Chapter 5

Discussion

Control region

<table>
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<th>TRN4Aphe</th>
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<td>16365</td>
<td>173</td>
<td>340</td>
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16024 → 16569/10 D310 C-tract 576

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CAAACCCCTCCCTCCCCCGGTTTCT

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Chapter 5

DISCUSSION

This chapter has been divided into three sections based on the objectives followed by the result section. The three sections are namely Section 5.1 where the discussion is on the results of the mitochondrial mutation, GSTS polymorphism and HPV infection in head and neck squamous cell carcinoma, Section 5.2 deals with the complete mitochondrial genome sequences by latest Ion torrent sequencing technology and find out the somatic Mutations and Head and neck Squamous Cell Carcinoma and last Section 5.3 discusses on mitochondrial content variation in head and neck cancer patients and controls for early detection.

5.1 Mitochondrial Mutation, GSTs Polymorphism and HPV Infection in head and neck Squamous Cell Carcinoma

The escalated number of tobacco related HNSCC 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 selected region of mitochondrial genome in HNSCC patients from North East India.

The present study investigated the D-loop region in 25 head and neck squamous cell carcinoma 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 cause mtDNA mutations which seems to be an important biological consequence and can also initiate or promote oral carcinogenesis (Lievre et al., 2006). In the D-loop region overall 24 mutations at different nucleotide positions were found. Among these mutations, nucleotide position at 146, 152 and 196 occurred repeatedly in most of the patients under this study. Although mutations at nt 146 and 152 have already been reported as hotspot HNSCC (Prior et al., 2006) and ovarian carcinoma (Liu et al., 2001) whereas hotspot mutation at the nucleotide position 196 is novel finding in our
study and has not yet been reported elsewhere in case of HNSCC. The mutations reported in oesophageal SCC (Kumimoto et al., 2004) 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 eight 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 the characteristics that accounts for a number of potent mutagens known to occur in tobacco smoking and also for oxidative damage (Prior et al., 2006). We were successful in establishing correlation between the D-loop mutation and clinical parameters. Reports are very few where correlation of mtDNA mutations with that of clinical parameters. In this study, there is significant association between the increasing number of mutations in D-loop excluding the D310 region with the increasing tumor stage of the patients \((P<0.001, r =0.74)\) was established which may be helpful in the prognosis of the disease (Matsuyama et al., 2003).

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 an important role in regulation of mtDNA metabolism (Mambo et al., 2003). In this study we observed that the patients with D310 alterations in tumour DNA showed higher levels of heteroplasmy. Heteroplasmy, at specific mitochondrial genes, have been reported in mitochondrial diseases, most likely because homoplasmic mutations would be lethal(Sanchez-Cespedes et al., 2001), and in polymorphic sites such as D310 (Marchington et al., 1997, Sanchez-Cespedes et al., 2001). MtDNA mutations enriched to a certain level of heteroplasmy would enhance tumor progression owing to either the elevated ROS generation which in turn activates the oncogenic pathways or to the increase in genome instability or to both (Park et al., 2009). Both homoplasmic and heteroplasmic mutations have been frequently observed in cancer cells, the exact mechanism by which homoplasmic arises from heteroplasmic mutations is uncertain. Possible mechanisms include the selection or clonal expansion of the mutants with growth.
or survival advantage or a random segregation of the mutant (Fliss et al., 2000, Carew et al., 2003). 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 (Park et al., 2009). However, the majority of the mutations found in our study are 2bp expansion and this alteration of 2bp still remains a part of wild-type distribution and functional difference of this mutation remains unclear. Furthermore, an alterations in this region is less likely to be hot spot region as because C-tract region as is known to be polymorphic and contain between seven to nine cytosine residues within the normal population (Lievre et al., 2006).

Presence of both GSTM1 and GSTT1 are essential for detoxication of carcinogenic compound. The most important risk factors for head and neck cancer is smoking, tobacco chewing and betel quid. Tobacco smoke contains pyrolysis products, which are generated due to high temperatures at the burning tip, whereas smokeless tobacco is rich in nitrosamines, PAHs aldehydes and ketones (Anantharaman et al., 2007). The concomitant use of betel quid leads to a 50-fold increase in reactive oxygen species generated (Nair et al., 1999). The increased risk factor of null GSTM1 in head and neck 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 ROS. 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 (Yadav et al., 2010, Agrawal et al., 2010). The distribution of these genotypes suggested a potential influence in the incidence of head and neck squamous cell carcinoma (HNSCC). 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.
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 (Finkel, 2000, Finkel, 2003). Generally it is believed that ROS are a relevant class of carcinogens (Park et al., 2009), 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 tumorigenic expansion of the cell clone]; and progression [the malignant conversion of the tumor to cancer]. Interestingly, in some systems, ROS mediate both pro- and anti-apoptosis effects, depending on the ROS concentration (Park et al., 2009). Mitochondrial defects have been implicated in the development and progression of cancer for several decades (Mambo et al., 2003, Ha et al., 2002, Jiang & Califano, 2004, Kumimoto et al., 2004).

We report for the first time the prevalence of HPV in HNSCC in Northeast India. HPV infection, particularly, high-risk HPV is a known independent causative factor for head and neck cancer. When a cell is infected with HPV, the $E7$ gene binds to $Rb$ so that the $Rb$ releases $E2F$ and the other proteins. This is a signal for the cell cycle to progress. As long as the $E7$ stays attached to $Rb$, the cell cycle will continue to happen, thus causing a cycle of uncontrolled cell reproduction (Wiest et al., 2002). Similarly, viral $E6$ protein binds to $p53$, and makes it inactive. This allows the virus to take over the cell and reproduce itself, since the virally inhibited $p53$ cannot stop it, or begin the process of cell death. The repeated replication of cells with erroneous DNA information is the beginning of malignant tumour formation. Along with blocking the cell's $p53$, the viral $E6$ protein activates telomerase, an enzyme that synthesizes the telomere repeat sequences. Activating this enzyme maintains a repeated cell cycle that continues to produce viral cells (zur Hausen, 2002). This leads to malignancy as the mutant cells continue uncontrolled reproduce. In the present study, we have found significant correlation of HPV infection with mitochondrial mutation which was also reported in a study of cervical cancer (Sharma et al., 2005). Bak protein is a pro-apoptotic member which localizes in mitochondria, and functions to induce apoptosis. Elimination of Bak protein by HPV $E6$ leads to a decrease in apoptosis. This $E6$ activity towards Bak is a key factor in promoting the survival of HPV-infected cells which in turn facilitates the tumour development.
5.2 Complete mitochondrial genome somatic mutations and head and neck squamous cell Carcinoma.

The present study investigated the 10 HNSCC tumour tissues and matched peripheral blood to detect mutations throughout the 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 (Lievre et al., 2006). In most of the cases in our study in mitochondrial mutations, we found transition 93 (31%) were of T>C and 73 (24.5%) of C>T, which have the characteristics that account for a number of potent mutagens known to occur in tobacco smoking and also for oxidative damage (Prior et al., 2006). The observation of the study suggests that the consumption of tobacco and betel quid, which generates increased ROS production and in turn causing mutation to mitochondria, thereby can eventually lead to the progression of cancer.

Mutations in mtDNA can be heteroplasmy or homoplasmy. The identification of heteroplasmy or homoplasmy and mixtures are technically challenging for analysis of mitochondrial DNA in cancer cells but with the PGM™ sequencer the reads and results generated gives a clear idea of definitive heteroplasmy and homoplasmy mutation. In this study, we observed that the patients with somatic mutations in tumour showed higher levels of homoplasmy and very less number of heteroplasmic mutation in specific nucleotide position. However, heteroplasmy at specific mitochondrial genes, have been reported in mitochondrial related diseases, most likely because homoplasmic mutations would be lethal (Sanchez-Cespedes et al., 2001). The mtDNA mutations enriched to a certain level of heteroplasmy would enhance tumor progression owing to either the elevated ROS generation which in turn activates the oncogenic pathways or to increase in genome instability or to both (Park et al., 2009). Both homoplasmic and heteroplasmic mutations have been frequently observed in cancer cells, the exact mechanism by which homoplasmcy arises from heteroplasmic mutations is uncertain. Possible mechanisms include the selection or clonal expansion of the mutants with growth or survival advantage or a random segregation of the mutant (Carew et al., 2003, Fliss et al., 2000).
In the D-loop region mutations at nucleotide positions T16294C, C16311T and C16325T of HVR1, G16463A and T16519C of VR1, T146C, T150C, A185G and T195C of HVR2 occurred more frequently in the patients of this study. The mutation in the nucleotide positions 16294, 16325 and 16463 are not previously been reported in any cancer in the MITOMAP somatic mutations however, reports are there for mutations at nucleotide positions 146 in ovarian cancer (Liu et al., 2001), 150 in breast (Tseng et al., 2006), prostate (Chen et al., 2002), thyroid (Maximo et al., 2002), lung cancers (Fliss et al., 2000), 185 in thyroid (Maximo et al., 2002) and glioblastoma (Kirches et al., 2001) and 195 in elderly fibroblasts and aging/AD brains (Coskun et al., 2004), also in tumors of lung (Liu et al., 2001), thyroid (Maximo et al., 2002), ovarian (Bragoszewski et al., 2008), prostate (Chen et al., 2002) and glioblastoma (Kirches et al., 2001). In our study, these nucleotide positions can be considered hot spot for D-loop region in HNSCC.

We found that almost all of the tumors with mtDNA mutations contained at least one of a D-loop, tRNA, ribosomal RNA, or non-synonymous coding mutation, thus possessing a potential for altering mitochondrial function via alterations in transcription, translation, or replication. Certain hot spot mutation are there in ND1 occurrence of mutation at nt4136 (60%) with amino acid change Cys to Tyr, ND5 at nt13542 (60%) and nt13869 (60%) and appeared simultaneously. The mutations in nucleotide position 13542 and 13869 are novel and have not yet been reported in any type of cancer in the MITOMAP.

Complex I have been found to play a role in apoptosis induction (Ricci et al., 2003, Lemarie & Grimm, 2011). Impairment of mitochondrial function by oxidative damage enhances the ROS production as a result of electron leakage within the aerobic respiration apparatus. The function is impaired particularly at Complex I (NADH dehydrogenase) and III (succinate-CoQ reductase) (Prior et al., 2006). These functional changes result in increased subcellular damage as antioxidant defenses are influenced (Prior et al., 2006, Staniek et al., 2002). Additionally, mitochondria that exhibit respiratory deficiencies as a result of mtDNA mutation, may thus release abnormally high levels of ROS into the cytosol, exposing the nucleus and other organelles of the cell to these cytotoxic compounds, potentially contributing to carcinogenesis (Staniek et al., 2002).
In the Complex III, mutation at nucleotide positions 15229, 15299 and 15721 occurred simultaneously in the patients which have the probability of being hotspot mutation for CYTB. These mutations are novel and not reported so far in any cancer in the MITOMAP. Synonymous mutations were found in ND1, ND5, COX1, ATP6 and CYTB (Table 1). It has been recently demonstrated that variations in synonymous codons in a defined gene can affect in vivo protein structure and thus alter function (Komar, 2007, Kimchi-Sarfaty et al., 2007). If this is the case for mitochondrial genes, the role of synonymous mtDNA mutations in cancer may not be neglected, given the fact that most mutations are synonymous mutations.

Although we have found a positive correlation between the increasing number of mitochondrial mutations with advancement in tumor stage of the patients, which may be helpful in the prognosis of the disease (Matsuyama et al., 2003), but more samples with different stages are needed to be studied. In our study most of the cases were at stage IV. Reports are very few where the correlation of mtDNA mutations with that of clinical parameters has been shown.

ROS have been identified as important mediators in the regulation of signal transduction processes which is involved in cell growth, differentiation and cell death (Finkel, 2000, Finkel, 2003). Generally, it is believed that ROS is a relevant class of carcinogens (Park et al., 2009), 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 tumorigenic expansion of the cell clone) and progression (the malignant conversion of the tumor to cancer). Interestingly, in some systems, ROS mediated both pro- and anti-apoptosis effects, depending on the ROS concentration (Park et al., 2009). Mitochondrial defects have been implicated in the development and progression of cancer for several decades (Ha et al., 2002, Jiang & Califano, 2004, Kumimoto et al., 2004). Also the alterations and mutations found in respiratory complexes permits the malignant cells to continuously grow thereby inhibition of apoptosis which is regarded as the hallmark of cancer cells.

All of these results discussed above suggest that mitochondrial mutations may play a role in tumor development. The knowledge of the role of mtDNA mutation in tumor
biology and in particular, mutations present in specific type of tumor may be helpful in assessing cancer risk, distinguishing between new primary cancer and recurrence. Our study is preliminary with small sample size, therefore difficult to give any statistical significance test for these mutations to be hot spot however, cannot be ignored in clinical point of view. Large-scale studies are needed to be done. Further research will be also needed to determine if mtDNA analysis has the specificity for detecting changes in smoke exposure or if the estimation of an individual’s risk could be improved by coupling of mitochondrial mutations to other markers for diseases associated to tobacco and betel quid chewing or smoking-related harm.

5.3 Mitochondrial DNA content variation in head and neck cancer patients and controls

The habit of chewing tobacco and betel quid 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 HNSCC 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 (Bagchi et al., 1999, Mahimkar et al., 2001). Antioxidant-rich foods such as green-leafy vegetables and fruits that may help reduce the oxidative stress caused by tobacco (Chopra et al., 2000, Poljsak, 2011) are usually lacking in the diet (Keusch, 2003, Saikat & Raja, 2011), the reasons may be the poor socio-economic condition and also customary practice of oral consumption.

HPV infection, particularly, high-risk HPV is a known independent causative factor in head and neck cancer. In the present study, we examined for HPV infection in the
individuals and found a significant difference with mtDNA content in cases and controls with or without HPV infection ($P<0.001$). 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 (Sharma et al., 2005). Bak protein is pro-apoptotic member which localizes in mitochondria, and functions to induce apoptosis. Elimination of Bak protein by HPV $E6$ leads to a decrease in apoptosis. This $E6$ activity towards Bak is a key factor in promoting the survival of HPV-infected cells which in turn facilitates the tumour development. Therefore, we can say that HPV play some role in mtDNA copy number variation for which the exact mechanism is yet to be unrevealed. 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 $P=0.001$ respectively) was observed. The presence of both $GSTM1$ and $GSTT1$ are essential for detoxication of carcinogenic compound. The most important risk factor for head and neck 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 (Nair et al., 1999). The increased risk factor of null $GST$s 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 HNSCC as well. No reports are there, until now, where the associations of $GST$s null genotypes and mtDNA content have been reported. 

In this study we demonstrated that low levels of mtDNA copy number in tobacco-betel quid chewers are associated with risk of HNSCC. During chewing substantial amounts of ROS (Nair et al., 1992) which in turn increase mtDNA mutation in human oral tissues and that accumulation of mtDNA deletions and subsequent cytoplasmic segregation of these mutations during cell division could be important contributors to the early phase of HNSCC (Lee et al., 2001, Sharan et al., 2012). The depletion in mtDNA may be result of the repression of mitochondrial biogenesis. The mtDNA copy number in cancer might depend on several factors, including the site of mutation in the mitochondrial genome. For
example, mutation in the D-loop region of the mtDNA may result in decreases copy number. As it has been demonstrated that the D-loop region was highly susceptible to oxidative damage compared with the other regions of the mtDNA (Yu et al., 2008). The findings of the present study well demonstrate the risk of HNSCC and mtDNA copy number to tobacco-betel quid chewers in this region. We did not evaluate the cancer tissue specimens before treatment due to its non availability from the biorepository. 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. Furthermore, ROS are an important determinant of cancer risk. Tobacco smoke or smokeless induces oxidative stress by creating ROS within the human body (Church & Pryor, 1985, Pryor & Stone, 1993). Given that mitochondria are highly susceptible to ROS (Kroemer, 2003), mtDNA copy number may serve as a biomarker for exogenous and endogenous exposures that are associated with subsequent tobacco-related cancer risk (Hosgood et al., 2010).

The mtDNA content correlated with histopathological tumour stage and observed that the mtDNA content decreases with the advancement in tumour stage ($P<0.001$). Similar results were reported in posttreatment salivary rinses in head and neck squamous cell carcinoma (Jiang et al., 2006). However, decrease of mtDNA copy number in tumour tissues have been reported in a variety of human cancers, including HCC (Yamada et al., 2006, Wong et al., 2004), breast (Tseng et al., 2006, Yu et al., 2007), gastric (Wu et al., 2005), osteosarcoma (Yu et al., 2012) and other cancers (Tan et al., 2006, Lee et al., 2005). 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 (Simonnet et al., 2002). In general, decreased mitochondrial activity seems to be an adaptation to environmental conditions of solid tumours, which have to undergo hypoxia during their development. Oxygen can initiate respiration and mitochondrial biogenesis (Howell et al., 2007). Low mitochondrial activity leads to lower oxidative stress under hypoxic conditions and hypoxia inducible factor (HIF) inhibits mitochondrial biogenesis (Zhang et al., 2007) or disrupts mitochondria by mitophagy (Zhang et al., 2008). When tumour is growing in size, cells are becoming more hypoxic, mitochondrial biogenesis is decreased (Zhang et al., 2008). 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. The intake of fruits and vegetables can also protect against oral cancer. 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. 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.