This is the first study that consisted of NPC cases, their first degree relatives (FDRs) and controls from the three highly NPC prone ethnic groups in Northeast India, i.e. Manipuri, Naga and Mizo. The lifestyle and environment have a huge impact on human health. This is what we have observed in this study. The first and foremost thing to be observed was the housing pattern with poor or no ventilation, especially in the economically poor people and those who are obviously more susceptible to NPC. These houses even do not have a separate kitchen; as a consequence, the smoke generated from firewood, charcoal, etc. is inhaled by the members in the house which can be one of the contributing factors to NPC. Smoked meat and fermented fish are traditional staple food in several regions of Northeast India, especially NPC-endemic areas. Increased risks of NPC were observed in both patients and their FDRs with the intake of smoked meat and fermented fish. The process of cooking and preserving fish and meat results in the accumulation of significant levels of nitrosamines, which are known carcinogens in human health. Smoked meat consumption is very common in Nagaland, which is linked with high prevalence of NPC, and in our study, we observed a twofold increased risk to NPC in smoked meat consumers. Smoking process contaminates the meat with nitrosodimethylamine (NDMA), nitrosodiethylamine (NDEA) and nitrosopyrrolidine (NPYR), which are known carcinogens, which affects human health and has proven to be risk factors for NPC (Chelleng et al., 2000; Sugimura et al., 2004). The habits of tobacco-betel quid (with/without tobacco) chewing and/or other forms of tobacco are endemic throughout India (Mondal et al., 2013a; Choudhury and Ghosh, 2014). In this study, individuals with tobacco chewing were at an increased risk of NPC, and are in consistent with previous study of cancer in northeast India (Mondal and Ghosh, 2013; Choudhury et al., 2014). Tobacco and betel-quid constitute large number of polycyclic aromatic hydrocarbon (PAH), alkaloids and other phenolic

compounds which are considered as prime risk factors of cancer (Nagaraj et al., 2006; Sabitha et al., 2010; Talukdar et al., 2013). These compounds not only cause single strand DNA breaks but also result in the oxidation of protein thiols and lipid peroxidation, thereby triggering damage to both nuclear and mtDNA.

CYP1A1, GSTT1 and *GSTM1* belong to a superfamily of Phase I and Phase II xenobiotics metabolizing enzymes. These enzymes help in the detoxification of numerous toxic compounds that are probably associated with cancer risk (McIlwain et al., 2006; Androutsopoulos et al., 2009). Many studies showed conflicting role of *GSTT1* null and *GSTM1* null genotypes on NPC risk (Guo et al., 2008; Jiang et al., 2011). In the present study, multiplex PCR was done for the detection of *GSTM1* and *GSTT1* null genotype. In both cases (2.76 fold) and FDRs (2.1 fold), we found a significant risk of *GSTM1* null genotype associated with NPC whereas no association was found with *GSTT1* null genotype. However, recent meta-analysis showed higher incidence of NPC in individuals carrying the defective *GSTT1* and *GSTM1* genes (Wei et al., 2013). As reported earlier, *CYP1A1* T3801C polymorphism was not associated with NPC risk (Cheng et al., 2003) and other cancers (Anantharaman et al., 2007). NPC is polygenic disease and polymorphism in individual genes cannot explain the underlying pathogenic mechanism. To understand such complex diseases the cumulative effect of many polymorphisms is more likely important. To date, no studies have examined the risk conferred by the combination of *CYP1A1, GSTT1* and *GSTM1* polymorphisms in the endemic part of northeast Indian population. Studies in an endemic region have shown the elevated risk of NPC in both *GSTT1* null and *GSTM1* null genotypes (Guo et al., 2008; Jiang et al., 2011). Similarly, we found that *GSTM1* null genotype in the absence of *GSTT1* genotype had a 3.77 fold increased risk of NPC. Significant interaction was also observed between *GSTMI* and *CYP1A1* genes

(*P*=0.001). However, highest risk of NPC (5.71 folds) was observed in individual carrying the defective genotypes of *GSTT1, GSTM1* and *CYP1A1* T3801C, suggesting that cross talk between these genes might modulate susceptibility towards NPC. Similar results were reported in head and neck cancers (HNC) (Soya et al., 2007; Choudhury et al., 2014).

Furthermore, significant gene-environment interactions that further modify the risk of NPC were noted. When a combine effect of diet (smoked meat and fermented fish) and genotypes were considered, highest joint effect was observed in individual with *GSTM1* null or *GSTT1* null genotypes (*P*<0.0001). Significant interaction was also observed with *CYP1A1* T3801C polymorphic variants, which modulate the risk of NPC (*P*=0.001). As mentioned, earlier these foods are highly contaminated by nitrosamines and nitrosamine precursors as a result of processing. Therefore, such an interaction is biologically possible as individual with the defective genotypes do not have proper enzyme activity and are more susceptible to carcinogens present in the preserved foods. To our knowledge, we reported for the first time a strong effect modification by diet of the association between metabolic genes and NPC.

In addition, we observed a significant interaction of metabolic gene with tobacco habits. Recent studies conducted in India, showed that *GSTM1, GSTT1* and *CYP1A1* genes are associated with cancer among chewers (Soya et al., 2007; Sam et al., 2008; Sam et al., 2010). In our study, chewers carrying the defective *GSTM1* gene had 5.86 fold increased risk of NPC. Significance interaction was observed in chewers with *GSTT1* null or *CYP1A1* T3801C polymorphic variants (P<0.05). However, highest risk (12.67 fold) of NPC was observed in *GSTM1* null individual with habits of smoking. Similarly, smokers without *GSTT1* gene (4.46 fold) were associated with NPC risk. A recent study conducted on head and neck cancer has also reported significant

interactions of *GSTT1* and *GSTM1* gene polymorphisms with smoking (Choudhury and Ghosh, 2014). NPC is strongly associated with smoking, and no study has been conducted that explore the role of *CYP1A1* polymorphism in the risk of developing NPC in smokers. Here, we observed a significant increased (7.13 fold) risk of NPC in smokers carrying the *CYP1A1* TC+CC genotypes. Our result is supported by previous studies conducted on HNC in northern and southern India (Sam et al., 2010; Sharma et al., 2011). However, other studies (Matthias et al., 1998; Varela-Lema et al., 2008) did not support any association between smoking and *CYP1A1* polymorphisms in cancer risk. Our findings confirm the role of environmental factors along with genetic polymorphisms as risks enhancers in the etiology of NPC among the ethnic population of northeast India.

Beside the conventional LR method we also use advanced MDR approach to investigated high-level interaction between *GSTT1, GSTM1* and *CYP1A1 T3801C* genotypes and environmental factors in NPC risk (Table 4.2.3 - 4.2.6). MDR analysis identify the best model for NPC risk as the four-factor model combinations of GSTM1 null genotype, fermented fish, smoked meat and smoking having 100% CVC and highest TBA. Study in bladder cancer also showed four factor model of gene-environmental interaction as the best model to determine the cancer risk (Huang et al., 2007). Similarly, MDR analysis among the northeast Indian population showed four factor model had the best ability to predict HNC risk with TBA of 0.6737 maximum CVC (10/10; $p<0.0001$) (Choudhury and Ghosh, 2014). Interaction entropy graphs were drawn to determine whether the gene-environmental interactions shown by MDR analysis have synergistic or antagonistic effects. Tobacco smoking, smoked meat and fermented fish had the highest individual effects. GSTT1 (0.58%) and GSTM1 (1.34%) null genotype, both showed synergistic interactions in NPC risk. Similarly, GSTM1

(1.34%) null genotype, showed synergistic interaction with fermented fish and smoked meat. Our investigations indicated that gene-environment interaction plays vital role in NPC carcinogenesis.

Any kind of damage in DNA is rectified by primary defence system in our body which includes the *XRCC1* and *XRCC2* repair genes that eliminate such damage keeping the genomic integrity of the cells. *XRCC1* gene of the BER pathway encodes a protein having three functional domains that interact with different enzymes to initiate DNA repair of different stages and types (Dianova et al., 2004). *XRCC2* encoded protein play a key role in DNA repair pathway and is required for RAD51 focus formation. It is an essential part of the HRR pathway and a functional candidate to be involved in tumor progression (Jiao et al., 2008). Polymorphism in *XRCC1* Arg399Gln and *XRCC2* Arg188His may affect the DNA repair capacity of an individual, which can further cause genomic instability and finally leads to cancer. The polymorphisms in these genes have been well documented with cancers (Johnson et al., 1999; Flores-Obando et al., 2010; Sterpone et al., 2010; Choudhury et al., 2014). A recent study conducted in China and another meta-analysis reported for high association of *XRCC1* Arg399Gln polymorphism and NPC [69, 70]. Similarly, our finding suggests a high (2.76 fold) risk of NPC in patients carrying Gln399Gln genotype of *XRCC1* gene. However, studies conducted in Chinese (Cao et al., 2006), Taiwanese (Cho et al., 2003), Malaysian (Visuvanathan et al., 2014) and North African (Laantri et al., 2011) population did not report such association. No case-control study has ever reported link between *XRCC2* Arg188His polymorphisms and NPC. And in our study also, we did not find any association of *XRCC2* variant genotype and NPC risk. Further, there were no risk association of NPC with *XRCC1* and *XRCC2* polymorphisms in FDRs.

Environmental factors alone cannot explain the complex mechanism involved in NPC carcinogenesis. Therefore, the interaction of repair genes and dietary and tobacco habits were also investigated in the study population. Several studies have reported the association of tobacco and DNA repair genes in cancer (Saikia et al., 2014; Uppal et al., 2014). In contrary to studies conducted in north India and China (Cao et al., 2006; Kumar et al., 2012; Li et al., 2013), tobacco habits were found to increase the risk of HNC in individual carrying *XRCC1* Arg399Gln and *XRCC2* Arg188His polymorphic variants (Choudhury et al., 2014) in northeast India. Both LR method and advanced MDR approach reveal a high-level interaction between repair genes and environmental factors in NPC risk (Table 4.3.2, 4.3.3 & 4.3.4). Here, MDR analysis identify the best model as the five-factor model interaction of *XRCC1* Arg399Gln genotype, fermented fish, smoked meat, smoking and chewing having 100% CVC and highest TBA. Interaction entropy graphs determine tobacco smoking, smoked meat and fermented fish had the highest individual effects. Though *XRCC1* and tobacco betel-quid chewing had low effects, both showed synergistic interactions in NPC risk. Similarly, interaction was also observed between *XRCC1* and smoked meat. Our investigations indicated that besides environmental factors, individual genetic make-up also plays vital role in NPC carcinogenesis.

The involvement of EBV in NPC has been postulated since 1970s (Henle et al., 1970). Some hypothesis proposed that EBV played a critical role in transforming nasopharyngeal epithelial cells into invasive cancer (Lo et al., 2004). In the present study, we have analyzed the EBV *LMP1* gene in cases, controls and FDRs and found to be a risk factor in the NPC cases with EBV infection. *LMP1* is of special interest since it is generally considered to be the main EBV oncogene that is believed to be important in the pathogenesis of nasopharyngeal carcinoma (Hu et al., 1991; Lin et al., 2004).

EBV, in conjunction with environmental and genetic factors, plays a role in the development of NPC. *LMP1* localizes to cellular membranes and functions as a constitutively active tumour necrosis factor receptor homologue that propagates intracellular signalling, including the NF-κB, cJun N-terminal protein kinase/AP-1 and Janus kinase/STAT pathways (Eliopoulos et al., 1999; Higuchi et al., 2002). Through these signalling pathways, *LMP1* generates innumerable effects on host cell growth, differentiation and apoptosis, including growth promotion and survival in epithelial cells (Dawson et al., 2000). Although the mechanisms by which *LMP1* influences cell biology have been intensively studied, little is known about the mechanisms that regulate *LMP1* oncogenic activity (Pandya and Walling, 2006). In the present study, we found a significant difference with mtDNA content in cases and controls with or without EBV infection (P=0.002). However, there are no reports of association of EBV infection with the mtDNA copy number. The EBV codes for BamHI fragment H rightward open reading frame 1 (*BHRF1*) an early protein, which localizes to the mitochondrial outer membrane and co-localizes with Bcl-2 (Henderson et al., 1993; Hickish et al., 1994). The *BHRF1* interacts with the cellular protein *VRK2* (Li et al., 2006) and enhances the cell survival. Several EBV-associated proteins, such as *BALF1*, *BHRF1, EBNA* and *LMP1*, have been shown to interfere with fatty acid metabolism, mitochondrial function and apoptosis pathways (Kawanishi et al., 2002; Lu et al., 2005). It may be possible that by inhibition of apoptosis, thereby facilitating tumour development with corresponding variation to mtDNA content, for which the exact mechanism is unclear. We are reporting for the first time the association of EBV infection with mtDNA content variation.

We also analysed if the risk associated between EBV infection and NPC risk is modified by dietary and tobacco habits. No case-control study has been conducted to

determine an association of lifestyle factors and EBV infection in NPC carcinogenesis. Statistically significant increased risks of NPC were observed in individuals with EBV infection and consuming smoked meat and fermented fish ($P<0.0001$). Our results may be supported by experimental evidence that determine the presence of possible EBV inducers, activators, mutagens and volatile nitrosamines in preserved food samples from high-risk areas for nasopharyngeal carcinoma (Shao et al., 1988; Bouvier et al., 1991). Results from the present study indicate that cigarette smoking is also associated with risk of NPC. An etiological link between cigarette smoking and NPC risk is biologically plausible since the nasopharynx is a site directly exposed to smoke during cigarette smoking. Tobacco smoke also contains over 4,000 compounds; such as polycyclic aromatic hydrocarbons, aromatic amines, and N-nitrosamines, which are carcinogens (West et al., 1993) and it is possible that carcinogenic products in cigarettes could cause genetic mutations and methylation (Ronai et al., 1993) thereby resulting in the transformation of epithelial cells in the nasopharynx. Cigarette smoking may also play an alternative role by acting as a cofactor to EBV infection or via inducting EBV reactivation (Abdulamir et al., 2008; Xu et al., 2012). We observed consistent evidence that a strong association exists between EBV infection and tobacco smoking among our study population. We verified that risk associated with NPC was much higher among tobacco smokers and EBV infected subjects. Though not statistically significant a very high interaction of EBV infection and tobacco smoking was observed in previous studies (Lin et al., 1979; Hsu et al., 2009). In our study population EBV infected individuals with tobacco smoking were associated with 11.44 fold risk for NPC development and were statistically significant.

Plants commonly of the Euphorbiaceae and Thymelaeceae families are associated with NPC risk in endemic areas. It has been found that these plants contain

phorbol esters, which is thought to be linked to nasopharyngeal carcinoma, either through its ability to reactivate the Epstein-Barr virus (EBV) infection (Zeng et al., 1983) or through a direct promoting effect on cells transformed by the EBV (Tomei et al., 1987). In our study, it was observed that the consumption of herbal medicine is associated with 3.18 fold risk of developing NPC, similar to other endemic areas (Hildesheim et al., 1992; Chelleng et al., 2000) though another study denied this association (Yu et al., 1989). Of particular note is our finding of an apparent association between EBV and herbal medicines. Herbal medicine was found to act jointly with EBV infection. The risk associated with NPC increases to 9.15 fold among EBV positive subjects with herbal medicine use. We also assessed the identity of the herbal medicine use in our study areas. We could identify only a few herbal medicines use in common ailments, such as *Alnus nepalensis D. Don, Ananas comosus (L.) Merr., Asparagus racemosus Wilt., Callicarpa arborea Roxb., Curcuma caesia Roxb., Drymaria cordata Willd., Fagopyrum esculentum Moench., Phyllanthus fraternus Web., Ricinus communis Linn., Sonchus wightianus Linn., Adhatoda vasica Nees., Bombax ceiba Linn., Cassia alata Linn, Phlogacanthus thyrsiflorus, Croton caudatus* (variety not specify). Interestingly, use of plants of the Euphorbiaceae family was common, which have been observed to be linked to nasopharyngeal carcinoma (Zeng et al., 1983; Tomei et al., 1987). This could explain the increased risk of NPC among EBV infected individual with herbal medicine use in our study population.

Moreover, mitochondrial dysfunction has been reported in various cancers like oral cancer in our previous studies (Mondal et al., 2013a; Mondal et al., 2013b) as well as in NPC (Shao et al., 2004; Pang et al., 2008). In this study, low level of the mtDNA copy number in cases was found to be associated with high risk of NPC, which is in contrary to the findings in the FDRs (Table 4.5.2). It may be probably due to the release

of substantial amounts of ROS generated from smoke inhalation from cooking fuel in the houses with attached kitchen and improper ventilation and of course from the smoked, preserved food and tobacco products which in turn increase mtDNA mutation. This was further proved by the enhanced risk of NPC observed among smoked meat and fermented fish consumers and tobacco user with low mtDNA content (Table 4.5.3). Similar results were reported in OSCC but not in tobacco chewers (Mondal et al., 2013a). The accumulation of mtDNA deletions and subsequent cytoplasmic segregation of these mutations during cell division could be important contributors to the early phase of NPC (Lee et al., 2001; Mondal et al., 2013a).We evaluated patients treated with radiation therapy and without therapy and found that those who underwent radiation therapy have a low copy number than the non-radiated ones. This decrease of mtDNA after radiotherapy may reflect an effect of external beam radiation that influences the mitochondrial number in cells, effectively reducing mtDNA (Jiang et al., 2006). Alternatively, the depletion in mtDNA may be the result of the repression of mitochondrial biogenesis. The mtDNA copy number in cancer probably depends on several factors, including the site of mutation in the mitochondrial genome as demonstrated in D-loop region, a highly susceptible site for oxidative damage compared with the other regions of mtDNA (Mondal and Ghosh, 2013).

In consistent with previous studies in Thais (Fachiroh et al., 2012) and Chinese (Bei et al., 2010) population we found associations of *TNFRSF19*, and *MDS1-EVI1* loci in NPC susceptibility. However, there were no association of *CDNK2B-AS1* loci in NPC susceptibility. Over expression of *TNFRSF19* (Tumor necrosis factor receptor superfamily, member 19), induces the c-jun N-terminal (JNK) pathway, leading to the activation of caspase-independent apoptosis (Eby et al., 2000). Dysregulation on the JNK pathway may occurs in NPC pathogenesis, in addition to

EBV oncogenic and cellular transformer Latent Membrane Protein1 (*LMP1*) that works in mimicking TNF receptor superfamily (Tsao et al., 2002). On the other hand, *MDS1-EVI1* (myelodysplasia 1 and ecotropic viral insertion site 1 fusion proteins) encodes three proteins, *EVI1*, *MDS1*, and the fusion protein *MDS1-EVI1*. *EVI1* can suppress the effects of transforming growth factor (TGF)-b on growth inhibition and protect cells from stress-induced cell death by inhibiting c-JNK. When *EVI1* is fused with *MDS1*, its capacity to repress TGF-b signalling may be significantly impaired (Metais and Dunbar, 2008). The interruption of the balance between *EVI1* and *MDS1-EVI1* proteins may be important for the pathogenesis of NPC (Bei et al., 2010). Further, MDR analysis reveal the five-factor model combination of *TNFRSF19, GSTT1, GSTM1, CYP1A1, and XRCC1* variant genotype having maximum CVC of 100/100 and highest TBA of 0.675 (P<0.0001) as the best genetic model for NPC risk prediction (Table 4.4.2). For better understanding, the overall probable risk factors associated with the development of NPC and prevalence in the ethnic groups of Northeast Indian populations are presented by a schematic diagram in Figure 5.1.

The environment and genetic risk factors which were examined in this study with their role in the etiology of NPC were discussed above and reached to a conclusion that the understandings of the risk factors are helpful as preventive measures and screening. The association of genetic polymorphisms along with mtDNA copy number can be used as a preventive measure for the patient either at risk of NPC due to lifestyle or those who have heredity of NPC in their family. Furthermore, the understanding that the environment–genetic interactions related to NPC pathogenesis where EBV remains a constant factor using next-generation sequencing technology will be of great potential along with in-depth study of EBV interaction with mitochondria in inhibiting apoptosis can be an addition, which may give a new direction in treatment of this enigmatic NPC.

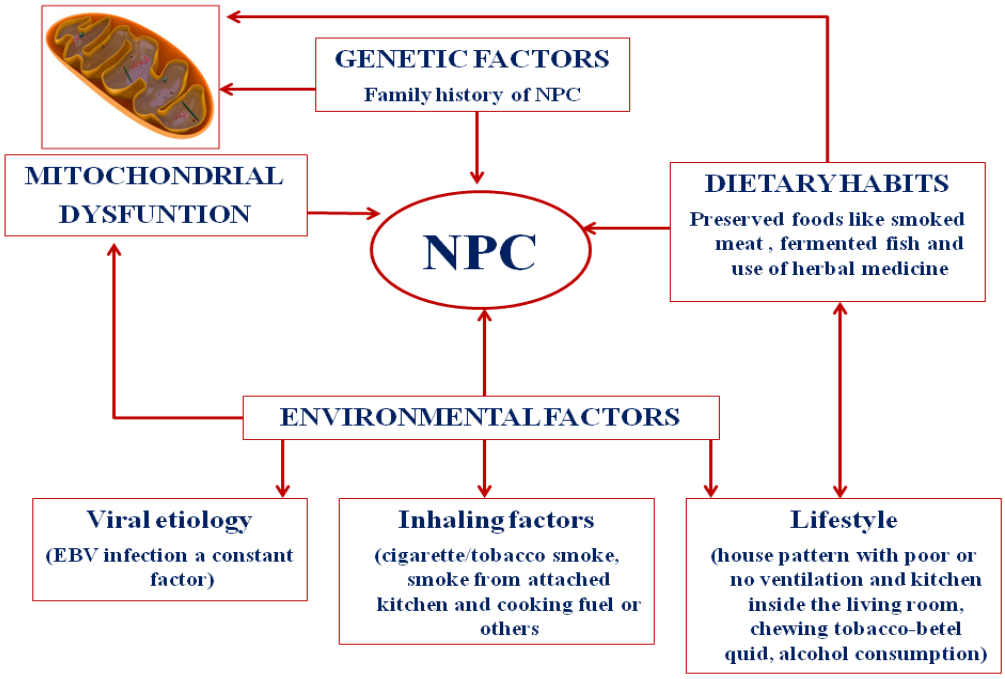

Figure 5.1 Schematic representation of the etiology of nasopharyngeal carcinoma (NPC). The combined effects of environmental factors and genetic factors led to the development of NPC directly as well as caused mitochondrial dysfunction which is related to NPC in the ethnic group of Northeast India. The environmental factors include viral etiology, i.e. EBV infection remains a constant factor for the development of NPC. The EBV-associated proteins like *LMP1, BALF1*, *BHRF1* and *EBNA* interfere with fatty acid metabolism, mitochondrial function and more importantly apoptosis pathways, thereby facilitating tumour development. The dietary factors, inhaling factors and lifestyle were few environmental factors which contain significant levels of nitrosamines, HCAs and PAHs which are involved in the development of NPC and responsible for mitochondrial dysfunction by production of ROS, which also cause oxidation of protein thiols and lipid peroxidation triggering mtDNA damage. Genetic factors-The family history of NPC has consistently been associated with an increased risk of NPC; it may be because of inherited genes, shared environmental factors (such as the same diet or living quarters) or combination of these. The polymorphism of carcinogen-metabolizing genes and DNA repair genes are another genetic factor, which has a potential role in cancer susceptibility. Phase 1 (*CYP1A1*) and phae II (*GSTM1* and *GSTT1*) gene are essential for the detoxication of carcinogenic compounds. Polymorphic variant of *CYP1A1* T3801C and the null GSTs increase the risk for NPC as incompetent to detoxify the carcinogens generated from different smoked foods, tobacco and betel quid consumption and also lead to the accumulation of mtDNA mutations as they play inside the mitochondrial matrix as mitochondrial protection factor from the damage caused by ROS. DNA repair genes also play important role in NPC carcinogenesis. Mutation in tumor suppressor genes (e.g., *p52, p21*) and proto-oncogenes (e.g., gain of function mutation of *ras* gene etc) may contribute to cancer development. Polymorphisms in DNA repair genes reduce the repair capacity and such mutation will go un-repair thereby increasing risk of NPC development.

Both conventional LR as well as advanced MDR approach used in our study further confirmed that the interaction of genetic and environmental factors further increased the risk of NPC. Moreover, implementation of advanced statistical tools in interaction study enables us to identify high-risk marker for cancer risk. Further studies with larger samples size and also in other populations are required before the clinical implications can be considered.