Introduction

Though no one can go back and make a brand new start, anyone can start from now and make a brand new ending – Carl Bard

CANCER: A DISEASE OF GREAT CONCERN IN INDIA

Cancer is the leading cause of deaths in economically developed countries and the second leading cause of deaths in developing countries (Jemal *et al*., 2011). The burden of cancer is increasing in developing countries as a result of population, aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets. Apart from this, the etiological factors such as HPV infection, alcohol consumption, areca nut chewing, pollution etc. also contribute to the burden of cancer (Jemal *et al*., 2011, Johnson *et al*., 2011, Madani *et al*., 2012). The estimated number of cancer cases are as high as 12.7 million and 7.6 million cancer deaths are reported worldwide (Jemal *et al*., 2011).

ORAL CANCER: Anatomy

Oral cancer is a type of head and neck cancer. The cancerous growth occurring within the structures of the oral cavity is known as oral cancer.

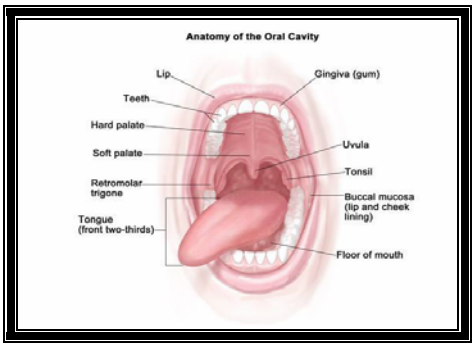

Figure 2.1: ***Anatomy of oral cavity.*** *Oral cavity comprises of lip, hard palate, soft palate, retromolar trigone, uvula, buccal mucosa, tongue and floor of mouth (http://seer.cancer.gov)*

As shown in **Figure 2.1** the **oral cavity** includes: The lips, teeth, and gums

- The lining inside the lips and cheeks (buccal mucosa)
- The floor of the mouth (under the tongue)
- The top of the mouth (hard palate)
- The small area behind the wisdom teeth

EPIDEMIOLOGY OF ORAL CANCER

Oral cancer is a major threat to public health. It is the eleventh most common cancer in the world and accounts for two-third of deaths in developing countries (Khan *et al*.,

2012). Smoking, alcohol use, smokeless tobacco products and HPV infections are the major risk factors for oral cavity cancer (Johnson *et al*., 2011).

Figures 2.2 shows the incidence and 5-year prevalence of lip and oral cavity cancer in various countries. Blue indicated 5-year prevalence, red indicates incidence.

Figure 2.3 indicates estimated 5-year prevalent cancer cases. Oral cavity cancer comprises of 6.6% of all cancer cases. Figure 2.4 indicate age standardised oral cavity incidence rates (both sexes) with highest incidence rates in dark blue colour. There is decrease in blue colour intensity as incidence rate decreases. The estimated numbers of new cancer (tongue and mouth) cases in U.S. for both sexes in 2014 are 25,510. Out of which 16,870 are males and 8,640 are females. The estimated deaths are 4,220, out of which 2,580 are males and 1,640 are females (Siegel *et al*., 2014)

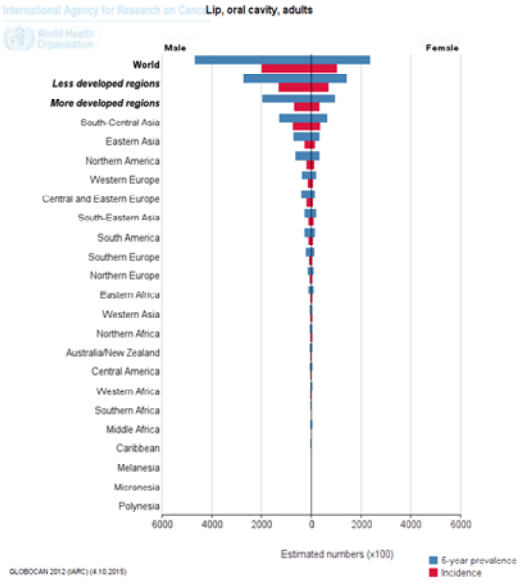

Figure 2.2

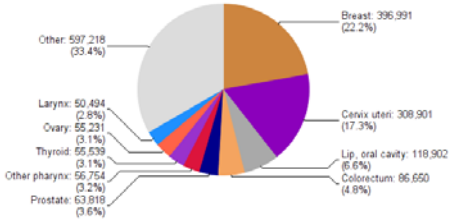

Figure 2.3

Figure 2.2: Incidence and 5-year prevalence of lip and oral cavity. *Blue indicated 5-year prevalence, red indicates incidence.*

Figure 2.3: Estimated 5-year prevalent cancer cases (adult population) in India-both sexes

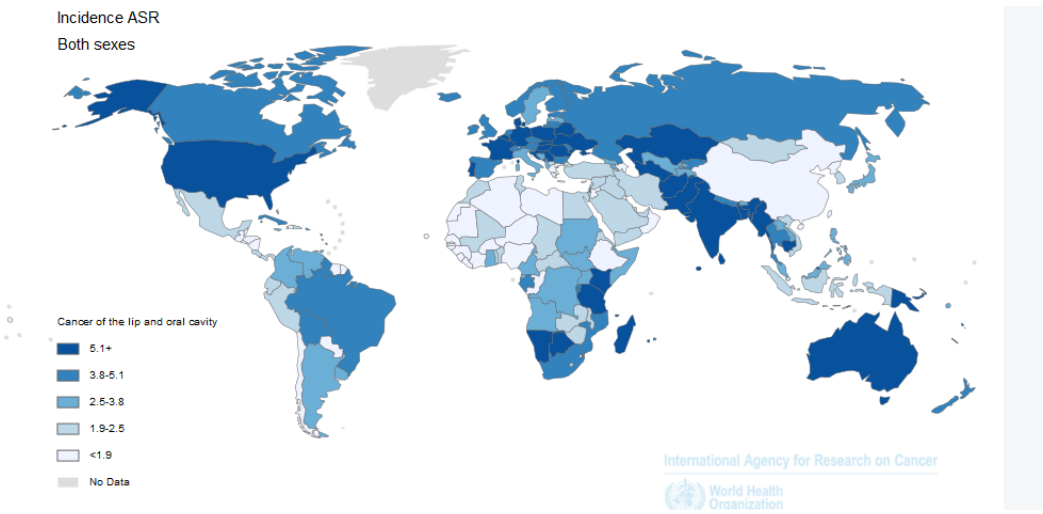

Figure 2.4: ***Age standardized oral cavity incidence rates by sex and world area. Source: GLOBOCON 2012***

Magnitude of problem in India:

In India, 47,653 deaths due to oral cancer were registered in 2008 according to Global Cancer Statistics, 2011 (Jemal *et al*., 2011). Nearly 80,000 oral cancer cases are diagnosed every year in India, which is mainly attributed to different forms of tobacco consumption. Out of all the cancers, head and neck is the leading cancer among males in India as depicted in **Figure 2.5** (Kulkarni *et al*., 2013). Nearly two-thirds of oral cancers are located in the buccogingival sulcus, where the betel quid is kept for long periods in the oral cavity. Most of the oral lesions are detected in their advanced stages, although oral cavity is accessible for visual examination. In fact, 60 to 80% of patients in India are present with advanced disease as compared to 40% in developed countries.

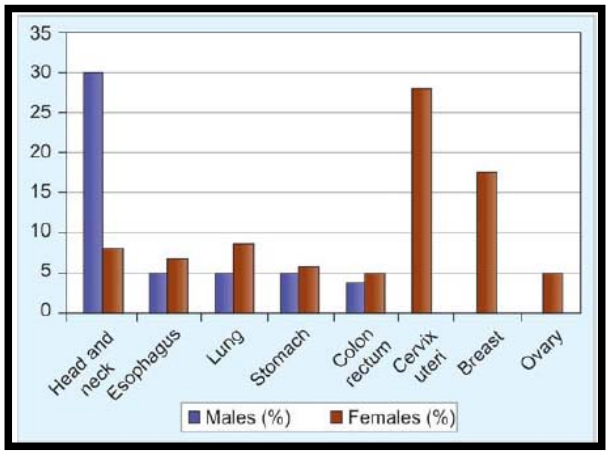

Figure 2.5: ***Top cancers in India in both sexes.*** *The highest cancer incidence depicted was head and neck cancer in males and cancer of cervix in females (Kulkarni et al., 2013).*

National Cancer Registry Programme (population based cancer registry-rural and urban of Ahmedabad, National cancer registry programme, Indian council of medical research, 2010) has indicated that Ahmedabad has the highest incidence of oral cancer in the country with 17.1 per 1 lakh population new cases of cancer registered every year. Tobacco related cancers (TRCs) accounted for 55.15% of all cancers in males and 17.90% of all cancers in females. Among the tobacco related cancer sites in males, cancer of oral cavity was the most common site (29.73%) followed by cancer of tongue (20.51%). In females, cancer of oesophagus alone accounted for 24.68% of the total TRCs followed by oral cavity (19.78%) and tongue (17.31%).

ETIOLOGY OF ORAL CANCER

Most oral cancer cases and deaths are due to both individual pre-disposition linked to certain genetic characteristics and exposure to carcinogens caused by lifestyle behaviors. In addition, exposure to two or more risk factors has a synergistic effect in increasing oral cancer risk (Petti *et al*., 2009). Around 57% of all men and 11% of women in India, between 15 and 49 years of age use some form of tobacco. Results from the global youth tobacco survey in India show that about 10 to 20% of students in 8^{th} and 10^{th} grades (about 13-15 years), currently use tobacco in some form. The strong association of cancers of the oral cavity with the tobacco use is well recognized. Epidemiological studies show that risk of developing oral cancer is five to

nine times greater for smokers than for non-smokers. More than 60 carcinogens are present in cigarette smoke and atleast 16 carcinogens have been identified in smokeless tobacco (Neville *et al.*, 2002). Worldwide, smoking accounts for 42% of deaths from cancers of the oral cavity (including the pharynx) (Danaei *et al*., 2005).

Besides smoking, use of smokeless tobacco is extremely prevalent. The use of betel quid (pan) is extremely widespread in many parts of India. In addition, *zarda, ghutka, kharra, mawa* and *khainni* are mixed by vendors and are all dry mixtures of areca nut flakes, lime, tobacco powder (Sarin *et al*., 2005). It is also used as a moist snuff containing lime with tobacco, vegetable oil and water and also in the form of dried tobacco applied on gums and teeth (Chaturvedi *et al*., 2009). A recent Population Based cancer Registry (PBCR) report shows that the proportion of cancers related to tobacco use among males in India is the highest in Ahmedabad i.e. 50.6% (Kulkarni *et al*., 2013).

Alcohol consumption regularly, is associated with an increased risk for oral cancer, which is dose-dependent. Regular consumption of 4 to 5 drinks daily increases the risk for oral cavity cancer to 2 to 3 fold as compared to non consumers. Overall, 7 to 19% oral cavity cancer cases are attributable to heavy alcohol drinking (Subapriya *et al*., 2007). By recognizing abuse associated factors, health policies and preventive frameworks can be effectively constructed to combat these oral preneoplasms (Lee *et al*., 2012).

Recent evidence suggests that human papilloma virus (HPV) may be linked with some oropharyngeal and oral cancer. HPV-16 has been detected in 22% and HPV-18 has been found in 14% of oral cancer cases (Heck *et al*., 2010). Iron deficiency, anemia in combination with dysphagia and postcricoid webs (Plummer-Vinson syndrome) is associated with a high risk for development of carcinoma of the oral cavity (Sankaranarayanan *et al.*, 1998).

ORAL CANCER PROGRESSION

Oral cancer precedes through various premalignant lesions like leukoplakia, erythroplakia, nicotine stomatits, tobacoo pouch keratosis etc. (Tanaka *et al*., 2011; Neville *et al*., 2002). Oral precancerous lesions are defined as ‘‘a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart”. Under this definition, leukoplakia, erythroplakia, and palatal keratosis associated with reverse smoking are categorized as precancerous lesions (Amagasa *et al*., 2011). According to Mishra *et al*., the transformation rate varies from 0 to 20% according to the nature of lesions and in India oral leukoplakia is considered to be potentially malignant (Mishra *et al*., 2005).

ORAL PRECANCEROUS LESIONS:

Premalignant and malignant lesions develop as a multi-step process within the mucosa (Brachman *et al*., 1992) **(Figure 2.6)**. The neoplastic process begins with normal epithelium processing through dysplasia to carcinoma *in situ* and invasive carcinoma. The earliest detectable morphological changes are the appearance of the ―premalignant lesions of leukoplakia and erythroplakia.

Leukoplakia:

A leukoplakia can be defined as a white patch or white lesion of oral mucosa that cannot be characterized clinically or pathologically as any other disease (Kramer *et al*., 1978). Rate of transformation of leukoplakia into malignancy is around 3 to 18%. The oral leukoplakia/erythroplakia is one of the most common epithelial precancerous lesions of oral squamous cell carcinoma (Pindborg *et al*., 1997).

Erythroplakia:

Another type of oral precancerous lesion is erythroplakia. An erythroplakia is a clinical term that refers to a red patch that cannot be defined clinically or pathologically as any other condition. It appears as a red plaque with a soft, velvety texture. Most common sites in oral cavity for erythroplakia are floor of mouth, lateral tongue and soft palate (Kramer *et al*., 1978). It exhibits high prevalence of dysplastic changes among all premalignant lesions and subsequent progression to invasive

carcinoma is also high. Some erythroplakias are smooth and some are granular or nodular. Often there is a well-defined margin adjacent to mucosa of normal appearance. Some lesions, composed of white and red areas, are termed as - erythroleukoplakia (Pindborg *et al*., 1997).

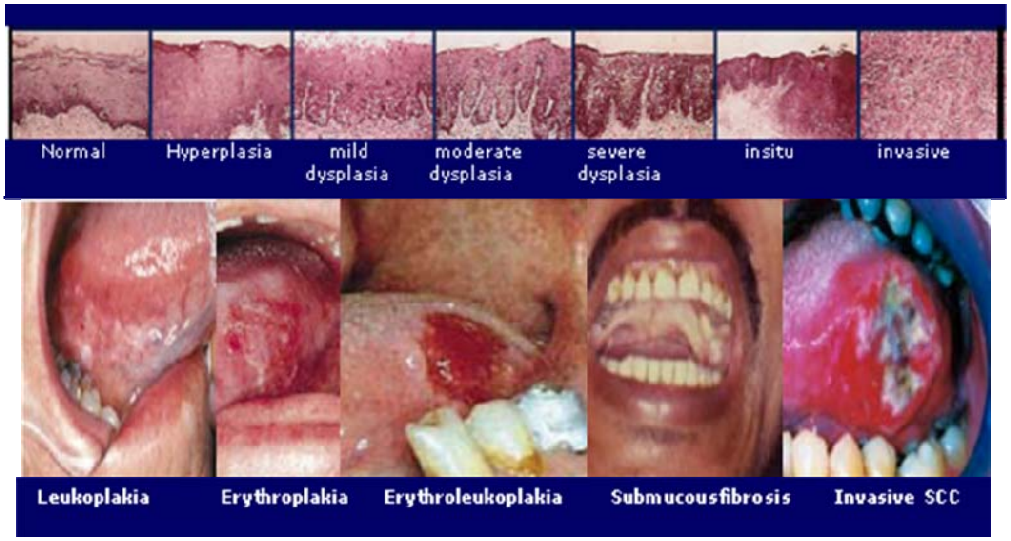

Figure 2.6: ***Sequential changes in oral cancer progression.*** *Oral cancer precedes through several precancerous lesions and conditions like leukoplakia, erythroplakia, erytholeukoplakia and sub mucous fibrosis. The apparent histopathological manifestations include hyperplasia, dysplasia, carcinoma in situ and finally invasive carcinoma develops. (Modified from Kumar et al., 2003).*

Dysplasia: Both types of precancerous lesions mentioned above show varying degree of dysplasia. The term dysplasia means different cytological abnormalities of cell. Such type of abnormalities mainly includes hyperchromatism, increased nuclear size, pleomorphism, dyskeratosis and increased abnormal mitotic index. Depending on the involvement of various layers of epithelium, dysplasia can be divided into three categories- mild, moderate, and severe. A severe dysplasia is also known as carcinoma *in situ* which ultimately results in invasive oral squamous cell carcinoma (OSCC) (Lumerman *et al*., 1995). In most cases, the dysplastic changes are more commonly found in erythroplakia than in leukoplakia. Therefore, true clinical erythroplakia is a much more worrisome lesion than leukoplakia. However, dysplastic leukoplakias have a high propensity to progress to invasive squamous cell carcinoma (Mashberg *et al*., 1995).

In addition to these lesions, other **Oral precancerous conditions** include lichen planus and oral sub mucous fibrosis.

Lichen planus:

Lichen planus is an inflammatory disease of skin and mucosa. Clinically, six types of oral lichen planus are described: popular, reticular, plaque-like, atrophic, ulcerative and bullous. Histologically, oral lichen planus is characterized by hyperkeratosis, epithelial atrophy and a dense, well defined infiltrate of lymphocytes in the superficial lamina propria. Dysplastic changes are sometimes seen (Pindborg *et al*., 1997) in lichen planus.

Oral submucous fibrosis:

Oral submucous fibrosis is characterized by epithelial atrophy, fibrosis of subepithelial connective tissue, resulting in stiffness of the oral mucosa. The principle etiological factors for oral submucous fibrosis in India include areca nut chewing and genetic traits. Histologically, oral submucous fibrosis is characterized by severe epithelial atrophy and an underlying dense collagenous tissue with coarse fibre formation. It may also display hyperkeratosis and epithelial dysplasia (Pindborg *et al*., 1997).

ORAL SQUAMOUS CELL CARCINOMA (OSCC): Gradually, all precancerous conditions progress to develop invasive OSCC. OSCC is a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges. The grading of the neoplasm is an attempt to predict their aggressiveness and hence it helps in prognosis and treatment of disease. This grading is generally based on assessment of the degree of keratinization, cellular, nuclear pleomorphism and mitotic activity (Pindborg *et al*., 1997). The grades are:

Grade 1: Well differentiated: There are varying proportions of basal and squamous cells with intercellular bridges; keratinization is a prominent feature (more than 75%);

few mitotic figures are seen and atypical mitoses or multinucleated epithelial cells are extremely rare; nuclear and cellular pleomorphism is minimal.

Grade 2: Moderately differentiated: This is a neoplasm with features intermediate between well differentiated and poorly differentiated. It shows less keratinization compared to well differentiated neoplasm (25-75%) and more nuclear, cellular pleomorphism; there are more mitotic figures and some are abnormal in form.

Grade 3: Poorly differentiated: Histologically, there is no resemblance to the normal squamous epithelium of the oral mucosa. Keratinization is rarely present (less than 25%) and intracellular bridges are extremely scarce; mitotic activity is frequent and atypical mitoses are readily found; cellular and nuclear pleomorphism are common and multinucleated cells are also frequent.

SCC of oral mucosa has capacity to extend by invasion into adjacent tissues through intravascular spread. Widespread dissemination of cancer cells also occurs through lymphatics which ultimately results in lymph node metastasis. Depending on the extent of invasion and metastasis, OSCC can be classified in to different stages. For such classification, a uniform system by American Joint Committee of Cancer (AJCC) (Greene *et al*., 2002) is established, called-TNM classification, where T represents the size of the primary tumor, N indicates the status of the regional lymph nodes and M indicates the presence or absence of distant metastases. Staging of oral cancer is important for establishing proper treatment and for determining the prognosis.

TREATMENT OF ORAL CANCER

A better treatment outcome is achieved in patients diagnosed with oral cancer in early stages. The main treatment approaches in patients with oral cancer are: surgery and radiotherapy for most early carcinoma of oral cavity. More advanced lesions require combination of surgery and radiotherapy to obtain optimal cure rates. Chemotherapy is mostly used for pre-operative patients having advanced stage disease, to reduce the tumor size. It has been used to improve survival or to reduce the distant metastases.

CHALLENGES IN ORAL CANCER

The most strenuous problems in oral cancer are late diagnosis, poor response of tumor to chemotherapy and radiotherapy as well as lack of reliable biomarkers for early diagnosis and post-therapeutic monitoring. The main reason for late stage presentation of the disease is due to patients or clinicians ignoring the lesions. This is also due to lack of awareness of malignant potential of small lesions of oral cancer. Hence there is a need to unravel early molecular changes during oral carcinogenesis process.

A NEED FOR IDENTIFICATION OF EARLY MOLECULAR EVENTS

Early diagnosis of oral cancer plays a key role in disease progression, treatment response, and ultimately, quality of life and patient survival. Therefore, identification of molecular changes for risk prediction of oral cancer holds much promise in this respect. Various oral lesions, including leukoplakia, erythroplakia, lichen planus and submucous fibrosis, are considered potentially malignant oral disorders that may contain precancerous cells, which could malignantly transform into cancerous cells (Mithani *et al*., 2007). Currently, these lesions are treated surgically, with or without cellular and tissue changes (dysplasia). However, it is unknown if surgery can really prevent transformation into OSCC. Although the term "potentially malignant disorders" was recommended by the WHO to describe precancerous lesions, the clinical and histological features alone cannot accurately predict whether these precancers of the oral mucosa remain stable, regress or progress to malignancy (Pitiyagi *et al*., 2009). Furthermore, evaluation of an asymptomatic patient for early-stage cancer, based on its physical features alone, is frequently compromised because malignant and benign lesions may not be clinically distinguishable (Rethman *et al*., 2010). Consequently, approximately 60% of oral cancers are advanced by the time they are detected, and approximately 15% of patients have another cancer in a nearby area such as the larynx, esophagus or lungs (Gonsalves *et al*., 2007; Weinberg *et al*., 2002). Therefore, there is a need to identify the molecular events to evaluate individuals with potentially malignant disorders who are at a high risk of developing OSCC and those with early-stage malignant lesions. Hence, identification of molecular changes that can predict disease progression and techniques that can be

applicable in routine practices will improve the management of these disorders (Jin *et al.*, 2010).

ORAL CANCER AND GLYCOSYLATION CHANGES

Oral cancer precedes through various preneoplastic stages (Tanaka *et al*., 2011; Neville *et al*., 2011; Mishra *et al.*, 2005) and the alteration in cell-membrane glycosylation is often associated with neoplastic transformation.

The protein sequence is completely encoded by the genome; however, diversity of proteins can be achieved by different sequences and structures of the sugar moieties or glycan attachment. Glycosylation is the most ubiquitous form of post-translational modifications of proteins. The diversity of the proteins can be achieved by different sequence and structure of sugar moieties or glycan attachment **(Figure 2.7)**. Protein glycosylation is increasingly being recognized as one of the most prominent biochemical alterations associated with malignant transformation and tumorigenesis (Guo *et al*., 2010; Goldman *et al*., 2009; Shah *et al*., 2008; Rajpura *et al*., 2005; Raval *et al.*, 2004). Cancer being a cellular disease, changes in the cell surface glycoconjugates and enzymes involved in cellular metabolism are of major interest in clinical oncology (Reis *et al.*, 2010).

Glycosylation has been studied intensively for the past two decades as the most common covalent protein modification in eukaryotic cells (Varki *et al.*, 2009; Marchal *et al*., 2003; Kam *et al*., 2008; Taniguchi *et al*., 2009; Drake *et al*., 2010; Hart *et al*., 2010 Hizal *et al*., 2014). Sophisticated oligosaccharide analysis has revealed a remarkable complexity and diversity of this post-translational modification. About 1-2% of the human transcriptome (about 250-500 glycogenes) has been predicted to encode proteins that are involved in glycosylation processing (Campbell *et al*., 2005).Glycoproteins are a family of complex proteins that have oligosaccharide chains covalently linked to their polypeptide backbones. The monosaccharide moieties in the oligosaccharides are hexosamines in the acyl form, neutral sugar such as glucose, galactose, fucose, and mannose and various derivatives of sialic acid (N-acetyl neuraminic acid). Sialic acid or fucose may exist as terminal units of oligosaccharides **(Figure 2.8)**.

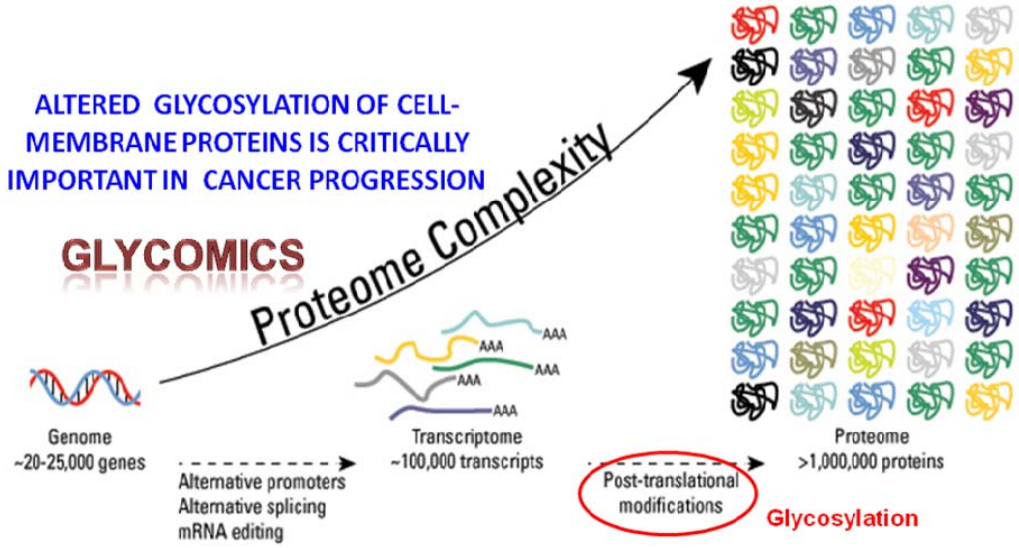

Figure 2.7: ***Glycosylation, the most abundant post-translational modification of proteins.*** *For every 20-25000 genes, there are approximately 100,000 transcripts and >1,000,000 proteins which are mainly due to post-translational modifications of proteins. Glycosylation being the most abundant post translational modification of proteins, it plays important role in malignant transformation, hence understanding glycomics is important in oral cancer pathogenesis. (Modified from www.lifetechnologies.com)*

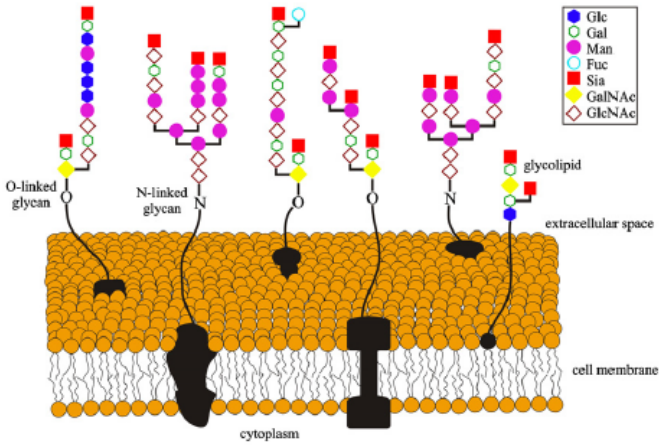

Figure 2.8: ***Schematic illustration of carbohydrate heterogeneity found on cell surface glycoproteins and glycolipids.*** *Sialic acid is usually found at the terminal residue of O-linked and N-linked glycans of glycoproteins and glycolipids. Glc, glucose; Gal, galactose; Man, Mannose; Fuc, Fucose; GalNAc, N-acetylgalactosamine, GlcNAc, N-acetyl glucosamine. (Ghazarian et al., 2010)*

It has been estimated that more than 50% of proteins in human are glycosylated (Wong *et al*., 2005). Protein glycosylation is an enzymatic process which takes place

inside the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus. N-linked glycosylation is a co-translational event accompanying protein synthesis in ribosomes located in the ER, whereas O-linked glycosylation is a post-translational process characterized by the stepwise addition of sugar residues directly to the protein. The most common type of O-linked glycans contain an initial N-acetylgalactosamine residue bound to the Ser/Thr residues (=mucine-type glycans). Other O-linked glycans are known to include glucosamine, xylose, galactose, fucose or mannose as the primary sugar. Furthermore, O-linked glycoproteins are very often large proteins (>200 kDa) with clusters of glycosylation chains which are comparatively less branching than N-glycans. Chain elongation and termination, if performed, are carried out by various glycosyltransferases localized in different regions of the Golgi. Termination of O-linked glycans usually includes Gal, GlcNAc, GalNAc, Fuc or sialic acid (Rek *et al*., 2009). Glycosylation reaction depends on the action of glycosyltransferases and glycosidases in different tissues or cells (Reis *et al*., 2010). Cancer being a cellular disease, changes in cell surface glycoconjugates and enzymes involved in cellular metabolism of these glycoconjugates are of major interest in clinical oncology. Cancer cells continuously shed their surface components, which can be used as tumor markers for various malignancies in bodily fluids (Bates *et al.*, 1991; Kam *et al.*, 2008). Sialylation and fucosylation; the major types of glycosylation changes are typical terminal modifications of proteins that mediate vital biological functions (Shah *et al*., 2008; Shah *et al*., 2008).

SIALYLATION

Sialylation affects the half-lives of many circulating glycoproteins and plays a role in a variety of biologic processes such as cell-cell communication, cell matrix interaction, adhesion, and protein targeting. The transfer of sialic acids from CMP-sialic acids to the acceptor carbohydrates is catalyzed by the sialyltransferase (ST) family. Aberrant sialylation in cancer cell is a characteristic feature associated with malignant properties including invasiveness and metastatic potential (Shah *et al*., 2008). Cellular sialic acid contents are mainly controlled by ST and sialidases **(Figure 2.9)**.

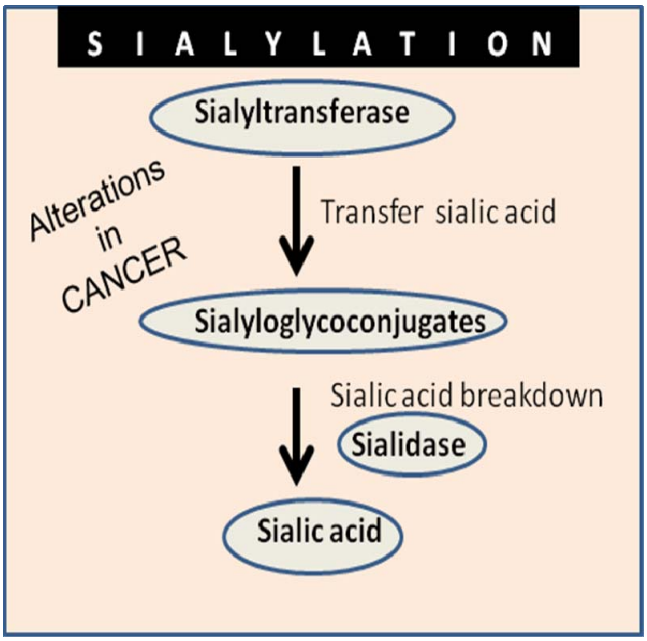

Figure 2.9: Sialylation alterations in cancer. *Sialylation alterations in cancer are accompanied by changes in sialyoglycoconjugates which are regulated by sialyltransferase and sialidase enzymes. Sialyltransferease enzyme catalyzes the transfer of sialic acid to terminal glycoconjugates. Sialidase enzyme is involved in breakdown of sialic acid from complex sialyloglyconjugates, and thus is involved in release of sialic acid.*

In cancer, there is alteration in sialylation pattern, which is mainly accompanied through changes in sialyloglycoconjugates by STs and sialidase enzymes. The amount and type of sialylation of tumor cell membrane depend on the activity of a number of different STs. Abnormally high levels of total ST are reported in tumor bearing cells and sera of cancer patients (Shah *et al*., 2008, Raval *et al*., 2004).

Sialic acid is linked either through α-2,3 or α-2,6 linkage to subterminal galactose or α-2,8 linkage to another sialic acid forming polysialic acid catalyzed by specific ST. The different STs can be distinguished on the basis of oligosaccharide sequence used as acceptors and anomeric linkage formed with the penultimate sugar residue (Mattox *et al.*, 1992; Harduin Lepers *et al*., 2001).

Expressions of STs are often de-regulated in cancer (Yamamoto *et al*., 1997; Vasquez-Martin *et al*., 2004; Dall'Olio *et al*., 2004). Moreover, altered expression of

sialylated glycoproteins during malignant transformation has been previously reported (Yamamoto *et al*., 1997; Dall'Olio *et al*., 2004; Shah *et al*., 2008; Alley *et al*., 2010). Alterations in the glycosylation machinery occurring as the consequence of abnormal glycosylation of proteins play a decisive role in malignant transformation. The altered glycoforms are released into the bloodstream due to increased shedding from the malignant cells (Drake *et al*., 2010).

TOTAL SIALIC ACID

Sialic acid is a generic term for N- or O- substituted derivation of neuraminic acid, a monosaccharide with a glycol carbon back bone **(Figure 2.10)**. Sialic acid (N-acetyl neuraminic acid) has variable roles in cell-cell recognition, protein targeting, protease resistance, conformation stabilization, adhesion and intracellular signaling events in biological systems. Sialic acids frequently occupy the terminal, non-reducing position on membrane glycoproteins. Researchers have led to a better understanding of the biological and pathological importance of sialic acid (Chen *et al*., 2010). Several studies have reported elevated levels of serum sialic acid in various types of cancers (Kokoglu *et al*., 1992; Patel *et al*., 1993; Paskowska *et al*., 1998; Shah *et al*., 2008; Raval *et al*., 2004; Suer Gokmen *et al*., 2004; Cylwik *et al*., 2005; Joshi *et al*., 2010; Siddhartha *et al*., 2011; Kadam *et al*., 2011).

Earlier reports have indicated elevated levels of salivary total sialic acid (TSA) in oral squamous cell carcinoma patients and breast cancer patients as compared to healthy individuals (Sanjay *et al*., 2008; Ozturk *et al*., 2011; Trivedi *et al*., 2012; Shubhada *et al*., 2012; Dhakar *et al*., 2013; Hemalatha *et al*., 2013; Rasool *et al*., 2014). Elevated serum TSA is reported in precancerous lesions of oral cancer (Shah *et al*., 2008; Joshi *et al*., 2010, Sawney *et al*., 2011).

Recently there are therapeutic approaches which target sialic acids in metastatic tissues (Lu *et al*., 2013). Earlier reports have also suggested that inhibition of serum sialic acid by antineoplastic drugs is due to tumor microenvironment (Lu *et al*., 2013).

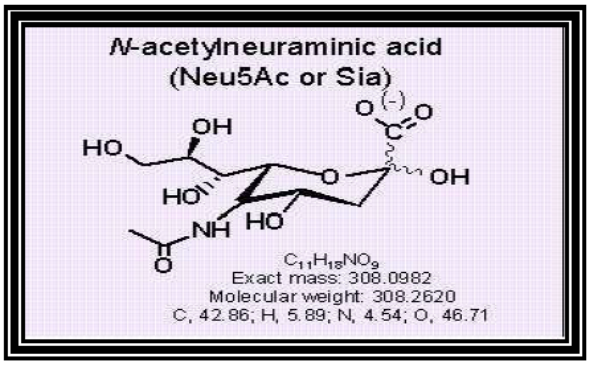

Figure 2.10: ***Structure of Sialic acid (N-acetylneuraminic acid)*** *(www.scienceblogs.com)*

SIALIDASE

Sialidase (neuramindase) enzyme (EC 3.2.1.18) catalyses the release of terminal sialic acid residue from complex carbohydrate moieties. The major function of sialidase is to hydrolyze glycosidic linkages between sialic acid and glycosyl residue of complex oligosaccharide and glycoconjugates. In cancer patients, increased sialic acid levels are found due to increased serum/tissue sialidase activity. Increased sialic acid activity also contributes to process of metastasis. Earlier studies have observed higher sialidase activity in serum and tissue of cancer patients (Sonmez *et al*., 1999; Miyagi *et al*., 2008; Miyagi *et al*., 2012). As there are lack of reports on evaluation of serum and salivary sialidase activity in patients with OPC and oral cancer patients, the present study evaluated serum and salivary sialidase activity from these patients.

There are four type of sialidases NEU1, NEU2, NEU3 and NEU4 (Miyagi *et al*., 2012). The major subcellular localization of NEU1 is lysosomal, NEU2 is cytosolic, NEU3 is on plasma membrane and NEU4 is in lysosomes or in mitochondria and ER. In human tissues, NEU1 generally shows the strongest expression, while NEU2 expression is extremely low (Hata *et al*., 2008).

SIALYLTRANSFERASES

Sialyltransferases are enzymes (EC 2.4.99.1-11) that transfer sialic acid to nascent oligosaccharide. Each sialyltransferase is specific for a particular sugar substrate. Sialyltransferases add sialic acid to the terminal portions of the sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins. Previous studies from our laboratory have indicated elevated serum and tissue ST enzyme activity in

oral cancer patients (Shah *et al*., 2008). However, salivary levels of α-2,3 and α-2,6 ST activity have not been explored earlier. Hence the present study also evaluated salivary α-2,3 and α-2,6 ST activity along with serum levels. Alterations in sialic acid have been reported in oral cancer, which might be due to increase in ST or sialidase activities. Various STs (*ST3GAL*, *ST6GAL*, *ST6GALNAC* and *ST8*) are named according to sialyl linkages they form (Carvalho *et al*., 2010). ST families are further sub-divided into 20 sub-families in mammals each of them have conserved amino acid positions (Harduin Lepers *et al*., 2010). *ST3GAL* family are α-2,3 STs which catalyzes the transfer of sialic acid residues via an α-2,3 linkage to galactose residue of terminal Galbeta1,3GalNAc structure on O-linked oligosaccharide of glycoproteins or on glycolipids. It has been predicted that *ST3GAL1* expression was mainly involved in biosynthesis of O-linked oligosaccharides of glycoproteins (Katsutoshi *et al*., 1996). The mRNA expression of *ST3GAL1* responsible for sialylation of O-glycans has been observed to be increased in colorectal cancer (Kemner *et al*., 1994; Schneider *et al*., 2001), breast carcinoma (Ito *et al*., 1997; Burchell *et al*., 1999) and bladder cancer (Videria *et al*., 2009). Wang *et al.* have observed significant down regulation of *ST3GAL1* with enhanced *ST6GAL1* mRNA expression in cervical cancer (Wang *et al*., 2001). Earlier reports have documented that suppression of ST by antisense DNA reduces invasiveness of human colon cancer cells *in vitro* (Zhu *et al*., 2001). Earlier studies have reported that anticancer effect of the Epidermal growth factor receptor (EGFR) kinase inhibitor, gefitinib, was increased in *ST6GAL1* deficient colon cancer cells. In contrast over-expression of *ST6GAL1* reduced the cytotoxic effect of gefitinib (Park *et al*., 2013). *ST3GAL3* and *ST6GAL1* have been shown to be associated with poor prognosis of human breast cancer (Recchi *et al*., 1998) and colorectal cancer (Petretti *et al*., 2000). Elevated levels of α-2,3 ST enzyme activity have been observed earlier in oral cancer patients (Shah *et al*., 2008). However, the studies on its transcript levels (*ST3GAL1*) are lacking in oral cancer patients. Therefore, the present study analyzed *ST3GAL1* transcript levels in malignant and adjacent normal tissues of oral cancer patients.

SIALOPROTEINS

In cancer, there is alteration in sialylation pattern, which is mainly accompanied through changes in sialyloglycoconjugates by STs and sialidase enzymes. Alterations in tissue and serum sialylated glycoproteins have been observed in cancer patients (Tian *et al*., 2012; Shetty *et al*., 2012). Earlier studies have observed association of α-2,3 and α-2,6 sialyloglycoconjugates with unfavorable outcome and worse prognosis (Tian *et al*., 2012). Recently high-throughput method for quantitative analysis of N-linked sialoglycoprotein using conditional hydrazide chemistry, liquid chromatography, and tandem mass spectrometry, has been developed which revealed an altered expression of sialoglycoprotein and total glycoprotein changes in breast cancer (Tian *et al*., 2012).

It has been reported that the levels of core-fucosylated biantennary glycans and α-2,3 linked sialic acids were significantly increased in prostate cancer patients as compared to patients with benign prostate hyperplasia (Saldova *et al*., 2011). Earlier evidences have reported that sialoglycoproteins patterns are not determined exclusively by the transcription of biosynthetic enzymes or the availability of N-glycan sequons; instead metabolic flux through the sialic acid pathway has a remarkable ability to increase the abundance of certain sialoglycoproteins. The results suggested that cancer cells can become more aggressively malignant in metastatic transformation via metabolic flux (Almaraz *et al*., 2012). Earlier reports have indicated aberrantly sialylated N-linked glycopeptides which have the potential to serve as biomarkers for ovarian cancer (Shetty *et al*., 2012). Studies from our laboratory have indicated higher serum and tissue α-2, 3 and α-2, 6 sialoproteins in patients with OPC and oral cancer patients (Shah *et al*. 2008). However, salivary estimation of α-2, 3 and α-2, 6 sialoproteins and its comparison between serum and salivary levels, have not been reported earlier in patients with OPC and oral cancer patients. Therefore, the present study also evaluated salivary α-2, 3 and α-2, 6 sialoproteins along with serum levels.

FUCOSYLATION

Fucosylation is the process of adding fucose sugar units to a molecule. It is performed by fucosyltransferase (FUT) enzymes. Fucosylation of glycoproteins is one of the

most important features that mediate several specific biological functions. It has been documented that tumor cells modulate their surface by increasing fucosylation level to escape recognition which contribute to several abnormal characteristic of tumor cell such as decreased adhesion and uncontrolled tumor growth.

FUCOSE

Fucose is a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$. It is found on N-linked glycans on the mammalian, insect and plant cell surface, and is the fundamental sub-unit of the fucoidan polysaccharide. It has two isomers, L and D, of which the D-isomer is rare and not found in mammals. L-fucose (6-deoxy-L-galactose) is an essential component of various mammalian glycan structures. It is present on *N*-and *O*-linked glycoproteins and glycolipids (Yan *et al*., 2005) or is covalently linked to some serine or threonine residues of proteins (Esko *et al*., 2002). Fucose can be linked via α1, 2-linkage to terminal Gal-α-1, 3 or α-1, 4 linkages to subterminal GlcNAc or via α1, 6-linkage to the innermost GlcNAc.

Fucose is a constituent of oligosaccharides and is notably associated with cancer and inflammation. It is known as an important constituent of glycoprotein. Increased fucose levels may lead to altered or unique glycoconjugates. The turnover of fucose residues in glycoconjugates can be implied by measuring the fucose levels and α-L-fucosidase activity. Several studies have suggested that monitoring serum/tissue fucose levels could be a promising approach for the early detection diagnosis and prognosis of various cancer types. Oligosaccharides are one of the most important factors in the post translational modification of proteins and lipids. Glycomics, the systematic study of glycans and glycan-binding proteins in various biological systems, is an emerging field in the post-genomics and post proteomics era. It is well known that oligosaccharide structures change during malignant transformation (Moriwaki *et al*., 2010). The remodeling of cell surface glycoproteins and glycolipids through modification of oligosaccharide structures is associated with the biological behavior of tumor cells.

FUCOSYLTRANSFERASE

Fucosylation is catalyzed by FUT [EC 2.4.1 (65, 68, 69, 152, 214, 221)], guanosine 5'-diphosphate (GDP)-fucose synthetic enzymes, and GDP-fucose transporter(s). FUT is a group of enzymes that catalyze incorporation of fucose from activated nucleotide donor GDP - fucose to reducing end of complex glycans in the linkage specific manner. The enzyme is expressed by many tissues and is increased in serum and tumors of cancer patients (Shah *et al*., 2008). It has been reported that increase fucosylation is associated with elevated fucosyl transferase activity, which is also associated with formation of various tumor antigens (Hansson *et al*., 1985). *FUT* genes from human genome are divided in three subfamilies, α-1,2 FUT, α-1,3/4 FUT and α-1,6 FUT (Petretti *et al*., 2000). *FUT3* to *FUT7* and *FUT9* to *FUT11* belong to the group of α-1,3/4 FUTs. *FUT3* predominantly exhibits α-1,4 FUT activity synthesizing Le^a, SLe^a, and Le^b, and a minor α-1,3 FUT activity, synthesizing Le^x, SLe^x, and Le^y. *FUT5* and *FUT6* synthesize Le^x and SLe^x, moreover, *FUT5* has been reported to produce Le^a, Le^b, and SLe^a (Carvalho *et al*., 2010). The various Lewis antigens and the transferases involved in biosynthesis are depicted in **Figure 2.11**. *FUT8* catalyzes the transfer of a fucose residue to the C6 position of the innermost GlcNAc residue of *N*-linked oligosaccharides on glycoproteins to produce core fucosylation.

The altered expression of FUT enzyme activities have been reported in oral cancer patients (Shah *et al*., 2008) however, expression of different types of *FUT* transcripts have not been studied earlier in oral cancer. Earlier reports have indicated no significant alterations of *FUT3* and *FUT5* and a moderate increase in *FUT6* in colon cancer (Ito *et al.*, 1997), while studies by Hiraiwa *et al*. have shown increase in *FUT3, FUT6* and *FUT8* transcripts in colon cancer tissues (Hiraiwa *et al*., 1995).

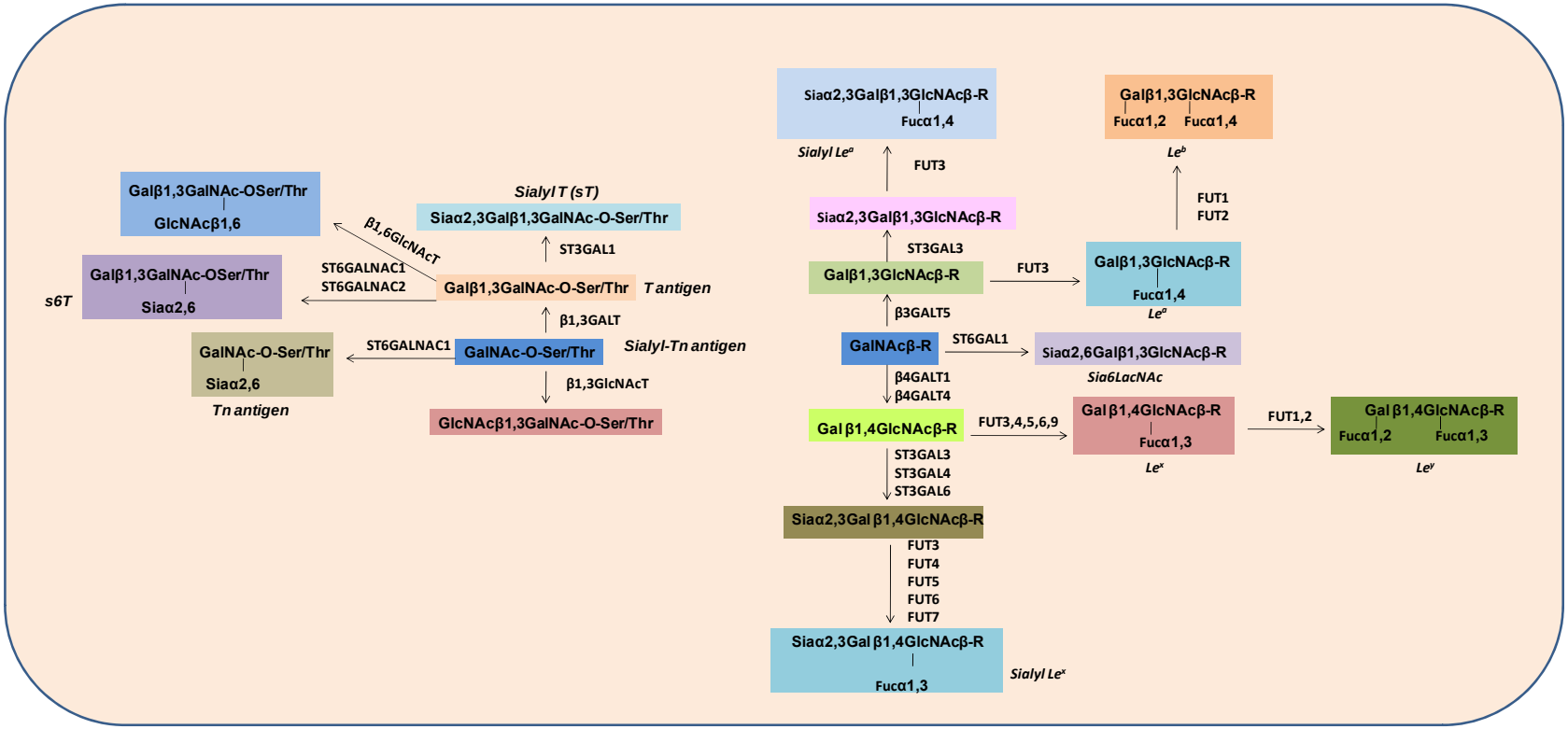

Figure 2.11: *Synthesis of various tumor antigens by different types of transferases*. *FUT3,4,5,6,9 are involved in synthesis of Le^X, FUT1,2 are involved in synthesis of Le^y, Sialyl Le^a is synthesized via β4GALT1/4 – β3GALT5-ST3GAL3-FUT3. Le^a is synthesized by FUT3 from Galβ1,3GlcNAcβ-R; FUT1,2 further synthesizes Le^b from Le^a. ST3GAL3,4,6 and further FUT3,4,5,6,7 are involved in synthesis of sLe^X. β1,3GALT is involved synthesis of T antigen, which further by ST3GAL1 is involved in synthesis of sialyl T. ST6GALNAC1 synthesizes Sialyl Tn antigen from Tn antigen. ST6GALNAC1, ST6GALNAC2 synthesizes s6T from T antigen*.

Increase in *FUT4* has been observed in colorectal adenomas and carcinomas (Petretti *et al*., 2000; Kudo *et al*., 1998). An increase in *FUT6* expression has been observed in breast cancer cells (Matsuura *et al*., 1998) and in colon cancer (Trinchera *et al*., 2011). There is dearth of reports on *FUT3*, *FUT5* and *FUT6* transcript levels in oral cancer. Hence the present study evaluated these transcript levels in normal and adjacent normal tissues of oral cancer patients.

α-L-FUCOSIDASE

α-L-fucosidase (EC: 3.2.1.51) is a lysosomal enzyme that catalyzes the hydrolytic cleavage of terminal fucose residue that is involved in maintaining the homeostasis of fucose metabolism. The presence of fucosidases is necessary for rapid turnover of N-glycans (including fucose) followed by reglycosylation and reinsertion of the proteins in plasma membrane (Kriesel *et al*., 1988; Horstkorte *et al*., 1996). It has been reported that alterations in serum and/or tissue α-L-fucosidase activity potentially may be useful in the diagnosis and management of cancer patients. (Youakim *et al*., 1985; Wang *et al*., 1995; Abdel-Aleem *et al*., 1996; Fernadez Rodriguez *et al*., 2000; Ayude *et al*., 2002; Ayude *et al*., 2004). Earlier reports have suggested that serum α-L-fucosidase activity may be used for the diagnosis of malignant diseases and as an indicator of tumor burden, metastasis, and response to anticancer treatments in oral cancer patients (Shah *et al*., 2008). However, salivary α-L-fucosidase activity has not been explored in oral cancer patients and patients with OPC. Moreover, comparison of serum and salivary levels has not been explored earlier, which was evaluated in present study.

FUCOPROTEINS

Fucoproteins are glycoproteins which contain fucose sugar units as one of their carbohydrates. They are often found on or in the cell or tissue membranes and participate in a variety of biological activities.

Cancer cells that are shed or released into circulation from the primary tumor, often over express fucosylated glycans on their surface. The expression of fucosylated glycoproteins (i.e. fucoproteins) has been detected by means of specific lectins (Thomson and Turner, 1987). Several lectin-based studies have indicated that

fucoproteins are increased in various cancers. Abnormally fucosylated serum haptoglobin and α-fetoprotein (AFP) are used widely as tumor markers of hepatocellular carcinoma (Aoyogi *et al*., 1985; Noda *et al*., 2002). Profound fucosylation of the serum microenvironment may be a factor that interrupts adhesion and influences the formation of metastases. For example, several fucose-containing 'natural ligands' are involved in the migration of tumor cells. Serum and tissue fucoproteins have been found to be elevated in oral cancer patients (Shah *et al*., 2008). Salivary fucoproteins have not been previously studied. Hence, the present study evaluated fucoproteins from saliva as well.

IMPLICATIONS OF SALIVARY GLYCOSYLATION CHANGES IN ORAL CANCER

Spurred in part by the sequencing of the human genome, there has been increasing emphasis on the importance of translational research to convert biomedical discoveries in cancer into useful clinical applications. Heightened interest in translational research is reflected more generally in the growing prominence of evidence-based medicine and the perspective of population health. Glycosylation changes on cell surface plays key role in sequential changes during oral carcinogenesis. We hope the changes in glycosylation are reflected in saliva as well due its direct contact with the lesions. Salivary diagnostic, an upcoming field has a vast potential in future for development of non-invasive biomarkers. It can prove wonders for screening, early detection, diagnosis and prognosis of patients.

Saliva offers an attractive non-invasive alternative to serum/tissue testing. Recently, there has been much advancement in salivary based genomics and proteomics biomarkers (Shah *et al*., 2011, Yakob *et al*., 2014, Cheng *et al*., 2014). The field of salivary (oral fluid-based) diagnostics is a broad, complex and crosscutting area of scientific research with enormous potential to impact the practicing dentist and health care in general. The role of rapid, in-office screening or chair-side diagnostic testing comes down to an important bottom line: improved access and health care outcomes for patients. Saliva-based tests also offer the following advantages for patients and health care providers:

- Ease of collection

- Elimination of the common fear of needle-sticks
- Lower costs for sample collection
- Reduced risks of percutaneous injury
- Availability of repeated samples during follow-up studies
- Reduced patient's discomfort

Salivary diagnostics could dramatically change clinical practice by introducing point-of-care testing and real-time disease surveillance and has advantage of being inexpensive, non-invasive, and simple collection procedure. Saliva offers some distinctive advantages when used for diagnosis of disease. Whole saliva can be collected non-invasively, and by individuals with limited training, including the patient. No special equipment is needed for collection of the fluid. Moreover, it is valuable for children, since collection of the fluid is associated with fewer compliance problems. Further, analysis of saliva may provide a cost-effective approach for the screening of large populations.

Elevated levels of sialylation and fucosylation have been observed from tissues and serum in various cancers. However, salivary estimation of total sialic acid, α-2,3 and α-2,6 sialyltransferase activities, α-2,3 and α-2,6 sialoproteins, sialidase activity, fucoproteins and α-L-fucosidase activity have not been explored earlier in oral cancer patients and patients with oral precancerous conditions. Simultaneous evaluation of all the markers from serum as well as saliva would aid in knowing the molecular alterations in oral cancer progression. The inclusion of patients with OPC would assist in monitoring early changes occurring during oral carcinogenesis and inclusion of post treatment follow-up patients would help in evaluating treatment response. Moreover, saliva being a non-invasive tool could be a better alternative to invasive procedures for early screening, diagnosis and prognosis of oral cancer patients.

GLYCOSYLATION AND MMPs: It has been suggested that alterations in glycoproteins on cell surface play a key role in progression and metastasis of tumors. Matrix metalloproteinases (MMPs) are the enzymes which plays important role in metastasis and invasion by proteolytic degradation of extracellular matrix (ECM), disruption of cell-cell and cell matrix adhesion, migration and angiogenesis (Vihenen

et al., 2002; Roy *et al*., 2009; Gialeli *et al*., 2011). Matrix metalloproteinases (MMPs) are a highly regulated superfamily of enzymes that degrade almost all extracellular matrix (ECM) and basement membrane components, processes which are essential for invasion and subsequent metastasis, which is the most strenuous problem in oral cancer.

Structures of the MMPs

Figure 2.12 shows the structures of different MMPs.

(A) The general domain structure of MMP family members. The signal peptide (Pre) guides the MMP into the rough endoplasmic reticulum during synthesis. The propeptide domain (pro) sustains the latency of MMPs. The catalytic domain houses a highly conserved Zn+2 binding region. The hemopexin-like-terminal domain (PEX) is linked to the catalytic domain by a short hinge region

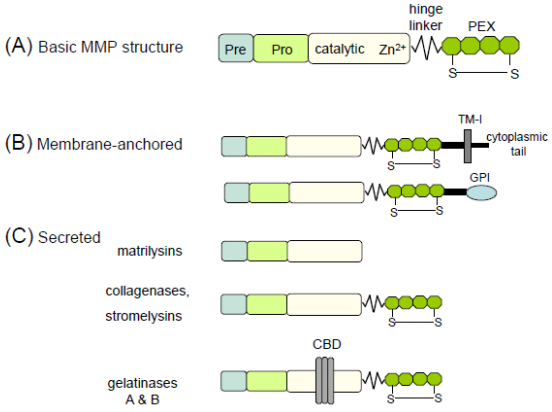

Figure 2.12: ***Structures of the MMPs.*** *(A) Basic MMP structure (B) Membrane-anchored MMPs (C) Secreted MMPs: Matrilysins, collagenases, stromelysin, gelatinases A and B (Bauvois et al, 2011).*

(B) MT-MMPs include membrane-anchored MMPs localized at the cell surface through a C-terminal (type I) transmembrane domain (TM-1) or by a glycosyl-phosphatidyl-inositol anchor (GPI)

(C) Secreted MMPs include stromelysins, matrilysins, collagenases and gelatinases. The gelatinases (MMP-2 and MMP-9) contain repeats of fibronectin type II-like domains (the collagen binding domain, CBD) that interact with collagen and gelatin (Bauvois *et al*., 2012).

Among the many MMPs that have been identified especially, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), play a key role in degradation of type IV collagen and gelatin, the two main components of ECM. MMP-2 and MMP-9 are secreted in their

latent zymogenic form. MMP-2 and MMP-9 are cleaved by other MMPs or proteases to yield the activated forms of 68, 58 and 54 kDa for MMP-2, and 84 kDa for MMP-9.

Activation of MMPs

MMPs are secreted in a latent form as pro-MMPs, which require activation. As depicted in **Figure 2.13**, when this Cys73-Zn^{+2}bonds are intact, the MMP is inactive. The activation of MMPs involves a disruption of the bond between the active Zn^{+2}site and the cysteine residue. This mechanism has been referred to as the "cysteine switch". A water molecule then binds to the Zn^{+2} ion and replaces the cysteine residue after the dissociation. The non catalytic zinc is then switched to a catalytic one, which results in an intermediate active enzyme. Additionally, the pro-domain of the MMP is removed by autolytic cleavage or by other proteases. This cleavage causes a reduction in molecular mass and results in a fully active enzyme. The mass reduction is generally in the range of 8–10 kDa. *In vivo*, MMPs are generally activated by other Proteinases. *In-vitro*, MMPs are also activated by chemical and physical agents such as aminophenylmercuric acetate (APMA), low pH, and heat treatment (Chaussain Miller *et al*., 2006).

MMP-9 levels are up-regulated in various malignancies including colorectal, gastric, thyroid, ovarian, bladder, lung, larynx, pancreatic, prostate cancer and oral cancer (Roomi *et al*., 2009;Vihenen *et al*., 2002; Patel *et al*., 2005; Singh *et al*., 2010; Singh *et al*., 2011; Varun *et al*., 2012; Farina *et al*., 2014).

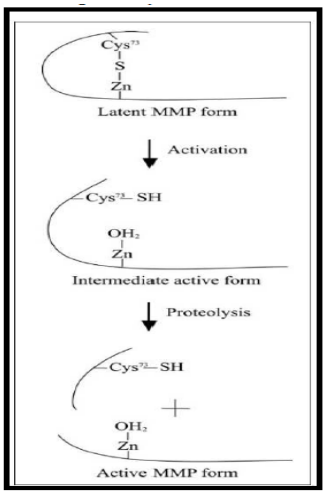

Figure 2.13: ***Activation of MMPs*** *when this Cys73-Zn^{+2}bonds are intact, the MMP is inactive. The activation of MMPs involves a disruption of the bond between the active Zn^{+2}site and the cysteine residue. This mechanism has been referred to as the "cysteine switch" (Chaussain-Miller et al., 2006).*

Earlier studies have indicated weak up-regulation of MMP-9 mRNA in tissues of oral lichen planus and dysplastic lesions (Jordan *et al*., 2004). Earlier reports have suggested the loss of E-cadherin, a cell adhesion molecule in oral precancerous conditions (OPC) (Shah *et al*., 2009). Although MMPs are known as key mediators of cancer invasion, their involvement in oral premalignant conditions is not documented. Saliva offers an attractive non-invasive tool for assessing biomarkers in oral cancer due to its direct contact with sites of the lesions. Recently there is eye catching advancement in salivary based diagnostics (Pfaffe *et al*., 2011; Nair *et al*., 2012; Wong *et al*., 2012; Yakob *et al*., 2014, Cheng *et al*., 2014). Various gelatinolytic activities were observed in saliva and studies have suggested a 42 kDa truncated form of enzyme which was due to autoactivation of gelatinolytic MMPs (Miyoshi *et al*., 2010).

On the basis of the pivotal roles that MMPs play in several steps of cancer progression, the pharmaceutical industry has invested considerable effort over the past 20 years aiming to develop safe and effective agents targeting MMPs. Various MMPs inhibitors have been developed, in an attempt to control the synthesis, secretion, activation and enzymatic activity of MMPs **(Table 2.1)**.

Table 2.1: Potential MMPs inhibitors (Gialeli *et al*., 2011)

MMP inhibitors	Type of drug/ source	Enzymes inhibited
Synthetic inhibitors		
Batimastat	Peptidomimetic	MMP 1, 2, 3,7, 9
Marimastat	Peptidomimetic	Broad spectrum
Tanomastat (BAY12-9666)	Non-peptidomimetic	MMP 2, 3, 9
Prinomastat (AG3340)	Non-peptidomimetic	MMP 2, 3, 7, 9, 13
BMS-275291	Non-peptidomimetic	MMP 2, -9
CGS27023A	Non-peptidomimetic	MMP-1, -2, -3
Minocycline	Chemically modified tetracycline	MMP-1, -2, -3
Metastat (COL-3)	Chemically modified tetracycline	MMP-1, -2, -8, -9, -13
SB-3CT	Reform proenzyme structure	MMP-2, -9
INCB7839	Small molecule sheddase inhibitor	ADAM-10, 17
Off-target inhibitors		
Bisphosphonates	Analogues of PPi	MMP-1,-2, -7, -9, MT1-MT2MMP
Letrozole	Non-steroidal inhibitor of aromatase	MMP-2, -9
Natural inhibitors		
Neovastat (AE-941)	Extract from shark cartilage	MMP-1, -2, -7, -9, -13
Genistein	Soy isoflavone	MMP-2, -9, MT1-, MT2, MT3-MMP

E-CADHERIN: THE COENHANCER OF MMPs FOR INVASION AND METASTASIS

The observation that malignant tumor cells leave the primary tumor to disseminate to distant organs and that tumor cells show marked changes in their interaction with extracellular matrix components, has led to the notion that changes in cell-cell and cell-matrix adhesion coincide with tumor progression.

As early as 1914, Theodor Boveri recognized the importance of changes in the adhesion of tumor cells to the development of cancer. Changes in the expression or function of cell-adhesion molecules can therefore contribute to tumor progression both by altering the adhesion status of the cell and by affecting cell signaling. The cadherin superfamily consists of classical cadherins, which are the main mediators of calcium-dependent cell-cell adhesion and non-classical cadherins, which include desmosomal cadherins and the large subfamily of protocadherins, which are implicated in neuronal plasticity. Most human cancer originates from epithelial tissue. E-cadherin – the prototype member of the classical cadherin family – is the key player in inducing cell polarity and organizing the epithelium. In most cancer of epithelial origin, E-cadherin mediated cell-cell adhesion is lost concomitantly with progress toward tumor malignancy (Cavallaro *et al*., 2004; Berx *et al*., 2009)

Homophillic binding between cadherins on adjacent cells is vital for the maintenance of strong cell-cell adhesion. The adherens junctions consist of E-cadherin intercellular components p120-catenin, the armadillo family protein β-catenin, and α-catenin. The E-cadherin intercellular domain interacts with β-catenin, and γ-catenin (plakoglobin) in a mutually exclusive way **(Figure 2.14).** The junctional complex mediates cell-cell adhesion and has long been assumed to be associated with actin cytoskeleton through α-catenin (Becker *et al*., 2010)

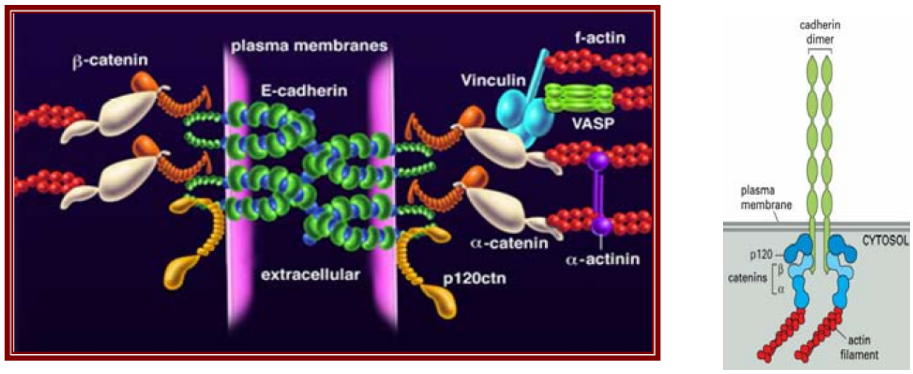

Figure 2.14: E-cadherin intercellular adhesion and cytoskeleton. *The adherens junctions consist of E-cadherin intercellular components p120-catenin, the armadillo family protein β-catenin, and α-catenin. The E-cadherin intercellular domain interacts with β-catenin, and γ-catenin. The junctional complex mediates cell-cell adhesion and has been associated with actin cytoskeleton through α-catenin (Jamora et al., 2002).*

Various types of carcinomas, OSCC, have been largely studied with regard to the immunohistochemical expression of E-cadherin, and it has been generally accepted that reduced expression is likely to be associated with poor prognosis (Berx *et al*., 2009; Luo *et al*., 2014). Disruption of E-cadherin- mediated intercellular adhesion is a hallmark of epithelial- mesenchymal transition (EMT), a phenomenon which occurs at certain stages of normal development and in the malignant progression of carcinoma (Thiery *et al*. 1999; Thiery *et al*., 2003; Andersen *et al*., 2005). Different molecular mechanisms including gene mutations (Berx *et al*., 1998; Guilford *et al*., 1998; Wang *et al*., 2004) hypermethylation of the promoter (Di Croce *et al*., 2003), and transcriptional silencing by transcriptional repressors (Snail, Slug, ZEB-2/SIP1, ZEB-1, and E12/E47) (Batle *et al*., 2000; Cano *et al*., 2000; Comjin *et al*., 2001; Perez Moreno *et al*., 2001; Bolos *et al*., 2003; Eger *et al*., 2005) contribute to the inactivation of E-cadherin linked with tumor progression. Re-expression of E-cadherin may induce morphological reversion and suppress cell growth and invasion suggesting an important function for E-cadherin in EMT (Takeichi *et al*., 1993; Croix *et al*., 1998; Gottardi *et al*., 2001; Wong *et al*., 2003).

Oral cancer, one of the most pernicious malignancies, has also been characterized by diminished E-cadherin levels (Downer *et al*., 1993; Schipper *et al*., 1993; Doki *et al*., 1993; Sakaki *et al*., 1999; Thomas *et al*., 2001; Diniz-Freitas *et al*., 2006; Luo *et al*., 2014; Kaur *et al*., 2009). Nonetheless, a subset of oral cancers prominently expresses

E-cadherin and maintains the ability to invade the surrounding tissue (Thomas *et al*., 2001; Tanaka *et al*., 2003). Because N-glycosylation of E-cadherin affects its ability to form mature adherens junctions, it is possible that aberrant N-glycosylation is the underlying cause for defective adhesion in some oral cancers. Indeed, N-glycosylation of cell-surface glycoproteins is frequently altered in cancer (Guo *et al*., 2002; Guo *et al*., 2003, Lau *et al*., 2007) with tumor progression linked to increase in the abundance of complex N-glycans (Dabelsteen *et al*., 1992; Dennis *et al*., 1999; Guo *et al*., 2007). A novel transmembrane protein, dysadherin, interferes with E-cadherin function by downregulating its protein levels, without affecting mRNA levels, and it induces the metastatic spread of tumor cells in xenograft tranplantation experiments (Ino *et al*., 2002). Hence the present study evaluated various glycosylation parameters along with E-cadherin mRNA and protein levels to understand its role in oral cancer progression.

MMPs, E-CADHERIN AND C-JUN WORK IN CONSORT:

Proteolytic degradation of E-cadherin by matrix metalloproteases (MMPs) is another mechanism by which E-cadherin-mediated cell–cell adhesion can be ablated (Cavallaro *et al*., 2004). A soluble 80-kDa form of E-cadherin, have an active part in the invasive process during tumor progression (Navrocki-Raby *et al*., 2003). Recently, a study using keratinocytes showed that the loss of E-cadherin-mediated cell–cell contacts leads to upregulation of the c-Jun protein. Here, the cytoskeleton network mediates a cell contact– dependent increase in c-Jun expression (Knirsh *et al*., 2009). It was shown that the disruption of the cytoskeletal network in intact retinal tissue and keratinocytes caused an increase in the cellular amount of the c-Jun protein (Polak *et al*., 2006; Knirsh *et al*., 2009). This induction was also E-cadherin dependent. Earlier result have shown that c-Jun is post-transcriptionally rather than transcriptionally regulated (Splanger *et al*., 2011), which was in accordance with the findings of Polak *et al*. (Polak *et al*., 2006), which depicted that translation of c-Jun was mediated by UTRs of c-Jun mRNA by IRES mediated translation.

c-Jun is the cellular counterpart of the transforming protein of the chicken retrovirus ASV17. Via a leucine zipper, c-Jun forms homodimers and heterodimers with Fos and other Jun-related proteins which, together, comprise the AP-1 transcription factor that binds TREs. c-Jun therefore mediates transcriptional regulation in response to a

variety of stimulants. It is known as a pivotal regulator of major biological events such as cell proliferation, differentiation, apoptosis, and tumorigenesis (Splanger *et al*., 2011).

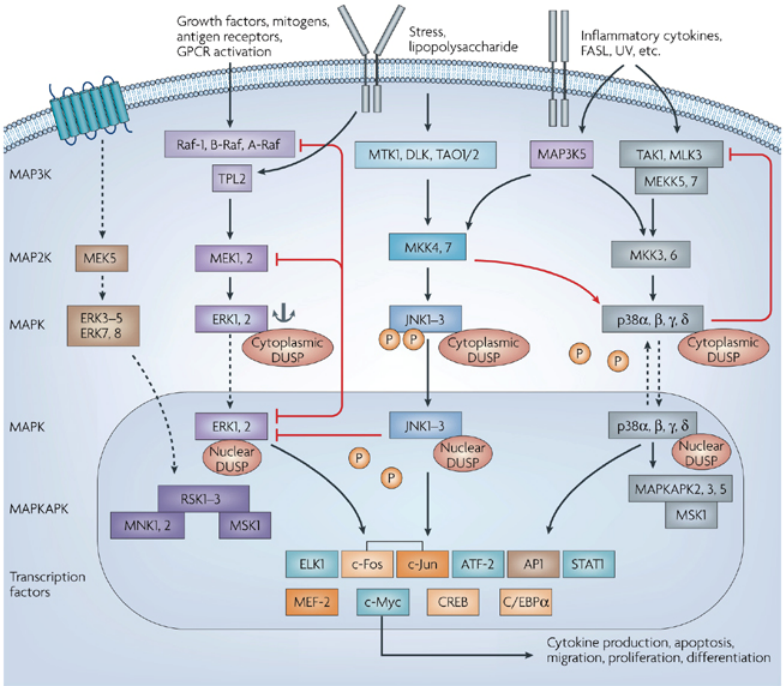

Figure 2.15: ***Pathway for c-Jun activation.*** *Several growth factors, cytokines or environmental stress activates different kinases. c-Jun is activated by c-Jun N-terminal kinase (JNK) dependent phosphorylation at Ser 63 or Ser 73 residues in transactivation domain near its N-terminus (Jeffrey et al., 2007).*

c-Jun is tightly regulated post-translationally and is phosphorylated in two distinct regions. c-Jun is also phosphorylated at two residues proximal to the major transactivation domain. These residues (Ser 63 and 73) are required to be phosphorylated for efficient transactivation function. The kinases responsible for this medication *in vivo* are the SAPK/JNKs **(Figure 2.15)**. These kinases bind with high affinity to a region in c-Jun termed the delta domain. This region is deleted in v-Jun. Mutation of the phosphorylation sites prevents dissociation thus resulting in inactive c-Jun (Vanhara *et al*., 2006). Earlier studies have reported elevated c-Jun protein expression in oral cancer tissues (Mishra *et al*., 2010; Kuo *et al*., 2006). However, simultaneous evaluation of c-Jun mRNA levels has not been carried out earlier. Hence the present study evaluated both c-Jun mRNA and protein levels along with E-cadherin in order to study the mechanism in oral carcinogenesis.

TYROSINE PHOSPHORYLATION AND REGULATION OF E-CADHERIN, C-JUN AND EPIDERMAL GROWTH FACTOR RECEPTOR

Recently, a study using keratinocytes showed that the loss of E-cadherin-mediated cell–cell contacts leads to upregulation of the c-Jun protein (Knirsh *et al*., 2009). Tyrosine phosphorylation has been previously implicated in the regulation of cadherin function: receptor tyrosine kinases (RTKs; which are frequently activated in cancer cells), such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-MET) and fibroblast growth-factor receptor (FGFR), and the non-RTK SRC, phosphorylate E-cadherin, neuronal (N)-cadherin, β-catenin, γ-catenin and p120-catenin, resulting in, the disruption of cadherin-mediated cell–cell adhesion (Behrens *et al*., 1993; Hamaguchi *et al*., 1993; Fujita *et al*., 2002).

The proto-oncogene referred to as EGFR is a well-known tyrosine kinase growth factor receptor. The EGFR family comprises four receptors: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 (**Figure 2.16**).

The EGFR consist of three regions, the extracellular ligand binding region, the intracellular region with tyrosine kinase activity and a transmembrane region with a single hydrophobic anchor sequence, by which the receptor traverses the cell membrane a single time.

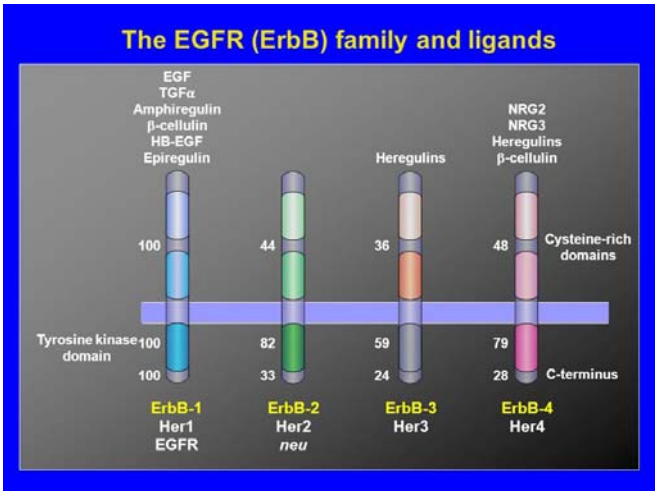

Figure 2.16: ***The EGFR family receptors and ligands.*** *EGFR family consists of receptors like ErbB-1, ErbB-2, ErbB-3, Erb-4 which are activated by several ligands like EGF, TGFα, Amphiregulin, β-cellullin, HB-EGF, Epiregulin, Heregulins, NRG2 and NRG3 (Wilding et al., 2002)*

Binding of a ligand to the receptor and the subsequent receptor hetero and mono dimerization leads to a phosphorylation cascade mediated via tyrosine kinase. EGFR can be hyperactive in a normal cell and provides signal for cellular proliferation, anti-apoptosis, angiogenesis and metastasis, which are the main characteristics of cancer (Sebastian *et al*., 2006).

EGFR has been reported to be expressed in a variety of human tumors of epithelial origin, and its over-expression has been documented in 80% of squamous cell carcinoma (Kumar *et al*., 2003). Abnormal amplification of EGFR gene has been observed widely in various human tumors, including lung carcinoma, laryngeal and oral squamous cell carcinoma (Kobayashi *et al*., 2013; Markman *et al*., 2010). Earlier reports have reported strong EGFR staining (75%) in the membrane of tumors tissues and also in 10% of histopathologically tumor-free margins in OSCC. This study highlighted that detection of EGFR may identify patients who are at a high risk for tumor recurrence and can benefit from anti-EGFR treatments (Vosoughhosseini *et al*., 2012). A study by Myers *et al*. (Myers *et al*., 2002) showed that targeted molecular therapy with EGFR blockade arrests the growth of oral cancer *in vitro* and reduces its proliferation in an experimental xenograft animal model.

EGFR has already been identified as an important target for cancer therapy, and various kinds of EGFR inhibitors are currently used in the treatment of several human cancers (Hiraishi *et al*., 2008, Yewale *et al*., 2013; Chong *et al*., 2013; Baselga *et al*., 2005). However, such treatments are lacking in oral cancer.

Role of Glycosylation, pEGFR, E-cadherin and MMPs in cancer progression

There is increased shedding of glycoproteins from malignant cells, which might be due to increased MMPs. MMPs, which are known to be involved in invasion and metastasis might be involved in subsequent loss of E-cadherin, due to degradation of ECM. Earlier studies have shown that cells expressing E-cadherin extracellular mutants displayed increased levels of pEGFR upon ligand stimulation (Mateus *et al*., 2007) which contributes to enhanced cell motility. It has been reported that down-regulation of E-cadherin expression resulted in a strong up-regulation of EGFR in

keratinocytes, whereas E-cadherin transfection reversed this effect (Wilding *et al*., 1996). Earlier results have shown that E-cadherins stimulate the MAPK pathway through the ligand independent activation of EGFRs and the consequent activation of a biochemical route leading to the stimulation of MAPKs. These findings suggest that E-cadherins can initiate outside-in signal transducing pathways through the engagement of tyrosine kinase receptors for epidermal growth factor, thus providing a novel molecular mechanism whereby these cell adhesion molecules may ultimately control the fate of normal and transformed epithelial cells (Pece *et al*., 2000).

Earlier reports have shown that EGFR activation down- regulates E-cadherin, and broad spectrum MMP inhibition ameliorates EGF-stimulated junctional disruption and loss of E-cadherin protein. MMP-9 involvement in EGF dependent down-regulation of E-cadherin was determined by siRNA specifically directed against MMP-9. The associations between EGFR activation, MMP-9 expression, and E-cadherin were investigated in human ovarian tumors and paired peritoneal metastases wherein immunohistochemical staining for activated (phospho) EGFR and MMP-9 colocalized with regions of reduced E-cadherin. These data suggested that regulation of MMP-9 by EGFR may represent a novel mechanism for downregulation of E-cadherin in ovarian cancer (Dahl *et al*., 2008). Malignant cell release glycoproteins carrying disease related epitopes into interstitial space, where they can enter the circulation. The shedding might be due increased MMPs. Reduction in cell-cell adhesion molecules like E-cadherin by MMPs together with activation of EGFR might cause increase in nuclear proteins like c-Jun.

There is lack of molecular markers in oral cancer. Multifaceted molecular evaluation may aid in estimation of the changes related to early, intermediate and late endpoints like prognosis and treatment outcome. There is a dearth of studies on simultaneous evaluation of glycosylation, pEGFR, E-cadherin, c-Jun mRNA and protein expression in oral cancer patients. Hence, the present study evaluated simultaneously all the parameters to study the mechanism in oral cancer development.