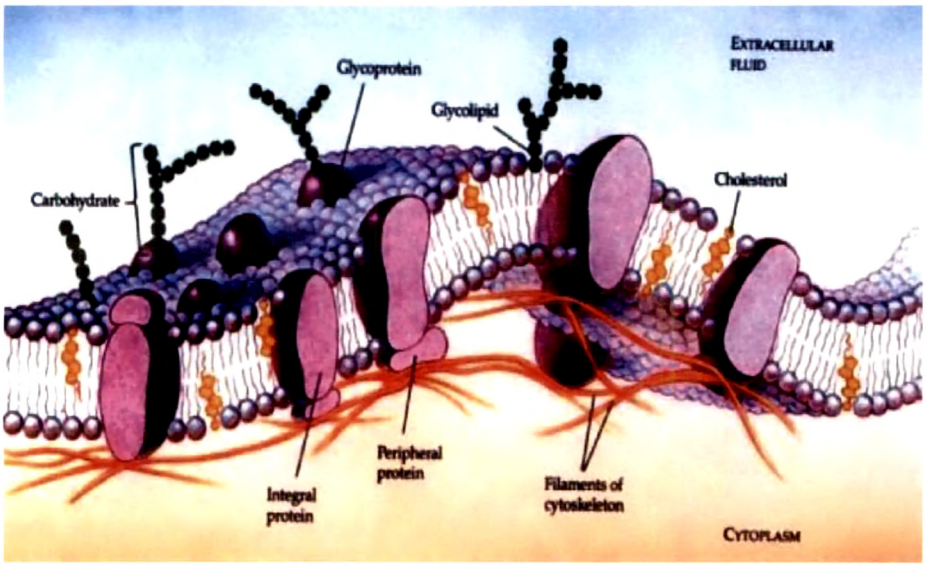
Introduction



Cancer cells speak different sugar dialect than the normal cells.

2. INTRODUCTION

2.1: CANCER- A DISFIGURING DISEASE

Cancer is an umbrella term covering a plethora of conditions characterized by unscheduled and uncontrolled cellular proliferation. Almost any mammalian organ and cell type can succumb to oncogenic transformation, giving rise to bewildering array of clinical outcomes. As the average age in many countries steadily rise, so do cancer related death, making it one of the most common causes of death in 21st century. The estimates of world cancer burden reported globally have shown that there are 10.1 million new cases, 6.2 million deaths and 22.4 million persons living with cancer in the year 2000. In Southeastern Asia, the annual estimate of cancer for the year 2000 was 251.2 cases per 100,000 (Parkin, 2005). The annual estimate of cancer in India, for the year 2001 was 0.98 million and the annual mortality in 2000 was 0.7 million (ICMR Bulletin, 2001). The incidence of cancer is rising in India, due to multiple risk factors that involve interplay between genetic and environmental components.

Oral cancer is one of the sixth most common malignancies and is a major cause of cancer morbidity and mortality worldwide. Globally, more than 3,00,000 new oral cancer cases are diagnosed every year (Parkin, 2001). In the year 2000, oral cancer caused more than 80,000 deaths worldwide (Ferlay et al., 2003). The highest rate of oral cancer in people of all ages occurs in developing countries including South and Southeast Asia (Parkin et al., 2005; Pindborg, 1980). Oral cavity is one of the major sites of cancer cause in India. Annually around 80,000 new cases are diagnosed in India (ICMR Bulletin, 2001).

Although it is well known that oral cancer increases with age, recent trends for a rising incidence particularly relates to cancer of the mouth and tongue in young males. Most studies and institutional reports have suggested that incidence of oral cancer in the patients under 40 years of age vary between

0.4 - 3.6% of all cases (Casumano and Persky, 1988), rising to 6.7% when the arbitrary cut off point is 45 years and below (Son and Kapp, 1985). Analysis of the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) database provided evidence for an increase in the percentage of cases of squamous cell carcinoma (SCC) of the oral tongue that occurred in adults younger than 40 years, from 3% in 1973 to approximately 6% in 1993 (Myers et al., 2000).

2.2: ORAL CANCER AND TOBACCO

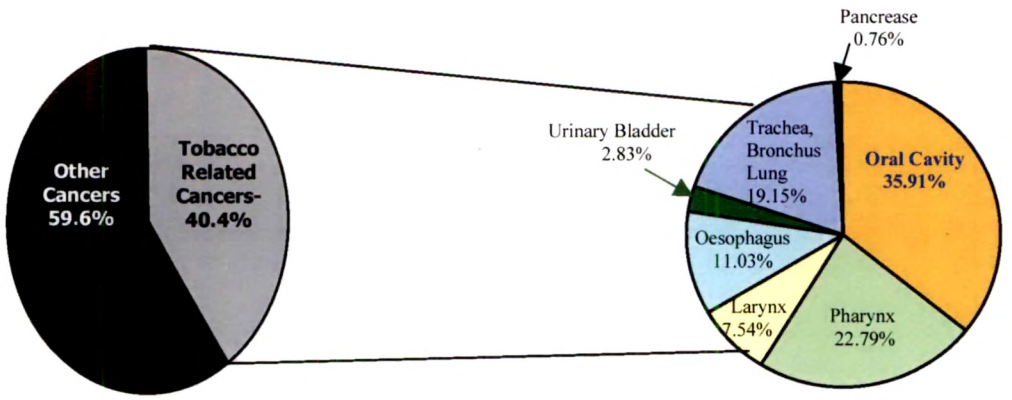
The tobacco problem is immense worldwide. Tobacco contains over thirty known carcinogens. Consumption of smokeless tobacco products also continues to be substantial and widespread. It is estimated that, of everyone alive today, 500 million people will eventually be killed by tobacco use, with cancer being one of the main causes (Mackay and Eriksen, 2002; WHO report, 2002). Oral cancer is highly prevalent in regions with high tobacco and alcohol consumption. Tobacco use is considered as the major risk factor in oral cancer development in a dose-dependent manner (Llewellyn et al., 2001). The occurrence of oral cancer is as high as 30-40 % in the Indian subcontinent whereas the rate is comparatively low in western countries at 2-6% of all malignancies (Parkin et al., 1993; National Cancer Registry, 1996). The exceptionally high incidence of oral cancer may be due to difference in the habit of tobacco consumption. The rising trends have been attributed to increased smokeless tobacco use (Davis and Severson, 1987) especially due to availability of areca products. In South-east Asian countries, people extensively use smokeless tobacco in the form of nass, naswar, khaini, pan masala, gutkha and betel quid (betel leaf coated with slaked line wrapped around areca nut and catechu) or tobacco smoking in the form of cigarette, bidi, chutta, reverse type of smoking and hooka (Nair et al., 2004). Consequently, in this part of world, buccal mucosa (cheek) represents the primary site for cancer development. Due to these major etiological factors and increasing incidence rate, Gupta (1999) has suggested that oral cancer in India should be considered as a "new epidemic". This rising incidence is also

reflected in the population under 40 years of age. It is estimated that around 16 to 28% of all oral cancer patients are seen at various institutions in India. This displays an alarming rise in the incidence rate in below 40 years of age (Padmanabhan et al., 1990; Patel and Dave, 1976). Kuriakose et al. (1992) noted that the lesions in young cases were predominantly invasive as compared with the exophytic lesions found in older patients.

2.3: INCIDENCE OF ORAL CANCER AT THE GUJARAT CANCER AND RESEARCH INSTITUTE

It is reported that oral and pharyngeal cancers are highest in Ahmedabad region in western India (Sanghavi et al., 1989). Hospital based cancer registry of the Gujarat Cancer and Research Institute reported 11,352 cancer cases in the year 2001 (GCRI registry, 2001). Among these, tobacco related cancers (TRC) consisted of 40.4%. Among all TRC, oral cavity (ICD10: C00-06) was the leading site accounting 35.9% cases (**Figure-2.1**). The male to female ratio for oral cancers was 4.55:1.

Figure-2.1: Incidence of Tobacco Related Cancers at GCRI, Year 2001.



2.4: MULTISTEP ORAL CARCINOGENESIS

Oral cancer evolves in a series of distinct multiple steps, each characterized by the sequential accumulation of additional genetic defects and cellular alterations induced by carcinogens followed by clonal expansion. The process is described as series of events including initiation, promotion and progression that lead to the development of invasive cancer (Farber, 1984). It is thought

to proceed in an orderly fashion from benign squamous hyperplasia (an increase in cell numbers of cells) to dysplasia (abnormal development of tissues or organs) to premalignant stage to carcinoma *in situ* to invasive carcinoma. The most common potentially malignant oral precancerous lesions of oral cavity are leukoplakia (persistent white patch on the mucous membrane which can not be scrapped off), erythroplakia (red patch) and precancerous conditions is oral submucous fibrosis (OSMF - fibroelastic changes with an epithelial dystrophy resulting into restricted mouth opening). However, the precise nature of the genetic alterations occurring at each step is still unclear. OPC are highly prevalent in India due to habit of tobacco chewing in younger population. OPC are known to represent an increased risk for cancer development with malignant transformation rates varying from 0.6 to 36% (Oliver et al. 2000).

The concept of "field cancerization" was introduced by Slaughter et al., (1953) and was proposed on the findings of multiple premalignant lesions or second primary tumours on the oral epithelium. The investigators reported that oral SCC originates in a multicentric fashion by a process of "field cancerization" in which an area of the epithelium has been preconditioned by spontaneous alterations or by carcinogenic agents. Thus, whole epithelium accumulates genetic damage over time leading to increased risk for developing multiple independent lesions that may become malignant (Califano, 1996; Shin 1994). The concept was mainly based on the fact that entire epithelial field is exposed to carcinogens and susceptible to the multiple genetic abnormalities passing through multistep carcinogenesis. The field cancerization is also evidenced by clinical, histopathological and molecular confirmation (Papadimitrakopoulou et al., 1996). Oral cancer includes cancers at the subsites i.e. lip, tongue, gum, floor of mouth, cheek mucosa and palate as per the International Classification of Disease (ICD-10: C00-06). The majority of malignant neoplasms of the oral region are squamous cell carcinoma of the buccal mucosa, tongue and lip.

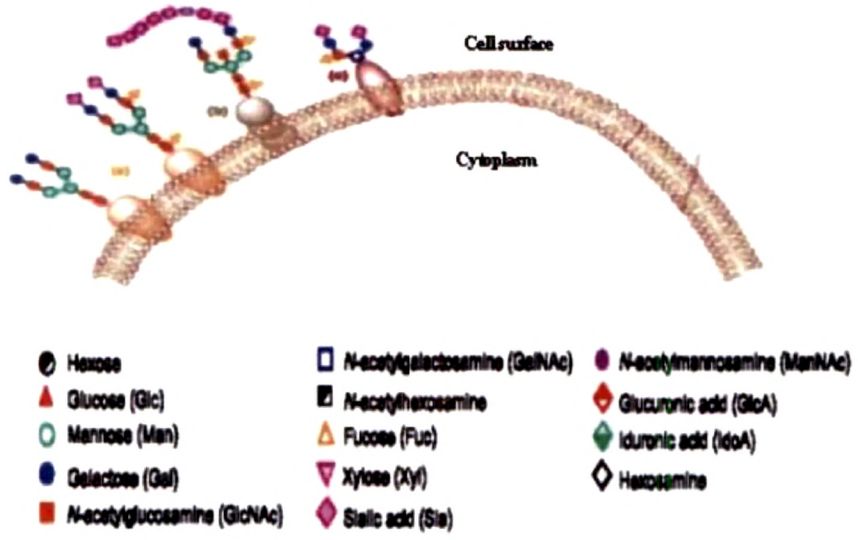
2.5: BIOCHEMICAL AND MOLECULAR CHANGES DURING ORAL CARCINOGENESIS

Oral carcinogenesis is characterized by genetic, epigenetic and phenotypic changes. Furthermore, carcinogenesis is not solely driven by the order in which genetic changes appear; the resultant cellular and tissue disorganization may also be important. This disorganization is an obvious manifestation of carcinogenesis and reflects the evolution from normal tissue to invasive cancer. Thus, molecular and cellular alterations during carcinogenesis hold a significant clinical implication.

The plasma membrane is one of the important components of cells, separating intra- and extracellular environments, which play a significant role in the behavior of cells. In particular, it is involved in regulating physiological processes and features like cell shape, growth rate, cellular recognition, communication, adhesiveness, migration, drug resistance, metastatic spread and immunological competence. As depicted in **figure-2.2**, the cell membrane is mainly composed of three chemically different classes of molecules: proteins (attached to the lipid fraction with hydrophobic bonds formed between non-polar amino acid residues and acyl chains); lipids (organized in phospholipid bilayers or incorporated in the membrane as distinct structures); and carbohydrates (attached covalently either to protein or lipid molecules and exposed to the aqueous environment). The surface of mammalian cell is decorated with complex carbohydrates (glycans). Glycans mediate a cell's communications with the outside environment. The abnormalities in glycan expression are implicated as causative or in both relatively rare congenital diseases (Schachter, 2000) and widespread acquired diseases, such as cancer (Sell, 1990). The recent revelation that fewer genes that originally thought comprise the human genome has further highlighted the importance of post-translational modifications, such as glycosylation, as determinants of higher eukaryotic functions (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001). For secreted as well as membrane proteins, glycosylation patterns represent most frequently

occurring post-translational modifications. The surface of eukaryotic cells is covered with carbohydrates, which show an enormous diversity of structure and recognition events. It is also reported that transformed tumour cells hide from normal immune surveillance by displaying different glycoproteins and glycolipids on their surfaces.

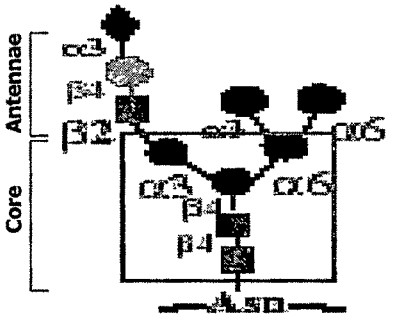
Figure-2.2: Plasma Membrane and its Components (Glycoproteins and Glycolipids)



(Adapted from Genome Biology 2, 2001)

Carbohydrate chains attached to proteins on the cell surfaces are known as glycoproteins. Protein-carbohydrate interactions are fundamental aspects in intercellular communication. The flexibility and dynamics of carbohydrate structure frequently play a key role in biological activity. Glycoprotein bound oligosaccharides fall into two well defined categories: (i) linked to the amide nitrogen of aspargine (N-linked) and (ii) linked to oxydril side group of serine or threonine (O-linked). The basic principle of glycan structure is that each class of glycans has a limited number of common core structures, to which a diversity of capping group is attached to a more terminal location of complex antennae (**Figure-2.3**). The microheterogeneity of antennae oligosaccharide structures has significant role in various biological activities of glycoproteins.

Figure-2.3: Oligosaccharide branching of glycoproteins



Under normal conditions glycoproteins improve solubility in water, contribute to the proper orientation of the molecule, cell-cell interaction, cell adhesion, protect molecules from proteases and in some cases are required for efficient intracellular transport. Some oligosaccharide sequences may also mediate highly specific molecular and cellular recognition. The biological functions of glycoproteins are most often carried out by the terminal elements of the glycans. Sialylation and fucosylation are typical terminal modifications that mediate specific functions. Both sialic acid and fucose contain structural features, which distinguish them from the monosaccharides that make up the common core structures. Most frequently described cancer related changes in the pattern of glycosylations include the synthesis of the highly branched and heavily sialylated and altered fucosylated glycans (Warren et al., 1978). The premature termination of oligosaccharide biosynthesis results into the expression of un-completed forms of antigens during cancer development. Same type of glycosidic antigens expression is also found during the fetal development (Feizi, 1985).

Terminal sugars, being most extended from the cell surface, play a very important role in the processes of neoplastic development and progression. The key postulate of theory of carcinogenesis is that "changes of membrane potential" during mitosis. The cell is postulated to become malignant when the outside surface reaches a threshold high electro-negative potential which may be due to highly negative sialic acid incorporation to cell surface glycoprotein and its transmembrane potential reaches to a threshold low value. At these values, its intracellular adhesion properties are decreased

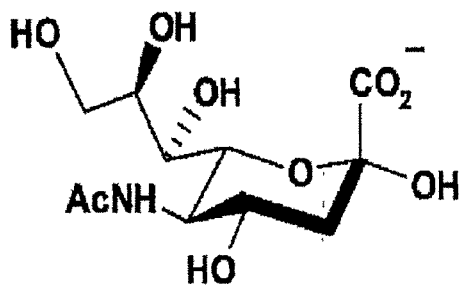
enough to permit it to leave its local environment and its cytoskeleton is disrupted which gives leukocyte-like invasive properties (Beech, 1989). Thus, alterations in glycan levels are the primary molecular phenotypes with functional significance in cancer rather than secondary effects of changes in the proteins to which they are attached.

2.6: SIALYLATION ASSOCIATED BIOCHEMICAL CHANGES

2.6.1 Sialic Acid (N-Acetyl Neuraminic Acid)

Sialic acids are a group of nine carbon sugars, which owing to the presence of carboxyl group bear a negative charge at physiological pH (Schauer, 2000). Sialic acids (N-acetyl neuraminic acid, NANA) are a family of monosaccharieds comprising about 40 members, which can be considered as derivatives of 2-keto-3-deoxy-nononic acid (Kdn), the 5-amino derivative representing the long-known neuraminic acid (Schauer and Kamerling, 1997). The main human sialic acid is N-acetyl neuraminic acid (shortly Neu5Ac/ NANA). It occupies the terminal, non-reducing positions of oligosaccharide chains of membrane surfaces in α-glycosidic linkage to galactose, N-acetylgalactosmine and to sialic acid itself. Thus, sialic acid are among the first molecules encountered by other cells or by compounds coming into contact with cells, a feature that is important in the expression of their biological role (Schauer, 2000).

Structure of Sialic acid



Sialic acid plays a major role in the chemical and biological diversity of glycoconjugates. Due to unique structural features (negative charge due to carboxyl group) enable it to mediate wide array of cellular functions such as the transport of positively charged molecules, cell-to-cell repulsion, influence

on the conformation of glycoproteins of cell membranes and masking of antigenic determinants on receptor molecules (Narayanan, 1994). Cell-type-specific expression of sialyltransferases (van den Eijnden and Joziasse, 1993; Paulson and Colley, 1989), leads to specific sialylation patterns of oligosaccharides, which can be considered as key determinants in the make-up of cells. The dual role of sialic acids, being receptors or masking recognition sites, makes them very fascinating molecules of great physiological importance.

Striking differences have been found in the sialoglycosylation patterns during development, activation, aging and oncogenesis of cells. On tumour cells this sugar plays a crucial role in the formation of metastasis and direct tissue-specificity of the process. It is theorized that sialic acid does not allow to generate host immune response against a tumour and therefore in promoting metastasis. This theory is strongly supported by the presence of sialylated glycoconjugates in the blood and by the fact that the sialic acid residues hide the expression of tissue-specific antigen determinants (Ladisch et al., 1992). Moreover, studies *in vivo* and *in vitro* showed the importance of sialic acid in the adhesion of cells to matrix (Yamada et al., 1995). Research on the biological and clinical importance of sialic acids of the glycoconjugates, has therefore intensified during the past several years. There are a number of reports on the clinical importance of this molecule. The ability of murine cells to metastasize spontaneously is positively associated with total sialic acid content of the cell culture and degree of sialic acid exposed on the tumour cell surface (Yogeeswaran and Salk, 1981). Elevated sialylation of membrane constituents of cells with hampered aggregation from metastasizing colon tumours has been demonstrated (Kemmner et al., 1992). Researchers have investigated possible correlation between serum and tissue sialic acid in endometrial and colorectal cancer patients (Paszakowska et al., 1998; Feijoo et al., 1997). Elevations in the serum sialic acid levels have been proposed as an important tumour marker (Tewarson et al., 1993). Rao et al. (1998) and Raval et al. (2003) from India have evaluated different forms of sialic acid in

serum in patients with oral cancer and OPC. They found significantly high sialic acid levels in patients with oral cancer and OPC than controls group, which suggested its utility in predicting early malignant changes and assessing the spread and invasiveness. Sialic acid measurements have value in comparing tumours at different stages of development, prognostication and monitoring of cancer patients during treatment (Raval et al., 2003; Patel et al., 1994).

2.6.2 Sialoproteins

Sialylation is one of the most versatile types of terminal glycosylation found in animal glycoconjugates (Reuter and Schauer, 1994). During post-translational modifications, the glycoproteins having sialic acid as non-reducing terminus in a variety of linkages, which are established by a large family of specific sialyltransferases known as sialoproteins. Although a universal "metastasis-related cell surface phenotype" has not been fully understood, differences in the sialoglycoconjugates are common when comparing cells of low and high metastatic potential (Passaniti and Hart, 1988; Collard et al. 1986; Yogeeswaran and Salk, 1981). Alterations in cell surface sialylation have been implicated in affecting some diverse characteristics as invasive potential (Collard et al. 1986), tumour cell-mediated platelet aggregation (Kijima-Suda et al., 1988; Bastida et al., 1987), resistance to T cell-mediated immune destruction (Yogeeswaran et al., 1983; Werkmeister et al., 1983) and alterations in tumour cell adherence to basement membrane components and target cells (Roos, 1984; Dennis et al., 1982). Osteopontin (OPN) and bone sialoprotein (BSP), phosphoglycoproteins are involved in various aspects of bone physiology. It has been reported that serum BSP and OPN expressions are ectopically associated with colon, breast, prostate and lung cancers as well as multiple myeloma and may offer promise as prognostic markers (Fedarko et al., 2001). The presence and abundance of glycosylated proteins can be determined by means of lectins. Lectins are the plant proteins that recognize and bind to specific structural features of carbohydrate chains of glycoproteins (Lis and Sharon, 1986). Hence, lectins have been used as an

important tool for exploring the structure and function of complex carbohydrates of glycoproteins during physiological and pathological processes (Montreuil et al., 1997). Glycoproteins with α2,6- and α2,3-linked sialic acid recognized by means of *sambucus nigra* (SNA) and *maackia amurensis leukoagglutinin* (MAL) lectins respectively. Clinical (Bresalier et al., 1996; Vierbuchen et al., 1995; Gassner et al., 1993) and experimental studies (Bresalier et al., 1990; Dall'Olio et al., 1992a) have pointed to a relationship between high levels of ST6Gal.I activity and SNA reactivity with a more malignant phenotype. Previous studies using lectins have been reported that aberrant glycosylation expressed in human cancer can define stage, direction and the fate of tumour progression (Dall'olio and Trere, 1993; Mostafapour and Glodstein, 1993).

2.6.3 Sialyltransferases (EC 2.4.99.1-11)

Cellular contents of sialic acid are mainly controlled metabolically by sialyltransferases (SiT) and sialidase. Sialylation is mediated by SiT, a family of 20 enzymes, which catalyze incorporation of sialic acid from activated sugar donor CMP-sialic acid to acceptors usually terminating with galactose (Gal) or N-acetylgalactosamine (GalNAc) or another sialic acid. Thus, it contributes to the diversity of carbohydrate structures. Sialic acid is linked either through α2,3- or α2,6- linkage to subterminal galactose or α2,8-linkage to another sialic acid forming polysialic acid catalyzed by specific SiT (Dall'Olio and Chiricolo, 2001; Harduin-Lepers et al., 2001). The different SiTs can be distinguished on the basis of the oligosaccharide sequence, they use as acceptors and the anomeric linkage they form with the penultimate sugar residue. To date, six α2,3-SiT, seven α2,6-SiT and five α2,8-SiT have been cloned. All SiTs cloned to date contain a conserved region called a "sialylmotif", which is involved in binding the donor substrate CMP-Neu5Ac (Datta and Paulson, 1995). In 1996, a systemic nomenclature of SiT family was proposed (Tsuji et al., 1996), and since then it has become universally accepted. This system mainly comprises four elements, for example the CMP-Neu5Ac: Galβ1-4GlcNAc α2,6-SiT is abbreviated as ST6Gal I, where ST

denotes sialyltransferase, 6 means that it is α2,6-sialyltransferase, Gal is the acceptor sugar to which sialic acid is transferred, and I is the numeral assigned consecutively to each new gene in the subgroup. ST6Gal I is the only known SiT that sialylates the 6-position of galactose. Although this SiT expressed by a single gene, show a complex pattern of regulation that allows its tissue- and stage-specific modulation. There are six different types of α2,3-SiT, ST3Gal I, II, III, IV, V and VI. They differ in their acceptor as well as tissue specificity. The addition of sialic acid in α2,3- linkage to Gal by ST3Gal I/II yields the sialyl-T antigen. ST8Sia I, ST8Sia II (STX) and ST8Sia IV are α2,8-SiT capable of synthesizing polysialic acid.

Expressions of SiT are more often deregulated in cancer. Up-regulation of a SiT in a cancer cell might indicate activation of a specific signaling pathway (Easton et al., 1991). The amount and type of sialylation of tumour cell membranes depend on the activity of a number of different SiTs. Abnormally high levels of total SiT are reported in tumour-bearing cells and sera of cancer patients (Martin et al., 2003; Kessel and Allen, 1975). Increased activity of specific SiT depends on the stage of tumour progression (Martin et al., 2003). It is also reported that not only the activities of enzyme but altered mRNA expression of SiTs (ST3Gal and ST6Gal) have clinical relevance with colorectal carcinomas, liver metastases and gastric cancer (Gretschel et al., 2003; Petretti et al., 2000). Furthermore, ST6Gal.I is one of the most frequently up-regulated glycosyltransferases in human cancers (Dall'Olio, 2000). In several malignancies, increased expression is reported in nearly 100% of the patients (Dall'Olio et al., 2000, 1989), whereas in other reports only a subset of patients displayed these types of modifications (Recchi et al., 1998). ST6Gal I regulation is mainly achieved transcriptionally through tissue-specific promoters. The contribution of these multiple transcripts in the regulation of enzyme activity and reactivity of lectin *sambucus nigra* with α2,6-sialoproteins has been described in hepatocarcinoma and colon cancer by Dall'Olio et al. (2004, 2000). An enhancement in the SiT activity of different cell lines from colorectal adenocarcinoma was associated with high sialic acid levels and the

spread of malignancy (Dimitroff et al., 1999; Saitoh et al., 1992; Kijima-Suda et al., 1986). Recently, reports from our laboratory have demonstrated association of serum total sialyltransferase activity and sialic acid levels with malignant transformation as well as treatment monitoring of oral cancer patients (Raval et al., 2003). However, the role of specific isoform of SiT has not been explored in oral cancer and OPC, which would be of keen interest. Due to its valuable role in cell-cell interaction and metastasis, it has great importance in the development of pharmacological modulators. More recently, it has been shown that SiT inhibitor KI-8110 reduces the metastatic potentials of cancer cells (Wang et al., 2003).

2.6.4 Sialidases (EC 3.2.1.18)

Sialidase (neuraminidase) catalyze the release of terminal sialic acid residue from complex carbohydrates. It is widely distributed from microorganisms to animal tissues (Gottschalk and Bhargava, 1971). Its activity in higher organisms was first time described by Warren and Spearing (1960). It has been suggested to play an important role in biological processes through regulation of cellular sialic acid contents. The major function of sialidase is to hydrolyze glycosidic linkages between sialic acid and glycosyl residue of complex oligosaccharides and glycoconjugates. Biochemical characterization of mammalian sialidase has demonstrated existence of multiple forms of sialidase. They are classified according to their major intracellular locations as intra-lysosomal (Miyagi and Tsuiki, 1984), cytosolic (Miyagi and Tsuiki, 1985), lysosomal membrane and plasma membrane-associated sialidases (Miyagi et al., 1990). However, their functional aspects are not fully explored and understood, probably due to their instability and low activity. The altered expression of sialidase observed in cancer suggests its involvement in malignant process. Sialidase is also involved in carcinogenesis and metastasis. Different isoforms of sialidase have different role in cancer progression like expression levels of lysosomal sialidase (Kato et al., 2001) may be critical and defining factors in malignancy. It is also found that increased expression of plasma membrane-associated sialidase may be essential for the survival of

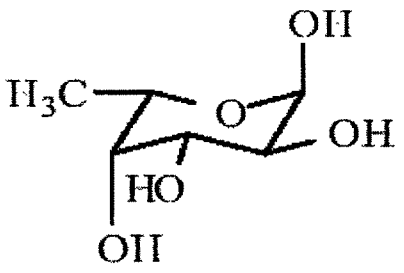
the various cancer cells (Kakugawa et al., 2002; Sun et al., 2002). However, very few studies have been reported to elucidate their functions and the significance of their altered expressions in cancer. Sonmez et al. (1999) have observed high sialidase activity in tissue and serum of breast cancer patients. In cancer patients, increased levels of serum sialic acid are found due to increased serum/tissue sialidase activity. Increased sialidase activity also contributes in process of metastasis. Due to its contribution in development of cancer it could be useful target for cancer diagnosis and therapy.

2.7: FUCOSYLATION ASSOCIATED BIOCHEMICAL CHANGES

2.7.1 Fucose

α-L-Fucose is a 6-carbon deoxyhexose that is commonly incorporated into human glycoconjugates. It is found at the terminal or preterminal positions of many cell-surface oligosaccharide ligands that mediate cell-recognition and adhesion-signaling pathways. These include such normal events as early embryonic development and blood group recognition and pathologic processes including inflammation and neoplastic progression. Fucosylated oligosaccharide ligands mediate cell-cell adhesion through binding to cell-surface selectins and calcium-dependant interactions with other cell-surface carbohydrate counter ligands. A number of fucose-containing "natural ligands" are common to inflammatory and malignant cell processes. A significant role of fucosylated glycans has been implicated in fertilization, embryonic development, induction of angiogenesis (Koch et al., 1995) and the regulation of cell signaling (Schiffer et al., 2001).

Structure of α-L-fucose



Fucose has been known for a long time to be a component of the glycocalyx of malignant cells. It has been documented that tumour cells modulate their surface to escape recognition by increased fucose levels (Macdougall et al., 1987; Alhadeff 1989; Hakomori 1989). The role of fucose in the process of metastasis is more obscure than of sialic acid. Fucosylated glycoproteins are characteristics of the surface of HeLa cells (Bosmann et al., 1968). Changes in the surface fucose-labeled glycoproteins are reported to be associated with the tumourigenic potentials of transformed cells (Smets et al., 1976). Studies involving labeling of membrane glycoproteins with radioactive fucose have showed strong relationship between structural and qualitative changes in these molecules and the process of cellular differentiation (Muramatsu et al., 1978; Herscovics et al., 1980). Increased cell surface fucosylation or incorporation of labeled fucose is found in metastatic variants of many tumours of transformed cells (Shirahama et al., 1993; Bruyneel et al., 1990). Furthermore, Kemmner et al., (1992) have demonstrated that metastasizing tumours are significantly more fucosylated than not-metastasizing tumours. Chatterjee and Kim (1978) documented significant correlation between fucosylation and metastatic properties of tumour cells. Several investigators have reported that monitoring serum or tissue fucose levels could be a promising approach for the early detection, diagnosis and prognosis of cancer (Fernandez-Rodriguez et al., 1997; Patel et al., 1994,1990; Rao et al., 1998; Wang et al., 1995).

2.7.2 Fucoproteins

The presence of fucose at the terminal or sub-terminal end of glycoproteins is known as fucoproteins. Fucose-containing glycoconjugates have been observed on cell surface as well as secreted proteins (Forstner and Wherrett, 1973; Gahmberg, 1971; Bennett and Lablond 1970). Studies by some investigators indicate that considerable alterations in fucose-containing glycoconjugates occur during malignant transformation (Baumann et al., 1979; Glick, 1978; Hakomori, 1975). However, it has not been much explored in oral cancer. The quantitative expressions of fucosylated proteins

(fucoproteins) can be recognized by means of lectin affinity chromatography using fucose-specific lectin *lotus tetragonolobus* (LTA). Only few reports based on lectin affinity have provided evidences that terminal fucose moieties of glycoproteins are modified during neoplastic transformation (Thompson and Turner, 1987; Thompson et al., 1991). Turner et al. (1985) have shown the association of elevated serum protein-bound fucose in cancer patients' especially abnormal fucosylation of serum haptoglobins as a potential cancer marker. Their findings were not due to the production of new glycoproteins, as very few new proteins were seen on one (1D) or two-dimensional (2D) electrophoretic patterns of cancer sera as compared to the patterns of healthy sera. Shirahama et al. (1993) have reported that high expression of fucose binding proteins in transitional cell carcinoma of urinary bladder could serve as a prognostic marker. High expressions of fucosylated cell surface antigens such as SLe^X and SLe^a are associated with malignant transformation, increased metastatic potentials of tumours and poor prognosis of cancer patients (Renkonen et al., 1997; Nakamori et al., 1993; Mushuhita et al., 1990). α1,6-fucosylated α-fetoprotein (AFP) is known to be useful as an early indicator and predictor of the poor prognosis as well as progression of the treatment for hepatoma (Noda et al., 2002; Ayogi et al., 1985). Thus, whether the fucosylation of serum proteins is altered in cancer still remains unclear; one of the objectives of the present investigation was to examine this possibility in oral cancer.

2.7.3 Fucosyltransferases [EC 2.4.1 (65, 68, 69, 152, 214, 221)]

Fucosyltransferases (FucT) are a family of enzymes distinguished by differences in acceptor specificities and other biochemical properties. FucT catalyze the transfer of fucose from activated nucleotide donor GDP-fucose in α-linkage to an appropriate acceptor substrate. In mammals, fucose is found at α1,3-, α1,4- or α1,6- linkage to the subterminal sugar N-acetylglucosamine and α1,2- linkage to galactose in glycan structures. The cloned FucT have been designated Fuc-T I-IX. FucT I and II are α1,2-FucT, III-VI and IX α1,3/4-FucT, and Fuc-TVIII is a α1,6-FucT that fucosylates the chitobiose

core of N-glycans. In addition, the protein O-FucT-I transfers fucose directly to serine or threonine of certain proteins (Wang et al., 2001). Each enzyme transfers only one sugar into one specific linkage to an appropriate acceptor.

α1,3-FucT, known as lewis enzyme, plays a key role in the synthesis of cell-surface antigen SLe^X, which is the minimal ligand for the selectin family of adhesion molecules (Lasky, 1992; Britten et al., 1998). There are specific receptors for these enzymes on plasma membranes determining the exact patterns of fucosylation of cell surface antigens. SLe^X, sialyl dimeric SLe^X and Sle^a have been proposed to have a role in hematogeneous metastasis of cancer, where malignant cells would adhere to endothelial lining via E-/ P-selectin in a manner analogous to leukocyte adhesion. High expression of α1,3-FucT, thereby causing increased fucosylation of surface antigens is associated with malignant transformation. The expression of these antigens has been correlated with increased metastatic potentials of tumours and poor prognosis of cancer patients (Martin-Satue et al., 1998; Yamada et al., 1997; Ogawa et al., 1996). Higher α1,6-FucT and α1,2-FucT expressions and activity have been found to be associated with various malignancies (Takahashi et al., 2000; Fukushima et al., 1998). The increased activity of α1,6-FucT was observed in tissues and sera of patients with hepatoma, as compared to those patients with liver cirrhosis and no evidence of hepatoma (Hutchinson et al., 1991). Several investigators have found that fucosylated protein levels are elevated in patients with malignant diseases (Campion et al., 1989; Tatsumura et al., 1977). Also, decreased activity of FucT is reported to accompany the process of differentiation of many malignant cell systems (Ambros and Kurman, 1993; Muramatsu and Muramatsu, 1983; Ronquist and Nou, 1983).

2.7.4 α-L-Fucosidase (EC 3.2.1.51)

α-L-fucosidase (α-L-fucoside fucohydrolase) is an acid hydrolase that is ubiquitously found in human tissues and extracellular fluids. Cellular enzyme apparently is located in lysosomes, involved in hydrolytic degradation of

fucose-containing oligosaccharides and glycoconjugates. Mammalian α-L-fucosidases are multimeric forms of glycoproteins (Cordero et al., 2001; Alhadeff 1998). They appear as having considerable degree of structural heterogeneity, both tissue-specific and within the tissue. The majority (90-100%) of the α-L-fucosidase activity is in the soluble fraction in almost all mammalian tissues (Alhadeff 1998). The presence of fucosidases is necessary for rapid turnover of N-glycans (including L-fucose) followed by reglycosylation and reinsertion of the proteins in plasma membrane (Horstkorte et al., 1996; Kreisel et al., 1988; Tauber et al., 1983). The biological significance of reprocessing is related to the regulation of cell surface carbohydrate information bearing function in cell-cell recognition and adhesion (Drickamer and Taylor 1998; Smith et al., 1998). In broad-spectrum, tissue differentiation and development through cell-cell recognition are modulated by sequential changes of the sugar chains of cell surface glycoproteins. Thus, the expression and deletion of α-fucosyl residues linked at various positions of sugar chains of glycoproteins is one of the important phenomenon, the role of α-L-fucosidase in these processes is of great interest. Alterations in serum and/or tissue α-L-fucosidase activity have been reported to be potentially useful in early detection, diagnosis, staging, and monitoring of malignancy as well as in the management of cancer patients (Ayude et al., 2003, 2000; Abdel-Aleem et al., 1996).

2.8: INVASION AND METASTASIS

Cancer cells invade into neighboring tissues and survive in this ectopic site. The term invasion indicates penetration into neighboring territories and their occupation. Cancer cells invade beyond the constraints of the normal tissue from which they originate; this invasion permits them to enter into the circulation from where they travel distant organs and eventually form secondary tumours, called metastasis. Epithelial tumours are the predominant form of cancer metastasis. The metastatic dissemination of tumour cells is the primary cause of morbidity and mortality in cancer patients (Ewans, 1991). In traditional metastasis models, metastatic cells are rare and arise during late

stages of tumour progression (Fidler IJ, 2003). These models have been challenged by recent expression profiling studies on human tumours, such as breast carcinomas, that focused on the formation of overt metastasis as the end point of their analyses (Van't Veer et al., 2002; Van de Vijver et al., 2002; Bernards and Weinberg, 2002). These studies reported that most cancer cells in a primary tumour have a 'metastatic phenotype', indicating that metastatic spread is an early event in tumorigenesis.

Metastases do not result from random survival of cells released from the primary tumour but from the selective growth of specialized subpopulations of highly metastatic cells. These cells are endowed with specific properties that befit them to complete each step of the metastatic process. For a tumour cell to metastasize successfully, a number of highly specialized events must occur to attain and proliferate at secondary site. Several critical steps in this metastatic cascade depend on cell-to-cell or cell-to-matrix interactions that involve cell surface molecules. Consequently, changes in oligosaccharide structures on the cell surface may have a strong influence on the behavior of malignant cells. Numerous cell surface glycoproteins play diverse roles in adhesion, intracellular signal transduction, cell-to-cell communication, and cytokine receptor function (Hakomori, 1996). Therefore, alterations in glycoprotein moieties can affect variety of processes in normal, as well as malignant cells. Hence, it is rational to say that these highly selective changes in tumour cell glycosylation are not random accidents.

Carbohydrates are known to play a vital role in cell-cell recognition processes. The importance of carbohydrates in molecular recognition originates from their unique structural diversity. The specific recognition of carbohydrate moieties by a protein is dependent on a number of more or less well defined structural motifs of saccharide. Carbohydrates are also important for tumour development because cancer cells alter their surface glycoprotein expression to evade the immune system. By cloaking themselves with the right

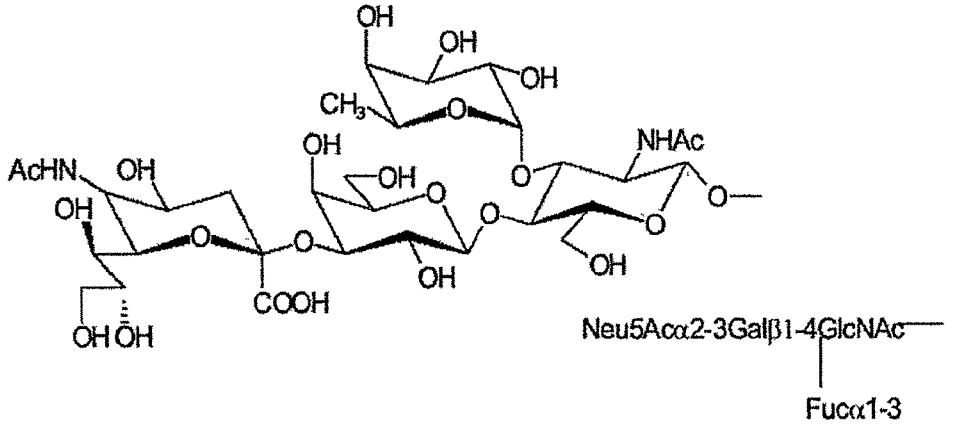
assortment of glycoproteins, the tumour cells can invade other tissues without being identified as aliens.

Hence, understanding the molecular mechanisms that underlie tumour progression, local invasion and the formation of tumour metastasis represents one of the great challenges in exploratory cancer research. Surprisingly though, in spite of the clinical importance of metastasis, much remains to be learned about the biology of the metastatic process. In part, the knowledge is limited because metastasis is a 'hidden and complicated' process. Many molecular factors have been identified as contributing to the formation of detectable metastasis. Inhibition of the growth of metastasis in secondary sites offers a promising approach for cancer therapy.

2.8.1 Sialyl Lewis X- Cell Surface Antigen

Sialyl Lewis X (SLe^X), sialic acid and fucose containing tetrasaccharide, is present on the terminus of cell surface glycoconjugates. It is one of the important ligand of lewis family and tumour-associated carbohydrate antigens. Altered blood group antigens represent a family of carbohydrate epitopes frequently overexpressed in malignant tissues.

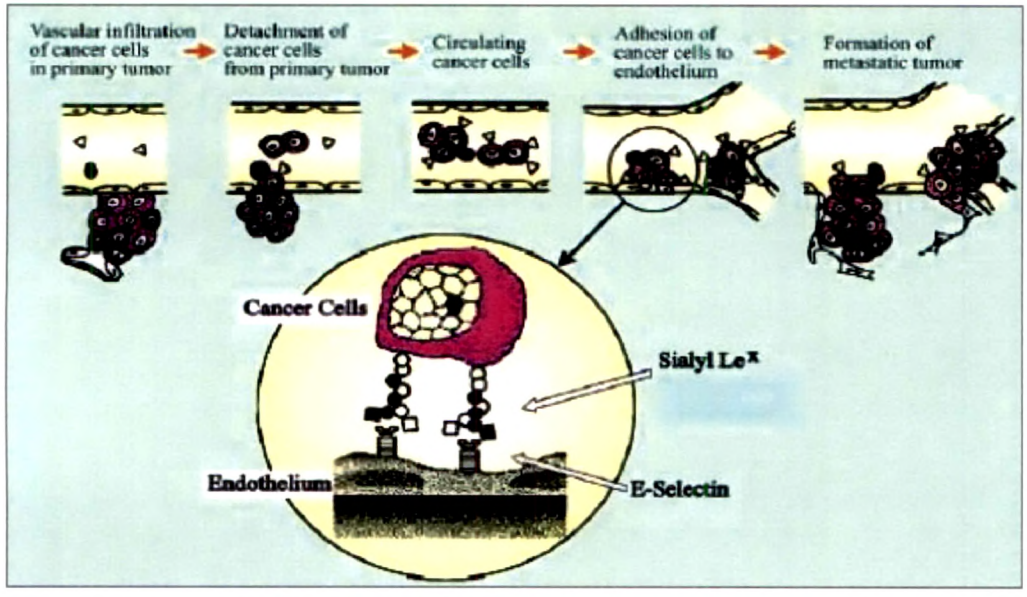
Structure of SLe^X



Synthesis of complex carbohydrate determinants well developed on normal epithelial cells tends to be impaired upon malignant transformation,

predisposing the cells to express less complicated carbohydrate determinants (Kannagi, 2004). As shown in **figure-2.4**, the carbohydrate determinants, SLe^X, which are frequently expressed on human cancer cells, serve as ligands for a cell adhesion molecule of the selectin family, E-selectin, which is expressed on vascular endothelial cells. These carbohydrate determinants are involved in the adhesion of cancer cells to vascular endothelium and thus contribute to hematogenous metastasis of cancer (Kannagi, 1997). The initial adhesion mediated by these molecules triggers activation of integrin molecules through the action of several cytokines and leads to the extravasation of cancer cells. Cancer cells also produce humoral factors that facilitate E-selectin expression on endothelial cells. The degree of expression of the carbohydrate ligands at the surface of cancer cells is well correlated with the frequency of hematogenous metastasis and prognostic outcome of patients with cancers. The alteration of fucosyltransferase activities that leads to the enhanced expression of these carbohydrate ligands on cancer cell surface is currently being investigated.

Figure-2.4: Schematic representation of the SLe^X mediated complex, multi-step process of hematogenous metastasis of cancer



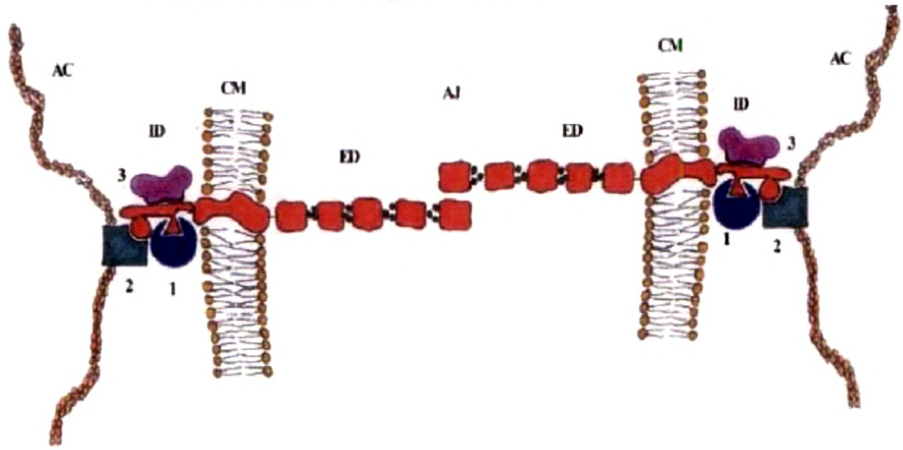
(Adapted from Cancer Science, 95, 2004)

Numahata et al. (2002) have revealed SLe^X expression as a potential predictor of invasive and metastatic outcome of bladder carcinoma. No other carbohydrate epitope examined to date has equal prognostic value. Mouse melanoma B16-F1 cells were made to express SLe^X by transfection of α1,3-FucT and then injected intravenously through tail veins of mice. B16-F1 cells expressing a moderate amount of SLe^X formed a large number of lung tumour nodules. Several clinical statistics support the role of E-selectin-carbohydrate interactions in hematogenous metastasis and poor prognosis in patients with cancer (Ohyama, 1999; Nakayama et al., 1995; Shimono et al., 1994; Nakamori et al., 1993; Narita et al., 1993; Matsushita et al., 1990).

2.8.2 E-cadherin- cell adhesion molecule

E-cadherin, 120 kD ($E\text{-}cad^{120}$) transmembrane cell surface molecule, have a key function in epithelial cell adhesion (**Figure-2.5**), through the establishment of calcium dependent homophilic interactions at sites of cell-to-cell contact known as adherens junctions (Gumbiner, 1996). As a member of a large family of genes coding for calcium-dependent cell adhesion molecules (CAMs), the cadherin glycoproteins are expressed by a variety of tissues, mediating adhesion through homotypic binding. Classical cadherins– E- and N-cadherins are best characterized and found to play important role in the formation of various tissues (Barth et al., 1997). E-cadherin has been studied in greater depth. Earlier reports indicate that it is essential for the formation and maintenance of epithelia. It was first identified in chicken, and was originally called L-CAM (Gallin et al., 1987). The mouse counterpart of this protein, uvomorulin (Ringwald et al., 1991), has 80% identity in both nucleotide and amino acid sequences to the human counterpart (Mansouri et al., 1998).

Figure-2.5: Schematic illustration of E-cadherin in adherens junction. E-cadherin homodimer on the cytoplasmic membranes of adjacent cells



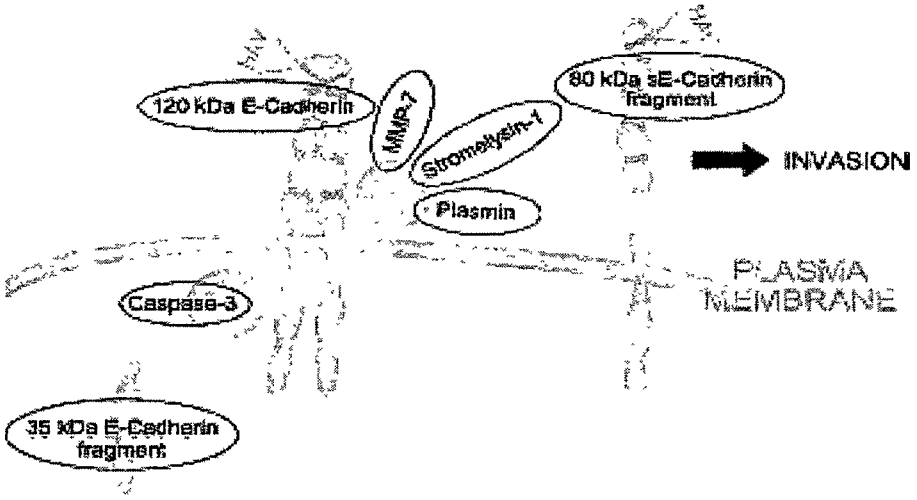
(Adapted from Cancer Cell International 3, 2003)
(CM– cytoplasmic membrane; AJ– adherens junction; ED– extracellular domain; ID– intracellular domain; AC– actin cytoskeleton; 1-beta-catenin; 2-alpha-catenin)

Besides its role in normal cells, this highly conserved gene can play a major role in malignant cell transformation, especially in tumour development and progression (Yap, 1998). The suppression of E-cadherin expression is regarded as one of the main molecular events responsible for dysfunction in cell-cell adhesion. Most tumours have abnormal cellular architecture, and loss of tissue integrity, which lead to local invasion. Thus, loss of function of E-cadherin correlates with increased invasiveness and metastasis of tumours (Vleminckx et al., 1991), resulting in it being referred to as the "suppressor of invasion" gene.

The control of cellular adhesion and motility is one of the crucial mechanisms responsible for tumour initiation and progression (Guilford, 1999). Loss of E-cadherin-mediated-adhesion characterizes the transition from benign lesions to invasive, metastatic cancer. Hence, E-cadherin tumour suppressor genes are particularly active area of research in development and tumorigenesis. Nevertheless, there is evidence that E-cadherins may also play a role in the *wnt* signal transduction pathway implicated in cancer (Peifer and Polakis, 2000). Proteolytic degradation of E-cadherin by matrix metalloproteases (MMPs) is a mechanism by which E-cadherin-mediated cell–cell adhesion can

be ablated. The mechanism of E-cadherin degradation is shown in **figure-2.6**. A soluble 80 kD form of E-cadherin, produced by the degradation of the full–length protein, is frequently found in cultured tumour cell lines and in tumour biopsy samples. This soluble form of E-cadherin promotes tumour cell invasion by up-regulating MMPs, such as MMP-2, MMP-9 and MMP-14. Such ectodomain shedding of E-cadherin might have an active part in the invasive process during tumour progression (Nawrocki-Raby et al., 2003). Recently, Rashid and colleagues (2001) have observed that the post-translational cleavage of native E-cad^{120} is a membrane-bound 97 kD (E-cad^{97}) protein. This cleavage removes its binding domain and renders functionless E-cad^{97}. This inactivating truncated protein E-cad^{97} is found to be accumulated during malignant transformation in prostate cancer as compared to the native protein.

Figure-2.6: E-cadherin ectodomain shedding resulting in the formation of an invasion-promoting sE-cadherin fragment



(Adapted from Physiol Rev. 83, 2003)

2.9: SCOPE OF THE PRESENT STUDY

Oral cancer incidence rates are reaching high proportions in India. Particularly the rate of leukoplakia and submucous fibrosis is rising in the young population due to increase in chewing pan masala products containing tobacco. In spite of number of studies aimed at the improvement of the early diagnosis and treatment of patients with oral squamous cell carcinoma, their

prognosis remains poor. Only about 15% of the patients are diagnosed at an early stage. Very poor 5-year survival rate of this disease has been found over the last several decades due to more advanced disease at presentation (Nagler, 2002; Brunin et al., 1999; Mark, 1998). The study conducted by Friedlander et al. (1998) has stated that the younger patients had a significantly increased risk of loco-regional failure ($p<0.05$) than the older patients. Thus, from the studies done so far, it is clear that cancer mortality can be reduced if lesions are detected, diagnosed and treated at precancerous or early stage. Early diagnosis and treatment of oral cancers are essential to achieve a good prognosis. Hence, search for newer combination of markers for early detection and therapy in the fight against cancer remains the top priority in biomedical research.

Cancer cell speak different sugar dialect than do normal cells. A common phenotypic change in malignancy is a dramatic transformation of cellular glycosylation. Indeed, alterations in terminal glycosylation can be considered a universal feature of cancer cells. What remains mysterious is how these molecules work in concert to convert non-malignant cells into malignant cell that gives each cell type a unique identity for the invasive and metastatic potential. Answering questions about glycan biosynthesis will lead to insight into basic biological processes and may open the door to therapeutics interventions in disease processes. The process of terminal glycosylation is far from a decorative function. There has been relatively modest attention paid, to date, to the various ways in which proteins are "tweaked" through the attachment of sugars. Carbohydrates help to determine the three-dimensional structures of proteins, which are inherently linked their functions and their efficacy as therapeutics. Moreover, in contrast to some of the other chemical tags employed by cells, carbohydrates exhibit a mind-boggling diversity of structures; can confer cell-type specificity, and crucial components of cell-to-cell signaling. At the same time, carbohydrates make problematic drug targets; they are the most difficult biological molecules to analyze and synthesize, and are rapidly broken down in the bloodstream. Despite these

challenges, recent technological advances have enabled to pursue new carbohydrate-based products, sweetening the outlook for glycobiology. Also, the pivotal roles that carbohydrates play in the regulation of biological activities, there is a great promise that a comprehensive and thorough study of glycome can contribute the unraveling of the functions of the proteins and eventually those of genes.

2.10: AIM AND OBJECTIVES

Earlier studies have shown significant role of aberrant sialylation and fucosylation in various cancers and their importance in invasion and metastasis. However, there are very few reports on importance of terminal glycoprotein changes in oral cancer from India. Few efforts are done to review the importance of these parameters either in serum or tissue specimens. None of the studies have shown simultaneous evaluation of multiple parameters in serum as well as in the tissues of oral cancer patients. Previous studies have compared the levels between healthy individuals and cancer patients. To assess the specificity of the markers for cancer, individuals at high risk of cancer should be distinguished from those having cancer. Hence, patients with OPC were included in the study to observe the early biochemical changes taking place during oral carcinogenesis and to serve as pathological controls. The treatment monitoring aspects of these parameters was also included and post-treatment follow-up samples of oral cancer patients were studied. Considering functional characteristics of glycoproteins, the present work would represent a step towards the evaluation of terminal glycosylation alterations in oral cancer. The study thus was aimed to determine the significance of terminal glycoprotein changes in patients with OPC and oral cancer as well as follow-ups of oral cancer patients. The major focal points of the study were as follows:

2.10.1 Serum analysis

- ✓ Comparison of serum TSA, α2,6-sialoproteins and α2,3-sialoproteins levels as well as α2,6-SiT and α2,3-SiT and sialidase activities between

healthy individuals, patients with OPC and untreated oral cancer patients.

- ✓ Correlation of serum sialylation markers in patients with OPC and oral cancer.
- ✓ Comparison of serum TSA, α2,6-sialoproteins and α2,3-sialoproteins levels as well as α2,6-SiT and α2,3-SiT activities in untreated oral cancer patients with post-treatment follow-up samples of responders and non-responders.
- ✓ Comparison of serum fucose and fucoproteins levels as well as α-L-fucosidase activity between healthy individuals, patients with OPC and untreated oral cancer patients.
- ✓ Comparison of pretreatment serum fucose and fucoproteins levels as well as α-L-fucosidase activity with post-treatment follow-up samples of responders and non-responders in oral cancer patients.
- ✓ Evaluate the association of serum sialylation and fucosylation markers with clinico-pathological characteristics of the oral cancer.
- ✓ Alterations in serum protein and glycoprotein profiles.

2.10.2 Tissue analysis

- ✓ Comparison of TSA, α2,6-sialoproteins and α2,3-sialoproteins levels as well as α2,6-SiT and α2,3-SiT and sialidase activities between malignant/ precancerous and their adjacent normal tissues of oral cancer and patients with OPC.
- ✓ Establish the association of sialylation markers with clinico-pathological characteristics in malignant tissue of the oral cancer patients.
- ✓ Comparison of α-L-fucosidase activity between malignant/ precancerous and adjacent normal tissues in patients with oral cancer and OPC.
- ✓ Comparison of E-cadherin and SLe^X expressions between malignant/ precancerous and adjacent normal tissues in patients with oral cancer and OPC.
- ✓ Determine the clinical significance of E-cadherin and SLe^X expressions in oral cancer.