Chapter 2

Review of Literature
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2.1 Overview of Head and Neck Cancer

The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviours, particularly smoking, in economically developing countries. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 (Ferlay et al., 2010); of these, 56% of the cases and 64% of the deaths occurred in the economically developing world.

An estimated 263,900 new cases and 128,000 deaths from oral cavity cancer (including lip cancer) occurred in 2008 worldwide. Generally, the highest oral cavity cancer rates are found in Melanesia, South-Central Asia, and Central and Eastern Europe and the lowest in Africa, Central America, and Eastern Asia for both males and females. Smoking, alcohol use, smokeless tobacco products, and HPV infections are the major risk factors for oral cavity cancer, with smoking and alcohol having synergistic effects (Blot et al., 1988, Hashibe et al., 2009). The rise in the incidence rate of oral cancer in Taiwan may have been in part due to the increased consumption of betel quid and alcohol (Ho et al., 2002). There were an estimated 84,400 incident cases of NPC and 51,600 deaths in 2008, representing about 0.7% of the global cancer burden, and the disease may be considered one of the rarer cancer forms globally, ranking as the 24th most frequently diagnosed cancer form worldwide and 22nd within the developing world.

The geographical disparities in the burden of NPC in relation to resource are noteworthy, with an estimated 92% of new cases occurring within economically developing countries. According to world area, incidence rates are highest in South-Eastern Asia, in both sexes, with the disease being the sixth most common among males in the region. Indeed in global terms, the 3 highest national incidence rates are estimated in Malaysia, Indonesia, and Singapore, where rates are high among the Chinese and Malay populations (Parkin et al., 2005). Elsewhere in Asia, high incidence rates are observed in a number of provinces in South-Eastern China, including Guangdong and Hong Kong, and in other parts of Southern Asia (the Philippines and Thailand) (Curado et al., 2007, Parkin et al., 2003).
2.2 Tobacco, betel quid and oral cancer

A risk factor is anything that increases a person's chance of developing cancer. Different cancers have different risk factors. Use of tobacco, certain diets, alcohol, exposure to ultraviolet (UV) radiation, and to a lesser extent, exposure to cancer causing agents (carcinogens) in the environment and the workplace are some of the potential catalysts of cancer. It is however, important to remember, that these factors increase a person's risk but do not always "cause" the disease. Oral cancer is the most common cancer in India, Pakistan, and Sri Lanka and ranks high in several Southeast Asian countries. The association of these cancers with cultural practices like chewing was recognized almost a century ago. Continued work since then has identified tobacco use as the most important avoidable cause of oral cancer (Jayant & Deo, 1986). Areca nut usage is widespread in Asian countries, especially India and Taiwan. Shah et al. (2012) reviewed that the incidence of oral cancer is increasing day by day, but there is no exponential increase with tobacco usage. Especially in the country like Taiwan where betel quid mostly do not contain tobacco, areca nut can be correlated with the increased incidence of cancer. There are different studies in the literature about areca nut and oral cancer but none of them have concluded with the definite pathway for carcinogenesis. Sharan et al., (2012) made a systemic review on betel nut (BN), betel quid (BQ) and products derived from them that widely used as a socially endorsed masticatory product. This addictive practice has been shown to have strong etiological correlation with human susceptibility to cancer, particularly oral and oropharyngeal cancers. The PUBMED database was searched to retrieve all relevant published studies in English on BN and BQ, and its association with oral and oropharyngeal cancers. Only complete studies directly dealing with BN/BQ induced carcinogenesis using statistically valid and acceptable sample size were analyzed. They observed that the BN/BQ mastication is significantly associated with susceptibility to oral and oropharyngeal cancers. Addition of tobacco to BN has been found to only marginally increase the cancer risk. Despite the widespread usage of BN/BQ and its strong association with human susceptibility to cancer, no serious strategy seems to exist to control this habit.

The role of diet in cancer risk has been investigated by Helen-Ng et al. (2012) based on intake of individual food items. However, food consumption is made up of a combination of various food items. The study aimed to determine the association of dietary patterns with oral cancer risk. A total of 306 matched cases and controls were recruited in this study.
Data on dietary intake were obtained using food frequency questionnaire (FFQ). Factor analysis (FA) was performed to identify dietary patterns based on the intake of nine major food groups, resulting in four factors/components being retained. The first pattern labelled as 'modern' was loaded with processed foods and snacks, whereas the second pattern termed as 'prudent' was characterized by intake of fruits and vegetables. The third pattern labelled as 'traditional' consisted of beverages and starches, while the fourth pattern termed as 'combination' was loaded with intakes of dairy, fermented/salted and meat/by-products. A significant reduced risk was found for 'prudent', whereas an increased risk was found for both 'combination' and 'traditional' patterns. They observed that the consumption in the highest tertile of 'traditional' and 'combination' patterns may induce twice and thrice the risk of oral cancer, respectively.

2.3 Mitochondria and Cancer

Cytological studies indicated the presence of subcellular granules similar in size and shape to bacteria in a variety of different cell types. In 1890, Altman postulated that these granules, which he termed 'bioblasts', were the basic units of cellular activity. Interestingly, Altman further speculated that bioblasts were capable of an independent existence, yet formed a colonial association with the cytoplasm of a host cell, and that it was through this association that the host cell acquired the properties of life. The term 'mitochondrion', meaning thread-like granule, was first applied to these subcellular structures by Benda in 1898 (Ernster & Schatz, 1981). Mitochondria are dynamic intracellular organelles that play a central role in oxidative metabolism and apoptosis. The recent resurgence of interest in the study of mitochondria has been fuelled in large part by the recognition that genetic and/or metabolic alterations in this organelle are causative or contributing factors in a variety of human diseases including cancer (Wallace, 2010).

Marchington et al. (1997) have demonstrated the length polymorphisms in homopolymeric tracts in the large noncoding region of mtDNA. They developed a new method, T-PCR (trimmed PCR), to quantify heteroplasmy for two of these tracts (D310 and D16189). D310 variation was sufficient to indicate clonal origins of tissues and single oocytes. Tissues from normal individuals often possessed more than one length variant (heteroplasmy). However, there was no difference in the pattern of the length variants between somatic tissues in any control individual when bulk samples were taken. Their data suggests that a restriction/amplification event, which they attribute to clonal expansion of
founder mtDNA(s), has occurred by the time oocytes are mature, although further segregation may occur at a later stage. In contrast to controls, where the length distribution of the D310 tract varied between tissues in a patient with heteroplastic mtDNA rearrangements, suggesting that these mutants influence segregation. Their findings have important implications for the genetic counseling of patients with pathogenic mtDNA mutations.

Fliss et al. (2000) examined the human bladder, head and neck, and lung primary tumours and revealed a high frequency of mtDNA mutations. The majority of these somatic mutations were homoplasmic in nature, indicating that the mutant mtDNA became dominant in tumour cells. The mutated mtDNA was detectable in paired bodily fluids from each type of cancer and was 19 to 220 times as abundant as mutated nuclear p53 DNA. They suggested that by virtue of the clonal nature and high copy number, mitochondrial mutations may provide a powerful molecular marker for noninvasive detection of cancer.

Liu et al. (2001) investigated the potential role of somatic mtDNA (mtDNA) mutations in tumourigenesis and the occurrence of mutations in mtDNA of ovarian carcinomas. They sequenced the D-loop region of mtDNA of 15 primary ovarian carcinomas and their matched normal controls and detected somatic mutation in 20% (3 of 15). Complete sequence analysis of the mtDNA genomes of another 10 pairs of primary ovarian carcinomas and control tissues revealed somatic mtDNA mutations in 60% (6 of 10) of tumour samples. Most of these mutations were homoplasmic, and most were T>C or G>A transitions, but one represented a differential length within a run of identical C residues. Similar study was carried out by Kirches et al. (2001), and showed that heteroplasmy in the mitochondrial genome of gliomas sometimes occurs in a D-loop polycytosine tract. They used a combination of laser microdissection and PCR to detect and quantify variations in the polycytosine tract. Their results suggested that all base substitutions appeared to be homoplasmic upon sequencing, and 89% occurred at known polymorphic sites in humans and the same mechanisms that generate inherited mtDNA polymorphisms are strongly enhanced in gliomas and produce somatic mutations. The high incidence of mtDNA mutations found in ovarian carcinomas and other human cancers suggests that genetic instability of mtDNA might play a significant role in tumourigenesis.

A specific and highly polymorphic homopolymeric C stretch (D310), located within the D-loop, as a mutational hotspot in primary tumours was identified by Sanchez-Cespedes
et al. (2001). Sequencing analysis of individual clones from lymphocytes revealed that patients with D310 mutations in the tumours had statistically significant higher levels of D310 heteroplasmy (more than one length variant) in the lymphocyte mtDNA as compared with the patients without D310 mutations in the tumour mtDNA. Their observations suggest that D310 alterations are already present in normal cells and achieve homoplasmy in the tumour through a restriction/amplification event attributable to random genetic drift and clonal expansion.

Chen et al. (2002) isolated histologically defined cell populations from prostate cancer and its preinvasive lesions using laser capture microdissection, and performed genetic analysis on the mitochondrial genome and observed an extremely high incidence of somatic mutation (90% of prostatectomy cancer specimens) in the D-loop of mtDNA. In their study they concluded that the massive induction of lesion-associated mutations suggestive of active mitochondrial mutagenesis in both prostate cancer and its preinvasive lesions. Inspection of these mutations provides new insights into prostate cancer genetics and reveals unique patterns of somatic mutations in prostatic neoplastic lesions.

The role of mtDNA mutations in carcinogenesis was examined by Ha et al. (2002) for which they analyzed 137 premalignant lesions of the head and neck from 93 patients for DNA alterations in the poly-cytosine tract (C-tract) of the displacement loop. They tested somatic microsatellites at six loci on a subset of patients with metachronous or synchronous lesions. They observed that in most of the cases, the mitochondrial C-tract status identified a clonal relationship between these lesions. Genomic microsatellites also confirmed that a clonal relationship was present in many of these cases. They concluded from their study that mtDNA alterations in the head and neck occur in the earliest premalignant lesions and demonstrate a rising incidence that parallels histological severity and these alterations were valuable as additional markers of histopathological progression.

Maximo et al. (2002) studied the relationship of mtDNA alterations and thyroid tumourigenesis, where they analyzed 79 benign and malignant tumours (43 Hurthle and 36 non-Hurthle cell neoplasms) and respective normal parenchyma. They found 57 somatic mutations, mostly transitions, in 34 tumours and 253 sequence variants in 59 patients. Follicular and papillary carcinomas carried a significantly higher prevalence of non-silent point mutations of complex I genes than adenomas and also a significantly higher prevalence of complex I and complex IV sequence variants in the normal parenchyma.
adjacent to the malignant tumours. They concluded that mtDNA variants and mtDNA somatic mutations of complex I and complex IV genes seem to be involved in thyroid tumourigenesis. Germline polymorphisms of the ATPase 6 gene are associated with the occurrence of mtDNA CD, the hallmark of Hurthle cell tumours.

The hindrance of the interaction of ubiquinol with the cytochrome bc1 complex is the regulator of single electron diversion to oxygen examined by Staniek et al. (2002) where the hindrance of electron bifurcation was observed following alterations of the physical state of membrane phospholipids in which the cytochrome bc1 complex is inserted. Irrespective of whether the fluidity of the membrane lipids was elevated or decreased, electron flow rates to the Rieske iron-sulfur protein were drastically reduced. Concomitantly superoxide radicals were released from these mitochondria, strongly suggesting an effect on the mobility of the head domain of the Rieske iron-sulfur protein. This revealed the involvement of the ubiquinol cytochrome bc1 redox couple in mitochondrial superoxide formation. The regulator, which controls leakage of electrons to oxygen, appears to be the electron-branching activity of the cytochrome bc1 complex.

MtDNA mutations occur in a wide variety of degenerative diseases and cancer. MtDNA seems to be more susceptible to DNA damage and consequently sustains higher rates of mutation than does nuclear DNA (nDNA). Mambo et al. (2003) used real-time quantitative PCR to analyze mtDNA integrity, damage repair, and induced mutations after exposure of human adult retinal pigment epithelial (ARPE)-19 cells to 4-nitroquinoline 1-oxide, a UV-mimetic and adduct-forming carcinogen, and tert-butyl hydroperoxide, an oxidant. It was observed that the time course of repair of mutations of the D-loop and especially the D310 region after exposure to DNA-damaging agents was delayed when compared with other regions and gave rise to common D310 C-tract frame-shift mutations. The induced mutations in the D310 region were predominantly homoplasmic only 7 days after exposure to damage. The findings may explain the high frequency of homoplasmic D310 somatic mutations in many tumour types.

The relationship between mtDNA mutations, reactive oxygen species (ROS) generation, and clinical outcomes in chronic lymphocytic leukemia (CLL) patients was investigated by Carew et al. (2003). An analysis of mtDNA from 20 CLL patients revealed that primary CLL cells from patients with prior chemotherapy had a significantly higher frequency of heteroplasmic mutations than did those from untreated patients. Overall,
mtDNA mutations appeared to be associated with increased ROS generation. Analysis of paired blood samples from the same patient led to the identification of a heteroplasmic mutation in the cytochrome c oxidase II gene several months after chemotherapy. The mutation was associated with increased ROS generation. The results suggest for the first time that chemotherapy with DNA-damaging agents may cause mtDNA mutations in primary leukemia cells, which often exist in heteroplasmy, and are associated with increased ROS generation.

Defects in mitochondrial oxidative phosphorylation have frequently been associated with Alzheimer’s disease (AD), and both inherited and somatic mtDNA mutations have been reported in certain AD cases. MtDNA mutations contribute more generally to the etiology of AD. Coskun et al. (2004) examined the sequence of the mtDNA control region (CR) from AD brains for possible disease-causing mutations. Sixty-five percent of the AD brains harboured the T414G mutation, whereas this mutation was absent from all controls. Moreover, cloning and sequencing of the mtDNA CR from patient and control brains revealed that all AD brains had an average 63% increase in heteroplasmic mtDNA CR mutations and that AD brains from patients 80 years and older had a 130% increase in heteroplasmic CR mutations. Findings suggest that reduced ND6 mRNA and mtDNA copy numbers would reduce brain oxidative phosphorylation. These CR mutations could account for some of the mitochondrial defects observed in AD.

Kumimoto et al. (2004) analyzed the somatic mutations for hypervariable regions (HVR-I and HVR-II) in the D-loop of mtDNA to reevaluate mitochondrial genetic instability in esophageal cancer. They observed 14 somatic mutations in 13 patients (34.2%), eleven mutations were at the C consecutive stretch from position 303 to 309, 1 at position 215 in HVR-II and 2 at positions 16304 and 16324 in HVR-I and 41 types of germ line variations in HVR-I including 2 and 17 in HVR-II including 1. They determined nuclear genome instability of these 38 specimens by analyzing 3 independent microsatellite sequences. Their results suggested that mtDNA mutations might show a genetic instability in esophageal cancer independently from nuclear genome instability.

Jakupciak et al. (2005) examined the heteroplasmic and homoplasmic sequence variants in the mitochondrial genome in patient tumours. The sequence variants were distributed throughout the coding region. In the forensic community, the sequence variations used for identification are localized to the D-loop region because this region appears to have
a higher rate of mutation. However, in lung tumours the majority of sequence variation occurred in the coding region. Hence, incomplete mitochondrial genome sequencing, designed to scan discrete portions of the genome, misses potentially important sequence variants associated with cancer or other diseases.

Oral squamous cell carcinoma (OSCC) is strongly related to cigarette smoking and mtDNA mutations presence in OSCC which could be used as additional biomarkers for smoking-associated DNA damage (Prior et al., 2006). Three mutation hotspots were observed in the D-Loop at nt 146, 152 and 186, two of which (nt 146 and 152) have also been implicated in oesophageal SCC, another smoking-related cancer. It was found that D-Loop mutations occur predominantly in male smokers and female non-smokers and that this association with gender is statistically significant ($P=0.003$). The mtDNA mutation hotspots found in this study showed to be in particular nt 186, are potential biomarkers for oral SCC.

The noncoding region of the D-Loop has emerged as a mutational hotspot and Lievre et al. (2006) has found the association with prognosis and response to 5 fluorouracil (5FU) in colon cancers. The evaluated the frequency of D-Loop mutations in a large series of HNSCC and correlated with clinicopathologic parameters, sequencing the D-Loop of 109 HNSCC before a treatment by neoadjuvant 5FU-cisplatin-based chemotherapy and the results found suggests that D-Loop mutations should be considered as a cancer biomarker that may be useful for the early detection of HNSCC in individuals at risk of this cancer. Similarly, Pietka et al. (2008) has shown mitochondrial D-loop to be mutation hot-spot with majority of mutations in the positions 303 to 315 of poly-C tract. Data showed that 37% of patients with premalignant lesions and 62% with carcinoma in situ are positive for mtDNA mutations and correlated mtDNA content with the stage of the disease. They concluded from their finding that the mtDNA mutations facilitates cell proliferation and inhibit apoptosis by increasing the production of ROS. Cells harbouring mutated mtDNA have increased proliferation rate, as increased ROS concentration may act as an endogenous growth factor.

Yu et al. (2008) explored the sequence variations of mitochondrial D-loop region in familial breast cancer and their possible associations with breast cancer risk. PCR-SSCP and direct DNA sequencing methods were used to detect the variants of the mtDNA D-loop in 23 familial breast cancer patients as well as three high-risk cancer families. Compared to that in sporadic breast tumours (53.3%) and healthy blood donors (6.7%), they identified a total of 126 sequence alterations in 23/23 (100%) of familial breast cancer patients,
including eight novel nucleotide variants. Their results indicated that sequence variants within the mtDNA D-Loop region are frequent events in Chinese familial breast cancer patients. Some of these nucleotide abnormalities, particularly those in D310 segment, might be involved in the breast carcinogenesis and could be included in a panel of molecular biomarkers for cancer susceptibility early-detection strategy.

In colorectal cancer the relationship between p53, D-loop mutation, and mtDNA content in colorectal cancer (CRC) in 194 patients with sporadic CRC without microsatellite instability who underwent surgery was examined by Chang et al. (2009). They quantified mtDNA content and D-loop mutation using real-time PCR and sequencing and observed that the D-loop mutation occurred at significantly higher frequency in tumours with p53 mutation than in tumours without p53 mutation. They observed that the frequency of the decreased mtDNA content was significantly associated with TNM stage and p53 mutation. They concluded that the change of mtDNA is a common event in colorectal cancer with p53 mutation, but is not associated with prognosis of CRC patients.

The contribution of mtDNA mutation and mitochondrial dysfunction in tumourigenesis using human cell lines carrying a frame-shift at NADH dehydrogenase (respiratory complex I) subunit 5 gene (ND5) was examined by Park et al. (2009). They observed that with increasing mutant ND5 mtDNA content, respiratory function including oxygen consumption and ATP generation through oxidative phosphorylation declined progressively, while lactate production and dependence on glucose increased. The cell line carrying the heteroplasmic ND5 mtDNA mutation showed significantly enhanced tumour growth, while cells with homoplasmic form of the same mutation inhibited tumour formation. Their results indicate that the mtDNA mutations might play an important role in the early stage of cancer development, possibly through alteration of ROS generation and apoptosis.

The level of mtDNA damage (deletions, mutations and changes in copy number) in bronchoalveolar lavage (BAL) cells from 10 preterm infants (27-30 weeks) was investigated by Zoer et al., (2010). A first BAL (BAL1) was done within 24 h of endotracheal incubation and BAL2 was performed 30-103 h thereafter. Deletions were analyzed by long range PCR, point mutations by heteroduplex analysis of the D-loop region, and copy number changes by real-time PCR. When BAL1 and BAL2 samples were compared no new mutations were found. They concluded with their results that the exposure of preterm infants to short term
mechanical ventilation did not lead to detrimental consequences for the mtDNA in the form of mutations or deletions.

The role of mtDNA in breast cancer evaluated that the mtDNA as the major control region and the largest mtDNA protein-coding gene. NADH dehydrogenase subunit 5 (ND5) was investigated together with a mitochondrial haplogroup analysis in 64 patients with breast cancer (BC) and 54 patients with benign breast disease (BBD) as controls. Mutations in D-loop region were found in 10/64 of patients with BC and 14/54 of patients with BBD, while mutations in ND5 were detected in 6/64 of patients with BC and 5/54 of patients with BBD. The study concluded that mtDNA mutation may play a role in early stage of tumourigenesis, and mitochondrial haplogroup can also modulate breast cancer occurrence (Shen et al., 2011).

The evidence for the hypothesis reviewed by Lemarie & Grimm. (2011) particularly emerged from work on how complex I and II mediate signals for apoptosis. Both protein aggregates are specifically inhibited for apoptosis induction through different means by exploiting with protease activation and pH change, two widespread but independent features of dying cells. Nevertheless, both converge on forming reactive oxygen species for the demise of the cell. They concluded from their findings that the investigations into these mitochondrial processes will remain a rewarding area for unravelling the causes of tumourigenesis and for discovering interference options.

The entire mitochondrial genome sequence (16.5 kb) in matched normal and tumours was investigated by Dasgupta et al. (2012b) obtained from 30 never-smoker and 30 current-smoker lung cancer patients, and determined the mtDNA content. All the patients' samples were sequenced for KRAS (exon2) and EGFR (exon19 and 21) gene mutation. The impact of forced overexpression of a respiratory complex-I gene mutation was evaluated in a lung cancer cell line. They found a significantly higher mtDNA mutation in the never-smokers compared to the current-smoker lung cancer patients. The majority of the coding mtDNA mutations targeted respiratory complex-I and forced overexpression of one of these mutations resulted in increased in vitro proliferation, invasion, and superoxide production in lung cancer cells. They concluded from their findings that the signature mtDNA mutations provide a basis to develop novel biomarkers and therapeutic strategies for never-smoker lung cancer patients.
Somatic mtDNA mutations have been reported in some human tumours, but their spectrum in different malignancies and their role in cancer development remain incompletely understood. Larman et al. (2012) described the breadth of somatic and inherited mutations across the mitochondrial genome by sequence analyses of paired tumour and normal tissue samples from 226 individuals with five types of cancer using whole-genome data generated by The Cancer Genome Atlas Research Network and found that the frequencies of deleterious tumour-specific somatic mutations in mtDNA varied across tumour types and were predicted to functionally impact the encoded protein. In summary, their data suggested that the damaging somatic mtDNA mutations occur frequently (13-63%) in the five tumour types in their study and likely confer a selective advantage in oncogenesis.

MitDNA mutations have been found in many cancers but the physiological derangements caused by such mutations have remained elusive. Prostate cancer is associated with both inherited and somatic mutations in the cytochrome c oxidase (COI) gene. A study on prostate cancer patient-derived rare heteroplasmic mutation of this gene, part of mitochondrial respiratory complex IV was analyzed. Functional studies indicate that this mutation leads to the simultaneous decrease in cytochrome oxidation, increase in reactive oxygen, and increased reactive nitrogen. The data suggested that mitochondrial DNA mutations resulting in increased reactive oxygen and reactive nitrogen generation may be involved in prostate cancer biology (Arnold et al., 2013).

### 2.4 COLD-PCR in Mutation Enrichment and Detection

Li et al. (2008) describe co-amplification at lower denaturation temperature PCR (COLD-PCR), a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence. They replaced regular PCR with COLD-PCR before sequencing or genotyping assays to improve mutation detection sensitivity by up to 100-fold and identified new mutations in the genes encoding p53, KRAS and epidermal growth factor in heterogeneous cancer samples that had been missed by the currently used methods. For clinically relevant micro deletions, COLD-PCR enabled exclusive amplification and isolation of the mutants. COLD-PCR will transform the capabilities of PCR-based genetic testing, including applications in cancer, infectious diseases and prenatal identification of fetal alleles in blood.
COLD-PCR provides a general platform to improve the sensitivity of essentially all DNA variation detection technologies including Sanger sequencing, pyrosequencing, single molecule sequencing, mutation scanning, mutation genotyping or methylation assays elucidated by Li et al. (2009). COLD-PCR combined with real-time PCR provides a new approach to boost the capabilities of existing real-time mutation detection methods. COLD-PCR is expected to have diverse applications in the fields of biomarker identification and tracing, genomic instability, infectious diseases, DNA methylation testing and prenatal identification of fetal alleles in maternal blood.

Milbury et al. (2011) have developed a novel platform that incorporates a synthetic reference sequence within a PCR reaction, designed to enhance amplification of unknown mutant sequences during COLD-PCR. This new platform enables an Improved and Complete Enrichment (ice-COLD-PCR) for all mutation types and eliminates shortcomings of previous formats of COLD-PCR. Conventional-PCR, COLD-PCR and ice-COLD-PCR amplicons were run in parallel and sequenced to determine final mutation abundance for a range of mutations representing all possible single base changes. Amplification by ice-COLD-PCR enriched all mutation types and allowed identification of mutation abundances down to 1%, and 0.1% by Sanger sequencing or pyrosequencing, respectively, surpassing the capabilities of other forms of PCR. Finally they concluded that Ice-COLD-PCR help to elucidate the clinical significance of low-abundance mutations and our understanding of cancer origin, evolution, recurrence-risk and treatment diagnostics.

A temperature-tolerant (TT) approach (TT-COLD-PCR) that reduces the requirement for strict control of the denaturation temperature for a given sequence was designed by Castellanos-Rizaldos et al. (2012). They also described thermocycling programs featuring a gradual increase of the denaturation temperature during COLD-PCR. This approach enabled enrichment of mutations when the cycling achieves the appropriate critical denaturation temperature of each DNA amplicon that is being amplified. Validation was provided for KRAS and TP53 (tumour protein p53) exons 6-9 by use of dilutions of mutated DNA, clinical cancer samples, and plasma-circulating DNA. Low-level mutations in diverse amplicons with different T(m)s can be mutation enriched via TT-COLD-PCR provided that their T(m) s fall within the denaturation-temperature window applied during amplification. This approach enables simultaneous enrichment of mutations in several amplicons and increases significantly the versatility of COLD-PCR.
Prognosis of solid cancers is generally more favourable if the disease is treated early and efficiently. With recent development in methodologies, screening for a presence of cell-free DNA (cfDNA) brings a new viable tool in early detection and management of major cancers. It is believed that cfDNA is released from tumours primarily due to necrotization, whereas the origin of nontumourous cfDNA is mostly apoptotic. The important steps include cfDNA extraction from plasma and its detection and/or quantification. To distinguish tumour cfDNA from nontumourous cfDNA, specific somatic DNA mutations, previously localized in the primary tumour tissue, are identified in the extracted cfDNA. Apart from conventional mutation detection approaches, techniques such as mutant-enriched PCR and COLD-PCR, were used (Benesova et al., 2013).

Point mutations in isocitrate dehydrogenase 1 (IDH1) have been identified in many gliomas. The detection of IDH1 mutations becomes challenging on suboptimal glioma biopsies when a limited number of tumour cells is available for analysis. Pang et al. (2013) developed a novel COLD-PCR assay on the LightCycler platform (Roche, Applied Science, Indianapolis, IN), using post-PCR fluorescent melting curve analysis (FMCA) for the detection of mutant IDH1 with a detection limit of 1%. Thirty-five WHO grade I to IV gliomas and 9 non-neoplastic brain and spinal cord biopsies were analyzed with this technique and the results were compared with the conventional real-time PCR and the Sanger sequencing analysis. COLD-PCR/FMCA was able to detect the most common IDH1 R132H mutation and rare mutation types including R132H, R132C, R132L, R132S, and R132G mutations. In summary, they reported a novel COLD-PCR/FMCA method which provides rapid and sensitive detection of IDH1 mutations in formalin-fixed paraffin-embedded tissue and can be used in the clinical setting to assess the small brain biopsies.

2.5 Mitochondrial Copy Number and Cancer

The mitochondrial enzymes, DNA and OXPHOS protein content in three types of renal tumours from 25 patients was studied. Renal cell carcinomas (RCCs) of clear cell type (CCRCCs) originate from the proximal tubule and are most aggressive. Mitochondrial enzyme and DNA contents in all tumour types or grades differed significantly from normal tissue. Mitochondrial impairment increased from the less aggressive to the most aggressive RCCs, and correlated with a considerably decreased content of OXPHOS complexes (complexes II, III, and IV of the respiratory chain, and ATPase/ATP synthase) rather than to the mitochondrial content (citrate synthase and mtDNA). In benign oncocytoma, some
mitochondrial parameters (mtDNA, citrate synthase, and complex IV) were increased 4- to 7-fold, and some were slightly increased by a factor of 2 (complex V) or close to normal (complexes II and III). A low content of complex V protein was found in all CCRCC and chromophilic tumours studied. All results are in agreement with the hypothesis that a decreased OXPHOS capacity favours faster growth or increased invasiveness (Simonnet et al., 2002).

Wu et al. (2005) examined the somatic mutations in the D-loop region and mitochondrial content in gastric carcinomas. 15 of the 31 (48%) gastric carcinomas displayed somatic mutations in the D-loop region. Ten (67%) cancers with the somatic mutations in the D-loop had insertion or deletion mutations in nucleotide position (np) 303-309 in the mononucleotide repeat region and also detected the common 4,977-bp deletion in 17 (55%) of the noncancerous tissue samples, but only in three (9%) carcinomas. Using competitive PCR technique they analyzed mtDNA content and found that mtDNA depletion occurred in 17 (55%) of the gastric carcinomas. Their results suggest that somatic mtDNA mutations and mtDNA depletion occur in gastric cancer and that mtDNA depletion is involved in carcinogenesis and/or cancer progression of gastric carcinoma. Similar study was performed by (Tseng et al., 2006) in various tumours including breast cancer in 60 Taiwanese patients and found similar result as in gastric cancer.

Lee et al. (2005) analyzed the nucleotide sequence of the D-loop and the copy number of mtDNA in 54 hepatocellular carcinomas (HCCs), 31 gastric, 31 lung, and 25 colorectal cancers as well as their corresponding non-tumourous tissues. The results revealed that 42.6% of the HCCs, 51.6% of the gastric cancers, 22.6% of the lung cancers, and 40.0% of the colorectal cancers harboured mutation(s) in the D-loop of mtDNA. The mtDNA mutations in HCCs gastric cancers, lung cancers, and colorectal cancers were having changes in the mononucleotide or dinucleotide repeats, deletions, or multiple insertions. Moreover, they found that there is a significant decrease in mtDNA copy number in HCCs, gastric cancers, lung cancers, and colorectal cancers compared with the corresponding non-tumourous tissues. Their finding suggests that instability in the D-loop region of mtDNA, together with the decrease in mtDNA copy number, is involved in the carcinogenesis of human cancers.

Jiang et al. (2006) did quantitative PCR analysis of cytochrome c oxidase (Cox) I and Cox II genes to measure changes in mtDNA content in pretreatment and posttreatment
salivary rinses obtained from 76 patients undergoing surgical resection for primary head and neck squamous cell carcinoma. Overall, mtDNA content in posttreatment saliva was significantly decreased. Patients in the radiation therapy group exhibited a significant decrease compared with the nonradiated group. In addition, significant decreases in Cox I and Cox II were found in never-smoking patients but not in former or current smokers. Their data suggests that salivary mtDNA content is decreased in never smokers and in response to radiation therapy after primary surgical resection.

Tan et al. (2006) found that the entire mitochondrial genome for somatic mutations detection in 20 pairs of tumour/surrounding normal tissue of esophageal cancers, using TTGE. Fourteen somatic mtDNA mutations were identified in 55% of tumours analyzed, including 2 novel missense mutations and a frameshift mutation in ND4L, ATP6 subunit, and ND4 genes respectively. Nine mutations were in the D-loop region. Using real-time quantitative PCR analysis, the mtDNA content was found to increase in some tumours and decrease in others. The results demonstrated that somatic mtDNA mutations in esophageal cancers are frequent. Some missense and frameshift mutations may play an important role in the tumourigenesis of esophageal carcinoma.

The association of mtDNA copy number and lung cancer risk in 227 prospectively collected cases and 227 matched controls from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study was assessed by Hosgood et al. (2010). There was suggestion of a dose-dependent relationship between mtDNA copy number and subsequent risk of lung cancer, with a prominent effect observed in the highest mtDNA copy number. This is the first report, to suggest that mtDNA copy number may be positively associated with subsequent risk of lung cancer in a prospective cohort study; however, replication is needed in other studies and populations.

2.5.1 Mitochondrial DNA Copy Number Association with Clinicopathological Parameters

King & Attardi (1989) had detected that mtDNA-depleted (p0) cells were established by long-term exposure to low concentrations of ethidium bromide. The cause–consequence relationship of mtDNA copy number changes with tumourigenesis has been mainly evaluated by using these mutant cell models. In current years, a growing body of functional experiments has suggested that mtDNA content variations have enough capability to affect many aspects of malignant cells' behaviours, such as cell growth and apoptosis,
anti-cancer drug sensitivity, hormone dependence as well as their invasive and metastatic potentials. Park et al. (2004) observed that mtDNA copy number alterations have been revealed to facilitate cancer cells in acquiring enhanced resistance to cytotoxicity of many anti-tumour chemotherapeutic agents, a major impediment over the course of cancer treatment. SK-Hep1 hepatoma cells lacking mtDNA exhibited markedly reduced apoptotic death when exposed to serial treatments of doxorubicin and two other oxidative stressors, menadione and paraquat.

The possible correlations of the mtDNA copy number in HCC with the pathological findings and prognosis was studied in 31 HCC specimens using quantitative real-time polymerase chain reaction analysis, and examined the correlation between the mtDNA copy number and the clinicopathologic parameters and mutations in the D-loop region of the mitochondrial genome. The mtDNA copy number was reduced in HCCs compared with the corresponding non-cancerous liver tissues, and significantly correlated with tumour size and cirrhosis. The copy number of HCC with mtDNA D-loop mutation or deletion was lower. The results indicated that a reduced copy number of mtDNA is correlated with HCC and associated with malignant potential (Yamada et al., 2006).

The copy number of mtDNA in 59 cases of invasive breast tumours and paired non-tumourous tissues using quantitative real-time PCR was analyzed by Yu et al. (2007) and the data showed that the level of mtDNA was significantly decreased in tumour tissues as compared to the adjacent non-tumourous counterparts \( (P = 0.001) \). The reduced copy number in mtDNA was associated with an older onset age as well as a higher histological grade. Together, the results suggested that reduced copy number of mtDNA may be involved in breast neoplastic transformation or progression and mtDNA content might be potentially used as a tool to predict prognosis. Somatic mutation in the D-loop region probably is one of key contributing factors leading to decreased mtDNA level in breast tumours.

2.5.2 Smoking, Tobacco chewing and Oxidative Damage Association with Mitochondrial DNA Copy Number

The causative roles of altered mtDNA levels in the etiology of cancer have been extensively evaluated by many approaches and substantial supportive evidence has been gathered from clinicopathological association and molecular functional studies. The mutated mtDNAs are readily detectable in easily procured bodily fluids (e.g. peripheral whole blood,
salivary rinse, sputum, urine and nipple aspirate fluid) from early-stage patients suffering from different malignancies opening up an excellent window of opportunity for non-invasive assessment of tumour-associated genetic materials was observed by Fliss et al. (2000) and Jeronimo et al. (2001). Likewise, Qu et al. (2011) detected that the extent of mtDNA content changes in extracted blood samples has been examined for the ability as a molecular predictor to screen individuals with higher future cancer susceptibility and evaluate malignant progression during the carcinogenic process. In a prospective cohort study consisting of 104 male non-hodgkin lymphoma cases and 104 matched healthy subjects provided clues that higher mtDNA amount in peripheral white blood cells was statistically significantly related with subsequent occurrence of non-hodgkin lymphoma and the effect was most pronounced for the subtype of chronic/small lymphocytic lymphoma. Consistent with these observations, several recent case–control studies in Lung cancer, breast cancer (Shen et al., 2010), CRC (Qu et al. 2011) reported that increased mtDNA content in peripheral blood specimens is in a positive association with elevated cancer risk, with a significant dose–response relationship in trend analysis.

The alteration of the mtDNA in human tumours and in other tissues in association with smoking exposure was reported by Jiang & Califano (2004). After performing quantitative PCR of cytochrome c oxidasel (Cox I) and cytochrome c oxidase II (Cox II) genes on oral rinse samples obtained from 94 patients with primary HNSCC and a control group of 656 subjects. MtDNA levels were elevated in primary tumours when compared with matched, pre-treatment saliva and significant correlation was noted. MtDNA content alteration is associated with HNSC independently of age and smoking exposure, can be detected in saliva, and may be due to elevation in mtDNA content in primary HNSCC.

Jiang & Califano (2004) reported that alterations in mtDNA sequence and content have been described in human tissues and tumours in association with smoking exposure. They have done the quantitative PCR analysis of cytochrome c oxidase (Cox) I and Cox II genes to measure changes in mtDNA content in pre-treatment and post treatment salivary rinses obtained from 76 patients undergoing surgical resection for primary head and neck squamous cell carcinoma, also examined the relationship between changes in mtDNA content and post operative radiation therapy, smoking exposure, alcohol intake, and other clinical characteristics. Patients in the radiation therapy group exhibited a significant
decrease compared with the non radiated group. In addition, significant decreases in Cox I and Cox II were found in never-smoking patients but not in former or current smokers.

The mitochondria are highly susceptible to oxidative damage. Although mitochondrial function decreases with oxidative damage, overall mtDNA content increases to compensate for general mitochondrial dysfunction. After performing quantitative polymerase chain reaction for genes specific to mitochondrial and nuclear genomes to investigate relative mitochondrial abundance in a spectrum of dysplastic head and neck lesions and it was found that mean of CoxIβ-actin DNA ratios for mild, moderate, and severe premalignant lesions were 0.0529, 0.0607, and 0.1021, respectively (Kim et al., 2004).

The increase in mtDNA content and decline in mitochondrial function occurs with aging and in response to DNA-damaging agents, including tobacco smoke was reported by Masayesva et al. (2006). MtDNA content was correlated with age, exposure history, and other variables using multivariate regression analyses. A significant increase in mtDNA content was noted in smokers (31% and 29% increase for Cox I and Cox II, respectively) and former smokers (31% and 34%) when compared with never smokers. Smoking is associated with increased mtDNA content in a dose-dependent fashion. Mitochondrial DNA alterations in response to smoking persist for several decades after smoking cessation, consistent with long-term, smoking-related damage.

Docetaxel is one of the most effective chemotherapeutic agents against cancer; nevertheless, some patients develop resistance. Unfortunately, their causes and mechanisms remain unknown. Mizumachi et al. (2008) created docetaxel-resistant DRHEp2 from human laryngeal cancer HEp2 and investigated the roles of mtDNA and ROS on docetaxel resistance. DRHEp2 had greatly increased mtDNA content. Reduction of mtDNA content in DRHEp2 by ethidium bromide treatment reduced the resistance. These results indicated the possible roles of mtDNA-coded enzymes in mitochondrial respiratory chain (MRC) in resistant mechanisms. Oligomycin A, an Fo-ATPase inhibitor, eliminated docetaxel resistance in DRHEp2. These results indicate the roles of Fo-ATPase for resistant mechanisms. Docetaxel induced ROS generation in HEp2 but not in DRHEp2 and antioxidant pyrrolidine dithiocarbamate eliminated docetaxel-induced cytotoxicity, suggesting roles of ROS in docetaxel-induced cell death.
The over-expression of inhibitors of apoptosis proteins (IAP) contributes to therapeutic resistance as observed by Sun et al. (2011). Second mitochondria-derived activator of caspase (Smac) promotes caspase activation by binding to IAPs upon release from the mitochondria. IAP antagonists, also called SMAC mimetics, are promising anticancer agents modeled after this mechanism. It was found that SMAC mediates apoptosis induced by several classes of therapeutic agents through the mitochondrial pathway. SMAC knockdown led to impaired caspase activation, mitochondrial membrane depolarization, and release of cytochrome c. A small molecule SMAC mimetic, at nanomolar concentrations, significantly sensitized HNSCC cells to gemcitabine-induced apoptosis and restored gemcitabine sensitivity in SMAC knockdown cells, through caspase activation, X-linked IAP dissociation, and mt-associated events, but not the TNF-a pathway.

Lin et al. (2010) reported the roles of mitochondrial DNA alterations in esophageal squamous cell carcinoma, with emphasis on the changes in the copy number and D310 variants of mitochondrial DNA. They screened paired samples microdissected from esophageal muscles, noncancerous esophageal mucosa, cancerous esophageal squamous cell carcinoma nests, and metastatic lymph nodes of 72 patients with esophageal squamous cell carcinoma. The copy number and D310 variants of mitochondrial DNA were determined by quantitative real-time polymerase chain reaction and direct sequencing, respectively. It was found that fifty-six patients (77.8%) with somatic D310 mutations had lower survival probability \( (P = .027) \). Concurrently, the mitochondrial DNA copy number was increased from 0.159 to 0.192 and 0.206, respectively, \( (P = .024) \), especially in cigarette smokers \( (P = .014) \) and heavy wine drinkers \( (P = .005) \). Notably, a decrease in D310 variants \( (1.5, P <.001) \) and an increase in the incidence of the homoplasmic D310 pattern \( (P = .005) \) were observed in the matched esophageal muscle tissues.

Chatterjee et al. (2011) reported that mitochondria control essential cellular activities including generation of ATP via oxidative phosphorylation. Mitochondrial DNA (mtDNA) mutations in the regulatory D-loop region and somatic mtDNA mutations are common in primary human cancers. The biological impact of a given mutation may vary, depending on the nature of the mutation and the proportion of mutant mtDNAs carried by the cell. Identification of mtDNA mutations in precancerous lesions supports their early contribution to cell transformation and cancer progression. Introduction of mtDNA mutations in transformed cells has been associated with increased ROS production and tumour growth.
Studies reveal that increased and altered mtDNA plays a role in the development of cancer but much more attention is required to establish the functional significance of specific mitochondrial mutations in cancer and disease progression.

Challen et al. (2011) reported that mtDNA mutations occur in HNSCC and are most frequently found in the D-loop region. The D-loop is considered to be important because it controls mitochondrial gene expression and mtDNA replication. There is currently no evidence that mtDNA mutations can be used as prognostic or predictive biomarkers in HNSCC. They screened the entire mitochondrial genome of six oral squamous cell carcinoma-derived cell lines and then focused on detecting D-loop abnormalities in 34 HNSCC tissue samples by applying denaturing high performance liquid chromatography. It was found that mitochondrial DNA mutations are not ubiquitous in HNSCC because only half of the cell lines had detectable mtDNA abnormalities following screening of the entire mitochondrial genome and only 18% of tissue samples had D-loop mutations. There was no correlation between D-loop mutations and determinates of clinical outcome.

Yu et al. (2012) determined quantitative mtDNA levels in 31 primary osteosarcoma specimens and 5 normal bone tissue samples using a real-time polymerase chain reaction assay. The resultant data showed that the average mtDNA amount was significantly reduced in osteosarcoma tissues compared with normal bone controls. The copy number of mtDNA was statistically associated with tumour metastasis. There was an approximately 2-fold decrease of mtDNA quantity in tumours with metastasis than that in low-grade tumours without metastasis. Furthermore, change in mtDNA content was linked with somatic mutations in the D-loop regulatory region. Taken together, these results provide evidence for the first time that reduced mtDNA content may be critically implicated in the development and/or progression of osteosarcoma. Somatic D-loop mutation is likely one key factor among others leading to altered mtDNA amount in osteosarcoma.

2.6 HPV and Head and Neck Cancer

Over the last decade it has become clear that human papilloma virus (HPV) not only causes genital and anal cancers, but also causes a subset of head and neck squamous cell carcinoma (HNSCC) observed by Kreimer et al. (2005). In addition to the estimated ~492,800 cervical cancers caused worldwide by HPV each year, HPV also causes an estimated ~30,000 oropharyngeal cancers, HPV is detected in ~25% of all HNSCC, and the majority of these HPV-associated HNSCC are oropharyngeal (tonsillar and base of tongue)
squamous cell cancers. In fact, HPV is now the major cause of oropharyngeal cancer in developed countries, detected in 45–90% of cases (D’Souza et al., 2007, Kreimer et al., 2005, Nasman et al., 2009).

According to PCR, the prevalence of HPV DNA were 6.3% (13 of 206) in tonsillitis or hypertrophic tonsillar tissues and 0.6% (1 of 174) in exfoliated cells from normal tonsils. HPV-16 was the only type detected in tonsillar tissues, but it did not appear to lead to L1 antibody production examined by Chen et al. (2005). An investigation on high incidence of mutations in mtDNA of cervical cancer patients was done by Sharma et al. (2005) where they examined the frequency of mutations in the D-loop region in 19 patients of cervical cancer. Mutations, often multiple, were detected in 18 of 19 (95%) patients. The presence of mutations was correlated with HPV infection in these patients. Mutations were also detected in normal samples and lymphocytes obtained from cervical cancer patients, but their frequency of occurrence was much lower as compared to the cervical cancer tissues. Their findings indicated that D-loop alterations are frequent in cervical cancers and are possibly caused by HPV infection.

The relationship between HPV status and known prognostic markers for head and neck cancers including tumour hypoxia, EGFR expression and intra-tumoural T-cell levels was evaluated by Kong et al. (2009) and determined the prognostic impact of these markers by HPV status. HPV status in 82 evaluable HNSCC patients was determined by pyrosequencing and related to p16INK4a staining and treatment outcomes. It was correlated with tumour hypoxia, EGFR status, and intratumoural lymphocyte expression (CD3 staining). There was a significant relationship between strong HPV signal and p16INK4a staining as well as oropharynx location. The strong HPV signal group fared significantly better than others, both in time to progression (TTP, \( p = 0.008 \)) and overall survival (OS, \( p = 0.004 \)) for all patients and for the oropharyngeal subset. However, HPV status correlated inversely with EGFR reactivity and directly with CD3 (+) T-lymphocyte level (\( p = 0.03 \)). Whereas CAIX and EGFR overexpression were negative prognostic factors regardless of HPV status, CD3 (+) T-cell levels was prognostic only in HPV (-) tumours. They concluded that HPV status was a prognostic factor for progression and survival.

Syrjanen et al. (2011) examined the pooled risk estimates for the association of HPV with OSCC and potentially malignant disorders (OPMD) when compared with healthy oral mucosa as controls. They evaluated the effects of sampling techniques on HPV detection
rates. Pooled data were analyzed by calculating odds ratios, using a random effects model. Risk of bias was based on characteristics of study group, appropriateness of the control group and prospective design. Of the 1121 publications identified, 39 cross-sectional studies met the inclusion criteria. Collectively, 1885 cases and 2248 controls of OSCC and 956 cases and 675 controls of OPMD were available for analysis. Significant association was found between pooled HPV-DNA detection and OSCC (OR = 3.98; 95% CI: 2.62–6.02) and even for HPV16 only (OR = 3.86; 95% CI: 2.16–6.86). The results suggest a potentially important causal association between HPV and OSCC and OPMD.

A study was performed by Turner et al. (2011) with an objective of HPV screening of normal healthy adults to assess oral HPV prevalence. DNA was isolated from saliva samples and screened for high-risk HPV strains HPV16 and HPV18 and further processed using qPCR for quantification and to confirm analytical sensitivity and specificity. Four patient’s samples were found to harbour HPV16 DNA, representing 2.6% of the total (n = 151). No samples tested positive for HPV18. The successful recruitment and screening of healthy adult patients revealed HPV16, but not HPV18, was present in a small subset. These results provide new information about oral HPV status, which may help to contextualize results from other studies that demonstrate oral cancer rates have risen in the US among both females and minorities and in some geographic areas that are not solely explained by rates of tobacco and alcohol use.

Ghosh et al. (2011) conducted HPV detection by Fast-PCR (within 15 min.) based diagnosis of HPV 16 and HPV 18 infection amongst patients of suspected cervical cancer, confirmed by cytological methods. Twelve women, out of a total of fifty studied cases who had positive cervical pap smears (24%) were found to be positive for HPV 16/HPV 18 infection when PCR based technique was applied. The results indicate, perhaps, a greater specificity of PCR based diagnosis, or presence of other HPV subtypes as etiological factors in the present study group confined to Southern Assam.

A pilot study by Nichols et al. (2012) was conducted to compare the genetic changes in a HPV-positive and an HPV-negative head and neck tumour. DNA was extracted from the blood and primary tumour of a patient with an HPV-positive tonsillar cancer and those of a patient with an HPV-negative oral tongue tumour. They performed exome enrichment using the Agilent SureSelect All Exon Kit, followed by sequencing on the ABI SOLiD platform. Exome sequencing revealed slightly more mutations in the HPV-negative tumour
(73) in contrast to the HPV-positive tumour (58). Multiple mutations were noted in zinc finger genes (ZNF3, 10, 229, 470, 543, 616, 664, 638, 716, and 799) and mucin genes (MUC4, 6, 12, and 16). Mutations were noted in MUC12 in both tumours. HPV-positive HNSCC is distinct from HPV-negative disease in terms of evidence of viral infection, p16 status, and frequency of mutations. Next-generation sequencing has the potential to identify novel therapeutic targets and biomarkers in HNSCC.

In the study of Marklund et al. (2012), the prevalence of HPV and p16 and their impact on survival in other OSCC sites (OOSCC) was examined. A total of 69 patients were and 61 were included in the survival analysis. HPV and p16 were present in only 17% and 25% of the OOSCC cases, respectively, while the majority 69% was both HPV and p16 negative. In conclusion, the prevalence of HPV and/or p16 is much lower in OOSCC compared to earlier reports including all OSCC, or tonsillar- and base of tongue cancer and HPV and p16 had no impact on clinical outcome in OSCC in this study. Their data highlighted the diversity of head neck cancer sub-sites and the importance of taking OSCC sub-sites in consideration in future clinical trials and treatment.

A prospective study was made by De Stefani et al. (2013) to analyze the HPV infection status and its impact on the outcome in a consecutive series of patients affected by oropharyngeal cancer. They obtained specimens of consecutive subjects surgically treated for oropharyngeal squamous cell carcinoma. Samples were collected by broom-type cell sampling devices and they underwent the Roche Linear Array HPV Genotyping Test to identify the presence of HPV types. In all, 52 patients were enrolled. The presence of HPV was detected in 13 samples, with HPV type 16 as the most frequently encountered type. Statistically significant associations were found between HPV-positive patients and a higher tumour grading, and between HPV-positive patients and a higher number of negative prognostic factors. They made the conclusion from their findings that a subset of oropharyngeal squamous cell carcinomas with a higher tumour grading is strongly linked to HPV16 infection.

A single institutional experience with definitive radiation therapy alone for HPV-positive head and neck cancer was reported by Chen et al. (2013). A total of 67 patients were treated by radiation therapy alone to a median dose of 70 Gy (range, 66-72 Gy) for HNSCC. Paraffin-embedded, formalin-fixed pretreated tumour tissues were used to establish HPV-positivity using standardized techniques of immunohistochemistry for p16 and
polymerase chain reaction for HPV. In all 23 patients with HPV-positive cancers were identified. With a median follow-up of 28 months (range, 6-85 months), the 3-year actuarial rates of overall survival, locoregional control, and distant metastasis-free survival were 83%, 90%, and 88%, respectively. These findings suggestive to the exquisite radiosensitivity of HPV-positive head and neck cancer.

2.7 Glutathione S-Transferase Polymorphism and Cancer

Nair et al. (1999) examined the prevalence of GSTM1 and GSTT1 null genotypes (GSTM1*2 and GSTT1*2) in oral premalignant leukoplakia cases and controls was ascertained in genomic DNA by a multiplex PCR technique. Biopsies taken from 98 oral leukoplakia patients and exfoliated cells from 82 healthy controls both of Indian ethnicity were analyzed. GSTM1*1 (active) was present in 83% and GSTT1*1 (active) was present in 78% of all control subjects, while prevalence of GSTM1*2 and GSTT1*2 null genotypes was significantly higher among oral leukoplakia cases. The prevalence of GSTM1*2 in leukoplakia cases was 81.6% compared with 17% in controls and GSTT1*2 was 75.5% in the cases versus 22% in controls. They concluded that their results still needs confirmation by a larger study, demonstrate that the null genotypes of both GSTM1 and GSTT1 increase with high penetrance, separately or in combination, the risk for developing leukoplakia in an Indian ethnic population.

Anantharaman et al. (2007) investigated the role of polymorphisms at CYP1A1, GSTM1 and GSTT1 to OSCC in a case-control study involving 155 patients with precancerous lesions, 458 cancer patients and 729 age and habit-matched controls. Risk to oral cancer was estimated among different tobacco exposure groups and doses using logistic regression analysis. GSTM1 null genotype conferred 1.29-fold increased risk and GSTT1 null genotype, however, conferred 0.57 times reduced risk to OSCC, specifically among tobacco chewers. These results support the finding that GSTM1 null genotype is a risk factor to OSCC among Indian tobacco habits; GSTT1 null genotype, however, emerged as a protective factor.

The association of GST variants with the risk of oral submucous fibrosis (OSF), the distribution of polymorphisms in GSTM1 and GSTT1 in 90 OSF patients and 130 healthy controls was evaluated by Agrawal et al. (2010). The frequency of both the GSTM1 and GSTT1 null genotypes was higher in the OSF cases than in the controls. The prevalence of the GSTM1 null genotype in the OSF cases was 46.6% as compared to 29.2% in the controls.
and GSTT1 null was 24.4% in the OSF cases versus 10.7% in the controls. There was evidence of an increased risk with the absence of both genotypes (7.5-fold; OR 7.5, 95% CI 2.3-24). Their findings suggest that the GSTM1 and GSTT1 null genotypes, separately or in combination, increase the risk of developing OSF in the North Indian population.

To establish the clinical and demographic profile and identify risk factors among patients with head and neck cancer and relate them to the polymorphism of GSTT1 and GSTM1. One hundred patients with head and neck cancer and 100 control group individuals without history of neoplasm were analyzed. There was prevalence of smokers and alcohol drinkers in head and neck cancer patients. The GSTT1 null genotype was found in 47% of the patient and 41% of the control. Likewise, the GSTM1 null genotype was found in 66% of the patient and 75% of the control group. The combined GSTT1 and GSTM1 gene null genotype shown association between GSTM1 0/GSTT1 and occurrence of head and neck carcinoma (OR = 7.64; CI 95%= 1.72-34.04; p = 0.0076). Analysis of clinical-pathological features showed association between GSTT1 null genotype and larynx, the inverse relation between this genotype and pharynx. In our study it was possible to establish association between GSTM1 null genotypes and head and neck cancer (Leme et al., 2010).

Yadav et al. (2010) explored the possibility of phase II metabolic enzymes being responsible for the high prevalence of cancers in this region of Northeast India. Samples from 370 cases with oral, gastric, and lung cancers and 270 controls were analyzed for polymorphism of GST genes using PCR-RFLP. The variant genotypes of GSTP1 were not associated with any of the aerodigestive tract cancers. GSTT1 and GSTM1 null genotypes appeared to play a protective role for lung cancer, but they were not associated with oral and gastric cancers. However, when data was analyzed in different geographic regions the GSTT1 null genotype was found to be a significant risk factor for oral as well as gastric cancer in samples obtained from the Assam region of Northeast India. This is the first study on the association of GST polymorphisms and aerodigestive tract cancers in the high-risk region of Northeast India.

The influence of the CYPIA1 A4889G and T6235C, GSTM1 and GSTT1 polymorphisms, involved in carcinogen metabolism, on the HNSCC risk was analyzed. Excesses of the CYPIA1 4889AG+GG plus GSTT1 null genotype were seen in patients with heavy tobacco habit compared with controls. Carriers of the referred genotypes and heavy tobacco consumption were under a 2.0-fold and 2.8-fold increased risk for HNSCC than
others, respectively. The CYP1A1 6235TC+CC plus GSTM1 and GSTT1 null genotypes were more common in pharyngeal SCC patients than in controls. The data presented an preliminary evidence that inherited combined CYP1A1 A4889G and T6235C abnormalities and GSTM1 and GSTT1 pathways are important determinants of HNSCC, particularly pharyngeal SCC in heavy smoking individuals observed by Lourenco et al. (2011).

A study was investigated by Ruwali et al. (2011) to examine the association of polymorphism in GSTs with susceptibility for HNSCC and its sites as well as treatment response in cases receiving chemotherapy and combination of CT-radiotherapy. The case-control study included 500 male cases and an equal number of healthy male controls. An increase in the risk for HNSCC and cancers of oral cavity, larynx or pharynx was observed in cases with null genotypes of GSTM1 or GSTT1. The interaction of alcohol or tobacco with variant genotypes of GSTM1 or GSTT1 also resulted in a significant increase in the risk for HNSCC. The present data thus provided evidence for the association of polymorphisms in GSTs with risk for HNSCC and its treatment response.

Mahimkar et al. (2012) retrospectively assessed whether polymorphisms of glutathione-S-transferase genes GSTM1 (deletion), GSTT1 (deletion), GSTP1 (Ile105Val, rs1695), and DNA repair genes hOGG1 (Ser326Cys, rs1052133), XRCCI (Arg194Trp, rs1799782, and Arg399Gln, rs25487), XPD (Asp312Asn, rs1799793, and Lys751Gln, rs13181) can predict clinical outcome in 187 oral squamous cell carcinoma patients treated with postoperative radiotherapy. Deletion polymorphism of GSTM1 gene was significantly associated with DSS. The rs1799793 variant allele showed significant protection in both disease-specific (DSS) and relapse-free (RFS). The combined analysis of GSTM1 and XPD polymorphisms revealed favorable effect on survival. GSTM1 and XPD variant alleles, independently as well as in combination may serve as important predictors of clinical outcome in radiotherapy-treated OSCC patients.