CHAPTER-5

DISCUSSION

Head and neck cancer (HNC) is among the most common cancer in the world with an estimated 650,000 new cases and 350,000 cancer deaths worldwide every year (Argiris et al. 2008). Despite considerable advancement in treatment, HNC has a high mortality rate and 40–50% of patients with HNC will survive for five years (Leemans et al. 2011). The development of head and neck squamous cell carcinoma (HNSCC) is the result of a multistep process characterized by both genetic and epigenetic alterations. The genetic alterations associated with HNSCC are governed by a variety of different pathway. The accumulation and selection of these altered pathways more commonly endorsed by diverse lifetime environmental exposures such as tobacco, alcohol intake and HPV infection. Genetic alterations, including copy number variations (CNV), deletion/null genotype, gains or losses of heterozygosity (LOH) may cause the inactivation of tumour suppressor genes and the activation of oncogenes, consequently lead to abnormal cell growth and metastasis. To identify the genetic alterations, such as chromosomal deletions, single nucleotide polymorphisms (SNPs) or mutations in tumour-related genes different techniques have been used, which ranges from various low throughput to high throughput methods (Ha et al. 2009).

5.1 Prevalence of HPV in Head and Neck Cancer

In Northeast (NE) India, incidence ratio of HNC is incredibly high with a serious increase of morbidity and mortality rates (Bhattacharjee et al. 2006). Several studies have been carried out from NE India to demonstrate the role of major risk factors associated with cancer (Mondal and Ghosh 2013, Sharan et al. 2012, Talukdar et al. 2013). These studies revealed that tobacco consumption in various forms (cigarette/beedi smoking, betel quid/tobacco chewing) is associated with increase cancer susceptibility. Betel quid chewing with tobacco or without tobacco is tradition in NE India. Further, tobacco products like *gutkha*, *paan-masala*, *khaini* are regularly consumed by people and is very popular in this part of India. In present study, we also found a very high incidence of HNC in tobacco-betel quid chewers and smokers, in comparison to never smokers and never chewers among the population of NE India.

The causal role of high-risk HPV in the carcinogenesis of HNSCC has been demonstrated in recent studies (Bussu et al. 2013, Chaudhary et al. 2009, Lajer and

von Buchwald 2010). The patients with HPV-positive tumour differ from HPV-negative regarding to the pathogenesis, risk factors and the prognosis and thus represent a distinct tumour entity (Dayyani et al. 2010, Jung et al. 2010). High-risk HPV16 plays the most significant role in the carcinogenesis of these HNC. Generally the tonsils are suitable for a HPV-infection in regard to the particular composition of the squamous epithelium (Glombitza et al. 2010). We found overall prevalence of HPV in NE Indian population was 30.7%, and the prevalence of HPV in HNSCC patients was 46.47%. We also found 3.43 fold increased the risk of HNSCC in HPV positive patients compared to HPV negative (**Table 4.1.4**). However, earlier studies in South India, Eastern Indian and Western India population reported 67%, 33.6% and 15% HPV in oral squamous cell carcinoma (OSCC) cases (Chocolatewala and Chaturvedi 2009). Earlier study found that HPV positive HNSCC patients showed better survival compared to HPV-negative, and may be considered as predictive marker (Genden et al. 2013). Our results also revealed that HPV-positive HNSCC patients showed better survival compared to HPV-negative patients (p=0.041). Previous study from India reported the combined effect of HPV and tobacco in predicting survival in HNSCC, although the study found HPV as marker of improved survival, however the combined HPV and tobacco habit showed poorer survival (Ghosh et al. 2009). HPV positive tumours are more radiosensitive and mediated by intact p53 gene. Studies have shown low level EGFR expression and gene copy number in HPV-positive tumours and higher viral load may be associated with a better survival. Based on the p16 expression and viral load, tumours of HNSCC can be categorized into three classes; p16 low and HPV-positive, p16 low and HPV-negative, P16 over expressed and HPV-positive. It is this last category of tumours that show significantly better five years overall survival and decreased local recurrence rate when compared to the tumours of the first two categories. However, there is a subset of HPV positive tumours with poorer outcomes when compared to other HPV-positive patients and that have similarity to clinical outcome of HPV negative patients. This might due to extensive smoking habits, p53 mutations, higher EGFR, BCL-xl expressions in this subset of HPV-positive tumours, and supposed to suggesting that HPV status alone may not be adequate to categorize these tumours. It is at present not recommended to modify the treatment for either HPV-positive or HPV-negative OPSCC as high-quality indication to support separate approach is lacking. Therefore, well defined

biomarkers and more high quality trials are necessary to develop diagnostic tools and to correct existing clinical management available for both HPV-positive and HPV-negative oropharyngeal cancers (Ramshankar and Krishnamurthy 2013).

5.2 Genetic Polymorphisms of Metabolic and DNA Repair Genes and Risk of Head and Neck Cancer

Xenobiotic metabolizing enzymes (Phase-I and Phase-II) are responsible for metabolic activation of various exogenous chemicals that are toxic, mutagenic or carcinogenic in nature. Genetic polymorphisms (mainly SNPs and deletions) of xenobiotic metabolizing gene affect the carcinogen-metabolizing pathway. The *CYP1A1* gene plays an important role in Phase-I metabolism pathway and activates major carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines into reactive (epoxide) intermediates (Bartsch et al. 2000). Polymorphism in *CYP1A1* (3801T→C) may affect the level of gene expression or mRNA stability, that alter the enzyme activity (He et al. 2014, Zhou et al. 2009). Consequently, polymorphism of *CYP1A1* gene which may cause enhanced enzymatic activity appear to play a vital role in susceptibility to DNA adduct formation and which can further cause genomic instability and may lead to cancer (Rojas et al. 2000). Earlier studies have reported the role of *CYP1A1* polymorphisms and the risk of various cancers (Shaffi et al. 2009, Shen et al. 2013, Singh et al. 2009). We found CC variant genotype of *CYP1A1* showed significant HNC risk in NE Indian population when compare with the wild TT-genotype.

The *GSTM1* and *GSTT1* are phase-II carcinogen-metabolizing pathway genes and play a central role in detoxification of tobacco carcinogens through conjugation reaction. Null genotype or deletion of *GSTM1* and *GSTT1* would decrease the capacity of the detoxification process. Many earlier studies reported the association between *GSTM1* and *GST1* null genotype and HNC in India (Chatterjee et al. 2009, Singh et al. 2008, Soya et al. 2007). One of the studies conducted on NE Indian population, found a possible role of *GSTM1* and *GSTT1* null genotypes in oral squamous cell carcinoma (OSCC) (Mondal et al. 2013). Meta-analysis studies also revealed the association between *GSTM1* and *GSTT1* polymorphisms and HNSCC risk (Hashibe et al. 2003, Zhang et al. 2012). Our present study further provided strong evidence that *GSTM1* and *GSTT1* null genotypes increased the

susceptibility to HNC. We found a 2.18-fold increase in HNC risk with the *GSTM1* gene deletion, whereas a moderate increase in HNC risk was found in case of *GSTT1* null genotypes (**Table 4.2.2**). However, many studies carried out on different population failed to support the association of *GSTM1*/*GSTT1* null genotypes and HNC (Anantharaman et al. 2007, Biselli et al. 2006). Moreover, several studies showed that a family history of HNC in first-degree relatives was strongly associated with the risk of HNC (Foulkes et al. 1996, Garavello et al. 2008). A recent study reported the increased risk among first-degree relatives having a family history of HNC (Negri et al. 2009). However, no prior study was carried out to evaluate the *GSTs* genes polymorphisms in first-degree relatives of HNSCC patients. In the present study, we observed almost similar pattern of *GSTM1* and *GSTT1* null (deletion) genotypes distribution in patients having HNC and their first-degree relatives. In addition, the *GSTM1* and *GSTT1* null genotype frequencies were higher in first-degree relatives of HNC patients compared with controls. Further, the frequencies of both null genotypes of *GSTM1* and *GSTT1* were significantly higher (p<0.001) in first-degree relatives compared with controls, therefore suggesting an increased susceptibility toward HNC in first-degree relatives. Numerous studies investigated the interaction between *GST*s genes polymorphisms and tobacco habits in various cancers (Lavender et al. 2009, Singh et al. 2014). In the present study, logistic regression (LR) analysis showed that interaction between *GSTM1* null genotype and tobacco chewing had 4.34-fold increase in the risk of cancer. A strong interaction between tobacco habits and *GSTT1* null genotype was observed. Tobacco chewing and *GSTM1* null genotype showed the highest individual effects as well as stronger synergistic interaction in HNC, supporting the role of tobacco carcinogens and *GSTM1* gene polymorphism toward the development of HNC.

We also investigated the interaction of combined genotypes of tobacco carcinogens-metabolizing genes (*CYP1A1*, *GSTM1* and *GSTT1*) and tobacco exposure (smoking and chewing) to the susceptibility HNC. As per our knowledge, this is the first report on an interaction effect of smoking, tobacco-betel quid chewing, and combined carcinogen metabolizing genes polymorphisms to the risk of HNC in NE Indian population. Many studies also investigated the possible role of *CYP1A1, GSTM1* and *GSTT1* polymorphisms and environmental factors in HNC

progression, though inconsistent results have been reported (Boccia et al. 2008, Gattas et al. 2006, Hiyama et al. 2008, Olivieri et al. 2009).

Many epidemiologic studies in various world populations showed an association of *CYP1A*, *GSTM1* and *GSTT1* polymorphisms with susceptibility to HNC (Anantharaman et al. 2007, Lourenco et al. 2011, Sharma et al. 2006, Suzen et al. 2007, Wei et al. 2013). However, other studies have shown no association between *CYP1A1* or *GSTM1* or *GSTT1* polymorphism and HNC (Anantharaman et al. 2007, Biselli et al. 2006). A strong correlation between HNC and polymorphism of *CYP1A1* and *GSTM1* was found in North Indian population (Sharma et al. 2010b). Similar results have been reported in South Indian population (Sam et al. 2008, Soya et al. 2007). One earlier study also showed association of *CYP1A1* and *GST*s polymorphisms and oral cancer susceptibility in NE Indian population (Chatterjee et al. 2009). We are the first to study and found that individuals carrying combined variant genotypes of *CYP1A1* T3801C and *GSTM1* null genotypes had a 3.52 fold increased the risk of HNC. This increase risk was higher than the risk of HNC reported in other studies that considered single genotype at a time. The present study also revealed that the combination of *CYP1A1* TC/CC and *GSTT1* null genotypes increased the susceptibility of HNC. These findings suggest that cross talk between these tobacco carcinogens-metabolizing genes might modulate susceptibility of HNC.

Tobacco contains more than 50 potent carcinogens, including tobacco specific nitrosamines and other carcinogens. The detoxification processes present in cellular system protects the cells from DNA damage caused by various carcinogens. Since *CYP1A1* plays an important role in the bioactivation of pro-carcinogens and *GSTs* genes take part in detoxification of carcinogens present in tobacco products. Thus, it is rational to study the combined effect of *CYP1A1, GSTM1* and *GSTT1* genes and tobacco with the susceptibility to tobacco-related cancers. Many previous studies investigated the combined association between tobacco and *CYP1A1, GSTM1* and *GSTT1* polymorphisms (Lourenco et al. 2011, Sabitha et al. 2010, Sam et al. 2010, Sharma et al. 2010b), but in a too simplest way. In addition, no previous studies have found that investigated the association between polymorphisms of *CYP1A1* T3801C*, GSTM1* and *GSTT1* metabolizing genes and various tobacco habits with HNC risk using MDR analysis. Therefore, we are the first to investigated

the association of smoking and tobacco-betel quid chewing and metabolism genes polymorphisms and HNC risk in a combined way (genotypes combination × tobacco uses). The data showed that, combination of smoking and tobacco chewing and *CYP1A1* variant genotype*, GSTM1* and *GSTT1* null genotype increased the risk of HNC. Smokers carrying *CYP1A1* TC/CC and *GSTM1* null genotype had been significantly increased the risk of HNC compared to non-smokers with wild-type *CYP1A1* and *GSTM1* genotypes. However, tobacco-betel quid chewers carrying both *CYP1A1* TC/CC and *GSTM1* null genotype showed the highest risk of HNC compared to non-chewers. The MDR analysis further validated the results of gene-environment interaction obtained by multiple comparisons analysis; while, the best model for HNC risk in the MDR analysis was the interaction of tobacco-betel quid chewing, smoking, *CYP1A1* TC/CC and *GSTM1* null genotype. Interaction entropy graphs were drawn for visualization and interpretation of MDR. Tobacco-betel quid chewing, *CYP1A1* and *GSTM1* genes polymorphism showed significant individual effects with strongest synergistic interactions among each other in HNC risk (**Figure 4.2.5**). These findings demonstrated that besides tobacco exposure, individual detoxification capacity also plays a crucial role in the development of HNC.

The DNA repair genes play a vital role in the maintenance of genomic integrity of cell. Many DNA repair genes have SNPs, which may cause changes in amino acid. This alteration of amino acid affects the pre-mRNA molecule splicing, and may have an impact in the function of the expressed protein. Therefore, the polymorphisms of DNA repair genes that affect the DNA repair system might be the key factors in cancer susceptibility. Several epidemiologic studies have shown the role of DNA repair genes on the risk of cancer (Benhamou et al. 2004, Krupa et al. 2011, Kumar et al. 2012, Perez et al. 2013, Yen et al. 2008). In the current study, we investigated the role of single nucleotide polymorphisms (SNPs) in DNA repair genes (*XRCC1* codon399 G>A and *XRCC2* codon188 G>A) to the risk of HNSCC in NE Indian population. Furthermore, we assessed the combined effect of smoking, tobacco-betel quid chewing, and *XRCC1* and *XRCC2* polymorphisms in HNSCC. *XRCC1* gene plays a key role in DNA repair pathway, and encoded a scaffolding protein that directly associates with other DNA repair proteins in multifaceted processes of single strand break and base excision repair (BER) pathway (Dianova et al. 2004). Whereas, *XRCC2* is one of the Rad51-related protein and function

through complex interaction with other relevant proteins to repair double-strand breaks and maintain genome integrity in multiple phases of a homologous recombination repair (HRR) pathway (Jiao et al. 2008).

Consistent with the earlier study from India (Ramachandran et al. 2006), our data also showed that polymorphism in *XRCC1* Arg399Gln was found to be associated with increase the risk of HNSCC. There appeared to be differences in frequency distribution of genotypes of *XRCC1* in our population, when we compared the data with North Indian population (Kumar et al. 2012). Several epidemiologic studies on world population also demonstrated positive association between *XRCC1* Arg399Gln polymorphisms and HNSCC (Khlifi et al. 2014, Yuan et al. 2012). Whereas, many studies did not find any association between *XRCC1* Arg399Gln polymorphisms and HNSCC (Al-Hadyan et al. 2012, Kostrzewska-Poczekaj et al. 2013, Tae et al. 2004). These differences in genotypes frequency distribution may due to the diverse ethnic variation among the Indian population.

The *XRCC2* Arg188His polymorphism has been anticipated to be a genetic modifier for tobacco-related cancer, and was associated with an increased risk of upper aero-digestive tract cancers (Benhamou et al. 2004). Earlier studies also documented that polymorphism of *XRCC2* Arg188His was associated with cancer of breast (Rafii et al. 2002), colorectal (Krupa et al. 2011), lung (Zienolddiny et al. 2006), larynx (Romanowicz-Makowska et al. 2012), pancreatic cancer (Jiao et al. 2008) cervical cancer (Perez et al. 2013). Unfortunately, there are no reports so far that directly demonstrate *XRCC2* Arg188His polymorphism and head and neck cancer risk. In present study, we found *XRCC2* GA (Arg/His) genotype increased the risk of HNSCC in NE Indian population. We also focus gene-gene interactions to explore the joint effect of multiple genotypes towards the risk of HNSCC. The finding revealed that the genotype combination of *XRCC1* 399 GA and *XRCC2* 188 GA higher the risk of HNSCC. Interaction of *XRCC1* 399 AA and *XRCC2* 188 GA genotypes increased the risk 5.15 folds, when compared with combined low-risk genotypes (*XRCC1* 399GG and *XRCC2* 188GG) (**Table 4.3.2**), suggesting that cross talk between these DNA repair genes might play key role towards HNSCC susceptibility.

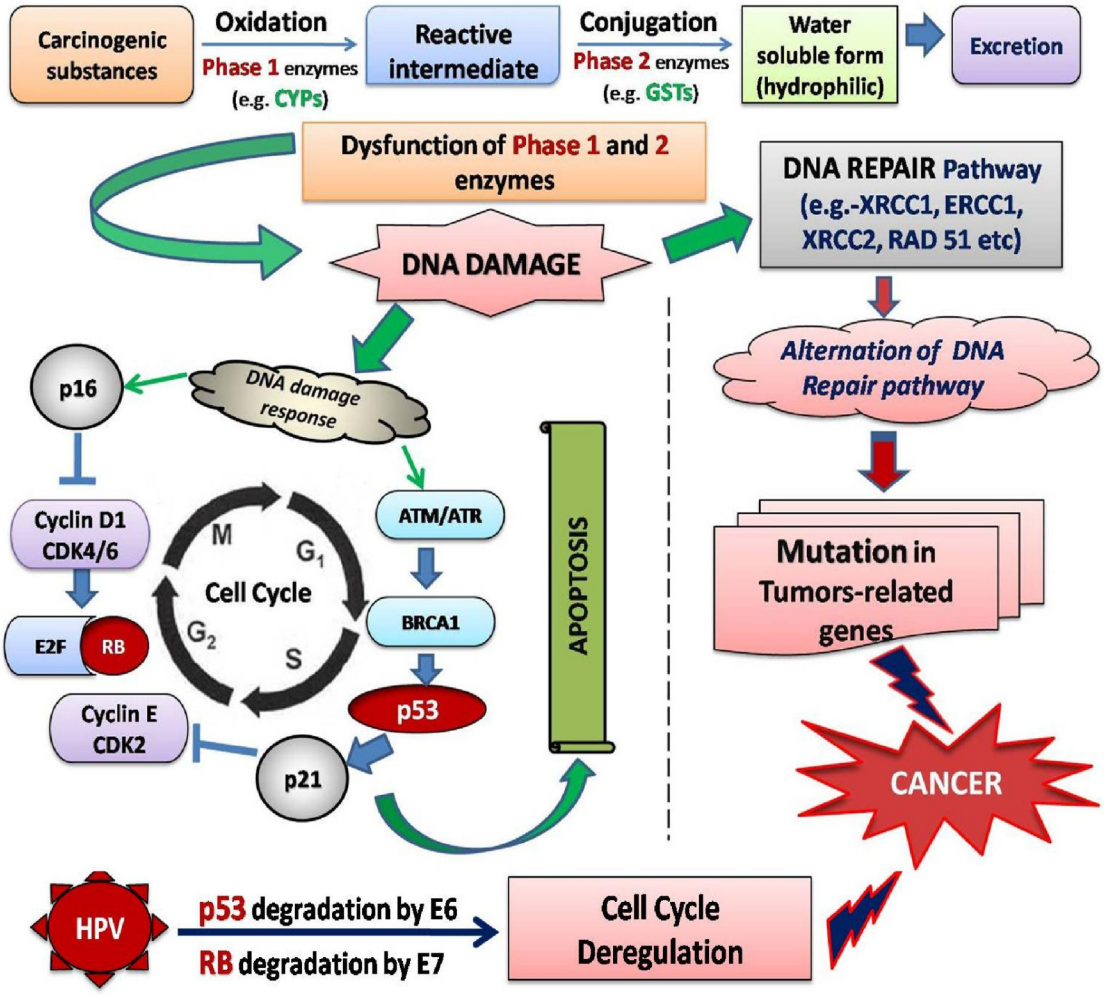

Figure 5.1: A mechanism of DNA damage response and cancer progression. The DNA damage response (DDR) manages the repair of DNA and the activation of cell cycle checkpoints to arrest the cell to allow time for repair. The interplay between the cyclins, CDKs and their inhibitors controls the cell cycle checkpoints. In reply to a mitogenic signal, the cyclin D1–CDK4/CDK6 complexes are activated and phosphorylate the RB pocket proteins, causing release of E2Fs (transcription factors), initiates entry into S phase. The inhibitor for the cyclin D1–CDK4/CDK6 complexes is p16INK4A, which is encoded by *CDKN2A* gene. A second important control mechanism of the cell cycle occurs during G2 phase, when replication errors and other DNA damage are repaired by p53 protein. The human papillomavirus (HPV) genome encodes two viral oncoproteins: E6 and E7. The E6 protein degrade p53, whereas the E7 protein binds with RB pocket proteins and inactivates its function, which leads to abnormal cell proliferations. Normally, functional DNA repair system respond appropriately to DNA damage by regulating various DNA repair proteins (XRCC1, ERCC1, XRCC2, RAD51 etc). In addition, Phase-I (CYPs) and Phase-II (GSTs) metabolizing enzymes activates and transformed carcinogens in water-soluble form for easy excretion. Any genetic polymorphisms or mutations in DNA repair and metabolic-pathway genes may predispose to cancer development.

The carcinogens present in tobacco can affect DNA repair processes by providing a strong free radical generating environment, which could lead to oxidation of DNA to form DNA adducts and single strand breaks (Bartsch et al. 1999, Rouissi et al. 2011). Many earlier studies also investigated the combined association of tobacco and DNA repair genes polymorphisms (Kumar et al. 2012, Ramachandran et al. 2006), but in a too simplest bi-modal way (absence/presence). Therefore, we investigated the association of smoking and tobacco-betel quid chewing, polymorphism of *XRCC1* Arg399Gln and *XRCC2* Arg188His, and HNSCC risk in a gene-dose dependent manner. Present study revealed that, combination of smoking and tobacco chewing and variants of *XRCC1* and *XRCC2* genes increased the risk of HNSCC. We found a clear trend in increase the risk of HNSCC, when stratified tobacco habits data of study population into never, light and heavy smokers or chewers. Heavy smokers carrying *XRCC1* AA (Gln/Gln) genotype had been significantly associated with increased the HNSCC risk compared to other tobacco-genotype combination. However, the *XRCC2* GA (Arg/His) genotype showed significant association with heavy tobacco-betel quid chewing in HNSCC risk These results indicated that besides tobacco exposure, individual DNA repair capacity also plays a crucial role in the development of HNSCC.

5.3 Promoter Methylation Profiles of Head and Neck Cancer

DNA methylation is one of the most significant epigenetic mechanisms regulating gene transcription, chromatin structure and genomic stability. Global DNA hypomethylation accompanied by hypermethylation of CpG islands in the promoter region of genes involved in detoxification, DNA damage repair, cell cycle regulation and apoptosis is a common phenomenon in cancer cells. Increased methylation in the promoter region of tumour suppressor genes can reduce their expression and give rise to growth advantage of some cells. Recently many epigenetic events in carcinogenic pathways have been studied and discovered the methods for detecting CpG island promoter methylation pattern to classify high risk groups among different cancers (Dong et al. 2014, Laskar et al. 2014, Teodoridis et al. 2004). This helps in detection of early onset of tumours, and predicts clinical status. Promoter methylation profile of tumour-related genes was likely to be crucial

and common in different cancers. Therefore, it is very essential to study the promoter hypermethylation status of a panel of representative genes in HNSCC. In this study, we analyzed the promoter hypermethylation profile of HNSCC using seven important tumour-related pathway genes and three methylated loci. We also correlated aberrant promoter methylation status of patients with genetic and environmental factor as well as with survival status. Present study has covered a broad range of tumour-related pathway genes, including *p16* (cell-cycle control), *BRCA1*, *MLH1* (DNA repair pathway), *ECAD* (cell-cell adhesion), *GSTP1* (carcinogen metabolism), *DAPK*, *RASSF1* (apoptosis control pathway), *MINT1*, *MINT2* and *MINT31* (methylated loci in tumours).

We found a significantly high level of hypermethylation in *p16*, *DAPK*, *ECAD*, *RASSF1*, *MINT1*, *MINT2* and *MINT31* in HNSCC tumour tissues compared to normal counterpart, reflecting the possible involvement of epigenetic alteration toward the development and progression of HNSCC (**Figure 4.4.2**). Earlier, many epigenetic studies explained existence of HPV mediated DNA-methylation in HNSCC (Lleras et al. 2013, Worsham et al. 2013). A recent study from the Northeast India, also explained the role of HPV and tobacco in the genesis of UADT cancer, using a panel of 10 genes, (Talukdar et al. 2014). However, we further investigated the hypermethylation of individual genes/loci separately in HPV-positive and HPV-negative tumours. We observed that promoter methylation of *DAPK*, *p*16, *RASSF1* and *MINT31* was significantly associated with HPV-positive HNSCC tumours. Therefore, HPV appeared to be a causal mediator for alterations of CpG island methylation of tumour suppressive genes in HNSCC. In most cases, HPV-positive HNSCC is associated with a better survival (Perez-Ordonez et al. 2006), again smoking related HNSCC harbouring a methylated p16 promoter compared to HPV-positive HNSCC harbouring an unmethylated promoter (Ramshankar and Krishnamurthy 2013). A recent study analyzed the role of promoter hypermethylation of 24 tumour suppressor genes in OPSCC and showed a significantly higher cumulative methylation index in HPV-positive compared to HPV-negative tumours. This study also found *CADM1* and *TIMP3* genes significantly more hypermethylated in HPV-positive OPSCCs and while *CHFR* specifically hypermethylated in HPV-negative tumours (van Kempen et al. 2014). In many cancer types, mostly in colorectal cancer, CpG Island Methylator Phenotype

(CIMP) was used to identify clinically and pathologically relevant subsets of tumours (Toyota et al. 1999a). CIMP-positive had diverse epidemiologic features, BRAF mutations, microsatellite instability (MSI) profile and survival, as compared to CIMP-negative colorectal cancer (Lochhead et al. 2013, Ogino et al. 2009). CIMPs also have been reported in other cancers, including upper aerodigestive tract (UADT) (Talukdar et al. 2014), oral cancer (Jithesh et al. 2013), and oesophageal squamous cell carcinoma (Ling et al. 2011) and breast cancer (Roessler et al. 2014). However, only one CIMP study particularly on HNSCC, which explain CIMP in the sub-group of HPV-positive tumour of HNSCC (Lechner et al. 2013). The hypermethylation profile of gene promoters is diverse for each type of cancers and the detection method and multiple gene selection for CIMP-panel varies among the studies. On the base of hypermethylation pattern of 10 tumour-related genes/loci, we stratified HNSCC in three groups: CIMP-high (CIMP-H), CIMP-low (CIMP-L) and CIMP-negative. We find distinct characteristics of tumour within the CIMP-high and CIMP-negative (CIMP-N) groups. Tobacco chewing, smoking and *GSTM1* null genotypes frequencies of patients were significantly higher in CIMP-H groups compared to CIMP-N. This may explain that genetic and environmental factors may direct to CIMP characteristics of HNSCC tumours. We also found a poor survival rate in CIMP-high tumours compared to CIMP-N group; indicating CIMP-H may be a predictor for a poor prognosis of HNSCC in Northeast Indian population. Perhaps, it was not unexpected that we have found association between the CIMP-high and patient poor survival, if we compared our present data of the CIMP and survival in HNSCC with other previously described cancers (Kang et al. 2014, Li et al. 2014). Therefore, the designing of enhanced treatment strategies for HNSCC, a profound understanding of CIMP could help.

Hierarchical cluster analysis identified two diverse subsets of HNSCC, one subset with high frequency of smoking, tobacco chewing habits, *GSTM1* null and *CYP1A1* (CC) variant genotype (**Figure 4.5.4**). The Cluster-1 also characterized by HPV-positive HNSCC and contained CIMP-H group, reflecting a possible association between DNA methylation and genetic and environmental factors in HPV-positive tumours. Therefore, epigenetic and genetic alteration along with a combination of environmental factors may play an important role in a sub-setting of HNSCC. In previous section, we discussed the strong interaction between

carcinogen metabolizing genes (*GSTM1* and *GSTT1*) and environmental factors in HNSCC. Since the interaction of Phase-I (*CYP1A1*) and Phase-II (*GSTM1* & *GSTT1*) tobacco carcinogens metabolizing genes may elucidate the accumulation of the larger amount of toxic substances inside the body, thus might play the key role during the progression of HNSCC. We further explored the interaction between tobacco and *XRCC1* and *XRCC2* DNA repair gene polymorphisms, and cross talk between these two DNA repair genes towards susceptibility of HNSCC. A recent study showed that association between *GSTM1* null genotype and increased susceptibility to CpG hypermethylation, however they further found reduced susceptibility to CpG hypermethylation of *DAPK* gene with *XRCC1* codon 399 Gln/ Gln genotype and further concluded that *GSTM1* null genotype may have a role in CpG hypermethylation related gastric carcinogenesis (Tahara et al. 2011). We are the first, to explore a significant association between promoter hypermethylation and *GSTM1* null genotype in HNSCC. Hierarchical cluster analysis further confirmed this association and therefore indicated a possible role of carcinogen metabolizing genes in promoter hypermethylation of CpG island. A better understanding of the epigenetic and genetic mechanisms underlying the carcinogenic processes of HNSCC is indispensable for development of new diagnostic tools and effective treatments. Our current study identified new genetic and epigenetic signatures for head and neck cancers, therefore opens up new opportunities for future research to develop better strategies for diagnosis and targeted therapies.

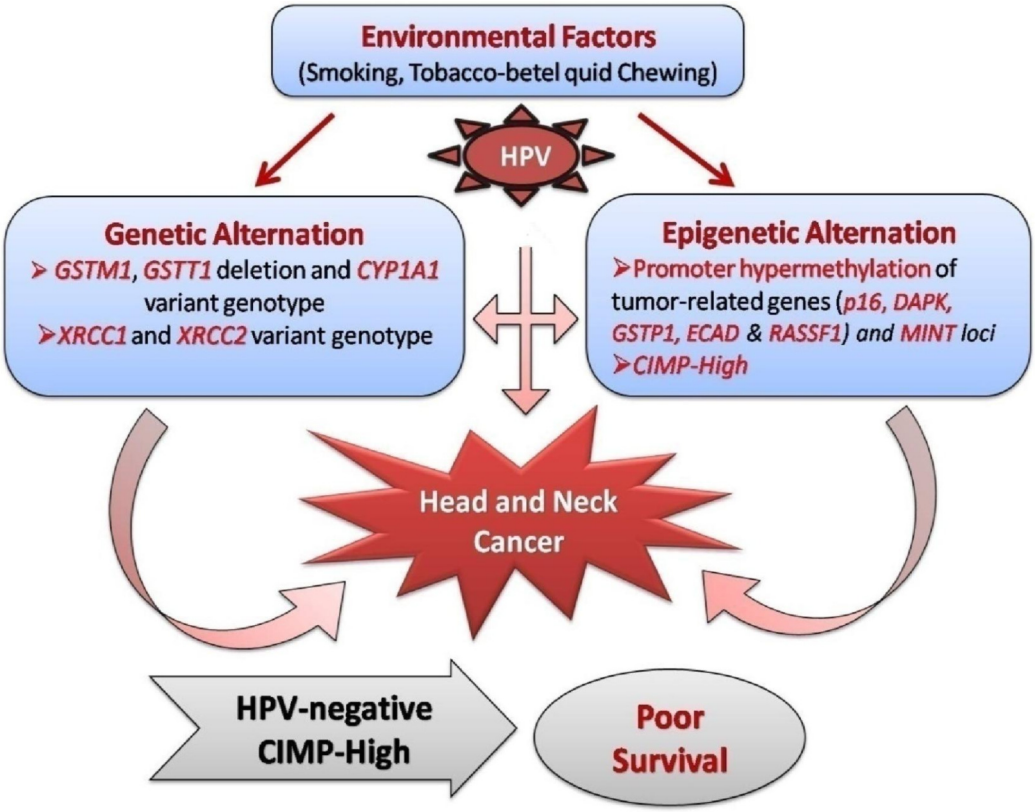

Figure 5.2: Proposed model for head and neck cancer development as evident from our present study.

SUMMARY

The development of head and neck cancer (HNC) is a multi-step process modulated by genetic, epigenetic and environmental factors. The environmental risk factors such as tobacco smoking and chewing in addition to HPV infection may be directly to a wide range of genetic and epigenetic events that promote genomic instability and endorse tumour development. In Northeast (NE) India, HNC accounts for the highest percentage of the cancer with oral cancer being the most frequent. However, very little is known about the environmental, clinical, genetic and epigenetic aspects of the head and neck cancer in NE India. We therefore, performed a comprehensive study on the environmental and viral risk factors, genetic alteration, epigenetic alteration (promoter hypermethylation), their combined effect (gene-gene interaction and gene-environment interaction) and the survival status in HNC from NE India. The current study consisted of all total 780 subjects, which include 180 patients with pathologically confirmed HNC, 300 first-degree relatives and 300 healthy individuals as controls. Methylation specific polymerase chain reaction (MSP) was used to detect the methylation status of 10 tumour-related genes/loci (*p16, DAPK, RASSF1, BRAC1, GSTP1, ECAD, MLH1, MINT1, MINT2 and MINT31*). Genotyping of *CYP1A1*, *XRCC1*, *XRCC2* and *GST* (M1 & T1), genes were carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and using multiplex PCR respectively. Logistic regression and multifactor dimensionality reduction (MDR) approach were used for statistical analysis.

The overall prevalence of HPV detected in Northeast Indian population was 30.7%, and the prevalence of HPV in HNC patients was 46.47%. We also found 3.43 fold increased the risk of HNC in HPV-positive patients compared to HPV-negative cases, in NE Indian population. In general, HPV-positive HNSCC is associated with a more favourable survival, present study also explored HPV-positive HNC patients had a better survival rate compared to HPV-negative patients (p=0.041) reflecting a possible role in prognosis.

Analysis revealed that *GSTM1* and *GSTT1* null genotype frequencies were significantly associated with HNC [adjusted odds ratio (OR) =2.18; p<0.001 and OR=1.61; p=0.031 respectively]. Smokers or tobacco-betel quid chewers carrying

CYP1A1 TC/CC + GSTM1 null genotypes had several fold increased risk of HNC (p<0.001). In MDR analysis, the best model for HNC risk was four-factors model of tobacco-betel quid chewing, smoking, *CYP1A1* TC/CC and *GSTM1* null genotypes (TBA=0.6292; CVC=9/10 and p<0.0001).

The result showed that variant homozygote AA (Gln/Gln) genotype of the *XRCC1* Arg399Gln was associated with increased the risk of HNC that was statistically significant (OR= 2.43 and p=0.031). Again, the present study suggests that tobacco habits, *XRCC1* and *XRCC2* gene polymorphisms and cross talk between these two DNA repair genes might modulate susceptibility towards HNC and may have an impact on identification of a high-risk population.

We found a significantly high level of *p16*, *DAPK*, *ECAD*, *RASSF1*, *MINT1*, *MINT2* and *MINT31* hypermethylation in head and neck squamous cell carcinoma (HNSCC) tumours, compared to normal tissue samples, reflecting the possible involvement of epigenetic alteration toward the development and progression of HNSCC. Result also indicates HPV appeared to be a causative agent for alterations of CpG island methylation of tumour-related genes/loci in HNSCC. CpG Island Methylator Phenotype (CIMP) was used to identify clinically and pathologically relevant subsets of HNSCC. We observed distinct characteristics of the tumour within the CIMP-high and CIMP-negative groups. Hierarchical cluster analysis identified two distinct subsets, one subset of HNSCC patients with high frequency of tobacco chewing, smoking, *GSTM1* null and *CYP1A1* (CC) variant genotype. Moreover, a CIMP-high and Cluster-1 characteristic was associated with poor survival.

In conclusion, findings of the present thesis revealed that genetic and epigenetic alteration play as a major mechanism in the development of HNSCC. The findings also suggest that polymorphisms of DNA repair and carcinogens-metabolizing genes and their interaction with environmental factors might modulate susceptibility of HNSCC in Northeast Indian population. We also identified discrete subsets of HNSCC based on differential genetic, epigenetic, HPV and environmental characteristics. Furthermore, we also assessed the outcome of patients, based on HPV and promoter methylation profiles. Overall, this type of study would be helpful in identification of diagnostic and prognostic biomarkers and therapy.