Chapter 1

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1.1 Overview of Cancer

The word cancer came from the father of medicine, Hippocrates, a Greek physician. Hippocrates used the Greek words, carcinos and carcinoma to describe tumours, thus calling cancer “karkinos” (Lyons & Petrucelli, 1978). The Greek terms were words to describe a crab, which Hippocrates thought a tumour resembled. Although Hippocrates may have named “Cancer,” but was certainly not the first to discover the disease. The history of cancer actually begins much earlier. The world’s oldest documented case of cancer hails from ancient Egypt, in 1500 B.C. (Sudhakar, 2009).

Cancer, by definition, is a disease of the genes. A gene is a small part of DNA, which is the master molecule of the cell. Genes make "proteins," which are the ultimate workhorses of the cells. It is these proteins that allow our bodies to carry out all the processes that permit us to breathe, think, move, etc. (Kumar et al., 2010). Many genes produce proteins that are involved in controlling the processes of cell growth and division. An alteration (mutation) to the DNA molecule can disrupt the genes and produce faulty proteins. This causes the cell to become abnormal and lose its restraints on growth. The abnormal cell begins to divide uncontrollably and eventually forms a new growth known as a "tumour" or neoplasm (medical term for cancer meaning "new growth"). As it grows, it may damage and invade nearby tissue. If a cancerous tumour outgrows its birthplace (called the primary cancer site) and moves on to another place (called the secondary cancer site), it's referred to as metastasizing. In a healthy individual, the immune system can recognize the neoplastic cells and destroy them before they get a chance to divide. However, some mutant cells may escape immune detection and survive to become tumours or cancers.

1.2 Classification of Cancer

Cancers are classified by the type of cell that the tumour cells resemble and are therefore presumed to be the origin of the tumour (Downward, 2006, Lyons & Petrucelli, 1978). The general categories include:
Carcinomas are cancers that arise in the epithelium (the layer of cells covering the body's surface and lining of the internal organs and various glands). Ninety percent of human cancers fall into this category. Carcinomas can be subdivided into two types: Adenocarcinomas and squamous cell carcinomas. Adenocarcinomas are cancers that develop in an organ or a gland, while squamous cell carcinomas refer to cancers that originate in the skin.

Melanomas also originate in the skin, usually in the pigment cells (melanocytes).

Sarcomas are cancers of the supporting tissues of the body, such as bone, muscle and blood vessels.

Cancers of the blood and lymph glands are called leukemias and lymphomas respectively.

Gliomas are cancers of the nerve tissue.

Germ cell tumour: Tumours derived from totipotent cells. In adults most often found in the testicle and ovary; in foetus, babies, and young children most often found on the body midline, particularly at the tip of the tailbone.

Blastic tumour or blastoma: A tumour (usually malignant) which resembles an immature or embryonic tissue. Many of these tumours are most common in children.

1.3 Global Burden of 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 country.

1.4 Head and Neck Cancer

The term head and neck cancer refers to a group of biologically similar cancers originating from the upper aero digestive tract, including the lip, oral cavity (mouth), nasal cavity, paranasal sinuses, pharynx, larynx. Most head and neck cancers originates from the mucosal lining (epithelium) of these regions (Agulnik, 2012). Mucosal surfaces are moist tissues lining hollow organs and cavities of the body open to the
environment. Normal mucosal cells look like scales (squamous) under the microscope, so head and neck cancers are often referred to as head and neck squamous cell carcinomas (HNSCC).

**Table 1.1: Total Number and Percentage of New Cases Diagnosed per Year, Worldwide**

<table>
<thead>
<tr>
<th>Cancer site</th>
<th>Cases</th>
<th>Percentage of all cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>16,08,055</td>
<td>12.7</td>
</tr>
<tr>
<td>Breast</td>
<td>13,82,155</td>
<td>10.9</td>
</tr>
<tr>
<td>Colorectum*</td>
<td>12,35,108</td>
<td>9.8</td>
</tr>
<tr>
<td>Stomach</td>
<td>9,88,602</td>
<td>7.8</td>
</tr>
<tr>
<td>Prostate</td>
<td>8,99,102</td>
<td>7.1</td>
</tr>
<tr>
<td>Liver</td>
<td>7,49,744</td>
<td>5.9</td>
</tr>
<tr>
<td>Cervix Uteri</td>
<td>5,30,232</td>
<td>4.2</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>4,81,645</td>
<td>3.8</td>
</tr>
<tr>
<td>Bladder</td>
<td>3,82,660</td>
<td>3</td>
</tr>
<tr>
<td>Non-Hodgkin Lymphoma</td>
<td>3,56,431</td>
<td>2.8</td>
</tr>
<tr>
<td>Leukemia</td>
<td>3,50,434</td>
<td>2.8</td>
</tr>
<tr>
<td>Corpus Uteri</td>
<td>2,88,387</td>
<td>2.3</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2,73,518</td>
<td>2.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2,73,518</td>
<td>2.2</td>
</tr>
<tr>
<td>Lip and Oral Cavity</td>
<td>2,63,020</td>
<td>2.1</td>
</tr>
<tr>
<td>Brain &amp; Central Nervous System</td>
<td>2,37,913</td>
<td>1.9</td>
</tr>
<tr>
<td>Ovary</td>
<td>2,24,747</td>
<td>1.8</td>
</tr>
<tr>
<td>Thyroid</td>
<td>2,13,179</td>
<td>1.7</td>
</tr>
<tr>
<td>Malignant Melanoma</td>
<td>1,99,627</td>
<td>1.6</td>
</tr>
<tr>
<td>Larynx</td>
<td>1,50,677</td>
<td>1.2</td>
</tr>
<tr>
<td>Other Sites</td>
<td>15,66,634</td>
<td>12.4</td>
</tr>
</tbody>
</table>

*The 20 Most Commonly Diagnosed Cancers: 2008 Estimates

*Colorectum included anus*

**Note:** The ICD-10 (International Classification of Diseases) groupings for kidney, ovarian and uterine cancer data worldwide, differ from those usually reported by Cancer Research UK (Ferlay et al., 2010)

Some head and neck cancers begin in other types of cells. For example, cancers that begin in glandular cells are called adenocarcinomas. Head and neck cancers often spread to the lymph nodes of the neck, and this is often the first (and sometimes only) manifestation of the disease at the time of diagnosis. Head and neck cancer is a very common tumour worldwide. It is the fifth leading cause of cancer related deaths with an annual incidence of 500,000 cases worldwide. Approximately, 40,000 new cases of
head and neck squamous cell carcinoma (HNSCC) are diagnosed annually in the United States alone (Rousseau & Badoual, 2011). The statistical analysis by the International Agency for Research on Cancer (IARC) indicated that the lip and oral cavity is the tenth most common tumour site in the human (Mehrotra & Yadav, 2006).

In India, over one third of all cancers occurs in the head and neck compared to less than 10% in the Western world. The primary reason for this unusually high incidence of head and neck cancer in India is the indiscriminate use of chewing tobacco in its various forms. In tobacco users, the oral cavity bears the brunt of the carcinogen and nearly 80,000 – 100,000 oral cancers are diagnosed every year in the country. Head and neck cancer includes many different malignancies. According to various studies, the prevalence of head and neck cancer with respect to total body malignancies ranges from 9.8% to 42.7%. The most common head and neck cancer is oropharyngeal carcinoma (28.6%) followed by oesophageal and oral cavity cancers (19.4%) and (16.3%), respectively. Carcinoma of the ear is the least common (0.4%) (Mehrotra & Yadav, 2006).

Different modes of tobacco intake such as cigarettes, pipes, cigars, smokeless tobacco and tobacco chewing are implicated in HNSCC development and these mitigating factors have been found to be responsible for 90% of HNSCC related deaths in males (Cinciripini & McClure, 1998). Several potential risk factors have been identified that are associated with HNSCC development and progression. Notably, other than chewing tobacco, different chewing products such as betel nuts, pan, chaalia, gutka, naswar and areca that are widely used in the Indian subcontinent, South East Asia and South Pacific Islands, also increase the risk of HNSCC (Dasgupta et al., 2012a). Consumption of alcohol is the second most important correlative risk factor for HNSCC and above 30 grams of alcohol intake per day linearly increases risk of HNSCC (Dasgupta et al., 2012a). Despite significant improvements in therapeutic modalities, 5-year post therapeutic survival rates are still among the lowest of the major cancers, with locoregional relapse being the primary cause of death (Chung & Gillison, 2009, Dasgupta et al., 2010). Due to poor survival, improved methods for early disease detection and prevention are clearly warranted.

In India, the Northeastern states, viz. Assam and Meghalaya accounts for the highest prevalence of tobacco related oral cancer which is about 33% (Ihsan et al., 2011) (Bhattacharjee A et al., 2006). The Northeast region of India has different
customs, food habits, life-style, diverse ethnic groups and type and pattern of tobacco use as compared to the rest of the country. Consumption of very spicy foods, hot foods and beverages, a diet containing high amounts of chilli and stale food may be associated with the risk of oral and oesophageal cancer. The risk factors are associated with consumption of locally prepared food items, e.g., kalakhar and some dietary practices does not decrease, even after adjustments with different confounding factors (Phukan et al., 2001). It is very well known that the carcinogenicity of tobacco is attributed to nitrosamines, PAHs, benzene, Benzo(a)pyrene etc. Moreover, there is extensive use of pesticides in tea garden in Northeast which can lead to widespread occupational and environmental exposures. The morbidity and mortality associated with this disease is a cause of major concern in this region.

1.4.1 Various Regions of the Head and Neck (Agulnik, 2012)

Cancers of the head and neck are further identified by the area in which they begin:

➢ Oral cavity. The oral cavity includes the lips, the front two-thirds of the tongue, the gingiva (gums), the buccal mucosa (lining inside the cheeks and lips), the floor (bottom) of the mouth under the tongue, the hard palate (bony top of the mouth), and the small area behind the wisdom teeth.

➢ Salivary glands. The salivary glands produce saliva, the fluid that keeps mucosal surfaces in the mouth and throat moist. There are many salivary glands; the major ones are in the floor of the mouth, and near the jawbone.

➢ Paranasal sinuses and nasal cavity. The paranasal sinuses are small hollow spaces in the bones of the head surrounding the nose. The nasal cavity is the hollow space inside the nose.

Figure 1.1: Illustrates the location of different regions of head and neck cancer. Figure courtesy of (http://www.cancer.gov/cancertopics/factsheet/Sites-Types/head-and-neck)
Pharynx. The pharynx is a hollow tube about 5 inches long that starts behind the nose and leads to the oesophagus (the tube that goes to the stomach) and the trachea (the tube that goes to the lungs). The pharynx has three parts:

Nasopharynx: The nasopharynx, the upper part of the pharynx, is behind the nose.

Oropharynx: The oropharynx is the middle part of the pharynx. The oropharynx includes the soft palate (the back of the mouth), the base of the tongue, and the tonsils.

Hypopharynx: The hypopharynx is the lower part of the pharynx.

Larynx. The larynx, also called the voice box, is a short passageway formed by cartilage just below the pharynx in the neck. The larynx contains the vocal cords. It also has a small piece of tissue, called the epiglottis, which moves to cover the larynx to prevent food from entering the air passages.

Lymph nodes in the upper part of the neck. Sometimes, squamous cancer cells are found in the lymph nodes of the upper neck when there is no evidence of cancer in other parts of the head and neck. When this happens, the cancer is called metastatic squamous neck cancer with unknown (occult) primary.

1.4.2 Stages of Head and Neck Cancer

The ‘stage’ of the cancer defines whether the tumour is localized to the organ or invaded nearby structures and whether it has spread to the local lymph nodes or to other parts of the body. Staging is very important, because it helps healthcare professionals determine whether or not to try a particular treatment (Deschler & Day, 2008). The information needed to decide the stage is gathered from both clinical examination and scanning. An examination under anaesthetic is often done to obtain the most accurate stage, and to get a biopsy. Stages of head and neck cancer start at 0 and go up to 4, but they are written in Roman numerals (I–IV). Generally, a lower number means the cancer has spread less whereas a higher number means the cancer has spread more. The staging system of the American Joint Committee on Cancer, also referred to as the TNM system, is used most often by doctors to describe a patient's cancer (Fleskens & Slootweg, 2009). The TNM system involves three scores that describe:

T = the tumour type and extent; N = whether or not lymph nodes are involved; M = how far the cancer has spread (metastasized)
## Tumour, Node, and Metastasis (TNM) Staging

<table>
<thead>
<tr>
<th>Primary Tumour (T)</th>
<th>Nodes (N)</th>
<th>Metastasis (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T0</strong></td>
<td>There is no evidence of a tumour</td>
<td></td>
</tr>
<tr>
<td><strong>Tis:</strong></td>
<td>The tumour is “in situ,” meaning it has not spread to nearby tissues</td>
<td><strong>N0</strong></td>
</tr>
<tr>
<td><strong>T1</strong></td>
<td>The tumour cannot be seen without using imaging techniques</td>
<td><strong>N1-4</strong></td>
</tr>
<tr>
<td><strong>T2-4</strong></td>
<td>The higher numbers indicate the involvement of the lymph nodes and extent of the primary tumour</td>
<td><strong>M0</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>M1</strong></td>
</tr>
</tbody>
</table>

### Stage 0 -
The tumour is “in situ”. It stays in the part of the head and neck where it started, and is not invasive, meaning it has not spread. Still, carcinomas in situ can become malignant (dangerous), and doctors may recommend removing them.

### Stage I -
The tumour has not spread to the lymph nodes or to distant areas of the body.

### Stage II -
The tumour is larger, but may not have spread to the lymph nodes or to distant areas of the body.

### Stage III -
The tumour is either much larger, or it has begun to spread to the lymph nodes or to distant areas of the body.

### Stage IV -
The tumour is much larger and has spread to the lymph nodes. It may have also spread to other areas of the body.

### 1.4.3 Grading of Head and Neck Cancer

Grading refers to the appearance of the cancer cells under the microscope. The grade gives an idea of how quickly the cancer may develop. There are three histological grades based on the amount of keratinization:

- **High-grade** or grade 3 cancers the cells look very abnormal and are more likely to spread. It is well-differentiated tumour, characterized by > 75% keratinization.

- **Moderate-grade** or grade 2 cancers fall between these two grades and have a level of activity somewhere between. A moderately differentiated tumour, characterized by 25% to 50% keratinization.
Low-grade or grade 1 means that the cancer cells look very like normal cells in the head and neck area. A poorly differentiated tumour, characterized by < 25% keratinization.

Histological grade has not been a consistent predictor of clinical behaviour. Features that predict aggressive behaviour include perineural spread, lymphatic invasion, and tumour spread beyond the lymph node capsule. HPV-positive tumours tend to be non-keratinizing and poorly differentiated (Goon et al., 2009).

Other tumour types

Other, less common head and neck cancers include mucoepidermoid carcinoma, adenoid cystic carcinoma, and adenocarcinoma, all of which may arise in the salivary glands. Head and neck cancers with neuroendocrine features include small-cell undifferentiated cancer and esthesioneuroblastoma (olfactory neuroblastoma). Both Hodgkin lymphoma and non-Hodgkin lymphoma may also be diagnosed as head and neck tumours, often involving the lymph nodes of the neck or Waldeyer's ring (Ridge et al., 2011).

Precancerous Lesions

There is a sequence of disease progression from atypia/dysplasia through carcinoma in situ to frankly invasive cancer. Leukoplakia and erythroplakia are terms applied to clinically identifiable lesions that may harbour invasive cancer or undergo malignant transformation.

1.4.4 Pathology of Head and Neck Cancer

- **Leukoplakia** results from chronic irritation of mucous membranes by carcinogens; this irritation stimulates the proliferation of white epithelial and connective tissue. Clinically characterized by white patch or plaque. Histopathological examination reveals hyperkeratosis variably associated with underlying epithelial hyperplasia. In the absence of underlying dysplasia, leukoplakia rarely (< 5%) is associated with progression of disease to malignancy.

- **Erythroplakia** is characterized by bright red velvety patches adjacent to normal mucosa. It is commonly associated with underlying epithelial dysplasia and has a much greater potential for malignancy than leukoplakia. Carcinoma is found in nearly 40% of erythroplakia cases.
Dysplasia is characterized cytologically by nuclear marginal irregularity of the squamous cells, perinuclear cytoplasmic halo and peripheral condensation of the cytoplasm. It is graded as mild, moderate, or severe, based on the degree of nuclear abnormality present. In the transition from mild to severe dysplasia, nuclear abnormalities become more marked, mitosis become more apparent, and these changes involve increasing depth of epithelium. The likelihood of developing a carcinoma relates to the degree of dysplasia.

Carcinoma in situ is characterized by the presence of atypical changes throughout the epithelium, with complete loss of stratification. It is estimated that approximately 75% of invasive squamous cell carcinomas have an associated in situ component. Specific DNA mutations have also been identified in the sequence of disease progression from mild dysplasia to atypia to carcinoma in situ to invasive carcinoma.

Field cancerization is an important concept related to the natural history of head and neck cancer. This term describes the diffuse epithelial injury throughout the head and neck, lungs, and oesophagus that results from chronic exposure to carcinogens. Clinically, field cancerization is manifested by the frequent occurrence of:

1. Mucosal abnormalities, such as leukoplakia and dysplasia, beyond the margins of a head and neck cancer.
2. Second primary tumours within this exposed field. The lifetime risk of a patient with head and neck cancer developing a new cancer is 20% to 40%. Over time, as the risk of relapse of the initial cancer declines, the development of a new cancer represents the greatest risk for these patients.

Regional and distant metastasis:
The incidence of lymph node metastasis is related to the size and thickness of the primary tumour. If the primary site is near the midline, contralateral or bilateral metastasis should be anticipated. In the presence of lymph node metastases, extracapsular spread of tumour is an important prognostic factor.

1.5 Etiology of Head and Neck Cancer

Etiology implies a complex interaction of entities, their introduction, and interaction with a host to produce a malignancy. There are number of etiologic agents are associated with various degrees of risk in the development of HNSCC.
➢ Tobacco Use

The medical and epidemiologic literature contains numerous reports of the association between carcinomas of the head and neck and use of tobacco, including tobacco inhaled as smoke and the smokeless products, such as snuff and chewing tobacco. Although, the association is strong and dose related both smoked and smokeless forms of tobacco contain considerable numbers of putative carcinogens. Therefore, pinpointing the mechanism of tobacco-induced carcinogenesis is exceedingly difficult and largely speculative. Finally, smokers are likely to have other risk factors or etiologic agents in addition to their tobacco use. Factors such as poor dentition, ethanol use, and poor nutrition are associated with tobacco use.

➢ Alcohol Use

An association between alcohol use and human cancer has been observed since 1910, when it was noted in Paris that 80% of patients with oesophageal carcinomas were heavy drinkers. Even then, confounders were similar to those noted for tobacco. The fact that absinthe, a fermentation product of wormwood (Artemisia absinthium) accounted for much of the alcohol consumed at that time in Paris, suggests a possibility that other substances may have contributed to this association. Nevertheless, the incidence of head and neck carcinoma is undoubtedly associated with the use of ethanol. In 1998, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) concluded that the evidence was sufficient to show that alcoholic beverages are carcinogenic to humans.

➢ Dietary Factors

Dietary factors may contribute. Excessive consumption of processed meats and red meat were associated with increased rates of cancer of the head and neck in one study, while consumption of raw and cooked vegetables seemed to be protective. A study examined a combination of Vitamin E and beta carotene in smokers with early-stage cancer of the oropharynx, and found a poor prognosis in the vitamin users (Bairati et al., 2005).

➢ Betel Nut

Betel nut chewing is associated with an increased risk of HNSCC. This nut is chewed regularly by at least 10% of the world population, imported by immigrant users wherever they settle, and is the fourth most widely used addictive substance. Specific
arecal alkaloids act as competitive inhibitors of GABA receptors and have widespread
effects in the body, including actions on the brain, cardiovascular system, lungs, gut
and pancreas. Nitrosated derivatives of arecal alkaloids are proven carcinogens
inducing tumours and associated with increased tumour risks.

➢ Human Papillomavirus

The papillomavirus family (Papillomaviridae) is a highly diverse group of small
non enveloped DNA tumour viruses. In humans, over 120 HPV genotypes have been
fully sequenced (de Villiers et al., 2004, de Villiers & Gunst, 2009). HPVs have been
classified as high or low risk types based on the clinical behaviour of the virally
infected tissues. High-risk HPVs are also associated with cancers of the anus,
opharynx and oesophagus, identifying HPV as a risk factor for multiple human
cancers. The life cycle of HPV is dependent on cellular factors and epithelial
differentiation. HPV produces epithelial tumours of the skin and mucous membranes.
HPVs specifically target the undifferentiated proliferative basal cells of epithelial
mucosa that are exposed following tissue trauma. Some of the HPV "early" genes, such
as \( E6 \) and \( E7 \), are known to act as oncogenes that promote tumour growth and
malignant transformation. Oral infection with HPV increased the risk of HPV-positive
oropharyngeal cancer independent of tobacco and alcohol use. HPV oncoproteins E6
and E7 of the high risk HPVs (HR-HPVs) interacts with host cell proteins to disturb the
normal epithelial differentiation and apoptosis by stimulating cellular proliferation,
DNA synthesis and inhibition of cell cycle regulators. The virally encoded E6 binds to
a cellular ubiquitin / protein ligase, E6-AP, and simultaneously to the tumour
suppressor protein p53, resulting in ubiquitination of p53 and its subsequent proteolytic
degradation.

➢ Epstein - Barr virus

The Epstein–Barr virus (EBV), also called gammaherpesvirus and is one of the
most common viruses in humans. It is best known for causation of infectious
mononucleosis and also associated with particular forms of cancer, particularly
Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, and central
nervous system lymphomas associated with HIV. Two subtypes of EBV are known to
infect human beings: EBV-1 and EBV-2. The gene organization that code for the EBV
nuclear antigen (EBNA-2, EBNA- 3a, b, c) differs in the two types (Sample et al.,
1990). EBV primarily infects and replicates in the stratified squamous epithelium of the
oropharynx during acute infection. Besides, its well-known tropism for B cells, the targets of EBV infection may also include epithelial cells, T cells, and cells of the macrocytic, granulocytic, and natural killer lineages. Although most humans coexist with the virus without serious sequelae, a small proportion will develop tumors. During acute infection, EBV primarily infects and replicates in the stratified squamous epithelium of the oropharynx (Murray & Young, 2002). This is followed by a latent infection of the B lymphocytes in which generally the virus persists in circulating memory B cells (Sixbey et al., 1984). EBV was the first human virus to be directly implicated in carcinogenesis.

- Gastroesophageal Reflux Disease

The presence of acid reflux disease (GERD - gastroesophageal reflux disease) or larynx reflux disease can also be a major factor. In the case of acid reflux disease, stomach acids flow up into the oesophagus and damage its lining, making it more susceptible to throat cancer.

- Other Possible Causes

There are a wide variety of factors which can put someone at a heightened risk for throat cancer. Such factors include smoking or chewing tobacco or other things, like gutka, or paan, heavy alcohol consumption, poor diet resulting in vitamin deficiencies (deteriorate if this is caused by heavy alcohol intake), weakened immune system, asbestos exposure, prolonged exposure to wood dust or paint fumes etc. In addition xenobiotics metabolizing enzymes play an important role in cancer as they are responsible for metabolism of many exogenous chemicals that are toxic, mutagenic or carcinogenic.

1.6 Xenobiotic Metabolizing Gene Polymorphism in Cancer

The development of cancer is influenced by both the genetic and environmental factors. This environment-gene interaction on carcinogenesis has been well illustrated by phase I and phase II enzymes that are involved in the metabolism of carcinogenesis. The phase I enzymes are CYPs (Cytochrome P450) that are involved in activating the environmental procarcinogens adding or exposing their functional groups whereas phase II enzyme like GST (Glutathione S-transferase) are involved in detoxication of the activated metabolites of the carcinogens (Anantharaman et al., 2007). Phase I reactions expose functional groups of the substrates and therefore yield highly reactive
intermediates. These intermediates form the substrates for phase II reactions that involve their conjugation with endogenous molecules such as glutathione (GSH) and thus facilitate their elimination. Hence, the coordinated expression and regulation of phase I and II enzymes determines the outcome of carcinogen exposure. The CYPIA1 encodes an aromatic hydrocarbon hydroxylase enzyme that catalyzes the oxidation of PAHs to their phenolic metabolite or diol epoxide [e.g. Benzo [a] Pyrene (B (a) P) to Benzo [a] Pyrene -Diol-Epoxide (BPDE)] (Bartsch et al., 2000).

GST family is composed of many cytosolic, mitochondrial and microsomal [now designated as MAPEG (Membrane-Associated Proteins in Eicosanoids and Glutathione metabolism)] proteins. GSTs are present in eukaryotes and prokaryotes, where they catalyze a variety of reactions and accept endogenous and xenobiotic substrates. GSTs can constitute up to 10% of cytosolic protein in some mammalian organs. GSTs catalyze the conjugation of reduced glutathione — via a sulfhydryl group —to electrophilic centers on a wide variety of substrates. This activity detoxifies endogenous compounds such as peroxidised lipids, as well as breakdown of xenobiotics.

GSTs may also bind toxins and function as transport proteins, and therefore, an early term for GSTs was “ligandin”. GSTs are a superfamily of ubiquitous, multifunctional enzymes that facilitate detoxification thus protecting cells from oxidative stress. GSTM1 catalyzes the conjugation of the tripeptide GSH to PAH diol epoxides whereas GSTT1 participates in detoxification of monohalomethanes and reactive diol epoxides.

Polymorphisms in the genes that code for these enzymes may alter expression or function, thus increasing or decreasing the activation or detoxication of carcinogenic compounds. The concept of gene–gene and gene–environment interactions to oral cancer risk has long been postulated (Buch et al., 2002, Sato et al., 1999).

Understanding this phenomenon in the Indian context where oral cancers are most predominant not only becomes significant but also particularly difficult as the consumption of tobacco occurs in several forms (use of smokeless tobacco with or without additives and smoking of cigarettes and/or bidis) and most often, as mixed habits.

1.7 Mitochondrial DNA

Mitochondria are organelles in cells that generate energy for the cell and are hence referred to as the "powerhouses" of the cell. The term 'mitochondria' was coined
by Carl Benda in 1898. Friedrich Meves, in 1904, made the first recorded observation of mitochondria in plants (Nymphaea) B. F. Kingsbury, in 1912, first related them with cell respiration, but almost exclusively based on morphological observations. Philip Siekevitz, in 1957, dubbed them 'the powerhouse of the cell'.

Mitochondrion DNA (mtDNA) is present in mitochondria as a double stranded circular molecule and consists of 16,569 nucleotide pairs. Mitochondrial DNA was discovered in the 1960s by Margit M. K. Nass and Sylvan Nass by electron microscopy as DNase-sensitive thread inside mitochondria, and by Ellen Haslbrunner, Hans Tuppy and Gottfried Schatz by biochemical assays on highly purified mitochondrial fractions. The entire molecule is regulated by only one regulatory region which contains the origins of replication of both heavy and light strands. The two strands of mtDNA are differentiated by their nucleotide content with the guanine rich strand referred to as the heavy strand, and the cytosine rich strand referred to as the light strand. Mitochondrial replication is controlled by nuclear genes and is exclusively appropriate to make as many mitochondria as that particular cell needs at the time.

Human mitochondrial DNA (mtDNA) has three promoters, H1, H2, and L (heavy strand 1, heavy strand 2, and light strand promoters). The H2 promoter transcribes almost the entire heavy strand and the L promoter transcribes the entire light strand. The H1 promoter causes the transcription of the two mitochondrial rRNA molecules. When transcription takes place on the heavy strand a polycistronic transcript is created. The light strand produces either small transcripts, which can be used as primers, or one long transcript. The production of primers occurs by processing of light strand transcripts with the Mitochondrial RNase MRP (Mitochondrial RNA Processing). The requirement of transcription to produce primers links the process of transcription to mtDNA replication. Full length transcripts are cut into functional tRNA, rRNA, and mRNA molecules. The process of transcription initiation in mitochondria involves three types of proteins: the mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM), and mitochondrial transcription factors B1 and B2 (TFB1M, TFB2M). POLRMT, TFAM, and TFB1M or TFB2M assemble at the mitochondrial promoters and begin transcription. The actual molecular events that are involved in initiation are unknown, but these factors make up the basal transcription machinery and shown to function in vitro. Mitochondrial translation is still not very well understood.
The heavy strand encodes 28 genes, and the light strand encodes 9 genes for a total of 37 genes. Of these 37 genes, 13 are for proteins (polypeptides), 22 for transfer RNA (tRNA) and two are for the small and large subunits of ribosomal RNA (rRNA).

The number of proteins involved in the electron transfer chain is much larger than 13, but the others are encoded by the nuclear DNA. In total, the mitochondrion hosts about 3000 proteins, but only about 13 of them are coded on the mitochondrial DNA. Most of the 3000 proteins are involved in a variety of processes other than ATP production, such as porphyrin synthesis.
In humans, as in most multicellular organisms, mtDNA is inherited only from the mother's ovum. Mitochondrial inheritance is therefore non-Mendelian, as Mendelian inheritance presumes that half the genetic material of a fertilized egg (zygote) derives from each parent. Eighty percent of mtDNA codes for functional mitochondrial proteins, and therefore most mitochondrial DNA mutations lead to functional problems, which may be manifested as muscle disorders (myopathies). Understanding the genetic mutations that affect mitochondria can help us to understand the inner workings of cells and organisms, as well as helping to suggest methods for successful therapeutic tissue and organ cloning, and to treatments or possibly cures for many devastating muscular disorders.

1.8 Mitochondrial DNA and Cancer

More than 70 years ago, in early 1930, Warburg pioneered the research on alterations in mitochondrial respiration in context of cancer and proposed a mechanism to explain their development during the carcinogenic process (Modica-Napolitano et al., 2007). Warburg hypothesized that the key event in carcinogenesis involved the development of "damage" to the respiratory machinery which resulted in compensatory increase in glycolytic ATP production and eventually, malignant cells would satisfy their energy needs by producing a large fraction of their ATP through glycolytic mechanisms rather than oxidative phosphorylation. Due to the inherent incompetency of glycolytic ATP generation, it represents a unique metabolic state of the malignant cells and would require high consumption of glucose to fulfill cellular energy requirements. This is in contrast to many normal cells, which use oxidative phosphorylation as the preferred means of ATP generation with high efficiency. The differences in energy metabolism between normal and cancer cells constitute a biochemical basis to speculate that therapeutic strategies might be developed to selectively destroy cancer cells due to their inherently compromised respiratory state. Subsequently, a number of additional metabolic alterations associated with mitochondrial function have been observed in cancer cells, including increased gluconeogenesis (Lundholm et al., 1982), reduced pyruvate oxidation and increased lactic acid production (Mazurek et al., 1997), increased glutaminolytic activity (Fischer et al., 1998), and reduced fatty acid oxidation. Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer (Carew & Huang, 2002, Chatterjee et al., 2011).
Figure 1.4: Mitochondrial genes involved in different cancers (modified Figure, courtesy of “Mitochondrial DNA Polymorphism and Risk of Cancer”)

Mitochondrial respiratory activity is associated with the generation of reactive oxygen species (ROS). Under physiological conditions, a small fraction of reducing equivalents from complex I or complex III of the mitochondrial electron transport chain may be transferred directly to molecular oxygen, generating the superoxide anion $\text{O}_2^-$. This ROS is converted to $\text{H}_2\text{O}_2$ by mitochondrial manganese superoxide dismutase. $\text{H}_2\text{O}_2$ can be converted to water by glutathione peroxidase or catalase, or acquire an additional electron from a reduced transition metal to generate the highly reactive hydroxyl radical $\text{OH}$. ROS generation has been shown to increase in mitochondria under the conditions of excess electrons (e.g., increased calorie intake) or as a result of respiratory enzyme complex inhibition. High levels of ROS, or oxidative stress, can induce mutations in both mtDNA and nuclear DNA (nDNA), and damage intracellular protein and lipid components. The mitochondrial genome is especially susceptible to ROS damage due to its proximity to the site of ROS production (i.e. the electron transport chain), its “intronless” DNA, and its limited mtDNA repair capabilities. Oxidative stress is therefore mutagenic to mtDNA and can thus impair mitochondrial function. It is generally accepted that oxidative stress in mitochondria throughout the human life-span is likely an important factor in the aging process.
Metabolic aberrations specifically associated with mitochondrial bioenergetic functions in cancer cells have also been observed. These include differences between normal and malignant cells with regard to preference for respiratory substrates, rates of electron and anion transport, and the capacity to accumulate and retain calcium (Pedersen, 1978). The activities of certain enzymes integral to the process of oxidative phosphorylation are known to be decreased in cancer cells in comparison to normal cells. Numerous differences in the molecular composition of the mitochondrial inner membrane between normal and cancer cells have also been reported. Analysis of the inner membrane lipid composition of various tumour mitochondria has indicated elevated levels of cholesterol, varying total phospholipid content, and/or changes in the amount of individual phospholipids relative to normal controls (Garcea et al., 1980). Polypeptide profiles of normal versus cancer cells reveal a number of differences in the appearance and/or relative abundance of several proteins as well.

1.8.1 Mitochondrial DNA Mutations in Head and Neck Cancer

The role of mitochondria in cellular function has long been known - oxidative phosphorylation occurs in these structures, but they also have a role in carrying out apoptosis and cellular proliferation, and affect the balance of reactive oxygen species within a cell. Alterations in respiratory activity and mitochondrial DNA (mtDNA) appear to be a general feature of malignant cells. The presence of mtDNA mutations has been reported in various cancer cells, and the abundance of mtDNA damage is consistent with the intrinsic susceptibility to constitutive oxidative stress.

In human epithelial cells, tobacco products increase the production of ROS and induce free radical reactions that may be responsible for single strand breaks in DNA especially in the mitochondria where they preferentially accumulate (Kodama et al., 1997, Allegra et al., 2006). MtDNA is present in multiple copies in each mitochondrion. Damage is thought to occur more frequently to mtDNA than to nDNA, through the production of ROS during oxidative phosphorylation, as mtDNA lacks protective histones (Yakes & Van Houten, 1997, Zastawny et al., 1998). Cigarette smoking can cause an increase in ROS such as \( H_2O_2 \) and \( O^- \). In addition, many tobacco smoking related metabolic products contain DNA binding agents that can accumulate preferentially in the mitochondria and lead to DNA damage (Szyfter et al., 1993, Szyfter et al., 1999, Ozawa et al., 1995).
It is known that tobacco products, as well as environmental and dietary factors are involved in the carcinogenesis process of head and neck cancer and the upper aerodigestive mucosa. If these carcinogens are not fully metabolized to nonhazardous forms, then DNA damage may occur and the multi-step process of carcinogenesis can begin, leading to squamous cell carcinoma. For instance, if the electron transport chain (ETC) is inhibited, patients with mitochondrial diseases may be severely affected because oxidative-phosphorylation is disrupted. Also, harmful free radicals can be formed from ROS and disrupt mitochondrial functions.

In majority of cases, mitochondrial mutations were multiple, implying possible accumulation of mtDNA damage that might occur in a clonal expansion model, in which the mutant somatic mitochondrial genome replicates at a higher rate than the wild type (Polyak et al., 1998). Primarily, mutations are identified in the protein coding genes, rRNA genes and in the D-loop region. Most of these mutations are T to C and G to A base transitions, indicating possible exposure to mutagens that generate ROS (Cadet et al., 1997). In fact, it is generally accepted that mtDNA mutations are produced during oxidative phosphorylation through mechanisms involving ROS. The mitochondrial ETC is a major source of ROS. Elevated level of ROS are associated with various diseases including cancer, and are generally associated with a cascade of redox signaling that leads to DNA damage. MtDNA is continuously exposed to ROS produced by the mitochondria and is also predisposed to chemical damage, for example, by environmental factors such as UV, cigarette smoke, and ionizing radiation (Cadet et al., 1997, Dizdaroglu, 1991).

MtDNA has a 10-fold higher accumulation of mutations than nuclear DNA (Ingman et al., 2000). Cancer cells can carry mtDNA mutations and altered copy numbers of mtDNA, which in turn affect expression and activity of the ETC. A number of antioxidant defense mechanisms exist in mitochondria to remove ROS. Cells are normally able to defend themselves against ROS damage through ROS scavengers and antioxidant enzymes such as superoxide dismutases (SOD), catalases, and glutathione peroxidases, which are often deficient in tumour cells. As a result, persistent oxidative stress on cells leads to promotion of cancer growth and metastasis through induction of DNA damage.
1.8.2 Mitochondrial DNA D-Loop and C-Tract Mutation in Head and Neck Cancer

Mitochondrial DNA mutations have been reported in several types of tumours, including HNSCC. The D-loop, which is 1122 bp in size (positions 16024-576), is a non-coding region, and acts as a promoter for both the heavy and light strands of the mtDNA, and contains essential transcription and replication elements. The D-loop region is a hot spot for mtDNA alterations, and it contains two hypervariable regions (HV1 at positions 16024–16365 and HV2 at positions 73–340). The sequence analysis of these two regions is used not only in forensic analyses, but also in medical diagnosis (Levin et al., 1999). The alterations are scattered throughout the mitochondrial genome but the mutations in non-coding region (D-loop) of the mtDNA is more frequent, particularly in the polycytidine stretch (between nucleotides 303 and 315) also termed as the D310 region (Yu et al., 2008).

Association of C-tract mutation in HNSCC has been reported in premalignant lesion which can be proven to be a marker for progression and clonal proliferation (Ha et al., 2002). Moreover, the D310 of the D-loop region has been found to be a “hot spot” for somatic mutations in many cancer types (Mambo et al., 2003, Singh & Kulawiec, 2009). Severe mutations might be of biological significance, because the D310 region lies in a conserved sequence block that is hypothesized to be involved in some aspect of mtDNA replication and transcription (Shin et al., 2006, Mambo et al., 2003).

1.8.3 Mitochondrial DNA ND2 Gene Mutations in Head and Neck Cancer

Mitochondrial ND2 gene encodes for the NAD⁺ dehydrogenase subunit 2 protein, a hydrophobic subunit of respiratory chain complex I, that resides near the junction between the membrane and the peripheral arm which projects into the mitochondrial matrix of complex I (Brandt, 2006). In humans, the ND2 gene is 1041 bp in size and the amino-terminal methionine is encoded by AUU, which, as in the "universal" genetic code, also used for isoleucine codon in elongation. It is well recognized that the mitochondrial respiratory chain is solely responsible to transfer electrons from NAD⁺ dehydrogenase or FADH to a series of electron acceptors, until the final transfer to oxygen leads to the production of water. As an outcome of these biochemical reactions can result in electron leakage and lead to production of ROS (Flemming et al., 2005). The ND2 subunit is suggested to be involved in proton pumping across the inner mitochondrial membrane because of its sequence homology
with a class of Na\(^+\)/H\(^+\) antiporters (Brandt, 2006). Mutations in cancer cells affecting subunits of the respiratory chain indicate a central role of oxidative phosphorylation for tumorigenesis.

1.8.4 Heteroplasmy and Homoplasmy in Cancer

A cancer cell contains many mitochondria with multiple copies of mitochondrial DNA compared to only 2 copies of each nuclear DNA. Homoplasmy is a state in which all the mitochondria of a cell have the same genome, which may either be the wild type or a mutated one. As each cell contains many mitochondria and each mitochondrion contains 2 to 10 copies of mtDNA, it is possible that wild-type and mutant mtDNA can coexist in a state called heteroplasmy. Therefore, the biological impact of a given mutation could vary, depending on the proportion of mutant mtDNAs carried by each cell. In earlier studies no evidence of heteroplasmy was detected, probably due to the lower sensitivities of earlier techniques. However, using massively parallel sequencing-by-synthesis approach, one recent study reported profound heteroplasmy in the mtDNA of normal human cell (He et al., 2010, Fendt et al., 2011). MtDNA constantly undergoes mutation, with expansion or loss of mutated mtDNA copies, which may lead to homoplasmy (Coller et al., 2001, Taylor et al., 2003). Whether mtDNA point mutations occur by simple clonal expansion is a debate. Although a large number of somatic mtDNA deletions are capable of clonal expansion in individual cells (Khrapko et al., 1999). Somatic point mutations have not been shown to be able to clonally expand or reach homoplasmy in vivo, though there are some reports in cell culture (Dunbar et al., 1995) and germline cells (Jenuth et al., 1996). It has been indicated that a single cell with a mutant mitochondrial genome may acquire a selective growth advantage during tumor evolution, allowing it to become the predominant cell type in the tumour cell population (Polyak et al., 1998). Also, it is possible that some germline mtDNA mutations are actually somatic mutations that occurred early during prenatal development of the individual and drifted to homoplasmy. As such, heteroplasmic mutations may reflect an intermediate stage in this process.

For an mtDNA mutation to have significant effect on the physiology of the cell it must reach a threshold level of 60% or more (Hayashi et al., 1991), depending on the type of mutation. Also, it is insufficient for the cell to accumulate different mutations, as different mutations are likely to compensate each other’s deficiencies.
(Hayashi et al., 1994, Takai et al., 1999). However, there are limitations of transcomplementation (Enriquez et al., 2000). These studies strongly support the hypothesis that mutants would need to accumulate via clonal expansion of a single initial mutant.

1.9 Co-amplification at Lower Denaturation temperature PCR (COLD-PCR)

The significance of identifying these low-abundance mutations is critical in several fields of medicine, including cancer, (Kobayashi et al., 2005, Sjoholm et al., 2005) genetic and infectious diseases. The polymerase chain reaction is often utilized as the basis of most molecular applications that investigate DNA sequence variation. Unless specifically modified, PCR will amplify all alleles with approximately equal efficiency, comparable to their initial concentrations. As such, when analyzing these specimens within which the variant DNA can exist at low abundance in the presence of a large majority of wild-type alleles, the ability to identify the mutation is dependent upon the sensitivity of downstream assays, such as Sanger sequencing, among many others. Sanger sequencing is typically reliable for screening germline or prevalent (clonal) somatic mutations, and is widely available; however, the sensitivity of mutation detection is typically limited to detecting. To overcome these limitations, a novel PCR-based application – COLD-PCR was developed that allows detection of DNA mutations when present in very low copy number ratios. The technology has been licensed in late 2009/early 2010 by Transgenomic from the Dana Farber Cancer Institute with an exclusive license for Sanger sequencing applications. COLD-PCR preferentially enriches mutant DNA in a mutant/wild-type mixture by exploitation of the critical temperature, Tc, at which mutation-containing DNA is preferentially melted over wild-type. COLD-PCR promises to provide the enhanced sensitivity and analytical accuracy necessary to screen cancer patients earlier in their individual development of this disease.

**Figure 1.5:**
Schematic representation of COLD-PCR Thermal cycling (modified from Figure courtesy of *Nature Medicine* 2008)
1.10 Mitochondrial Copy Number Alterations in Cancer

Mitochondria, the key organelles in eukaryotic cells principally responsible for multiple cellular functions. Regulation of mitochondrial biogenesis is essential for proper cellular functioning. In addition to a plethora of somatic mutations as well as polymorphic sequence variations in mtDNA, the identification of increased or reduced mtDNA copy number has been increasingly reported in a broad range of primary human cancers. The accumulation of mtDNA content alterations may be a pivotal factor in eliciting persistent mitochondrial deficient activities and eventually contributing to cancer pathogenesis and progression (Yu, 2011).

Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer (Carew & Huang, 2002, Chatterjee et al., 2011). The mtDNA copy number per cell is maintained within a constant range to meet the energy requirement of the cell to sustain normal physiological functions. In normal cells, mitochondria have 2–10 copies of their genomes (mtDNA). It varies significantly among the population from 1000 to 10,000 per cell (Veltri et al., 1990) and also significantly varies by cell type. However, this number is altered during the aging process or under pathological states. During the aging process, the mtDNA copy number was found to increase in the brain and lung (Barrientos et al., 1997, Lee et al., 2000).

This increase in mitochondrial copy number has been viewed as a potential compensatory effect for the generalized decline in mitochondrial respiratory function (Shigenaga et al., 1994). Under pathological states, the conditions are complex. Subsequently, alterations in mtDNA copy number have been observed in a variety of human cancers. It is likely that the variations in the copy number of mitochondria reflect the net results of gene–environmental interactions between unknown hereditary factors and the levels of oxidative stress (an imbalance between ROS production and the antioxidant capacity). This variation is caused by a variety of endogenous and exogenous factors, such as, hormones, age, dietary and environmental oxidants/antioxidants, and reaction to oxidative damage, all of which are thought to be risk factors for various types of cancer development (Renis et al., 1989, Lee et al., 1998, Verma et al., 2007).
1.11 OBJECTIVES

The prevalence of head and neck cancer in Northeast India is very high especially incidence of tobacco related head and neck cancers. The area of our study is the southern part of Assam, North East India, Barak valley which derives its name from River Barak and comprises of three districts, viz. Cachar, Hailakandi and Karimganj. Tobacco usage in different patterns is very high as compared to the rest of the country. Tobacco smoke contains various carcinogens. These compounds not only cause single-strand breakage in DNA but also results in oxidation of protein thiols and lipid peroxidation thereby triggering damage to mitochondrial DNA. The detection of hot spot mutations in the mtDNA in patients of HNSCC can be a possible biomarker for the early detection and diagnosis of head and neck cancer. Furthermore, the tobacco - betel quid chewing, GSTM1-GSTT1 null genotypes, HPV infection and mtDNA content associated with HNSCC was studied in the present study which may serve as a possible molecular biomarker for early detection of head and neck cancer, and might prove clinically useful as unique molecular sites against which novel and selective chemotherapeutic agents might be targeted in the most prevalent cancer of Northeast region of India. Keeping in view the idea of development of early detection in HNSCC the following objectives have been carried out in this study.

2. Analysis of incidence of different cancers in Southern Assam.
3. Molecular detection of mutation based on mtDNA D-loop, ND2 gene and complete mitochondrial genome in oral cancer patients.
4. Occurrence of C-tract mutation in mtDNA from normal and cancer patients and analysis of heteroplasmy and homoplasmy mutations.
5. Detection of mutation from patients without DNA sequencing by means of co-amplification at lower denaturation temperature polymerase chain reaction (COLD-PCR).
6. Study of mtDNA copy number variation in oral cancer patients and controls along with analyzing the GSTs polymorphism and human papilloma virus infect