CHAPTER-2

REVIEW OF LITERATURE

2.1 Head and Neck Cancer Incidence

2.1.1 The Global Scenario

Globally head and neck cancer (HNC) is the sixth most common malignancy and representing about 6% of all cancers. Squamous cell carcinoma (SCC) is the most common histological type of head and neck malignancy, accounting for more than 90% of all head and neck cancers (Argiris et al. 2008, Parkin et al. 2005). The head and neck squamous cell carcinoma (HNSCC) incidence showed dramatic variation across the globe. The global incidence and mortality rates were estimated for different cancers are available in the GLOBOCAN database series of the International Agency for Research on Cancer (IARC). Ferlay et al. (2015) reviewed the fifth version of GLOBOCAN and estimated around 686,000 cases of head and neck cancer in 2012, comprising 300,000 oral, 157,000 laryngeal, 142,000 pharyngeal and 87,000 nasopharynx cancer. It was estimated that regions including South east Asia, Western Asia, South America, the Caribbean and Northern Africa would face sharp increases of cancer deaths in 2020 as compared to 2000 (Mishra and Meherotra 2014).

The regions of high incidence for oral cancer include Melanesia (a subregion of Oceania, Northeast of Australia), South-Central Asia, Western and Southern Europe, and Southern Africa, whereas regions of high incidence for laryngeal cancer are Southern and Eastern Europe, South America and Western Asia (Parkin et al. 2005). In the last decades, a decline incidence of HNC in the Western world was observed, due to reduced levels of smoking habit. However, the incidence of oropharyngeal cancer increased in the Western world including Denmark, the Netherlands, Norway, Sweden, the UK, Australia, Canada, and the USA. Among HNCs, nasopharyngeal carcinoma (NPC) represents a separate group of tumours due to its unusual characteristics in epidemiology, pathological and clinical history. This change in the epidemiology of a specific subgroup of head and neck cancers is suggestive of additional risk factors such as high-risk human papillomaviruse (HPV) infection (Pezzuto et al. 2015). NPC is rare in the US and Europe [age-standardized incidence rate (ASR) 0.5–2 per 100,000], at intermediate incidence is found in the Mediterranean Basin and the Arctic and in Southeast Asia, but is widespread in Southern China (ASR up to 25 per 100,000) (**Table 2.1**).

Table 2.1 Estimated numbers of new cancer cases in males and females and world ASR per 100,000 (Pezzuto et al. 2015)

Population	Lip and oral cavity		Larynx		Pharynx		Nasopharynx	
	Cases	ASR*	Cases	ASR	Cases	ASR	Cases	ASR
Africa	17,276	2.6	8,671	1.4	5,297	0.8	8,293	1.1
Eastern	6,998	3.6	2,167	1.2	1,473	0.8	3,138	1.5
Southern	1,871	4.0	1,217	2.7	1,176	2.5	132	0.2
Middle	1,764	2.6	470	0.8	793	1.1	765	0.9
Northern	3,804	2.3	3,569	2.2	1,232	0.7	2,979	1.6
Western	2,839	1.5	1,248	0.7	623	0.3	1,279	0.5
Asia	168,850	3.8	77,505	1.8	80,013	1.8	70,108	1.6
Eastern	38,077	1.7	26,075	1.2	15,325	0.7	36,420	1.8
South-Central	108,651	7.3	36,746	2.6	54,682	3.8	6,184	0.4
South-Eastern	18,071	3.2	8,259	1.5	8,898	1.6	25,596	4.3
Western	4,051	2.1	6,425	3.6	1,108	0.6	1,908	0.9
Europe	61,416	4.8	39,921	3.2	34,094	2.9	4,172	0.4
Central and Eastern	23,765	5.0	16,493	3.6	11,588	2.6	1,628	0.4
Northern	7,795	4.4	3,464	1.9	3,438	2.1	430	0.3
Southern	11,545	3.9	10,536	3.7	4,868	1.9	1,140	0.5
Western	18,311	5.5	9,428	2.7	14,200	4.5	974	0.3
Latin America	20,633	3.3	16,481	2.7	8,859	1.5	1,639	0.3
Northern America	28,567	5.1	13,474	2.4	13,249	2.5	2,278	0.5
Oceania	3,631	7.4	825	1.6	875	1.8	201	0.5

*ASR= age-standardized incidence rate per 100,000

Three different patterns of oropharyngeal and oral cancer incidence trends were observed (Chaturvedi et al. 2013):

(1) Countries with a substantial increase in the incidence of oropharyngeal carcinoma and with stable or declining incidence of the oral cancer (the US, Australia, Canada, Japan, and Slovakia)

(2) Countries with a significant increase in the incidence of both oropharyngeal and oral cancer but with higher trends for oropharyngeal cancer (Denmark and the UK)

(3) Countries with similar incidence trends in both conditions

Five years survival rates of head and neck cancer patients are about 40-50%. The majority are diagnosed with advanced stage cancer with lymph node metastases, whereas only one third of HNSCC patients are diagnosed with early-stage disease. Most of the early stage tumours are treated by surgery or radiotherapy ensuing more favourable prognosis. Surgery in combined with post-operative chemo and radiotherapy is the main source of treating advanced tumours. Despite advanced surgical and radiotheraupeutic techniques, survival has not noticeably improved, it may because, patients still frequently develop local-regional recurrences, distant metastases and second primary tumours (Leemans et al. 2011). The mortality rates were particularly high among middle-aged (35 to 64) men in countries like Hungary (55.3 per 100,000) and Slovakia (40.8 per 100,000). Over the period of 1975–2004 in Europe, oral and pharyngeal cancer mortality increased 2.1% annually between 1975 and 1984 and decline 1.3% annually between 1993 and 2004. However, during the same period, the oral and pharyngeal cancer mortality constantly increased in several central and eastern European countries with the highest peaks in Hungary (21.1 per 100,000) and Slovakia (16.9 per 100,000) (Pezzuto et al. 2015).

2.1.2 The Indian Scenario

India is a centre of a diverse ethnic population and thus disparities in pattern of cancer incidence rate found among different regions and ethnic groups. HNC is one of the most common cancers in India and account for 30-40% of all sites. Oral cancer is the most prevalent form of the HNCs in some region of India. In 1981, to generate reliable data on the magnitude and patterns of different cancers in India and to help in cancer control activities, the Indian Council of Medical Research (ICMR) has started the National Cancer Registry Programme (NCRP) across the country. Previous study reported that the ASR (age-standardized incidence rate) of HNC in Indian males to exceed 30 per 100,000 and in Indian females to exceed 10 per100,000. However, the highest ASR in males is reported from France, but the highest ASR in females is reported from India. (Sankaranarayanan et al. 1998).

Increase in incidence of HNC in young adults was also reported in Indian population (Elango et al., 2006).

The trends in incidence of oral squamous cell carcinoma (OSCC) from Western U.P, revealed a male to female ratio of 2.2:1 with the largest number of OSCCs developing in the fourth and fifth decades of life and the most common site was the buccal mucosa (63.75%) (Sharma et al. 2010a). There is an increased trend towards the incidence of tongue cancers in Indian population (Bhattacharjee et al. 2006, Mammas et al. 2011). Around 200,000 head and neck cancer occurs in India each year of which oral, pharyngeal cancer and laryngeal contributed around 80,000, 40,000 and 29,000 cases respectively (Kulkarni 2013). Larynx cancer was the commonest cancer in Bihar (North India) followed by oral cavity and oropharynx cancer. In Northeast India, the prevalence of head and neck cancers is significantly high and oropharyngeal along with oral cancer is the most common head and neck cancer (Bhattacharjee et al. 2006, Shunyu and Syiemlieh 2013). Hypopharynx cancer is second most occurring cancer in kamrup district of Assam (a major district in Northeast India), which comprised 8.25% of all cancer cases in males (Sharma et al. 2014). However, actual burden of HNC in India is much larger than revealed through the available literature and hence can be considered as a 'tip of iceberg' situation. India as compared to USA has a very limited number of registries and this further emphasizes the 'tip of iceberg' hypothesis for cancer incidence in Indian and offer a necessity for having more number of evenly circulated cancer registries across the country (Mishra and Meherotra 2014). On the whole, with changing lifestyle in India, it is estimated that future burden of cancer cases in 2020 will be around 13,20,928 (male 6,22,203, female 6,98,725) (Sharma et al. 2014).

2.2 Aetiology of Head and Neck Cancer

2.2.1 Environmental Risk Factors

Head and neck cancer (HNC) is one of the most widespread cancers in Southeast Asia and the major cause of death from cancer among males. These high incidence and mortality rates are due to lifestyle risk habits such as tobacco smoking, betel quid chewing and alcohol drinking (Baez 2008, Petti 2009), in addition genetic and infectious factors also played a vital role (Chen et al. 2008).

Numerous epidemiological studies have confirmed smoking and alcohol consumption as the major risk factors for HNSCC. A pooled analysis of 15 case-control studies performed by INHANCE (International Head and Neck Cancer Epidemiology) consortium, which included 10,244 HNC cases and 15,227 controls (1072 case and 5775 control were never tobacco users and 1598 case and 4051 control were never alcohol drinkers) (Hashibe et al. 2007). This pooled analysis evaluated the effect of smoking in never and in ever alcohol drinkers in addition to the effect of never alcohol users and ever cigarette smokers, and showed that smoking was a much stronger risk factor for HNC than alcohol. Another pooled analysis of 11,221 HNC cases and 16,168 controls, carried out by INHANCE consortium, showed a greater than multiplicative joint effect between tobacco and alcohol use and the risk of HNC. The population attributable risks (PAR) for tobacco or alcohol was 72% (95% confidence interval, 61-79%) for HNC, of which 4% of HNC was due to alcohol alone, 33% was contributed by tobacco alone, and 35% was due to combined tobacco and alcohol use. The total PAR varied by sub-site (64% for cancer of oral cavity, 72% for cancer of pharynx, and 89% for cancer of larynx), by age (33% for cases <45 years, 73% for cases >60 years) and by sex (74% for men, 57% for women). In addition, tobacco and alcohol together accounted for higher rates of HNC in Europe (84%), Latin America (83%) and than in the North America (51%) (Hashibe et al. 2009). The risk of HNSCC increases with smoking and chewing in a dose-response relationship with intensity and duration of consumption. In addition, it was observed that risk among former smokers is consistently lesser than among current smokers and there is a trend of declining risk with increasing number of years since quitting. This increased risk of cancers is mostly attributable to the genotoxic effects of carcinogens in tobacco smoke, including nitrosamines and polycyclic hydrocarbons. Tobacco smoking has also showed to have site-specific differences in the anatomical sub-regions (Werbrouck et al, 2008). Tobacco has also proven to be a significant prognostic marker (Ragin et al, 2007). Bidi smoking is prevalent in India, and in other South Asian countries and a case–control study established a strong association of bidi smoking with HNSCC (Rahman et al. 2003, Subapriya et al. 2007).

The major events in malignant transformation in tobacco-related cancer are mutations affecting the p53 tumour suppressor gene and the ras oncogene. Nearly

50% of HNSCCs have p53 mutations and more than 90% of the p53 mutations are observed in exons 5 to 8 and within the DNA-binding domain of the p53 protein. In oropharyngeal cancers, prevalence of p53 mutation was high in heavy smokers. The ras gene family (H, K and N-ras) encode the Ras protein, a GTPase activating protein that regulates several signal transduction pathways including cellular proliferation and differentiation. Mutations in ras genes have a higher frequency in HNSCC in India and Taiwan, while in the Western Hemisphere, low frequency has been reported (Baez 2008).

Betel quid chewing is an ancient and popular cultural practice in South-east Asian countries. The leading cause of cancer of the oral cavity in India is due to betel quid chewing (with or without tobacco). The causal association between betel quid chewing without tobacco and carcinogenesis has been recognized (Merchant et al. 2000). Addition of tobacco to betel quid further enhances the risk. A dose response relationship has been observed with daily frequency and duration of chewing and the risk persists even after quitting the chewing (Balaram et al. 2002, Subapriya et al. 2007). Worldwide, more than 50% of oral cancers are attributable to betel quid chewing in areas of high chewing prevalence, 25% to tobacco usage (smoking and/or chewing), 7-19% to alcohol drinking and 10-15% to micronutrient deficiency (Petti 2009). Betel quid chewing along with smoking and alcohol consuming further pump up the risk in men resulting in an early age of onset of oral cancer (Lee et al. 2011). Tobacco chewing exhibited a linear association with risk of development of oral cancer. Those who were tobacco chewer and smoke heavily further exaggerating the risk (Dikshit and Kanhere 2000, Jayalekshmi et al. 2011). Tobacco-specific nitrosoamines had been found in the saliva of betel quid chewers. Lime, an ingredient of betel quid, acts as a tumour promoter by hydrolysing alkaloids present in the areca nut (Shah et al. 2012).

Heavy alcohol consumption has also been recognized as an independent risk factor for HNSCC, particularly for the hypopharynx cancer (Sturgis et al, 2004). Alcohol has ability to enhance the effects of tobacco chewing and smoking, it is probably due to the nature of alcohol as a chemical solvent, increasing and prolonging mucosal contact with the carcinogens present in tobacco. A case–control study from India demonstrated that the risk increased 11-fold with combined

tobacco/betel quid chewing, smoking and heavy alcoholic drinking habits (Subapriya et al. 2007).

2.2.2 Human Papillomavirus (HPV)

Involvement of HPV in head and neck tumourigenesis was primarily reported 30 years ago (Syrjanen et al. 1982); however, it was recently recognized as an emerging risk factor for some of the HNC like oropharyngeal squamous cell carcinoma (OPSCC) and oral squamous cell carcinoma (OSCC) (Chaudhary et al. 2009). High-risk HPV types were found to be significantly higher in tonsillar cancers than in other head and neck cancers (Koskinen et al. 2003, Venuti et al. 2004). A systematic review of 60 studies that included 5,046 HNSCC cancer specimens, showed the overall HPV prevalence was 25.9%, and among the HNSCC, prevalence was significantly higher in OPSCC (35.6% of 969) than OSCC (23.5% of 2,642) and laryngeal cancer (24.0% of 1,435). HPV16 accounted for a larger majority of HPV-positive OPSCC (86.7%) compared with HPV-positive OSCC (68.2%) and laryngeal cancer (69.2%). On the other hand, HPV18 was rare in HPV-positive OPSCCs (2.8%) compared with other head and neck sites (34.1% of oral SCCs and 17.0% of laryngeal SCCs). HPV prevalence based on tumour site was higher among studies from North America compared with Asia and Europe. The high HPV16 prevalence and the lack of HPV18 in oropharyngeal compared with other head and neck sites may indicate specific virus-tissue interactions (Kreimer et al. 2005). A meta-analysis study on HPV in non site-specific HNSCC vs. OSCC samples, observed that the prevalence of HPV in the overall samples was 34.5%, whereas it was 24.1% in HNSCC and 38.1% in the non site-specific OSCC group (Termine et al. 2008). Incidence of HPV positive oral cancer varies worldwide from 25-80% and predicted to increase in the near future. There is a increase of oral and oropharyngeal cancer incidence mostly in individuals aged 40-55 years without environmental risk factors, and is associated with infection with high-risk HPVs (Chaturvedi et al. 2011, Lajer and von Buchwald 2010). The prevalence of oral and oropharyngeal cancers increased in male over the last 30 years, even though a decline in smoking habits, which may be associated to the increasing proportion of HPV positive cancers. HPV associated oral cancer represents about 60% of cases compared to 40% in the previous decade (Mehanna et al. 2010). An increasing

incidence of oral cancer was observed in the USA, Sweden, Finland and Czech Republic during the last decade (Hong et al. 2010, Tachezy et al. 2005). The prevalence of HPV infection rates were recently found to range between 12.6-90.9% in oropharyngeal carcinoma (Mammas et al. 2011).

Many authors found frequent HPV infection in oral cavity cancer by considering the overexpression of p16INK4A (Heath et al. 2012, Sethi et al. 2012). Among all the high-risk HPV types, HPV-16 is the most frequent in HPV-positive oropharyngeal cancers. Apart from high-risk HPV-16 type, other less frequent are HPV-33, HPV-35, HPV-45 and HPV-58, representing 10-15% of HPV-positive HNSCC (Deng et al. 2012, Glombitza et al. 2010, Koskinen et al. 2003). One of review studies on the association between smoking and HPV in causation of HNSCC, recognizes smoking has the potential to promote infection, persistence, and the carcinogenetic effect of HPV (Sinha et al. 2012).

Molecular detection of HPV DNA is the gold standard for the identification of HPV from exfoliated cell and tissue samples using a number of assays with different sensitivity and specificity, including polymerase chain reaction (PCR), in situ hybridization (ISH), Southern transfer hybridization, dot blot hybridization and hybrid capture (Zaravinos et al. 2009). PCR is very sensitive detection method for HPV subtypes since it detects less than one copy number of the viral DNA per cell. Common primers of conserved HPV DNA sequences have been designed for the L1 region (i.e. MY09/MY11 primers), for E1 region (i.e. CPI) and for E6 and E7 region (i.e. CPII). The mass of studies used the common primers MY09/11 for the detection of HPV having base pair 450 bp for different types of cancers (Chaudhary et al. 2009).

HPV positive and HPV negative OSCCs show different genetic characteristics, which most likely underlie differences in tumour development and progression. These differences may have implication for the patient's management (Jung et al. 2010, Lohavanichbutr et al. 2009). The detection of increased p16 INK4A protein levels by IHC is the most well known biomarker for the identification of biologically active HPV infection in HNSCC. p16INK4A is a cyclin-dependent kinase (CDK) inhibitor, which arrests the cell cycle in the G1 stage, pRb inactivation by E7 oncoprotein of HPV is associated with upregulation of

CDKN2A and subsequent protein overexpression (Chandarana et al. 2013). On the other hand, in HPV negative HNSCC, perturbation of the pRb-pathway is uncommon and CDKN2A expression is usually low. Thus, p16INK4A immunostaining in conjunction with HPV DNA detection is very a helpful to establish a diagnosis of HPV-related HNSCC. In the Danish Head and Neck Cancer Group (DAHANCA), five trial p16INK4A was assessed as prognostic biomarker of treatment response in a cohort of patients treated exclusively with conventional radiotherapy. However, some authors have argued that HPV status may reduce the overall prognostic significance of nodal category (Genden et al. 2013). Loss of p16INK4A has been consistently associated with a worse prognosis; however, the association of p14/ Arf/INK4B loss and HNSCC outcomes is still unclear. Missense mutations in the DNA-binding domain of p53 is most common in HPV-negative HNSCC, such mutations have been linked to reduced survival after surgery (Poeta et al. 2007, Rothenberg and Ellisen 2012). Very recently, a single-institutional experience with definitive radiation alone for HPV-positive HNSCC confirmed the inherent radio-sensitivity of these tumours (Chen et al. 2013).

2.3 Genetic Alterations in Head and Neck Cancer

Only small fraction of tobacco and alcohol users develop head and neck cancer (HNC) suggesting that genetic factors may play a key role in HNC. Molecular epidemiological studies have now provided evidence that mainly the combined effects of genetic and environmental factors mediate an individual's susceptibility toward cancer. Environmental carcinogens damage DNA through oxidative stress, alkylation, and formation of bulky adducts, and strand breaks. Metabolism of environmental carcinogens found in tobacco smoke and alcohol is the balance between metabolic activation and detoxification of potential carcinogens (Baez 2008). Therefore, alteration of carcinogen metabolizing enzymes (Cheng et al. 1999) and DNA repair proteins (Sturgis et al. 1999) will have an impact on cancer risk.

2.3.1 Genetic Polymorphisms of Metabolic Genes

Large number of studies had been conducted to screen the GST genotypic status, since GST enzymes provide protection to somatic cells from DNA damage by

carcinogens. Persons having homozygous deletions or null genotype of *GSTT1* or *GSTM1* genes lack the ability to detoxify the carcinogen and in turn increase the susceptibility of cancer. Glutathione S-transferases (GSTs) are a group of Phase-II xenobiotic metabolising enzymes that detoxify a variety of exogenous as well as endogenous reactive species by conjugating electrophilic substrates to glutathione, which can be hydrolyzed and easily excreted out from the body. The phase-I enzymes (such as CYP1A1 etc) first set up functional electrophilic intermediates (such as –OH,-SH, -NH2, etc.) in the xenobiotic molecules, which are soon detoxified by phase II enzymes (such as GSTs etc) (Kumar et al. 2009). The most commonly studied genes of the GST superfamily are *GSTT1*, *GSTM1* and *GSTP1*. The prevalence of the *GSTM1* null genotype in Asians, Caucasians and Africans was 42~54%, 47~57%, and 16~36%, respectively, whereas the prevalence of the *GSTT1* null genotype in Caucasians was rather low as 13~26% but relatively higher in Asians (35~52%) (Garte et al. 2001). Epidemiological studies have shown that the null genotypes of the *GSTM1* locus is associated with various types of metabolic disorder, including bladder cancer (Johns and Houlston 2000), lung cancer (Ford et al. 2000) and prostate cancer (Wei et al. 2012). It was also revealed that null genotype of these genes may be associated with the risk of HNSCC in different parts of the world (Ho et al. 2007). The studies on *GSTM1*, *GSTT1* and *GSTP1* polymorphisms in relation to HNC have been recently reviewed in a meta-analysis of 31 case-control studies that included 4635 patients and 5770 controls. This meta-analysis summarized that *GSTM1* null, *GSTT1* null and *GSTP1* Ile105Val genotype frequencies were highly variable in the HNSCC case populations (range 43-80% for *GSTM1* null, 12-58% for *GSTT1* null and 29-66% for the *GSTP1* Val105 allele frequencies) (Hashibe et al. 2003). Another meta-analysis of 42 published case-control studies explored that *GSTM1* null genotypes were associated with a self-effacing risk in HNSCC (OR=1.27, 95% confidence interval=1.13-1.42) (Ye et al. 2004).

A case control study on an American white population reported a modest risk associated with *GSTM1* null genotypes among 147 HNSCC patients (McWilliams et al. 2000). Similarly, in German population, *GSTM1* null genotypes frequency was high in patients with HNSCC compared to controls (Ko et al. 2001). A meta-analysis reported that the null genotypes of *GSTT1* has an increased risk of HNSCC

(OR=1.17, 95% CI=0.98-1.40) (Hashibe et al. 2003). Another case-control study reported that the presence of *GSTT1* gene (OR=1.6, 95% CI=1.1-2.5, p=0.03) was associated with a significant increased risk of HNSCC in US population (Evans et al. 2004). On the other hand, one study reported that the combined *GSTT1* and *GSTM1* null genotypes were twice as common in HNSCC patients as in controls (p<0.054) (Gronau et al. 2003). A case control study in an Italian population significant association between HNSCC and *GSTM1* null genotypes (Capoluongo et al. 2007). Another study found significantly more *GSTM1* null genotypes in HNSCC patients (OR=2.02; 95% CI: 1.32-3.10; p=0.001) than in control groups in the North Indian population (Singh et al. 2008). A case control study conducted in Brazil and found a significant association between *GSTM1* null genotypes and HNSCC (OR=2.25, 95% CI=1.05-4.84, p=0.0368) (Leme et al. 2010). Similarly, a case control study in a Pakistani population conducted on 388 HNSCC patients and 150 healthy controls reported *GSTM1* null genotypes (OR=2.3, CI=1.5-5.5) significantly (p<0.05) associated with HNSCC risk (Nosheen et al. 2010).

A case-control study demonstrated that combined *CYP1A1* A4889G and T6235C abnormalities and *GSTM1* and *GSTT1* alter pathways are important determinants of HNSCC, mainly pharyngeal cancer in heavy smoking individuals from South-Eastern Brazil (Lourenco et al. 2011). Previous studies have revealed that the null genotype or homozygous deletion at the *GSTT1* gene locus resulted in loss of enzyme function and may be linked to the risk of HNSCC (Cadoni et al. 2012). A recent meta-analysis including 42 studies for *GSTM1*, 32 for *GSTT1*, and 15 studies for *GSTM1* and *GSTT1* in combination, gives additional evidence of the association between polymorphisms of *GSTM1* and *GSTT1* and HNSCC. They also found elevated risks of HNSCC in individuals with *GSTT1* null genotype in South American, *GSTM1* null genotype in Asian, and dual null genotype in European and Asian. In addition, a significant association of *GSTM1* null genotype with HNSCC risk was found in smokers (Zhang et al. 2012).

A review study explained that, the oral cavity cancer was more influenced by *GSTM1* and *GSTT1* gene deletions. Relating to ethnicity, Asians are more susceptible to head and neck cancers with these null genotypes as compared to Europeans and Americans. The review also showed significant associations of

GSTM1 and *GSTT1* null genotypes with head and neck cancers (OR=9.0, 95%CI; 1.4-9.5 and OR=3.7, 95%CI; 1.4-9.5respectively) (Masood et al. 2013). Many previous studies reported the association between *GSTM1* and *GSTT1* null genotype and head and neck cancer in India (Anantharaman et al. 2007, Singh et al. 2008, Soya et al. 2007, Yadav et al. 2010). A case–control study in North Indian population, demonstrated the role of *CYP1A1* polymorphism in the development of HNSCC (Singh et al. 2009). A case-control study including 750 HNSCC and a equivalent number of healthy controls investigated the association of genetic polymorphisms in the metabolizing genes (*CYP1A1*, *CYP1B1*, *CYP2E1* and GSTM1) with the risk of developing cancer. This study pointed out the role of gene-gene and gene-environment interactions to the susceptibility to HNSCC. Polymorphisms in *CYP1A1* and GSTM1 showed modest associations with HNSCC risk. In addition, cases carrying a combination of *GSTM1*-null and *CYPs* variant genotype were at higher risk of developing HNSCC (up to 5-fold). HNSCC risk also increased several-fold in cases carrying *CYPs* variant genotypes, and were habitual tobacco smokers and tobacco chewers. These data therefore suggest that along with polymorphisms in carcinogen-metabolizing enzymes, gene-gene and gene-environment interactions play a major role in increasing the susceptibility toward HNSCC (Maurya et al. 2014). A meta-analysis also indicated that *GSTM1* polymorphism had a significant effect on the susceptibility of oral cancer in the Indian population (Peng et al. 2014).

2.3.2 Genetic Polymorphisms of DNA Repair Genes

Mammalian cells have several DNA repair mechanisms that each deal with a specific type of DNA damage to maintain integrity of the genome (Huet al. 2002; Kotniset al.2005). *XRCC1* is located on chromosome 19q13.2, which encodes a protein which interacts with many other components of the base excision DNA repair (BER) pathway, such as PARP-1(Poly-ADP ribose polymerase), PNK (Poly nucleotide kinase), Polβ (DNA polymerase β), LIG3 (Ligase 3α) etc (Ramachandran et al. 2006) thus playing an central role in the maintenance of genome integrity. More than 60 SNPs have been identified, and the most commonly investigated coding region SNPs are: rs25487 in exon 10 (G→A; Arg399Gln), rs1799782 in exon 6 (C→T; Arg194Trp), and rs25489 in exon 9 (G→A; Arg280His) (Huang et

al. 2005, Hung et al. 2005). These polymorphisms of *XRCC1* were investigated to find an association between the *XRCC1* Arg194Trp and Arg399Gln polymorphisms and the risk of HNSCC (Olshan et al. 2002, Sturgis et al. 1999). In spite of a good number of studies done in well-characterized populations, results of HNSCC are still puzzling. There was a moderately significant increase risk of HNSCC was found in variants of *XRCC1* genotype with Arg194Trp allele in a Thailand population (Kietthubthew et al. 2006). However, a statistically significant association was observed for *XRCC1* Arg399Gln (for Caucasians only) and *XRCC1* Arg194Trp variants and HNSCC (American population) (Flores-Obando et al. 2010). No significant association was found between HNSCC and polymorphisms of *XRCC1* (Arg194Trp and Arg399Gln) in a Hungarian population; whereas a significant difference was found between patients with different *XRCC1* Arg194Trp polymorphism in clinical stage III (Csejtei et al. 2009). Further, no altered risk of HNSCC was found with the *XRCC1* Arg399Gln genotype in non-Hispanic white population (Li et al. 2007) and in a Polish population (Jelonek et al. 2010). However, it was also observed that smokers carrying *XRCC1* risk genotype with dominant Arg399Gln allele were overrepresented in HNSCC patients from eastern Indian populations (Majumder et al. 2005). Other recent reports showed an association of the Arg399Arg variant with HNSCC (Kowalski et al. 2009). A case-control study, found that there was a 3.37-fold increased risk of laryngeal carcinoma for individuals carrying *XRCC1* Arg/Gln+Gln/Gln genotypes, compared with subjects carrying *XRCC1* Arg/Arg genotype at codon 399. (Yang et al. 2008).

Another study showed that the *XRCC1* Arg399Gln variant genotype have a risk of 3.9 fold (95% CI=1.76– 9.05) for smoking and 4.62 fold risk (95%, CI=1.24–17.2) for betel quid chewing (Matullo et al. 2006). These results were consistent with a previous report that showed a modest positive association with smoking and betel quid chewing for subjects with *XRCC1* Arg399Gln variant genotypes (Werbrouck et al. 2008). Presence of the variant allele of *XRCC1* codon 194 and 399 was associated with increased risk of oral cancer compared to the wild genotype. Moreover, smokers and betel quid chewers with the variant genotypes of *XRCC1* Arg399Gln exhibited increased risk of oral cancer (Ramachandran et al. 2006). Acetylation status could transform the risk of cancer for *XRCC1* genotypes variant at codon 399 (Majumder et al. 2005). Therefore, tobacco consumption especially

smoking and its carcinogen element could be interaction factors of risk of oral cancer affected by *XRCC1* genotype (Werbrouck et al. 2008). The *XRCC1* Arg399Gln polymorphism have a contributive role in DNA adduct formation, sister chromatid exchange and increased risk of cancer development. One of the studies suggests an important role for *XRCC1* Arg399Gln polymorphism in p53 gene mutation in OSCCs of Taiwanese population (Hsieh et al. 2003). However, studies also showed, no association between NPC and *XRCC1* Arg399Gln polymorphisms (Cao et al. 2006, Cho et al. 2003). On the contrary, a significant protective influence of the *XRCC1* 194Trp/Trp genotype (OR=0.34, 95% CI= 0.14–0.82) was found on NPC risk in smokers, suggesting effect modification by tobacco smoking. While, one study reported an association between *XRCC1* gene polymorphisms and susceptibility to NPC (Pan et al. 2007). Recently, genotyping of three SNPs in *XRCC1* was done in 598 NPC cases from Morocco, Algeria and Tunisia and 545 controls, found that the genotype and allele distributions for *XRCC1* 399Arg/Gln, 280Arg/His, and 194Arg/Trp SNPs did not vary significantly among NPC cases and controls (Laantri et al. 2011). Recently, a case–control study was conducted on HNSCC including the *XRCC1* Arg194Trp, *XRCC1* Arg399Gln and *XRCC1* Arg280His genetic variants (Gugatschka et al. 2011), found no significant difference between patients and controls in respect of the investigated polymorphisms in an Austrian Caucasian population. However in Indian population, a significant difference between patients and controls in respect of *XRCC1* Arg194Trp and *XRCC1* Gln399Arg SNPs was found, but no significant difference was found for *XRCC1* Arg280His (Kumar et al. 2012).

However, the meta-analysis of 29 studies consisting of 6,719 cases and 9,627 controls, demonstrated that the *XRCC1* Arg194Trp, Arg280His and Arg399Gln polymorphism might not involve in HNC susceptibility (Lou et al. 2013). Another meta-analysis study of total 27 case-control studies also showed that the *XRCC1* Arg399Gln polymorphism was not associated to HNC (the dominant model AA+AG vs. GG: OR=1.00, 95% CI=0.78-1.29; the recessive model AA vs. AG+GG: OR=0.91, 95% CI=0.71-1.16) (Liu et al. 2014). These meta-analysis studies further suggested that gene-gene and gene-environment interaction in diverse populations are required to establish HNC susceptibility.

2.4 Epigenetic Alterations and Head and Neck Cancer

The idea of epigenetics was first introduced by C.H. Waddington in 1939 and explained that, "the causal interactions between genes and their products, which bring the phenotype into being". Later epigenetics was defined as, any heritable changes in gene expression that are not coded by the DNA sequence. Both covalent and non-covalent modifications of chromatin are currently considered as trademark of changes in an epigenetic landscape. In broadest terms, epigenetics is a bridge between genotype and phenotype, a phenomenon that changes the ultimate outcome of a genetic locus without changing the underlying DNA sequence (Esteller 2008, Goldberg et al. 2007). Two basic components of chromatin include DNA and histones; undergo modifications to influence chromatin structure and gene expression. Depending upon the type and location, these modifications alter multiple cellular processes such as gene regulation, cellular differentiation, and stem cell development (Lindahl Allen et al. 2009, Margueron and Reinberg 2010). The key processes involved in epigenetic regulation are DNA methylation, histones modification and gene regulation by noncoding RNAs. Any Disruption of these three distinct epigenetic mechanisms leads to inappropriate gene expression, resulting in tumour growth and other "epigenetic diseases" (Sharma et al. 2010b). Hypomethylation of DNA is one of the first epigenetic alterations observed in human tumours (Feinberg and Vogelstein 1983), was soon followed by the detection of hypermethylated tumour-suppressor genes involved in cancer (Merlo et al. 1995, Sakai et al. 1991). Many studies also emphasized on deregulation of microRNA (miRNA) genes by DNA methylation in cancer. (Field et al. 1995, Lu et al. 2005). The explanation of how epigenetic changes can alter gene expression has leaded to initiate human epigenome projects and epigenetic therapies. (Jones and Martienssen 2005).

2.4.1 Promoter Methylation of Tumour-Related Genes

In past decades, there has been a rapid increase of interest in promoter hypermethylation in various human cancers including head and neck cancer. In HNSCC, the promoter hypermethylation of *p16*, *DAPK*, *MGMT* and *ECAD* are a frequently observed (Asokan et al. 2014, Kulkarni and Saranath 2004, Maruya et al. 2004, Rosas et al. 2001, Sanchez-Cespedes et al. 2000). A total of 12 genes,

including well-characterized tumour suppressor genes (*p16 INK4a*, *p15 INK4b*, *p14 ARF*, *p73*, *APC*, and *BRCA1*), DNA repair genes (*hMLH1*, *GSTP1* and *MGMT*), and genes related to metastasis and invasion (*CDH1*, *TIMP3*, and *DAPK*) were investigated. This study observed that the prevalence of *p16*, *p14*, *MGMT* and *DAPK* promoter hypermethylation methylation were 27%, 4%, 32% and 18%, however, *GSTP1* and *APC* found ummethylated (Esteller et al. 2001). Another study showed that the prevalence of *p16*, *DAPK*, *ECAD* and *RASSF1A* promoter methylation in HNSCC was 26/80 (32.5%), 19/80 (23.8%), 29/80 (36.3%), 6/80 (7.5%) respectively (Hasegawa et al. 2002). A large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypomethylated genomic regions in gene-poor areas (Weber et al. 2005). The Ataxia-telangiectasia-mutated (*ATM*) and glutathione S-transferase P1 (*GSTP1*) gene is a well-characterized tumour suppressor that plays a key role in maintenance of genomic stability. One of the study found a significant percentage (25%) of *ATM* promoter aberrant methylation in head and neck squamous cell carcinomas (Ai et al. 2004), but no promoter hypermethylation was found at the *GSTP1* gene (Sanchez-Cespedes et al. 2000). The frequency of methylation of *CDKN2A* (*p16*) has been commonly investigated and reported in various cancers, it is also mostly studied gene in HNSCC. The reported incidence of hypermethylation of *p16* ranges from 23% to 76% in OSCC (Mascolo et al. 2012). A study reported that, in HNC, promoter methylation of *RASSF1A* was 42.9% in cell lines and 15% in primary tumours but not found in the normal control DNA. The study also observed a significant inverse association between *RASSF1A* promoter methylation and HPV infection (p=0.038) (Dong et al. 2003). A significantly higher prevalence of *p15* methylation was found in histologically-normal surgical margin epithelia of HNSCC patients with chronic smoking and drinking habits compared with non-smokers and non-drinkers (Wong et al. 2003). Another study demonstrated that MGMT promoter hypermethylation and loss of protein expression were significantly associated to increased tumour recurrences and decreased patient survival, independent of other factors, such as tumour site and size, nodal status, age, and chemoradiation therapy (Zuo et al. 2004). Many studies shown an association between low expression of E-cadherin (*ECAD*) and a more aggressive behavior of OSCC (Maruya et al. 2004, Saito et al. 1998, Viswanathan et al. 2003). *ECAD* gene hypermethylation frequency

ranged between 7% and 46%. (Mascolo et al. 2012). The reported frequency of *DAPK* promoter hypermethylation ranges from 18% to 27% (Baylin et al. 2001, Dong et al. 2014, Hasegawa et al. 2002, Mascolo et al. 2012). A recent review article demonstrated that the methylation status of $p16^{INK4A}$ act as a promising candidate biomarker for predicting clinical outcome of OSCC, particularly for recurrence-free survival (Al-Kaabi et al. 2014).

The higher frequency of $p16^{INK4a}$ methylation (47.8%) was found in Indian cohort in comparison with a North-American cohort (37.5%) (Viswanathan et al. 2003). Another study suggesting that aberrant methylation of *EDNRB, KIF1A, DCC* and $p16^{INK4a}$ genes is a common event in Indian OSCC (Kaur et al. 2010). The inconsistency observed within and between cell lines and tumour specimens supports a heterogeneous and dynamic state of methylation in genes associated with HNSCC tumourigenesis (Maruya et al. 2004). The methylation status of 24 tumour suppressor genes was investigated by methylation-specific multiplex ligation-dependent probe amplification in matched tumour and normal tissue samples from patients with HNC and found *CHFR*, *RARβ*, *DAPK1*, and *RASSF1* genes were the most frequently methylated genes in tumour tissue, whereas eight genes were not methylated in any sample (Yalniz et al. 2011). In OSCC patients, 60%, 60%, 40%, 20%, and 10% of methylation were observed in the case of *p16*, *ECAD*, *MGMT*, *p15* and *hMLH* gene respectively. These results suggest that aberrant methylation of *p16* and *ECAD* genes occur early in head and neck cancer development and might play a role in the progression of these tumours (Asokan et al. 2014). A recent study identified *NOL4* and *IRX1* as a highly specific promoter methylated gene associated with HNSCC and may have potential as a biomarker for HNSCC (Demokan et al. 2014). Another recent study, highlight the importance of assessing tumour suppression genes at the genomic and epigenomic level to identify key pathways in HNSCC deregulated by simultaneous promoter methylation and somatic mutations using whole genome sequencing (Guerrero-Preston et al. 2014).

2.4.2 CpG Island Methylator Phenotype (CIMP)

CpG island methylator phenotype (CIMP) refers to the tumour subgroups with aberrant methylation levels at multiple loci. CIMP is associated with distinct clinicopathological characteristics (Toyota et al. 1999). A broad range of genetic and

environmental alteration including the increased expression and abnormal targeting of DNA methyltransferases (DNMTs), HPV and tobacco habits may lead to CIMP type (Etoh et al. 2004, Teodoridis et al. 2008). CpG island methylation has been linked to tumour suppressor gene inactivation in neoplasia and may serve as a useful marker to clone novel cancer-related genes. The concept of CIMP was first studied in colorectal cancer, although well explored in several other cancer types including upper aerodigestive tract cancer, oral cancer, gastric cancers and oesophageal squamous cell carcinoma and breast cancer. Previous study on OSCC, demonstrated that CIMP was associated with a mark inflammatory response and less aggressive tumour biology. The study found that CIMP-positive group had less aggressive tumours in term of thickness (p=0.015) and nodal metastasis (p=0.012). In addition, CIMP-positive tumours had a great host inflammatory response (p=0.019) (Shaw et al. 2007). However, in another study observed a trend for CIMP-high tumours to be involved with poor prognosis (Jithesh et al. 2013). Lechner et al. (2013) first time reported CIMP for HNSCC, and demonstrated that CIMP had been associated with potentially less favourable clinical outcome. In addition, they were able to demonstrate that CIMP signature was independent of gender and predictive for survival and smoking status, thus confirming previous findings. They also found consistent hypermethylation in the promoter regions of such genes, with a candidate CIMP in the HPV-positive samples. Another recent study by Talukdar et al. (2014) found that a significant association between tobacco consumption and CIMP-high tumours. They also reported that the combined effect of HPV and tobacco promote risk for developing CIMP-high tumours, which indicating to a possible synergistic role of HPV and tobacco in aberrant methylation leading towards tumourigenesis..