Prologue

Patience, persistence and perspiration make an unbeatable combination for success
- Napoleon Hill

ORAL CANCER FACTS

In 2011, Global Cancer Statistics reported 263,900 new oral cancer cases and 128,000 deaths worldwide due to oral cancer (Jemal *et al*., 2011). The Indian subcontinent accounts for one-third of the world burden, which is mainly attributed to different forms of tobacco consumption (Khan *et al*., 2012). The rising trend among younger population in India is the major concern. Therefore, there is an urgent need to identify molecular signatures of the disease that can be used for early detection, prognostication and post treatment monitoring of oral cancer in clinical set-up. Oral carcinogenesis is a sequential process which proceeds through several premalignant conditions and the molecular changes are accompanied by glycosylation changes.

IMPORTANCE OF GLYCOSYLATION

Glycosylation is the process of addition of glycans to glycoproteins and is the major posttranslational modification of proteins. The addition of one or more complex oligosaccharides (glycans) to the polypeptide backbone of majority of eukaryotic proteins makes the glycoproteome several orders of magnitude more complex than the proteome itself. Understanding glycomics is a bigger challenge due to the complexity of glycan processing pathways which are sensitive to genetic and environmental factors. The prominent peculiarity of the oligosaccharides/sugar moieties is that our genome does not contain genetic template to code for them. Instead, glycan structure results from the activity of a dynamic network of over 600 “glycogenes” which are in our genome that code for various glycosyltransferases, glycosidases, enzymes for sugar nucleotide biosynthesis, transporters etc. Novel glycan structures result from modulation of gene expression as well as changes in the activity and/or the localization of any of the enzymes involved in their production. These changes are liable to environmental influences. The alterations can lead to altered repertoire of glycan structures, as found in many diseases. Terminal glycosylation plays an important role in malignant transformation and metastasis. Alterations in glycoproteins mainly occur due to aberrant expression of different types of glycosyltransferases and glycosidases. Earlier, alterations in glycosylation from blood

and tissues have been reported in various cancers. However, salivary glycosylation changes are still an unexplored field for its clinical significance in oral cancer.

SALIVA: THE MIRROR OF HUMAN HEALTH

Oral saliva examination offers an attractive non-invasive alternative to blood/tissue testing. Saliva based tests offer the advantages for patients and health care providers due to non-invasiveness, less ethical complexity, ease of collection, elimination of the common fear of needle sticks, lower costs of sample collection, availability of repeated samples during follow-up studies and no risk of percutaneous injury. Apart from digestive and protective functions, saliva serves as an effective diagnostic modality for various genomics, transcriptomics and proteomics analysis **(Figure 1.1)**.

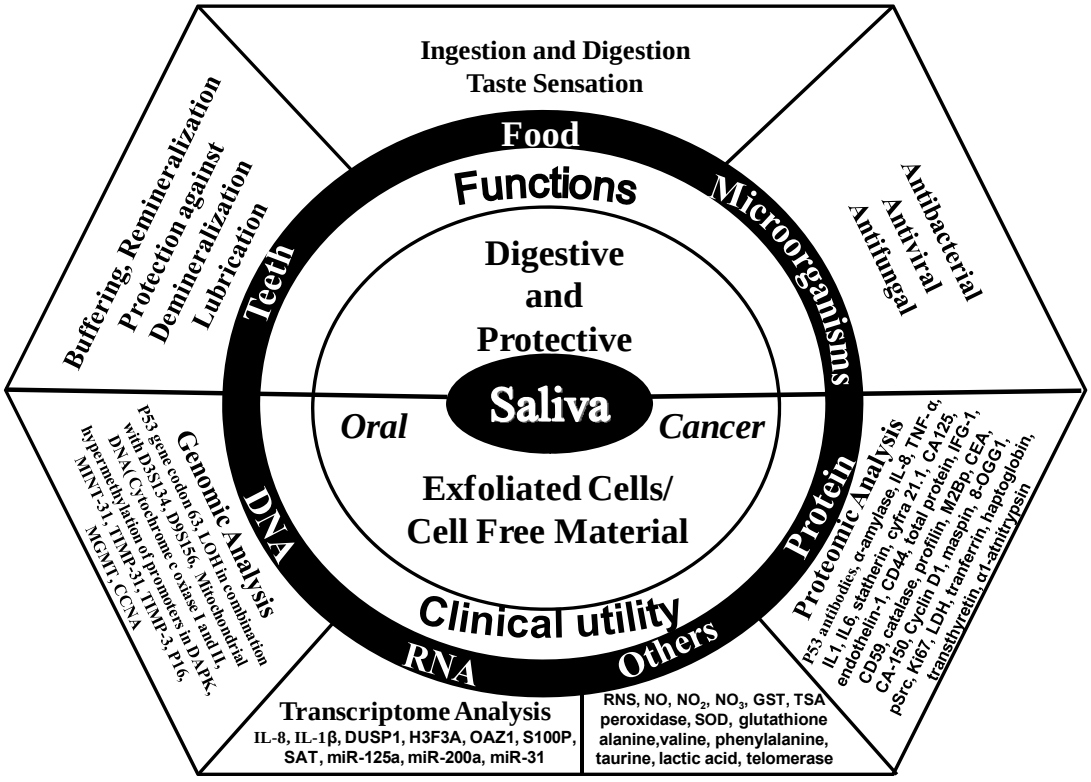

Figure 1.1: ***Various functions and clinical utility of saliva in oral cancer.*** *Saliva plays important role in digestion. It serves as protective functions due to its antibacterial, antiviral and antifungal properties. It helps in lubrication and remineralization of teeth. Apart from this, various genomics, transcriptomics, proteomics and other biomarkers including oxidative stress related molecules, metabolomics etc. are analyzed from exfoliated cell or cell free material, which might aid in diagnosis and monitoring of oral cancer.*

Saliva serves as an effective modality to detect biochemical changes occurring in cancer cells as it is in direct contact with oral cancer lesions. Glycoproteins and glycans are released into circulation due to increased shedding from the malignant cells **(Figure 1.2)**. Sialylation and fucosylation being the most abundant posttranslational modifications, it would be interesting to compare sialylation and fucosylation changes from saliva and serum. Along with glycosylation changes, further monitoring of other associated molecular markers might help in unraveling their role in sequential changes that takes place in oral cancer.

GLYCOSYLATION ASSOCIATED MOLECULAR MARKERS

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 play important role in metastasis and invasion by proteolytic degradation of extracellular matrix (ECM), disruption of cell-cell and cell matrix adhesion, migration and angiogenesis **(Figure 1.2)**. E-cadherin, a key molecule in cell-cell adhesion play an important role in malignant transformation. Earlier reports have documented loss of E-cadherin, a cell adhesion molecule in oral precancerous conditions (OPC). Initially, MMPs were thought to be important exclusively in invasion and metastasis, but recent studies have demonstrated that MMPs play a major role in several other steps also during carcinogenesis. Further, MMPs are well-known for modulating the cell-cell and cell-ECM interactions affecting both cell phenotype (epithelial mesenchymal transition) and increasing cell migration. Hence, understanding MMPs in OPC would help in monitoring early events in tumorigenesis.

Earlier studies have documented that MMP-9 is a mediator of Epidermal growth factor receptor (EGFR) dependent downmodulation of E-cadherin in ovarian cancer (Dahl *et al*., 2008). EFGR is a tyrosine kinase receptor of the ErbB family that plays an important role in cell proliferation, invasion and metastasis. Abnormal amplification of *EGFR* gene has been observed in various human tumors like lung, laryngeal, ovarian, cervical, breast, and oral squamous cell carcinoma. Earlier studies have reported that reduction of E-cadherin results in upregulation of EGFR transcriptionally in head and neck cancer (Pidone *et al*., 2014). Also, *in vitro* studies

have indicated that activation of EGFR promotes cell migration and invasion via inducing EMT-like phenotype change and MMP-9 mediated degradation of E-cadherin via ERK1/2 and PI3-K signaling pathway (Zuo *et al*., 2011; Bae *et al*., 2013). Recently, a novel pathway has been reported using keratinocytes which suggest that loss of E-cadherin mediated cell-cell adhesion is responsible for increase in proto-oncogene c-Jun levels, a key player in tumorigenesis (Knirsh *et al*., 2009).

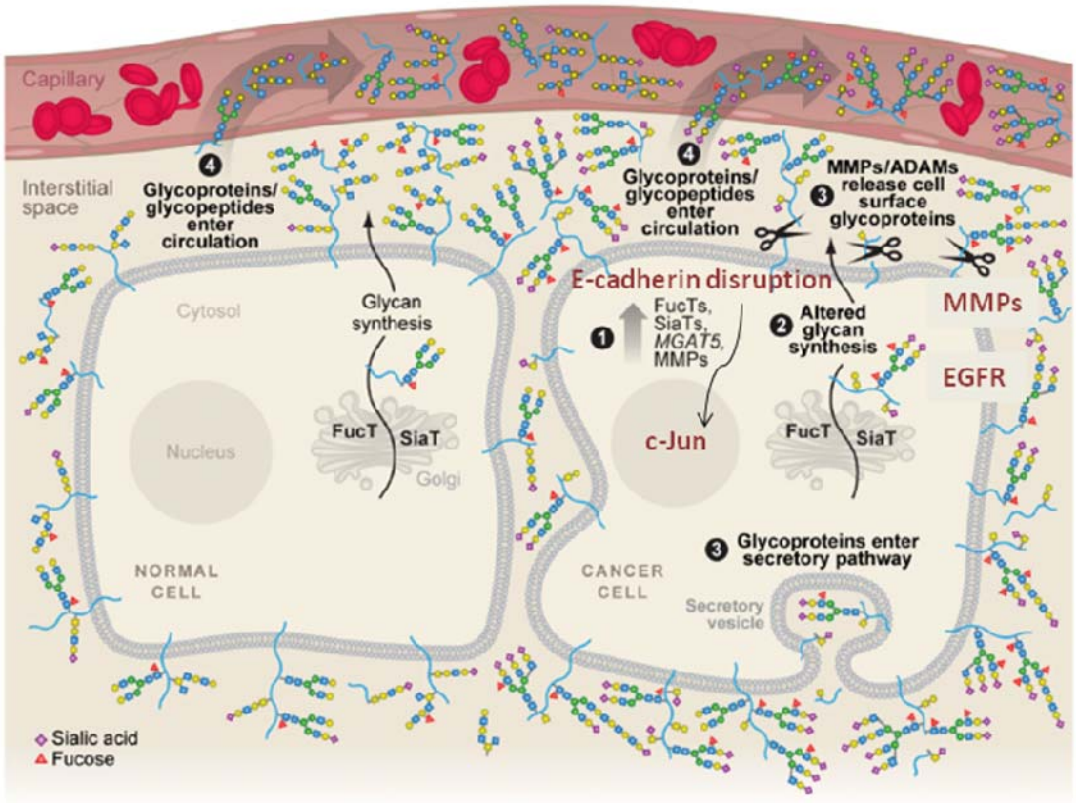

Figure 1.2: ***The hypothesized pathway for release of glycoproteins in circulation and correlation with associated molecular events.*** *Increase in sialyl and fucosyl transferases lead to the formation of altered glycan synthesis in malignant cells. These cells release glycoproteins carrying disease related epitopes into interstitial space, from where they can reach the circulation. This shedding might be due to 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 (Figure modified from Drake et al., 2010).*

It has been documented earlier that loss of E-cadherin mediates increase in c-Jun protein accumulation with no corresponding increase in *CJUN* mRNA (Spangler *et al*., 2011). Such a control was suggested to be due to internal ribosomal entry site

(IRES) mediated translation (Polak *et al*., 2005). However, there are lack of studies correlating E-cadherin, MMPs and c-Jun in oral cancer. Therefore, simultaneous evaluation of *ECAD* mRNA and protein, MMPs, *CJUN* mRNA and protein together with EGFR in oral cancer patients might aid in understanding the molecular basis of oral carcinogenesis.

In spite of the tremendous progress made in "OMICS" on bench side, the translational and applicability to clinical fields is limited. The hope of non-invasive tools like saliva to enter in clinics with appropriate molecular markers for early detection and disease monitoring has brought several upcoming researches in salivaomics.

HYPOTHESIS AND SCOPE OF THE STUDY:

We hypothesized that glycomic analysis i.e. salivary glycosylation together with MMPs can aid in early screening, treatment monitoring and prognosis in clinical set-up. The present study was therefore undertaken to establish non-invasive biomarkers and markers associated with progression and metastasis for its applicability to clinical fields and to understand the pathways of oral carcinogenesis.

Glycomics has come-up as a hot field in recent years and several glycoproteins has entered in clinical diagnostics (Kam *et al*., 2008; Taniguchi *et al*., 2009; Drake *et al*., 2010). As malignant cells release glycoproteins carrying disease related epitopes into circulation; the present study hypothesized that, understanding the glycoprotein electrophoretic profile can shed light on changes associated with neoplastic tranformation in oral cancer.

Recently, our laboratory and various other investigators have documented much advancement in salivary based genomics and proteomics biomarkers in oral cancer (Shah *et al*., 2011; Yakob *et al*., 2014, Cheng *et al*., 2014). There are very few studies on salivary glycosylation changes in oral cancer. Therefore, we hypothesized that as there is increased shedding of glycoconjugates from the tumor cells, the changes can be reflected in saliva as it is in direct contact with oral cancer lesions. The comparison of serum and salivary sialylation and fucosylation changes in patients with OPC, oral cancer patients and follow-ups during anticancer treatment, might give insights into

significance of salivary biomarkers for early screening, disease monitoring and prognosis of oral cancer.

Glycosylation is mainly controlled by glycosyltransferase and glycosidase enzymes. The present study hypothesized that evaluating different subtypes of sialyltransferase and fucosyltransferase might help in understanding its role in oral cancer development. We further hypothesized that increased glycosylation and alterations in glycoproteins might be involved in oral cancer progression by subsequent loss of E-cadherin through increase in Matrix metalloproteinase, MMP-2 and MMP-9 **(Figure 1.3)**.

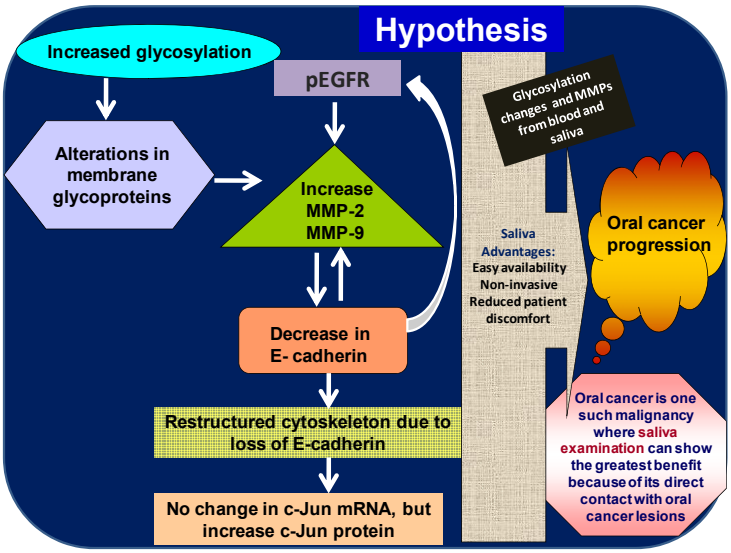

Figure 1.3: ***Hypothesis of the study.*** *Increased glycosylation of cell membrane proteins might cause alterations in cell membrane glycoproteins. The alterations in glycoproteins might activate MMPs which is involved in increased shedding of glycans in circulation. Increased MMPs might be involved in disruption of cell-cell adhesion and hence there is further decrease in E-cadherin protein. Moreover, loss of E-cadherin might be involved in upregulation of pEGFR. The loss of E-cadherin might be involved in upregulation of c-Jun protein with no changes in CJUN mRNA levels. Moreover, comparison of glycosylation changes and MMPs from blood and saliva can give insights for use of the non-invasive tools for monitoring of oral cancer.*

The increase in MMPs further causes disruption of basement membrane. In addition, loss of E-cadherin might be involved in increased activation of pEGFR. Loss of E-cadherin mediated cell-cell contacts (restructured cytoskeleton) may cause increase in c-Jun accumulation (c-Jun protein) with no corresponding increase in the transcript

levels (*CJUN* mRNA) **(Figure 1.3)**.The study on these interactions might signify that restructuring of cytoskeleton by loss of E-cadherin might also play a role in increased expression of c-Jun protein. The increase in c-Jun protein might be through internal ribosomal entry site (IRES) mediated translation and not dependent on mitogen activated protein kinase (MAPK) pathway. A comprehensive and thorough study of glycome and its association with other molecular markers like E-cadherin, pEGFR, MMPs and c-Jun can contribute to understanding the pathway of oral carcinogenesis process. Moreover, a systematic study on glycosylation and its associated molecular markers may open the doors for multiple drug targeting to combat oral cancer.

Considering these aspects, the major objectives were to study:

- Glycoprotein electrophoretic profile in controls, patients with OPC and oral cancer patients.
- Serum and salivary sialylation (total sialic acid, α-2,3 and α-2,6 sialoproteins, sialidase activity, α-2,3 and α-2,6 sialyltransferease activity) and fucosylation changes (α-L-fucosidase activity, fucoproteins) in controls, patients with OPC, oral cancer patients and post-treatment follow-ups.
- Transcript levels of different subtypes of sialyltransferases and fucosyltransferases.
- MMP-2 and MMP-9 from blood and saliva in controls, patients with OPC, oral cancer patients.
- Associated molecular markers like E-cadherin, c-Jun and pEGFR and to correlate with glycosylation and MMPs, to understand the molecular mechanism of oral carcinogenesis.