genes that code for these enzymes may alter expression or function, thus increasing or decreasing the activation or detoxication of carcinogenic compounds. Tobacco smoke is a complex mixture of carcinogenic compounds, and smokeless tobacco is rich in nitrates. Furthermore, the concomitant use of betel quid leads to 50-fold increase in reactive oxygen species generation. In phase II enzymes like GSTM1 catalyses with the conjugation of the tripeptide GSH to PAH diol epoxides whereas GSTT1 participates in conjugation of the monohalomethanes and reactive diol epoxides. A structural deletion in these genes represents a null genotype and has been associated with an increased risk to oral cancer.

The present study was aimed to investigate the oral cancer patients on the effect of betel quid and tobacco use on the D-loop region mutation, the association of D-loop mutations and GSTT1 and GSTM1 gene polymorphism to the risk towards oral cancer progression along with establishing the hot spot mutations in the D-loop region in patients of OSCC, which can be a possible biomarker for the early detection of oral cancer. We have also determined the correlation between the D-loop mutations with that of tumour stage of the OSCC patients.

Materials and methods

Ethics statement

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

Sample collection

The case–control study comprised of 25 OSCC patients and 25 control subjects. The sample collection started from 1st February–20th October 2010. Twenty five OSCC patients tumour tissue samples and matched blood were collected from the biorepository of Cachar Cancer Hospital and Research Centre (CCHRC) at Meherpur, Assam. We also obtained the control oral swab from inner cavity samples from 25 non-OSCC subjects. Written Informed consent was obtained from all subjects and the personal details were recorded using a structured questionnaire upon interview. 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. All possible precaution was taken to avoid any cross-contamination while collecting as well as processing of the samples. Tumours were located in the oral cavity (n = 12), gingiva (n = 5) and cheek (n = 8). They were also at following stages: 4 at stage I, 6 at stage II, 12 at stage III and 3 at stage IV respectively.

DNA isolation

For each sample, DNA was extracted from preselected regions of tumour tissue and matched blood. The tissue samples and matched blood were digested in TES buffer and incubated overnight at 55 °C. DNA was subsequently isolated by phenol/chloroform/isoamylalcohol method followed by ethanol precipitation and re-suspended in TE buffer and stored at −20 °C.

PCR amplification

PCR amplification was done using Fermentas High Fidelity PCR Enzyme Mix (Cat No. K01920). The total reaction volume was 20 µl containing 2 µl of 10× PCR buffer with MgCl2, 2 µl of 10 mM dNTPs, 0.2 µl (5 units/µl) of Taq DNA polymerase, 1 µl each of 20 pmol/µl forward and reverse primer, 50–100 ng of genomic DNA and the volume make up to 20 µl by adding nuclease-free water.

Primers for D-loop amplification

The published primers used for D-loop amplification study to accomplish the objective were: forward primer dLhuF S'-CAG-GCCTTACTGGTCTCTGATTTCTC-3' and reverse primer dLhuR 5'-GAG-GTAGTCATCTACATAAACCTGTC-3' which amplify an initial 649 bp PCR product. The PCR programme used for amplification was: initial denaturation step was done at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s; annealing at 55 °C for 45 s; extension at 72 °C for 90 s. The amplified product was observed in 1.5% agarose gel.

Primers for GSTM1 and GSTT1 gene polymorphism

For each sample, DNA was extracted from preselected regions of tumour tissue and matched blood. The tissue samples and matched blood were digested in TES buffer and incubated overnight at 55 °C. DNA was subsequently isolated by phenol/chloroform/isoamylalcohol method followed by ethanol precipitation and re-suspended in TE buffer and stored at −20 °C.

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. Statistical analysis for association studies were done with Spearman Rank Correlation. To establish statistical significance Chi square and Fischer exact test were performed. Relative risk was estimated in terms of Odds Ratio (OR) and the Cornfield
95% confidence interval (95% CI). P values (two sided) below 0.05 were considered statistically significant.

Results

Study of population

The characteristics of the study population were summarized in Table 1. There were no statistically significant differences between the cases and control subjects in terms of age (P = 0.99), gender (P = 0.76), smoking habit (P = 1) and alcohol consumption (P = 0.14). However, significant differences were observed in tobacco (P = 0.002) and betel quid chewing (P = 0.002) between the cases and controls.

Heteroplasmy and homoplasmy of mitochondrial DNA in cancer

A cancer cell contains many mitochondria with multiple copies of mitochondrial DNA; mutations in mtDNA can be heteroplasmic or homoplasmic. The frequencies of the D-loop mutations were very high. Of these mutations found in tumour tissue excluding the D310 region, 73% of the mutation were heteroplasmy and 27% of the mutation were homoplasmy whereas in the D310 region sequence in the tissue, 76% were heteroplasmic and 24% homoplasmic (Fig. 1A,1B). Here the majority of the mtDNA mutations in the D-loop region detected were heteroplastic. It has been reported that heteroplasmatic mitochondrial DNA mutation promotes tumorigenesis by making alteration in ROS generation and apoptosis. However, the mechanism of the causal homoplasmic mitochondrial DNA alterations and their potential role in the alterations of tumorigenesis is poorly understood.

Mutation detection in the D310 region

The C-tract region is highly polymorphic in the human population. The number of cytosines in the 7 bp tract varies from 6 to 13 bp. The most frequent sequences for the D310 region are C,TC, C,TC, and C,TC. In our study the distribution of number of cytosines in the first stretch of D310 tract has been shown in Fig. 1C. The most common variants in matched blood were 7-C and 8-C followed by 9-C whereas in case of tumour the most common occurrence was of 9-C and 8-C followed by 7-C. The insertion and deletion of cytosine in C-tract region from matched blood to
tumour tissue for each OSCC patients has been shown in Table 2. Of the 25 cases with the C-tract alterations, 1 of 25 (4%) had single base pair alterations and 24 of 25 (96%) of cases had multiple base pair alterations. In 5 (20%) patients out of 25, thymine at nt 310 was missing due to substitution of T → C which is referred to as D310 region with variation in numbers of cytosine in the C-stretch (GenBank ID: JN933656-86). Mutation with deletion T has been reported in MITOMAP. We did not find any strong association between the increased numbers of cytosines in the D310 region with that of the clinical parameters.

**Mutation detection in the D-loop**

Different types of mutations were observed in the D-loop region between nt 51 and 595. The mutations were base substitution 3(3.8%) transversions (A → T), transversions (C → G), 5(6.4%) transversions (C → A), 2(2.6%) transversions (G → C), 1(1.3%) transversion (T → G), 1(1.3%) transversion (T → A), 2(2.6%) transitions (C → T), 31(39.7%) transitions (T → C), 7(9%) transitions (A → G), 1(1.3%) and transition (G → A) (Table 1). Out of the 25 patients 16(64%) patients had mutation at nucleotide position 146, 17(68%) patients had mutation at nucleotide position 152 and 10(40%) patients had mutation at nucleotide position 196 and 7(28%) patients had mutation in nt 146, nt152 and nt 196 (Fig 2A) that occurred simultaneously and were significant mutational hotspots with (P < 0.001, P < 0.001 and P < 0.01 respectively). In case of the subject controls, we did not found any mutation in the D-loop region (GenBank ID: JN603607-25) for those who are non-chewers (betel quid and tobacco) whereas in ten control subjects with the habits of betel quid and tobacco consumption were found to have D-loop mutation but were non-cancerous. Among these ten controls six were males and four were females. Relationship of mtDNA D-loop mutation to clinical parameters has been established in this study, i.e., a significant association was observed between the increased number of mutations in D-loop with the advancement in tumour stage of the patients (P = 0.009, r = 0.48) (Fig 2B).

**GSTM1 and GSTTI polymorphism**

The null genotype was detected by the absence of the band either in case of GSTTI or GSTM1 or both (Fig 3A). The frequency of GSTM1 null genotype was found to be 15(60%), GSTTI null genotype 10(40%) and both GSTTI and GSTM1 null genotype 6(24%) of the patients respectively. High frequencies of somatic mutations are reported in the mtDNA for OSCC cases in our series, 56% of D-loop mutation occurs in the null GSTM1, whereas 36% of D-loop mutation occurs in the null GSTTI. In controls 32% of D-loop mutation occurs in the null GSTM1, whereas 36% of D-loop mutations occur in the null GSTTI. We observed a risk of 3.6-fold to mtDNA mutations (95% CI, 1.07-12.54, P = 0.03) due to null genotypes of GSTM1 and further the risk increases 2.8-fold (95% CI, 0.82-10.03, P = 0.09) due to null GSTTI (Table 3). Moreover, we have also found a significant association between GSTM1 and GSTTI null genotypes with the increased number of D-loop mutation (OR = 2.03; 95% CI, 1.04-3.96, P = 0.003 and OR = 1.73; 95% CI, 1.10-2.71, P = 0.0027 respectively) and the risk increases further with both GSTTI and GSTM1 null genotypes with the increased number of mutations (OR = 7.77; 95% CI, 1.13-53.28, P = 0.036) (Fig 3B).

**Discussion**

The escalated number of tobacco-related OSCC cases is a major concern. The reasons may be the poor socio-economic condition, oral consumption of tobacco in its various forms, use of lime with betel-leaf and betel nuts, alcohol and smoking habits and lack of awareness. We present for the first time a study of mutation in the selected region of mitochondrial genome in OSCC patients from Northeast India. The present study investigated the D-loop region in 25 OSCC tumour tissues and matched peripheral blood to detect mutations in mtDNA, which might be related to betel quid, tobacco chewing and smoking. Our study suggests that escalated consumption of tobacco in different forms result in increased ROS production that causes mtDNA mutations, which seem to be an important biological consequence and can also initiate or promote oral carcinogenesis. In the D-loop region overall 24 mutations at different nucleotide positions were found. Among these mutations, nucleotide position 146,152 and 196 occurred repeatedly in most of the patients in this study. Although mutations at nt 146 and 152 have already been reported as hotspot OSCC and ovarian carcinoma whereas hotspot mutation at the nucleotide position 196 is novel finding in our study, and has not yet been reported elsewhere in case of OSCC. The mutations reported in oesophageal SCC and our findings may have epidemiological relevance since both oral and oesophageal SCC occur in predominantly in tobacco users. Whereas in the control noncancerous subjects, the mutation found in the ten chewers may be at the risk of being affected by cancer because in nonchewers we did not found any mutation in the D-loop region. So from the observation we can say that consumption of tobacco and betel quid which generates increased ROS production and in turn causing mutation to D-loop, which can eventually lead to the progression of cancer. In most of the cases in our study in D-loop mutations, we found 26(33.3%) were transitions (C → T), 31(39.7%) were transitions (T → C) which have characteristics that accounts for a number of potent mutagens known to occur in tobacco smoking and also for oxidative damage. We were successful in establishing correlation between the increasing numbers of mutations in D-loop region with the increasing tumour stage of the patients which may be helpful in the prognosis of the disease. Reports are very few where the correlation of mtDNA mutations with that of clinical parameters has been shown. However, there are certain reports, which have mentioned regarding the lacking of prognostic utility of mtDNA mutations and in their study; they did not found any correlation of D-loop mutation with that of clinical parameters.

The D310 region is located in the conserved sequence block that is most likely controls the mtDNA replication and transcription, and it is possible that severe C-tract length variations may have increased ROS production and in turn causing mutarion to D-loop. which is known to occur in tobacco smoking and also for oxidative damage. Both homoplasmic and heteroplasmic mutations have been frequently observed in mitochondrial diseases, most likely because homoplasmic mutations would be lethal and in polymorphic sites such as D310.

Heteroplasmy is one of the distinctive features in mitochondrial DNA mutations. With increasing mutant mtDNA both endogenous respiration and ATP synthesis declined progressively and at the same time lactate level and dependence on glucose increases, which indicates a switch from mitochondrial oxidative phosphorylation to glycolysis from ATP production. However, the majority of the mutations found in our study are 2 bp expansion, and this alteration of 2 bp still remains a pan el
Table 2
Details of hotspot mitochondrial D-loop mutations and CSTM1-CSTM1 polymorphism in patients.

<table>
<thead>
<tr>
<th>St.No.</th>
<th>Age</th>
<th>Sex</th>
<th>Betel quid/tobacco use</th>
<th>Alcohol consumption</th>
<th>Nucleotide position in D-loop</th>
<th>Ins/del compared with matched blood</th>
<th>DNA (N=1T)</th>
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Table 2 (continued)

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<th>DNA (N-T)</th>
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</table>

Patients are identified by code number. F. female; M. male. Mutations are sectioned by column according to nucleotide position and described according to nucleotide change, e.g. A → C means A was present in matched blood but changed to C in tumour tissue. Betel quid chewer is denoted by alphabet - a, tobacco chewer - b, smoker - c and alcohol consumption - d. For insertions and deletions: + indicates a single insertion of C - T indicates a single deletion of T. NC indicates no change. Tumour stages denoted by the respective numerals e.g. stage II = 2. 'O' stands for absence and 'I' for presence in GSTT1 and GSTM1 polymorphisms. 'N'-matched blood; 'T' - tumour tissue. HM - homoplasmy, HT - heteroplasmy.

Figure 2 Hotspot mutations in D-loop and correlation with tumour stage. (A) Hotspot mutations at nucleotide positions 146, 152 and 196 in the D-loop region with nucleotide change from matched blood to tumour tissue. (B) Correlation between the increasing numbers of mutations in D-loop region with the increasing tumour stage (I-IV) of the patients (1-25).

wild-type distribution and functional difference of this mutation remains unclear. Furthermore, an alteration in this region is less likely to be the hot spot region because C-tract region as is known to be polymorphic and contain between seven to nine cytosine residues within the normal population. The increased risk factor of null GSTM1 and GSTT1 are essential for detoxification of carcinogenic compound. The most important risk factors 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. The increased risk factor of null GSTM1 in oral cancer is more than that of null GSTT1 as revealed from the results as GSTM1 enzyme possibly plays inside the mitochondrial matrix as mtDNA protection factor regarding damage caused by reactive oxygen species. Here we have found an risk of 3-4 folds in the patients with null GSTT1 and GSTM1 along with having increased number of D-loop mutation, which might have occurred due to ROS production by increased consumption of tobacco and betel quid and also individual having tobacco and betel quid practice with null genotypes have high risk of oral cancer. Finally, betel quid contains tender areca nuts and lime and smokeless tobacco that have been shown to generate ROS and induce oxidative damage, and also genetic polymorphism of certain genes can increase the risk of oral carcinogenesis as the development of cancer is influenced by both the genetic and environmental factors. A study has addressed the direct correlation of null genotypes of GSTs in HNSCC and impact of chemotherapy in null GSTs subjects. However, our study has addressed the correlation of D-loop mutations with null genotypes of GSTT1 and GSTM1 in the individuals with the betel quid-tobacco chewers which have been shown to generate ROS and induce oxidative damage, which in turn may be a cause of the increase mitochondrial mutation and thereby increases the risk of oral carcinogenesis as the development of cancer is influenced by both...
Oxidation-reduction (redox) reactions that generate ROS have been identified as important mediators in the regulation of signal transduction processes involved in cell growth, differentiation and cell death. Generally, it is believed that ROS is a relevant class of carcinogens and 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 growth of pre-malignant cells]; and progression [the stimulation of metastatic potential].

the genetic and environmental factors which is completely different from the study made by Ruwali et al. At present, we have insufficient information for non-availability of complete treatment records of the patients those were under treatment and most of the patients moved for alternative medicines available elsewhere to avoid repeated chemotherapy, which proved to be too costly to the patients.

Table 3

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Age</th>
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<th>Betel quid/tobacco use/alcohol consumption</th>
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<th>GSTT1</th>
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</tbody>
</table>

Controls gender is identified by alphabets. F - female; M - male. Betel quid chewer is denoted by alphabet - a. tobacco chewer - b. smoker - c and alcohol consumption - d. 'O' stands for absence and '1' for presence in GSTT1 and GSTMI polymorphisms.

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The figures and tables are as follows:

**Figures Polymorphism of CSTT1 and CSTM1 genes.** (A) Multiplex PCR of CSTM1 and CSTT1 polymorphism and CYPIA1 as internal control on 1.5% Gel. Lanes 2, 3, 6 and 8—CSTMI and CSTT1 positive; Lanes 5, 7, 9, 10, 12—null for CSTM1; Lanes 4 and 11—null for both CSTT1 and CSTM1. (B) Significant association between CSTT1 and CSTM1 null genotypes with the increased number of D-loop mutation (OR = 1.73; 95% CI = 1.10-2.71; P = 0.0027 and OR = 2.03; 95% CI = 1.04-3.96; P = 0.003 respectively) and further the risk increases with both CSTT1 and CSTM1 null genotypes with increased number of mutations (OR = 7.77; 95% CI = 1.13-53.28; P = 0.036).
Acknowledgements

about role, patterns and timing of mitochondrial mutations in

Conclusion

ities (BT/Med/NE-SFC/2009) for conducting research on Cancer and

Conflict of interest statement

results of the patients because of the fan that most of the patients in

References

Some systems, ROS mediates both pro- and anti-apoptosis effects,
depending on the ROS concentration. Although mitochondrial
defects have been implicated in the development and progression
of cancer for several decades, but in contrast, a report has shown
that somatic D-loop of mtDNA was associated with better survival.
However, our study lacks the follow-up re-

Author's personal copy

neither the role of mitochondrial mutation in tumour biology and in particular,
mutations present in specific type of tumour may be helpful in

Acknowledgements

Constitution of mitochondrial DNA. In this regard, our finding of

4. Gupta PC Ray CS. Smokeless tobacco and health in India and South Asia.

We are unable to afford the expenditure of treatment. Also, some patients due to their poor socio-economic

None declared.

Impact of conflict of interest

None declared.

Our humble acknowledgement goes to the Department of Bio-
technology (DBT), Govt. of India for providing infra-structural facil-
ities (BT/Med/INE-SFC/2009) for conducting research on Cancer and


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


Department of Biotechnology, Assam University, Silchar, Assam, India

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 mtDNA copy number was found positively correlated with smoking and alcohol having synergistic effects [9,10]. Mitochondrial DNA copy number and risk of OSCC increased with the decreased mtDNA copy number (P<0.0001). 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=0.005). Significant difference was observed between GSTM1/GSTT1 null genotypes (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.

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-gen 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 expounding their functional groups whereas phase II enzyme like GST (Glutathione S-transferase) are involved in detoxication of the activated derivatives of the carcinogens [11]. Tobacco smoke is a complex mixture of carcinogenic compounds, and smokeless tobacco is rich in nitroaromatics. 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 has 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 hospitals as well as from home with written Informed consent and approved by IRB. The availability of such controls alone in the hospitals was impossible as most of the patients come with their relations. All the cases were diagnosed as OSCE 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), Mcherpur, 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]. Biomide Isolate Genomic DNA minikit (Biolinc, UK) was used for isolation of genomic DNA from FFPE tissues following manufacturer's instructions.

Multiplex PCR for GSTM1 and GSTT1

Analysis for GSTM1 and 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 GSTM1 was 5'-TTGCTTACTGGTCCTCAGCATCCT-3' and R 5'-TACGGGAATCATGCGCCAGCA-3', GSTM1 was 5'-GAACTTCCCTGAAACGCCTAAAGC-3' and R 5'-GTGGGGCTCATAATACGGTGCG-3', CYP1A1 was 5'-ACTGGCAGCTTCAGCTGCT-3' and R 5'-GTTGCAGATTGGGAAGCTGCT-3' respectively [38,39]. The PCR programme used for amplification was: initial denaturation step at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30; 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 subvype 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 analysed 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 5'-CAGGGTCTAATAAGCTTAAATGG-3' and R 5'-CAGGGTCTAATAAGCTTAAATGG-3' respectively [38,39]. The PCR programme used for amplification was: initial denaturation step at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30; annealing at 59°C for 45s and elongation at 72°C for 90s. The amplified product was observed in 1.5% agarose gel.

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...