CHAPTER 5

DISCUSSION
The current study conducted on Northeast Indian population exhibited an age adjusted incidence rate of 12-13/100000 population making oral and esophageal squamous cell carcinomas the leading sites of cancer in the region. Similar rates are reported from developing countries of Southeast Asia, where oral and esophageal squamous cell carcinomas are among the leading cancers based on the incidence and mortality rate of the population (Chen et al., 2011; Guo et al., 2008). Head and neck cancer is a major health problem in India constituting up to 40% of all cancers. It is the most predominant cancer in males and the third most prevalent in females (Sharma et al., 2010). Similarly, ESCC is another prevalent form of cancer in Indian population responsible for highest cancer related mortalities (Dar et al., 2013). The high incidence and poor prognosis of ESCC contributes significantly to the cancer burden of India some other high-incidence countries (Merletti et al., 2011). The high incident countries of esophageal cancer includes developing regions of Central Asia stretching from Northern Iran through the central Asian republics to North-Central China, referred to as the “esophageal cancer belt”. Since, esophageal cancer exhibits high incidence and poor prognosis it contributes significantly to the cancer burden in the belt and some other high-incidence regions (Szumilo, 2009).

The high incidence of oral and esophageal cancers in developing countries is primarily thought to be due to high prevalence of tobacco related habits. In the current study, Betel quid and tobacco chewing as well as smoking were the most potent life-style related individual risk factors of OSCC. Both smoked and smokeless forms of tobacco consumption along with areca nut and betel leaf are customary in the population, as in other southeast Asian countries (Lee et al., 2011). Betel quid and tobacco consumption (including chewing in the form of betel quid, or khaini and smoking bidis and cigarettes) are considered as the major risk factor for oral cancers in India (Gupta et al., 1995; Ulap et al., 2011). A follow up cohort study from Jayalekshmi et al (Jayalekshmi et al., 2009) showed an increased risk of OSCC development with high frequency of daily intake of tobacco (smoked or smokeless forms). The cohort study also showed a significant association between oral cancer incidence and daily frequency of tobacco chewing \( P < 0.001 \), which increased 9.2
fold among women chewing tobacco 10 times or more a day, with the highest risk during the first twenty years of chewing.

Like oral cancers, the environmental and dietary risk factors like habit of smoking and smokeless tobacco consumption, betel quid chewing, alcohol intake, poor nutrition, etc., are considered to be associated with ESCC in the high risk areas (Ganesh et al., 2009). This study also confirmed tobacco smoking (beedi and cigarette) as a predominant risk factor for ESCC, but highest risk was associated with tobacco chewing in the concerned population. Tobacco is chewed in various forms either alone or with slaked lime or betel quid, and the spit is often swallowed. Like tobacco smoke, smokeless forms of tobacco are also known to contain several carcinogenic compounds, the most potent of which are the tobacco specific N-nitrosamines like N'-nitrosonornicotine (NNN), 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) etc (Humans, 2007). Previous studies have quantified the risks of esophageal cancer associated with betel quid chewing; consumption of alcohol and tobacco in Mumbai, Bangalore and North Indian populations (Jussawalla and Deshpande, 1971; Nagpal et al., 2014; Nandakumar et al., 1996). An earlier study was conducted by Phukan et al (Phukan et al., 2001) on the high incident population of Assam, where certain ingredients and methods of preparation of the betel quid and tobacco consumption differ from those common in other parts of India. They also reported similar findings that various forms of tobacco chewing were the most potent risk factor of ESCC in the population.

Although the environmental and lifestyle factors are undoubtedly associated with ESCC development, but only a minuscule proportion of the exposed individuals actually develop cancer in due course. This is largely due to the differences in inherent carcinogen detoxification capabilities of these individuals, defined by the potency of various carcinogen-metabolising enzymes that catalyzes the breakdown of the carcinogens present in the body. The GSTM1 and GSTT1 genes are responsible for the degradation of several carcinogenic compounds present in tobacco (Hecht, 2003). Null genotypes of GSTM1 and GSTT1 were considered to be associated with an increased risk of OSCC (Zhang et al., 2011; Zhao et al., 2014) and ESCC (Gao et al., 2002; Liu et al., 2010). In the present study, null genotypes of
both GSTM1 was significantly associated with 2.79 folds increased risk of OSCC and GSTT1 genes were imparted 1.74 folds risk towards developing ESCC. Meta-analysis conducted by Zhang et al. (Zhang et al., 2011) on the association of GSTM1 and GSTT1 revealed a pooled odds ratio of 1.43 for the GSTM1 null genotype was in Asians (95% confidence interval (CI): 1.14, 1.78; P < 0.01, I (2) = 73%) and 0.98 in Caucasians. Moreover, they concluded that GSTT1 null genotype may not be associated with oral cancer. A study from Chinese population documented 2.17 folds increased risk of ESCC in GSTM1 null individuals than GSTM1 carriers (Gao et al., 2002). However, a pooled analysis of 11 studies could ascertain only a modest increase in risk of ESCC in GSTM1 null genotype carriers [OR= 1.197 (95% CI=0.846 - 1.692)] (Huang, 2004), and two others failed to establish any association between GSTM1 and GSTT1 polymorphisms and risk of ESCC (Yang et al., 2005; Zendehdel et al., 2009).

Cancer risk is largely modified due to complex gene-environment interactions. In the current study, we studied the effect of the interaction between various habit related factors and carcinogen metabolizing gene polymorphisms in OSCC and ESCC using MDR. Betel quid chewing was the most potent individual risk factor predicted by MDR for OSCC, whereas, tobacco chewing was the best suited model for ESCC. Similar to findings in regression analysis, the interaction of GSTM1 null polymorphism, betel quid and tobacco consumption was the best interaction model for OSCC, whereas, the interaction of tobacco consumption and GSTT1 null genotypes was the best model predicted for ESCC. Although no prior report used MDR for studying these risk factors in ESCC, but, an earlier case-control study using logistic regression conducted on Indian betel quid/tobacco chewers, found that the null genotypes of GSTM1 and GSTT1 greatly increased the risk for developing oral leukoplakia (Bartsch et al., 1999). Moreover, another study conducted on Indian population exhibited that increased lifetime exposure to tobacco chewing appeared to be associated with a 2-fold increase in oral cancer risk in GSTM1 null individuals. An earlier study case-control from Northeast Indian population have documented highest risk of esophageal cancer in betel quid and tobacco chewers with smoking habit (OR=15.3 in males, OR=27.4 in
females) (Phukan et al., 2001). A recent study conducted on a South Asian population established a 21.4 fold increased risk of ESCC in betel quid and tobacco chewers who smoked cigarettes (Akhtar et al., 2012). However, both the studies did not take genetic factors into consideration.

In recent years, the role of promoter hypermethylation of tumour suppressor genes is recognized as one of the key events in instigation and progression of cancer by repressing the expression of the corresponding genes. Evidences suggest that the allocation of methylation at various gene promoters across a tumour series follows particular patterns. Tumours that have methylation at one gene are more prone to have methylation at other sites too. This has been described as concordant methylation and the CpG island methylation phenotype (CIMP) and was first described in 1999 in colorectal cancer (Toyota et al., 1999a), and shortly after in gastric cancer (Toyota et al., 1999b). Since then, CIMP has been recognised as a molecular subclass of colorectal cancers and extensive studies were performed to evaluate CIMP and associated clinical characteristics. However, studies of CIMP in OSCC and ESCC are rare. Here, in order to evaluate CIMP status, we classified samples based on the presence or absence of methylation of key tumour related genes/loci involved in different cellular pathways and consisting of the five classical CIMP panel loci and five other tumour-related genes, namely, P16 (cell cycle regulation), DAPK, RASSF1 (apoptosis), GSTP1 (protection of DNA), CDH1 (cell-adhesion), MLH1 and BRCA1 (DNA repair) and other frequently methylated loci in tumours viz, MINT1, MINT2 and MINT31. The overall frequency of methylation of these loci ranged from 4.38% (MLH1) to 53.44% (RASSF1). Frequency of the three MINT loci MINT1, MINT2 and MINT31 were 47.7%, 45.40% and 44.82% respectively. In an earlier study on OSCC, promoter hypermethylation was observed in 23% patients for p16 8% for MLH1, and 35% for E-cad (Viswanathan et al., 2003). However, the most frequently methylated genes in ESCC includes P16, ECAD, RASSF1, DAPK etc having an average methylation frequency of 50-60% (Sato and Meltzer, 2006). A study conducted by Guo et al. found a comparatively higher proportion of P16 (52%) and a lower percentage of DAPK (24%) promoter hypermethylation in ESCC tumours, which might be due to ethnic variations.
However they reported a similar proportion of *BRCA1* promoter methylation (28%) as ours.

In the present study, we identified three subclasses of oral and esophageal squamous cell carcinoma in hierarchal clustering based on the methylation level of the 10 genes and loci. The three clusters were characterised by predominance of CIMP-high (≥5 methylated loci), CIMP-intermediate or CIMP low cases. Similar classification of CIMP-high, CIMP intermediate or CIMP low/CIMP negative tumours was established for colorectal cancers in both marker based as well as genome based studies (Ang et al., 2010; Kaneda and Yagi, 2011; Shen et al., 2007). However, very few studies dealt with evaluating CIMP in OSCC or ESCC. The only prior marker based study to establish CIMP in oral cancer used promoter methylation of seven cancer associated genes viz, *P16, CCNA1, MGMT, ECAD, TIMP3, RARb* and *CYGB* genes(Shaw et al., 2007). They identified a cluster of tumours with a greater degree of promoter methylation associated with distinct clinical characteristics and designated CIMP+ve. Prior methylome analysis of 48 oral cancer tissues using hierarchical agglomerative clustering identified two visibly distinct clusters, one cluster with low β values (median=0.3) designated as ‘CIMP-low’ and other cluster with high β values (median=0.56) designated as ‘CIMP-high’(Jithesh et al., 2013). An additional genome-wide methylation study demonstrated that aberrant methylation of promoter CpG islands exists across oral precancer and OSCC genomes and clustering of all methylation data revealed distinct methylation patterns between the normal and the CIS/OSCC tissues (Towle et al., 2013). Although no prior study was conducted to establish CIMP in ESCC, however, global methylome analysis of ESCC and normal surrounding tissues identified a total of 37 CpG sites to be differentially methylated between tumours and normal surrounding tissues (Lima et al., 2011).

Increasing evidences are growing that tobacco smoke associated carcinogens and carcinogen metabolising gene polymorphisms are capable of modulating DNA methylation in cultures, animal models as well as certain tobacco-related cancers like lung cancer (Jin et al., 2010; Lin et al., 2010; Mani et al., 2012; Pulling et al., 2004). In the present study, smokeless tobacco chewing was found to be significantly
associated with promoter methylation in both univariate as well as multivariate analysis. The mean methylation index of tobacco chewers was significantly higher than non-chewers and it imparted 3-5 folds increase in methylation of all the genes under study, except MLH1. Further classifying the cases according to CIMP status, tobacco chewing had the highest risk of CIMP high as compared to CIMP low, followed by smoking with odds ratios of 6.67 and 2.64 respectively. Same was reflected in MDR, as tobacco chewing was the best one factor model in CIMP high cases. A similar study from Indian oral cancer patients found a significantly higher percentage of p16 and DAPK promoter methylation in tobacco chewers as compared to non-chewers (Kulkarni and Saranath, 2004). The fact that certain tobacco specific nitrosamines and poly-aromatic hydrocarbons (PAHs) like NNK, Benzo[a]pyrene etc are capable of modulating DNA methylation is evident from both in-vitro as well as human studies. In previous studies, NNK was found to induce hypermethylation of multiple tumour suppressor genes like P16, DAPK, Rarβ etc. in liver and lung tumours of rat and mouse models (Pulling et al., 2001; Pulling et al., 2004; Vuillemenot et al., 2006). A recent study combining cell, animal and clinical lung cancer tissues as model found that, NNK attenuates DNMT1 degradation and also induces its nuclear accumulation resulting in subsequent hypermethylation of promoters of tumour suppressor genes (Lin et al., 2010). Benzo[a]pyrene present in tobacco is converted to its carcinogenic form BPDE (benzo[a]pyrene diolepoxide) by the phase I enzymes CYP1A1, CYP1B1 etc., which are further metabolized by the GSTs. In a study on esophageal cancer cells, BPDE was found to suppress Rarβ expression via promoter hypermethylation by recruiting DNMT3A (Ye and Xu, 2010). Moreover, DNA repair and carcinogen metabolising gene polymorphisms are believed to predispose cells towards promoter hypermethylation of genes (Tahara et al., 2011). In this study, association was observed between GSTM1 null genotypes and CIMP high in OSCC, whereas, null genotypes of GSTT1 gene had association with CIMP high in ESCC. In a case-control study on lung cancer, null genotype of GSTM1 has been found to increase the risk of promoter hypermethylation of DAPK and Rarβ. Moreover, they also identified significant interaction of tobacco smoking and null GSTM1 genotype in modulating promoter hypermethylation of multiple TSGs (Jin et al., 2010). In our study, the interaction of tobacco and betel quid
chewing, smoking and null GSTM1 genotype was the best model for CIMP-low in MDR. Additionally, the interaction of tobacco chewing, smoking and GSTT1 null genotypes was the best model for CIMP high. This further supports the hypothesis that a complex interaction is likely to interplay between tobacco-related habits and carcinogen metabolising gene polymorphisms towards promoting aberrant DNA methylation in oral and esophageal squamous cell carcinoma.

Entropy graphs were drawn for visualization and interpretation of MDR interactions. Tobacco chewing and smoking showed highest individual effects as well as strongest synergistic effects among each other in CIMP high cases, supporting the role of tobacco carcinogens in promoting DNA methylation. In CIMP low cases however, synergistic interactions of betel quid chewing, tobacco and GSTM1 null genotype was most striking.

Studies have shown CIMP to be an independent negative prognostic factor in colorectal cancer (Juo et al., 2014), however the prognostic role of CIMP in oral and esophageal squamous cell carcinoma is very poorly understood. We report significantly poorer survival in CIMP high OSCC and ESCC cases in both univariate and multivariate analysis. In an earlier study on oral cancer patients CIMP high was an independent marker of poorer prognosis in methylome based assay (Jithesh et al., 2013). In addition a study on hypermethylation profile of 13 CpG loci characterized by polycomb group target genes, mammalian interspersed repeats, and transcription factor binding sites (PcG/MIR/TFBS), was associated with reduced survival (hazard ratio: 3.98, p=0.001) in head and neck squamous cell carcinoma patients. Moreover, multivariate analysis of the individual effects of 10 genes under study exhibited significant association of P16 and DAPK1 promoter hypermethylation after adjusting for other molecular and clinical parameters, however the latter exhibited only borderline significance. Similarly, P16 and DAPK methylation was earlier found to be individual marker for poor prognosis in oral cancers (Sailasree et al., 2008; Su et al., 2010; Supic et al., 2011).

The current study on OSCC and ESCC from a high-incidence region of Northeast India has shown an increased rate of HPV presence in tumour tissues
(36%). Similarly, findings from other high-risk areas reported a significantly higher percentage of these viruses in oral, pharyngeal, laryngeal and esophageal cancers (Jiron et al., 2013; Morshed et al., 2005; Nichols et al., 2013; Scudellari, 2013; Wang et al., 2010). Although HPV is recognised as a major risk factor for head and neck cancers, but its association with SCC of the esophagus has remained discrepant (Awerkiew et al., 2003; Lagergren et al., 1999). This discrepancy is likely due to the genetic susceptibility towards viral infection, geographical variations, detection technique used and sample origin (Wang et al., 2010). However, a recent meta-analysis, including 1223 cases and 1415 controls re-confirmed the aetiological role of HPV in esophageal squamous cell carcinoma, in addition to head and neck cancers (Liyanage et al., 2013). Usually, HPV 16 is found as the major sub-type (Herrero, 2003), but we found HPV 18 as the major sub type, which might be due to geographical difference (Damin et al., 2013).

HPV presence is known as a predictive marker for better survival in oropharyngeal cancers (Petrelli et al., 2013; Tahtali et al., 2013; Worsham et al., 2013). We failed to find a significant association of HPV and survival overall, although when site specific survival was considered, HPV presence showed significantly improved survival rates in OSCC cases; in ESCC cases, however, a non-significant trend for poorer survival was observed in HPV (+) cases. Association of HPV with better survival in OSCC was irrespective of tobacco habits; however tobacco consumption appeared to affect survival in HPV negative cases only in a non-significant manner. The combined effect of HPV and tobacco in predicting survival in HNSCC was reported an earlier study from India, where although they found HPV as marker of better survival, but the combined HPV and tobacco habit appeared to be associated with poorer survival(Ghosh et al., 2009).

HPV presence has earlier been reported to adversely affect survival in ESCC. Furihata et al. reported worse survival of HPV (+) patients as compared to HPV (-) patients with an over-expression of p53 in esophageal cancer (Furihata et al., 1993). But others failed to establish HPV as a predictor of ESCC survival, in both PCR based and quantitative studies (Dreilich et al., 2006).
Evidence are growing that virus induced aberrant DNA methylation of the host genome might act as an alternative route for the carcinogenic pathway in a variety of HPV-driven cancers (van Kempen et al., 2013). Our findings also exhibited higher frequency of methylation in tumours associated with HPV, indicating towards a potential role of HPV in enhancing aberrant DNA methylation of cancer related loci. In this study, we have used methylation markers widely employed and found to be representative of the overall CpG island methylation status in cancers like colorectal and gastric (An et al., 2005; Hughes et al., 2012; Toyota et al., 1999a; Zhang et al., 2008). Although these markers were not extensively studied in UADT cancers, but based on these markers we could classify the cases as CIMP-high, intermediate and low. HPV was significantly associated with CIMP-status, the highest frequency being observed in the CIMP-high group. The mean methylation index was also significantly higher in HPV (+) cases as compared to HPV (-) group. Moreover, multivariate logistic regression analysis revealed almost 11 folds higher association of HPV with CIMP-high after adjusting with all potential confounding factors. The fact that HPV can modulate aberrant DNA methylation is evident from both cell culture as well as clinical studies. In a comparative genome wide methylation study of HPV (+) and HPV (-) head and neck squamous cell carcinoma cell lines, higher CpG DNA methylation in both genic and LINE-1 regions were observed in HPV (+) cell lines as compared to the HPV(-) ones (Sartor et al., 2011). Another study on genome-wide methylation of HPV (+) and HPV (-) HNSCC patients identified distinct methylation signatures with preponderance of hypermethylation at transcription start sites leading to CIMP in HPV (+) cancer cases. During functional validation, ectopic expression of HPV E6 and E7 in an HPV (-) HNSCC cell line partially phenocopied the hypermethylation signature of the HPV (+) tumours further establishing the causal effect (Lechner et al., 2013). In an earlier study on cervical cancer, promoter hypermethylation of p16 gene was found associated with HPV infection and also reports the inactivation of Retinoblastoma (Rb) by viral E7 protein (Nuovo et al., 1999). However, we could not find any strong association between p16 aberrant methylation and HPV presence.
In developed countries, HPV associated HNSCCs are thought less likely to be tobacco users or harbour lower p53 mutations and comparatively homogenous in terms of harnessing overall mutations (Scudellari, 2013). Hence they are classified as either ‘tobacco-associated’ or ‘HPV-associated’. In contrast, the study published by India team of the International Cancer Genome Consortium on gingivo-buccal OSCC have reported a high proportion of HPV cases (61%) with P53 mutation and also the overall mutation rates did not vary significantly between HPV (+) and HPV (-) cases (India Project Team of the International Cancer Genome et al., 2013). This might be because almost all the HPV (+) patients were also tobacco chewers or smokers. Similarly, most of the HPV (+) patients in our study were tobacco consumers as the study is based on a unique population of Northeast India, where tobacco and areca-nut consumption is customary. Combined chewing of tobacco and areca-nut with slaked lime (a combination called betel quid) causes mucosal injury to the lining of upper aero digestive tract, and increases susceptibility for penetrance of tobacco-related carcinogens and reactive oxygen species. In addition, it might also pave a way for persistent HPV infection in the aberrated mucosa. The resultant interaction between HPV and tobacco might synergistically modulate the cancer risk (Tezal, 2012). Moreover, tobacco related carcinogens are now established as important DNA methylation modulators in cultures as well as human studies (Baba et al., 2010; Zeilinger et al., 2013). Earlier in the study, we reported the probable role of smokeless tobacco in modulating promoter hypermethylation of multiple tumour suppressor genes in ESCC. Here also, we find a significant association of tobacco with CIMP-high tumours. Furthermore, the combined effect of HPV and tobacco imparted the highest risk for developing CIMP-high after adjusting for all potential confounders.

In conclusion, our study not only confirmed tobacco consumption, especially the smokeless form as the main risk factor of OSCC and ESCC in Northeast India, but also indicated its possible interaction with carcinogen metabolizing genes towards modulating promoter hypermethylations of tumour related genes or loci.
Figure 5.1 Proposed interaction model of different epigenetic and environmental factors in OSCC and ESCC based on our findings

Moreover, our findings demonstrate a strong association of HPV in aberrant methylation of cancer-related genes, which might be the key mechanism for transcriptional deregulation of these crucial genes for malignant transformation in OSCC and ESCC. The results also indicate towards a possible synergistic role of HPV and tobacco in the genesis of these cancers probably by modulating DNA methylation signatures of the host genome. Therefore, understanding the interactions among risk factors and their effect on various cellular mechanisms could be vital towards effective prevention and treatment strategies of these cancers. The evaluation of HPV vaccination for oral and esophageal cancers is also an important question to be addressed. Moreover, mechanism of targeting specific cancer-related genes/loci in HPV induced epigenetic reprogramming has potential translational value and requires special attention.