Discussion

Individually we are one drop but together, we are an ocean – Ryunosuke Sataro

GLYCOPROTEIN PROFILING

In recent years, glycomics has emerged as a major field of interest in cancer research (Taniguchi *et al*., 2009; Hart *et al*., 2010; Hizal *et al*., 2014). Alterations in glycosylation machinery occurring as a consequence of abnormal glycosylation of proteins play a decisive role in malignant transformation. Also, protein-glycan interactions have became more amenable for therapeutic approaches, so that novel inhibitors of this interaction are currently in preclinical and clinical studies (Rek *et al*., 2009; Ghazarian *et al*., 2011). In glycoproteins, the branching chain of carbohydrates includes hexose (galactose, mannose, fucose), amino sugars (N-acetyl glucosamine, N-acetyl galactosamine) and sialic acid (N-acetyl neuraminic acid); which are linked to polypeptides. Elevations in one or more of these glycoconjugates have been shown to be related to tumor burden ([a]Shah *et al*., 2008; [b]Shah *et al*., 2008; Taneja *et al*., 2009). Since glycoproteins carry many carbohydrate chains, the signal they produce are highly amplified as compared to proteins, making them attractive candidates as biomarkers (Reis *et al*., 2010; Listinsky *et al*., 2011; Dall'Olio *et al*., 2012; Liu *et al*., 2014). Exploiting differences in glycoproteins in oral cancer patients and healthy subjects offer excellent opportunities to identify sensitive and specific biomarkers.

The subjects included in the study comprised of healthy controls, patients with OPC, oral cancer patients and post treatment follow-ups of oral cancer patients. The incidence of OPC continues to increase among the younger population. Therefore, the inclusion of patients with OPC (as pathological controls) in the current study established a link between normal and malignant conditions. We hypothesized that the inclusion of controls and 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. The statistical significance between different groups were calculated using unpaired *'t'* test. The significance of varying marker levels during favorable or poor treatment response was performed using paired *'t'* test. ROC curve is a meaningful statistical approach as it considers both sensitivity and specificity of the parameters. For ROC cut-off, uppermost and leftmost portion of the curve was selected as ideal cut-off value. The AUC of above

0.5 suggests valid discriminatory efficacy of the markers. As ROC curve is used to analyze diagnostic utility of the marker, ROC cut-off and Kaplan Meir's survival analysis was considered to evaluate its prognostic significance. The significance was calculated using Log rank Chi^2 test. Pearson's correlation analysis was performed to understand the correlation of various markers under study.

In spite of the progress made in genomics, proteomics and advanced glycoproteomics technologies, cancer is being missed at an early stage. Moreover, mass spectrometry and other advanced proteomics technologies are costly and sophisticated. There is a need for development of potential biomarkers that can monitor sequential changes in the oral cancer development. Earlier studies in our laboratory using polyacrylamide disc gel electrophoresis have indicated alterations in albumin, alpha and gamma regions of glycoproteins in patients with OPC and upper aerodigestive tract cancer (Patel *et al*., 1997). Further insight into the determination of molecular weights could give an idea of the characterization of specific glycoprotein bands. Native polyacrylamide gel electrophoretic separation of serum glycoproteins can provide a comprehensive view on various glycoproteins associated with cancer. Hence the present study explored the potentials of glycoprotein electrophoretic pattern in monitoring changes associated with neoplastic transformation in oral cancer.

In the present study, glycoprotein electrophoretic pattern depicted a significant increase in 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoprotein in patients with OPC and oral cancer patients when compared to the controls. Also, the levels were significantly higher in oral cancer patients as compared to patients with OPC. These results were also supported by ROC curve analysis which depicted significant discriminatory efficacy in distinguishing all the three groups. It has been reported earlier that the altered 44 kDa band in the current investigation was characterized to be α_1-acid glycoprotein (Glossman *et al*., 1971). Significantly elevated α1-antitrypsin, α_1-acid glycoprotein, haptoglobin have been reported in cancer patients (Baskies *et al*., 1980). The unusual glycoprotein "S" band found in our earlier studies (Patel *et al*., 1997) was also found in majority of tobacco consumers of all the groups in the present study and was characterized to be 230 kDa glycoprotein by molecular

weight marker analysis. In the present study, a 230 kDa consistently appeared in majority of the individuals with tobacco habits i.e. 57.8% of controls, 60% of oral cancer patients and 50% of patients with OPC. The absence among majority of non-habituates indicates that this 230 kDa glycoprotein is associated with alterations occurring due to tobacco consumption. An increasing trend in the expression of this 230 kDa band was observed from TH controls to TH patients with OPC to TH oral cancer patients, however the levels were non-significant. The increasing trend highlights the importance of 230 kDa glycoprotein in sequential changes due to tobacco consumption occurring during the neoplastic transformation. Moreover, it was observed that 5% of TNH controls and 4% of TNH oral cancer patients also showed the presence of this 230 kDa band. We hypothesize that owing to certain genetic polymorphism in carcinogen metabolizing enzymes, there is increasing susceptibility to certain carcinogens among the subjects and hence TNH individuals were showing the presence of 230 kDa band. These individual might be exposed to passive smoke or it is equally likely that tobacco history has been mentioned incorrectly by the subjects.

Earlier studies from our laboratory have indicated more number of glycoprotein bands in gamma region in patients with OPC and oral cancer patients (Patel *et al*., 1997). The gamma fraction of glycoproteins contains an appreciable amount of carbohydrates with higher amounts of sialic acid and fucose (Beckman *et al*., 1973). We have also reported that alterations in sialic acid, fucose and other glycoproteins are useful in diagnosis and treatment monitoring of oral cancer patients ([a]Shah *et al*., 2008; [b]Shah *et al*., 2008; Rajpura *et al*., 2005). There are numerous reports which indicate that α_1-acid glycoprotein, β_2-microglobin and other glycoproteins have great value as tumor markers (Chrostek *et al*., 2007; Shah *et al*., 2008; Drake *et al*., 2010; Zhang *et al*., 2014). Similarly, in the present study, we observed significantly elevated α_1-acid glycoprotein (44 kDa) in patients with OPC and oral cancer patients together with 192 kDa, 170 kDa, and 116 kDa glycoproteins. The differences in glycoprotein pattern in TNH and TH groups were also highlighted in the present study. A significant increase in 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins was observed in TNH and TH oral cancer patients when compared to TNH and TH

controls, respectively. Moreover, all the above mentioned glycoproteins were found to be higher in TH controls than in TNH controls. Earlier studies have also explored the role of tobacco in oral carcinogenesis (Castellsague *et al*., 2004; Lin *et al*., 2011). For further characterization of 230 kDa band, in-gel digestion was performed followed by SDS-PAGE. It revealed 3 bands corresponding to 139 kDa, 86 kDa and 61 kDa proteins. We assume that these 3 glycoproteins are involved in tobacco related changes taking place during oral carcinogenesis. However, further characterization by Mass spectrometry might aid in identification and function of these glycoproteins.

As there are no earlier reports on alterations of 192 kDa, 170 kDa 116 kDa and 230 kDa glycoproteins, further characterization of these glycoproteins by mass spectrometric analysis is required for identification of structure and function of these proteins. Recently, there has been considerable progress in the development towards an integrated proteomics and glycomics approach for finding cancer biomarkers (Zhang *et al*., 2008; Taylor *et al*., 2009; Liu *et al*., 2009; Goetz *et al*., 2009; Tian *et al*., 2010; Mann *et al*., 2010; Zeng *et al*., 2011). The development of methodologies for elucidating the structures and functions of glycoproteins has provided the opportunities to advance the glycomics approach.

SALIVARY BASED GLYCOMICS APPROACH

Salivary diagnostic tools is an effective non-invasive modality as an alternative to serum testing for detecting biomarkers in oral cancer (Jacob *et al*., 2011; Wong *et al*., 2012). Recently there is much advancement in salivary based biomarkers for detection of oral cancer (Shah *et al*., 2011; Yakob *et al*., 2014; Cheng *et al*., 2014). However, salivary glycomics has not been much explored in patients with OPC and oral cancer patients. This prompted us to monitor glycosylation changes from saliva. As alterations in membrane glycoproteins might also be involved in upregulating MMPs, the present study also aimed to estimate MMPs from saliva.

ALTERATIONS IN TSA/TP RATIO

The results depicted that serum and salivary levels of TSA/TP ratio were found to be significantly elevated in patients with OPC and oral cancer patients as compared to

the controls. The results of serum and salivary sialic acid in oral cancer patients were in accordance with several other studies (Joshi *et al*., 2010; Siddhartha *et al*., 2011; Kadam *et al*., 2011; Trivedi *et al*., 2012; Dhakar *et al*., 2013; Rasool *et al*., 2014; Dadhich *et al*., 2014). However, there are lack of reports on salivary TSA in patients with OPC and on comparison between serum and salivary levels. The results depicted an increasing trend of salivary TSA/TP ratio from controls to patients with early stage to advanced stage of disease. Serum and salivary TSA/TP ratio was found to be higher in patients with LN metastasis when compared to patients without LN metastasis. Joshi *et al*., (2010) indicated positive correlation of serum TSA levels with stage of malignancy, specifically with the tumor burden Also, it has been reported that serum TSA is important in prognosis and early detection of recurrence and metastasis (Suer Gokmen *et al*., 2004; Shah *et al*., 2008).

Madani *et al*., (2012) reported that gutkha, supari (areca nut), chewing tobacco, bidi smoking and mishiri (tobacco powder, applied as a tooth and gum cleaner) are independent risk factors for oral cancer in India where gutkha chewing is most prevalent among tobacco consumers. Earlier, it has been indicated that serum TSA concentration was significantly higher in users of smokeless tobacco products than those of control subjects (Kurtul *et al*., 2005). In the current investigation, majority of TH (38/50 controls, 31/50 OPC, 58/88) oral cancer patients were exclusive chewers and it was observed that serum and salivary TSA/TP ratio were higher in TH controls and oral cancer patients as compared to TNH controls and oral cancer patients, respectively. This might be due to tobacco associated changes which may also be correlated with molecular alterations in oral cancer progression. Moreover, the elevations in salivary levels of TSA/TP ratio as compared to serum, suggested better potentials of saliva in monitoring the changes associated with oral cancer development. The levels when assessed during post treatment follow-ups showed that serum and salivary levels were comparable between PT and CR while the levels were elevated in NR as compared to PT. The Kaplan Meir's survival analysis depicted lower overall survival with values above ROC cut-off of serum and salivary TSA/TP ratio and levels were significant for serum TSA/TP ratio. From literature search, we found that there are no earlier studies on correlation of TSA with overall survival. We

assume that increased levels of TSA/TP, might be involved in alterations of glycoproteins and metastatic spread of the disease, thus patients had lower overall survival.

ALTERATIONS IN SIALIDASE ACTIVITY

The present study revealed an increasing trend of serum and salivary sialidase activity from controls, to patients with OPC to oral cancer patients. Reuter *et al*., (1992) have reported elevated levels of sialidase activity in oral secretions of patients with upper aerodigestive tract tumors. Various other studies have observed higher sialidase activity in serum and tissues of various cancers (Sonmez *et al*., 1999; Miyagi *et al*., 2010; Miyagi *et al*., 2012). The results of ROC curve analysis depicted that serum and salivary sialidase activity significantly distinguished controls and oral cancer patients as well as patients with OPC and oral cancer patients. Serum sialidase activity also significantly distinguished controls and patients with OPC. Our screening from available data revealed elevation of plasma membrane sialidase Neu 3 in various cancers (Kakugawa *et al*., 2002; Ueno *et al*., 2006; Miyagi *et al*., 2008; Shiozaki *et al*., 2009; Li *et al*., 2011; Kawamura *et al*., 2011). However, to the best of our knowledge there are no earlier reports on evaluation of salivary sialidase activity in patients with OPC and oral cancer and also correlating with other sialylation changes.

It has been suggested that elevated levels of sialic acid in cancer patients is due to increased serum/tissue sialidase activity. Increased sialic acid activity also contributes to process of metastasis. Analysis in the present study depicted significant positive correlation between TSA and sialidase activity, which highlights the significance of increased sialidase activity which causes increased cleavage of sialic acid from sialoglycoconjugates. We observed higher levels of serum and salivary sialidase activities in patients with LN metastasis as compared to patients without LN metastasis. The results depicted an increasing trend of both serum and salivary sialidase activity from stage I to stage IV disease. Earlier studies have shown significant difference of serum and tissue sialidase activity between grade I, II and III tumors in breast cancer (Sonmez *et al*., 1999; Miyagi *et al*., 2012). Moreover, an increasing trend of serum sialidase activity was observed from well to moderate to

poorly differentiated tumors, in the present study. However, there are no earlier studies on serum and sialidase activity in oral cancer.

The oral cancer patients were followed after the anticancer treatment and results depicted that serum sialidase activity was significantly decreased in complete responders as compared to PT levels. While in case of non-responders the levels were found to be increased. Also, salivary sialidase activity was found to be decreased in CR as compared to PT levels while the levels were elevated in NR as compared to their corresponding PT levels. It was notable that marker levels increased prior to clinical detection of recurrence and decreased in patients with remission. Our literature survey revealed paucity of studies on serum and salivary sialidase activity in post-treatment follow-ups of oral cancer patients.

ALTERATIONS IN α-2, 3 AND α-2, 6 SIALOPROTEINS

We observed that serum and salivary α-2,6 sialoproteins were comparable between controls, patients with OPC and oral cancer patients. While an increasing trend of serum and salivary α-2,3 sialoproteins was observed from controls to patients with OPC to oral cancer patients and levels were significantly elevated in oral cancer patients as compared to the controls. Also ROC curve analysis revealed significant discriminatory efficacy between controls and oral cancer patients. Previous studies from our laboratory have indicated higher serum and tissue α-2,3 sialoproteins in patients with OPC and oral cancer patients ([a]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. In the present study, ROC curve analysis revealed that salivary α-2,3 sialoproteins significantly discriminated controls and patients with OPC. Also, salivary α-2,6 sialoproteins significantly distinguished patients with OPC and oral cancer patients. Various investigators have observed alterations in tissue and serum sialylated glycoproteins in oral cancer patients as well as other other malignancies (Yamamoto *et al*., 1997; Dall'Olio *et al*., 2004; [a]Shah *et al*., 2008; Tian *et al*., 2012; Shetty *et al*., 2012). Recently high-throughput method for quantitative analysis of N-linked sialoglycoproteins 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). Earlier reports have indicated aberrantly sialylated N-linked glycopeptides which have the potential to serve as biomarkers for ovarian cancer (Shetty *et al*., 2012). There is dearth of reports on simultaneous evaluation of salivary α-2,3 and α-2,6 sialoproteins in patients with OPC, oral cancer patients and also during post-treatment follow-up of oral cancer patients.

The results on serum and salivary α-2,3 and α-2,6 sialoproteins when compared between early and advanced stage revealed increased expression of serum and salivary α-2,3 sialoproteins in advanced stage of disease as compared to early stage of disease. These results signify increased sialylation of glycoproteins during advanced stage of disease. The linkage specific sialoprotein alterations have not been earlier correlated with stage of disease.

The results depicted that the salivary levels of α-2,3 and α-2,6 sialoproteins were found to be decreased in CR as compared to PT levels, which assess its usefulness in revealing of remission. Kaplan Meir's survival analysis revealed no significant association of serum and salivary α-2,3 and α-2,6 sialoproteins with overall survival. Tian *et al*., (2012) have observed association of sialyloglycoconjugates with unfavorable outcome and worse prognosis in breast cancer; however, it has not been explored in oral cancer.

ALTERATIONS IN α-2,3 AND α-2,6 ST ACTIVITIES

Earlier, we have observed alterations in enzyme activities of α-2,3 and α-2,6 STs in serum of oral cancer patients ([a]Shah *et al*., 2008). We hypothesized that same could be translated to saliva as well. Therefore, the present study investigated salivary α-2,3 and α-2,6 STs and also compared with serum levels. In the present investigation, an increasing trend of serum and salivary α-2,3 and α-2,6 ST was observed from controls to patients with OPC to oral cancer patients. Also the levels were found to be significantly elevated in saliva as compared to serum for both α-2,3 and α-2,6 ST. The levels of salivary α-2,6 ST activity were found to be significantly higher in oral cancer

patients as compared to controls. ROC curve depicted that serum α-2,6 ST significantly distinguish controls and oral cancer patients. Moreover, serum α-2,6 ST also significantly discriminated patients with OPC and oral cancer patients. Several studies have documented de-regulated expression of serum/tissue STs enzyme activities in various cancers like colorectal, liver, gliomas, oral cancer etc. (Yamamoto *et al*., 1997; Ito *et al*., 1997; Recchi *et al*., 1998; Burchell *et al*., 1999; Schneider *et al*., 2001; Dall'olio *et al*., 2001; Dall'Olio *et al*., 2004; Vasquez-Martin *et al*., 2004; [a]Shah *et al*., 2008). To the best of our knowledge there are no prior studies on salivary linkage specific ST in patients with OPC and oral cancer.

Earlier studies have documented higher serum/tissue ST activity in metastasis of cervix and colon cancer (Gessner *et al*., 1993; Wang *et al*., 2002). In agreement with these studies, we observed higher levels of salivary levels of α-2,3 ST in patients with metastasis as compared to the patients without metastasis and it was also higher in advanced stage of the disease as compared to early stage of the disease. We hypothesize that increased α-2,3 ST might be involved in increased sialylation during advanced disease.

It was observed that the levels of serum and salivary α-2,6 ST along with salivary α-2,3 ST were found to be significantly decreased in CR as compared to PT levels. The result highlights the importance of STs in treatment monitoring of oral cancer patients during follow-ups. The results further strengthened our earlier studies that reported significance of serum sialylation changes in treatment monitoring of oral cancer patients ([a]Shah *et al*., 2008) In the present study, significantly lower levels of α-2,3 sialoproteins in PT and NR samples were observed, while levels were markedly higher of α-2,3 ST activity in these samples. It is our assumption that sialidase activity is increased much more than ST activity which is responsible for the decreased expression of sialoproteins. This might be due to increased break down of sialic acid from sialoproteins by sialidase enzyme.

CORRELATION BETWEEN SIALYLATION PARAMETERS

Pearson's correlation analysis was performed to assess the correlation between various serum and salivary sialylation changes. It was observed that serum α-2,6 sialoproteins were positively correlated with serum α-2,6 ST. This indicated that increase in sialoproteins might be due to the increase in ST activity.

Serum α-2,3 ST and α-2,6 ST were found to be negatively associated with sialidase activity. Serum TSA/TP ratio was positively associated with sialidase activity and negatively associated with α-2,3 ST and α-2,6 ST activities. Salivary TSA/TP ratio was significantly positively correlated with sialidase activity. Moreover, a significant negative correlation was seen between salivary α-2,6 ST activity and sialidase activity. Also, salivary α-2,3 ST was negatively correlated with sialidase activity. We hypothesize that increased sialidase activity might cause increased breakdown of sialic acid from sialoproteins. Moreover, increased breakdown of sialic acid might also be correlated with decreased ST activity. Hence, increased sialidase activity might be correlated with decreased ST activity, which was observed in the present study. It was noteworthy that sialylation increased prior to clinical detection of recurrence and decreased in patients with remission. It was evident that salivary sialylation changes are reflected in patients with OPC, oral cancer patients and also during anticancer treatment. Thus, close monitoring of salivary sialylation changes may be a promising non-invasive approach to assess disease progression and treatment outcome during post-treatment follow-up.

ALTERATIONS IN FUCOPROTEINS

Exploring the structure and functions of carbohydrates of glycoproteins using lectins has contributed tremendously to the advancement of glycobiology (Montreuil *et al*., 1997). Increased fucose levels may lead to altered or unique glycoconjugates. In the current study, fucoprotein analysis by means of fucose specific lectin (*Lotus tetragonolobus*) revealed an increasing trend of both serum and salivary 44 kDa fucoprotein from controls, to patients with OPC to oral cancer patients. Salivary fucoprotein levels were found to be significantly higher in oral cancer patients as

compared to controls. The results were also strengthened by ROC curve analysis which indicated that salivary fucoprotein levels significantly discriminated between controls and oral cancer patients. Our results were in accordance with a previous study which has reported that 44 kDa fucoprotein is a haptoglobin, which was also observed to be increased in serum of cancer patients (Thomson *et al*., 1987).

The levels of fucoproteins were also assessed in follow-up samples and were compared with the PT values. The analysis of serum and salivary fucoproteins in follow-up samples revealed decreased expression in CR as compared to PT levels while levels were higher in NR as compared to PT levels. The data were consistent with our earlier studies from serum ([b]Shah *et al*., 2008). However, salivary estimation of fucoproteins has not been explored earlier in post-treatment follow-up of oral cancer patients.

The levels of serum and salivary 44 kDa fucoprotein were higher in advanced stage of disease as compared to early stage and levels were significant for saliva. Multivariate analysis showed significant association of salivary fucoprotein with stage of the disease. Various investigators have reported increased fucosylation of serum and tumor glycoproteins as a prognostic marker in patients with cancer that reflected tumor burden (Thomson *et al*., 1987; Shirahama *et al*., 1993; [b]Shah *et al*., 2008), which strongly support the current results. Moreover it was observed that serum and salivary fucoprotein levels were decreased in early stages as compared to controls. We assume that during early stage of disease α-L-fucosidase activity might be much more than fucosyltransferase activity, which resulted in increased breakdown of fucoproteins, and hence decreased in early stage of disease.

ALTERATIONS IN α-L-FUCOSIDASE ACTIVITY

Our earlier studies have observed significant clinical utility of serum α-L-fucosidase activity in monitoring of oral carcinogenesis ([b]Shah *et al*., 2008). As saliva is in direct contact with the lesions, and also there is lack of information on salivary α-L-fucosidase activity, it was estimated in patients with OPC and oral cancer patients. Moreover, study also aimed to compare serum and saliva levels. In the present study, serum α-L-fucosidase activity was found to be significantly higher in oral cancer

patients and patients with OPC as compared to the controls. Salivary α-L-fucosidase activity was found to be significantly higher in oral cancer patients as compared to controls. Moreover, an increasing trend of both serum and salivary α-L-fucosidase activity was observed from controls to patients with OPC to oral cancer patients. The ROC curve analysis results revealed that both serum and salivary α-L-fucosidase activity could significantly distinguish controls and oral cancer patients as well as controls and patients with OPC. Salivary α-L-fucosidase activity also significantly distinguished patients with OPC and oral cancer patients. Other investigators have found association of increased serum/tissue α-L-fucosidase with colorectal, endometrial, breast, cervical, ovarian, hepatocellular carcinomas and oral cancer (Wang *et al*., 1995; Abdel-Aleem *et al*., 1996; Giardina *et al*., 1998; Ayude *et al*., 2003; [b]Shah *et al*., 2008). There is paucity of data on comparison of serum and salivary α-L-fucosidase activity in TNH and TH sub-groups. Therefore, the serum and salivary α-L-fucosidase activity were compared between TNH and TH sub-groups. The results showed an increasing trend of both serum and salivary α-L-fucosidase activity from TNH to TH from controls to patients with OPC and further to oral cancer patients.

It was observed that serum and salivary α-L-fucosidase activities were higher in advanced stage of the disease as compared to early stage and were also higher in patients with LN metastasis as compared to patients without LN metastasis. Moreover, both serum and salivary levels were significantly higher in early and late stage of the malignant disease as compared to the controls. Also serum and salivary levels were significantly higher in patients with and without LN metastasis as compared to the controls. The results were in accordance with previous reports that documented increased levels of serum α-L-fucosidase activity in cancer which was correlated with stage of disease (Wei *et al*., 2000; Ayude *et al*., 2003; [b]Shah *et al*., 2008).

Serum α-L-fucosidase activity in CR decreased significantly as compared to the corresponding PT value. In supportive of our results, previous investigations have reported decreased levels of serum α-L-fucosidase activity after treatment in cancer

(Wei *et al*., 2000; [b]Shah *et al*., 2008). It was observed that values above ROC cut-off of serum α-L-fucosidase activity were significantly associated with lower overall survival. Ayude *et al*., (2003) have observed significant clinical utility of serum α-L-fucosidase activity in diagnosis, prognosis and early detection of recurrence as well as management of colorectal cancer. However, there is dearth of report on correlation with overall survival in oral cancer patients.

CORRELATION BETWEEN FUCOPROTEINS AND α-L-FUCOSIDASE ACTIVITY

By Pearson's correlation analysis, significant negative correlation between salivary α-L-fucosidase activity and salivary fucoproteins was observed. We hypothesize that α-L-fucosidase activity might cause increased breakdown of fucose from fucoproteins, and hence increased α-L-fucosidase activity might cause decrease in fucoproteins, and thus was negatively correlated. Moreover, our results depicted that serum and salivary fucoprotein levels were decreased in early stages as compared to controls. As we have observed significant negative correlation, we assume that during early stage of disease α-L-fucosidase activity might be much more than fucosyltransferase activity, which resulted in increased breakdown of fucoproteins, and hence decreased in early stage of disease of disease.

ST3GAL1, FUT3, FUT5 AND *FUT6* mRNA EXPRESSION

Carbohydrate determinants expressed preferentially on cancer cells contain sialylated and/or fucosylated structures. Synthesis of these sialylated and/or fucosylated carbohydrate determinants in cancer is regulated by a set of ST and FUT (Hanson *et al*., 1998; Homes *et al*., 1985). Alterations in enzyme activity of α-2,3 and α-2,6 STs and FUT have been reported in serum of oral cancer patients ([a]Shah *et al*., 2008; [b]Shah *et al*., 2008). However, there are no earlier reports on mRNA levels of different subtypes of *ST* and *FUT* in oral cancer. Therefore, the present study aimed to evaluate *ST3GAL1*, *FUT3, FUT5* and *FUT6* transcripts in malignant and adjacent normal tissues of oral cancer patients.

***ST3GAL1* mRNA expression:** The present study demonstrated an increase in *ST3GAL1* mRNA levels in malignant oral cancer tissues as compared to adjacent normal tissues. Enhanced *ST3GAL1* expression has been observed in various malignancies (Dall'olio *et al*., 2001; Zhu *et al*., 2001; Wang *et al*., 2005; Videria *et al*., 2009; Picco *et al*., 2010). It was hypothesized that the elevation of α-2,3 ST enzyme activity observed in oral cancer patients in the present study, might be due to increase in *ST3GAL1* expression. *ST3GAL1* and/ or S*T3GAL2* transcript levels along with enzyme activity of α-2,3ST have been reported to be significantly increased in colorectal, cervical and breast carcinomas (Burchell *et al*., 1999; Schneider *et al*., 2001; Lopes-Morales *et al*., 2009). On the other hand, Wang *et al*., (2001) have found down regulation of *ST3GAL1* expression along with increased *ST6GAL1* expression in squamous cell carcinoma of the cervix.

Our results indicated that *ST3GAL1* mRNA levels were higher in metastatic tumors as compared to the non-metastatic tumors of oral cancer patients. Schneider *et al.,* (2001) have shown that *ST3GAL1* mRNA expression was found to be significantly increased in cases showing invasion of lymph vessels in colorectal cancer. In the present study, *ST3GAL1* mRNA expression was found to be elevated in advanced disease as compared to the early disease. Moreover, multivariate analysis showed significant association of *ST3GAL1* mRNA levels with tumor infiltration. Earlier study by Wang *et al.*, (2005) have reported no correlation of *ST3GAL1* and *ST6GAL1* transcripts with stage, differentiation, amount of ascites and serum levels of CA125 in ovarian cancer (Wang *et al*., 2005). However, *ST3GAL1* transcript levels have not been explored earlier in oral cancer patients.

***FUT3, FUT5* and *FUT6* mRNA expression:** There are different types of *FUT* genes involved in synthesis of various tumor antigens. *FUT3* predominantly exhibits α-1,4 FUT activity synthesizing tumor antigens Lewis^a (Le^a), Sialyl Lewis^a (SLe^a) and Le^b, and a minor of 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). There are mixed reports on alterations of *FUTs* genes in various neoplastic diseases. However, *FUT* transcripts have not been explored earlier

in oral cancer patients. We observed that the expressions of *FUT3* and *FUT5* transcripts were found to be significantly decreased in malignant oral cancer tissues as compared to adjacent normal tissue. Hanski *et al*., (1996) have reported that human colon carcinomas showed equal or even lower expression of *FUT3* mRNA than normal mucosa. In colon cancer, *FUT3* and *FUT6* transcripts were not significantly altered while *FUT6* showed moderate increase in cancer tissues when compared to adjacent non-malignant colonic epithelia (Ito *et al*., 1997). In the current study, *FUT6* transcripts were comparable between oral cancer tissues and adjacent normal tissues, while moderate increase was observed in metastatic and advanced stage tumors as compared to non-metastatic and early stage tumors, respectively. We predict that a significant decrease in *FUT3* and *FUT5* transcripts observed in the present study, might be causing up-regulation of other subtypes of *FUT* transcripts, which may be further involved in increasing FUT enzyme activity. Moreover, survival analysis depicted that levels above cut-off of *FUT3* transcripts were significantly associated with lower survival of patients. It is expected that, in patients with values above cut-off of *FUT3* transcripts, there is increased production of SLe^a expression which is known to be involved in metastasis and aggressive behavior of disease. Earlier, increased expression of SLe^a has been observed in metastatic breast cancer (Renkonen *et al*., 1997). Increased *FUT7* expression has been reported to be associated with survival of patients in lung carcinoma (Ogawa *et al*., 1997). Hiraiwa *et al*., (1995) have shown increase in *FUT3*, *FUT6* and *FUT7* in colon cancer tissues along with increase in SLe^a antigen. Increase in *FUT4* has been observed in colorectal adenomas and carcinomas (Petretti *et al*., 2000; Kudo *et al*., 1998). Increased expression of *FUT5* and *FUT6*, and decreased expression of *FUT4* expression has been observed earlier in gastro intestinal carcinoma cells (Carvalho *et al*., 2010). *FUT6* expression has been shown to be involved in SLe^X expression on breast cancer cells (Matsura *et al*., 1998). The present study depicted higher expression of *FUT6* in metastatic and advanced tumors, which has been known to be involved in upregulation of SLe^X in metastatic disease. Earlier studies from our laboratory have indicated increased SLe^X in oral cancer and depicted a significant positive correlation with advanced stage of disease and metastasis (Shah *et al*., 2009). Trinchera *et al*., (2011) have reported an

increase in *FUT6* in colon cancer and have observed that its knockdown caused decrease in *FUT6* mRNA and inhibition of SLe^X expression. Our results demonstrated comparable levels of *FUT3* and *FUT5* transcripts between metastatic and non-metastatic tumors. Petretti *et al.,* (2000) showed that *FUT3* was less expressed in carcinomas exhibiting distant metastasis and in highly invasive tumors. The results warranted evaluation of other sub-types of *FUTs* and *STs* genes, which might give deeper insights into involvement of specific subtype of *FUTs* and STs in oral cancer pathogenesis. In addition, the correlation of *FUT3*, *FUT5* and *FUT6* transcripts and its associated molecules like SLe^X / SLe^a might elaborate the involvement of specific subtypes in oral carcinogenesis.

MATRIX METALLOPROTEINASES (PLASMA)

As per recent reports, metastatic potential of tumors are established at precancerous stage (Shrestha *et al*., 2014; Al-Rawi *et al*., 2014). Moreover, our earlier studies have observed loss of E-cadherin in patients with OPC. As MMPs being crucial to loss of E-cadherin protein, understanding MMPs in precancerous conditions might help in predicting metastatic behavior of the disease. The results of the present investigation depicted significantly higher levels of plasma pro, active and total MMP-2 and MMP-9 in patients with OPC as compared to controls. Active MMP-2 was observed to be significantly higher in oral cancer patients as compared to patients with OPC. Recent studies have demonstrated that MMPs play a role in several steps during carcinogenesis (Yadav *et al*., 2014). Increase in the expression of plasma MMP-2 from normal oral mucosa to dysplasia to oral cancer has been reported during oral carcinogenesis (Sutinen *et al*., 1998; Shrestha *et al*., 2014). Al-Rawi *et al*., (2014) have shown significant immunoreactivity of MMP-2 and MMP-9 in oral lichen planus. We demonstrated significantly higher levels of plasma active MMP-2, pro and active MMP-9, total MMP-2 and MMP-9 in oral cancer patients as compared to controls. The results were consistent with our previous data which depicted upregulation of MMP-2 and MMP-9 in oral cancer (Patel *et al*., 2005; Singh *et al*., 2010). An increase in tissue/plasma MMPs have been also observed in various

cancers like pancreas, lung, bladder, colorectal, ovarian, prostate, brain, oral etc. (Roy *et al*., 2009; Singh *et al*., 2010; Tao *et al*., 2014).

Higher levels of plasma pro MMP-2, active MMP-2, pro MMP-9, total MMP-2 and total MMP-9 were observed in advanced stage as compared to early stage of disease and levels were significant for pro MMP-2. These results indicated role of MMPs in tumor invasion and progression to advanced disease. Previous investigators have shown that higher expression of tumor and stromal MMP-2 and MMP-9 expression was significantly associated with positive lymph node metastasis (Fan *et al*., 2012; Zhang *et al*., 2012). In the current study, an increasing trend of plasma active MMP-2, pro MMP-9, active MMP-9, total MMP-2, total MMP-9 from well to moderate to poorly differentiated tumors was observed. Hong *et al*., (2006) have documented a close correlation of MMP-2 with differentiation, depth of invasion, lymph node metastasis and distant metastasis. Survival analysis depicted that values above ROC cut-off of plasma active MMP-2, pro MMP-9, total MMP-2 and total MMP-9 had lower overall survival as compared to those with values below ROC cut-off. This indicated that patients with values above cut-off represent metastatic spread of disease, and thus had lower overall survival. Fan *et al*., (2012) have demonstrated significant decreased overall survival in patients with high tumor and stromal MMP-2 and MMP-9 expression in tongue cancer.

MATRIX METALLOPROTEINASES (SALIVA)

Gelatin zymography is a cost effective technique which detects both pro and active forms of gelatinases. There is a dearth of study on salivary MMPs in patients with OPC and oral cancer. An increasing trend of salivary pro and active MMP-9 was observed from controls to patients with OPC to oral cancer patients. Earlier studies have indicated elevated levels of tissues MMP-9 in benign, premalignant and malignant laryngeal lesions (Peschos *et al*., 2006). Also, salivary MMP-9 levels have been examined in patients with periodontitis (Goncalves *et al*., 2009) and they also report bands corresponding to higher molecular mass complexes as well as low molecular sized components; representing truncated MMP species. Miyoshi *et al*., (2010) have suggested that 42 kDa MMP is the truncated form of MMP which is

expressed due to autoactivation of various other gelationolytic MMPs. In the present investigation, salivary protease activity was observed to be very high in saliva. Hence, a truncated species of 42 kDa MMP was observed which might be due to proteolytic activities of other MMPs. High molecular weight gelatinases and truncated lower molecular weight species of gelatinases were also observed by Ingman *et al*., (1994) in periodontitis patients, which represented *in-vivo* proteolytically activated truncated enzymes. Similarly in the present investigation, a truncated species of 42 kDa was observed and an increasing trend from controls to patients with OPC to oral cancer patients was observed. The levels of pro MMP-9, active MMP-9 and truncated 42 kDa MMP were found to be significantly higher in oral cancer patients as compared to the controls. Earlier investigators have reported that levels of MMP-9 were elevated after areca quid chewing in healthy volunteers and in oral cancer patients who were betel quid users (Liu *et al*., 2005; Chiu *et al*., 2008). However, no significant difference of pro and active MMP-9 was observed when compared between tobacco habituates and non-habituates sub-groups in the present investigation. Moreover, a band of 125 kDa was observed which possessed gelatinolytic activity. Triebel *et al*., (1992) reported that MMP-9 is associated with 25 kDa protein (microglobulin), giving a band at 125 kDa.

The results were compared between patients with metastasis and without metastasis to understand the role of gelatinases in metastatic spread of the disease. It was observed that salivary pro MMP-9, active MMP-9 and truncated MMP 42 kDa levels were higher in patients with LN metastasis and advanced disease as compared to patients without LN metastasis and early disease, respectively. The results confirmed the role of gelatinases in tumor invasion and metastasis, which was detected in saliva as well. However, there is scarcity of reports on salivary MMPs correlating with metastatic behavior of the disease. The results indicated significant involvement of salivary MMPs (gelatinases) in sequential changes occurring during oral cancer progression and metastatic spread.

E-CADHERIN: PROTEIN AND mRNA EXPRESSION

Western blot analysis of E-cadherin revealed significant increase of truncated 97 kDa E-cadherin protein in malignant tissues as compared to adjacent normal tissues. Rashid *et al*., (2001) have demonstrated significant increase of 97 kDa, a truncated form of mature 120 kDa E-cadherin in prostate cancer. Various studies have observed diminished E-cadherin levels in oral cancer (Thomas *et al*., 2001; Diniz-Freitas *et al*., 2006; Vered *et al*., 2012; Zhao *et al*., 2012; Luo *et al*., 2014; Wong *et al*., 2014). Earlier studies from our laboratory have reported loss of E-cadherin and an increase in 97 kDa E-cadherin protein in patients with OPC and oral cancer tissues (Shah *et al*., 2009). Disruption of E-cadherin has been documented to be a hallmark of epithelial-mesenchymal transition (EMT), a phenomenon which occurs at certain stages of normal development and in the malignant progression of carcinoma (Gonzalez-Moles *et al*., 2012; Gheldof *et al*., 2013; Scanlon *et al*., 2013; Adhikari *et al*., 2014). Previous investigations have reported decreased E-cadherin levels in oral leukoplakia and OSCC by immunohistochemical analysis (De Freiltas Silva *et al*., 2013; Von Zeidler *et al*., 2014). Moreover, a significant loss was observed from normal to hyperplasia to dysplasia to OSCC (Kaur *et al*., 2013; Yuwanati *et al*., 2011). Earlier immunohistochemical analysis has shown no significant difference of E-cadherin in SCC of lower lip and tongue (Cruz *et al*., 2009).

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). The ectodomain shedding of E-cadherin have been suggested to play an active part in the invasive process during tumour progression (Navrocki-Raby *et al*., 2003; Kuefer *et al*., 2003). In the present study, the levels of truncated E-cadherin protein (97 kDa) were found to be higher in advanced stage of the disease as compared to early stage of the disease. The levels of truncated E-cadherin protein were found to be increased in infiltrative tumors as compared to non-infiltrative tumors and were also significantly higher in metastatic tumors as compared to non-metastatic tumors. The results were consistent with our earlier studies, in which a positive correlation of 97 kDa truncated E-cadherin protein was observed with

advanced stage and lymphnode metastasis (Shah *et al*., 2009). Rosado *et al*., (2013) by immunohistochemical analysis have documented a significant association of E-cadherin with histological grade in OSCC and Zhi *et al*., (2004) have documented correlation of reduced expression of E-cadherin with LN metastatic tumor of nasopharyngeal carcinoma.

In the present investigation, the levels of *ECAD* mRNA were analyzed by semiquantitative RT-PCR using *ECAD* specific primers and levels were normalized to *β-ACTIN* expression. The results revealed that the *ECAD* mRNA levels were comparable between malignant and adjacent normal tissues, which have not been explored earlier in oral cancer patients. No significant difference was also observed between normal and tumor tissues in breast cancer patients by Goyal *et al*. (Goyal *et al*. 2008). Lower E-cadherin mRNA and protein levels have been reported in colorectal cancer (Garinis *et al*., 2003).

We observed lower *ECAD* mRNA expression in infiltrating tumors as compared to non-infiltrating tumors. Multivariate analysis showed significant association of *ECAD* mRNA with differentiation and stage of disease. A decreasing trend of *ECAD* mRNA levels was observed from well to moderate to poorly differentiated tumors. The decreased *ECAD* mRNA levels in infiltrating, advanced stage and poorly differentiated tumors signifies decreased E-cadherin protein which may be formed after translation, during metastatic disease. Moreover, in the present study, significant increased expression of truncated 97 kDa E-cadherin protein was observed in malignant oral cancer tissues, which signifies decrease in E-cadherin protein levels. Earlier studies have reported that transcript levels of *ECAD* decreased significantly in metastatic tumors as compared to non-metastatic tumors in nasopharyngeal carcinoma (Zhi *et al*., 2004; Kaur *et al*., 2009). In the present study, Kaplan Meir's survival analysis of *ECAD* mRNA depicted lower survival with values below ROC cut-off. Earlier studies by Dorudi *et al*., (1995) have documented that colorectal cancer patients with survival rate of 5 years and longer exhibited significantly higher levels of *ECAD* mRNA than those surviving less than 5 years. Earlier studies in various types of carcinomas, including OSCC, have revealed reduced expression of E-

cadherin protein which was associated with poor prognosis (Popov *et al*., 2000; Berx *et al*., 2009; Luo *et al*., 2014), however its mRNA levels were not studied.

CJUN: mRNA AND PROTEIN EXPRESSION

In the present study, the levels of *CJUN* mRNA were comparable between paired malignant and adjacent normal tissues. Our screening from available data revealed that *CJUN* mRNA expression has not been explored earlier in oral cancer patients. Recent cell lines based studies have revealed that there was no change in *CJUN* mRNA levels in glioblastoma and melanoma, but there was an increase in translatability of *CJUN* transcript which was suggested to be due IRES mediated translation (Splanger *et al*., 2011; Blau *et al*., 2012). It was observed that *CJUN* mRNA levels were observed to be higher in early disease as compared to advanced disease, in the present study. We hypothesize that this increase in *CJUN* mRNA expression might be due to MAPK pathway. However, during later stage of disease due to subsequent loss of E-cadherin by MMPs, the increase in c-Jun protein is by IRES mediated translation (Splanger *et al*., 2011; Blau *et al*., 2012), hence no change in *CJUN* mRNA was observed between malignant and adjacent normal tissues. To the best of our knowledge, there are no earlier studies on simultaneous evaluation of *CJUN* mRNA and protein in oral cancer.

We observed that the levels of c-Jun protein were found to be significantly higher in malignant tissues as compared to adjacent normal tissues. It has been reported that c-Jun protein expression was increased in oral cancer tissues as compared to adjacent normal (Vairaktaris *et al*., 2007; Mishra *et al*., 2010). De Sousa *et al*., (2002) have shown that nuclear localization of c-Jun protein was linked to development of oral cancer. Previous studies have shown predominant expression of activated c-Jun at the invasive front and metastasis of breast cancer which was associated with proliferation and angiogenesis (Vleugel *et al*., 2006; [a]Zhang *et al*., 2007; [b]Zhang *et al*., 2007). In the present study, the values of c-Jun protein above ROC-cutoff were significantly associated with reduced overall survival. The results indicated that increased c-Jun protein might contribute to increased cellular proliferation and malignant transformation, and therefore the patients with increased c-Jun expression exhibited

lower overall survival. In accordance with our data, earlier studies have reported increased positive c-Jun staining which served as a marker for worse prognosis in OSCC (Kuo *et al*., 2006). Decreased overall survival observed in the present study, in patients with values above cut-off indicated that c-Jun may serve as a predictive marker for evaluating prognosis of oral cancer patients and may serve as an important therapeutic drug target for future interventions.

EXPRESSION OF pEGFR

It was observed that the expression of pEGFR was significantly higher in malignant tissues as compared to adjacent normal tissues. Abnormal amplification of EGFR gene has been observed widely in various human tumors including oral squamous cell carcinoma (Sarkis *et al*., 2010; Masuda *et al*., 2012; Kobayashi *et al*., 2013; Mahendra *et al*., 2014; Ribeiro *et al*., 2014; Pannone *et al*., 2014). EGFR expression in premalignant lesions has been documented to be useful for predicting the neoplastic potential of dysplastic tissues. Mechanisms leading to constitutive activation of EGFR include increased production of ligands, elevated levels of EGFR protein, EGFR mutations and defective down-regulation of EGFR (Zandi *et al*., 2007).

In the present study, Kaplan Meir's survival analysis indicated that pEGFR levels with values above ROC-cut-off were associated with lower overall survival. Supporting this result, Chen *et al*., (2003) have observed positive correlation of EGFR over-expression with reduced overall survival in oral cancer. Increased EGFR expression has been found to be correlated with poor survival in various cancer including gastric, colorectal and glioblastoma cancer (Gamboa-Dominguez *et al*., 2002; Spano *et al*., 2005; Choi *et al*., 2013; Pannone *et al*., 2014).

The current investigation revealed increased expression of pEGFR in advanced stage and metastatic tumors as compared to early stage and non-metastatic tumors, respectively. Earlier studies have reported that EGFR expression was significantly associated with stage of disease (Chen *et al*., 2003; Spano *et al*., 2005). However, Sarkis *et al*., (2010) in OSCC observed no correlation of EGFR positive immunoreactivity with clinicopathological parameters. A study by 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. Since pEGFR overexpression was also observed in malignant oral cancer tissues in the present study, targeting EGFR may be a promising approach to combat the disease.

COMPREHENSIVE ROLE OF GLYCOSYLATION, MMPs, pEGFR, TRUNCATED E-CADHERIN, AND c-JUN EXPRESSION IN ORAL CARCINOGENESIS

The result depicted significant positive correlation of serum α-2,6 sialoproteins, α-2,6 ST and α-L-fucosidase activity with plasma MMPs. Moreover, salivary α-2,6 sialoproteins, α-2,3 sialoproteins were positively correlated with salivary MMPs. To the best of our knowledge, there are no previous reports on correlation of sialylation or fucosylation alterations with MMPs. Hence, supporting our hypothesis, we observed that increased glycosylation is involved in alteration of glycoproteins and thus is contributing to metastasis with corresponding increase in MMPs.

Loss of E-cadherin mediated cell-cell adhesion plays a key role in metastasis. Recently, *in vitro* studies have documented that loss of E-cadherin mediates increase in c-Jun protein with no corresponding increase in *CJUN* mRNA in melanoma and glioblastoma (Splanger *et al*., 2011; Blau *et al*., 2012). As MMPs play a crucial role in disruption of cell-cell adhesion, the present study was designed for simultaneous evaluation of E-cadherin and c-Jun, protein and mRNA expression along with MMPs in oral cancer patients. Our literature search revealed that there is lack of studies evaluating all these parameters simultaneously in oral cancer.

We depicted a significant positive correlation between truncated E-cadherin protein and *CJUN* mRNA. Moreover, *ECAD* mRNA was observed to be positively correlated with *CJUN* mRNA. However, there are paucity of reports on simultaneous evaluation of mRNA and protein expression of E-cadherin and c-Jun in oral cancer. Knirsh *et al*., (2009) have observed that loss of E-cadherin mediated cell-cell adhesion is responsible for upregulation of c-Jun protein, which might be due to IRES mediated translation. The present study revealed positive correlation of truncated E-cadherin protein with c-Jun protein. The results supported our hypothesis that increased

truncation of E-cadherin protein might be responsible for upregulation of c-Jun protein in oral cancer. Earlier studies have demonstrated that endogenous c-Jun enhances cell growth, invasion, and tumor stem cell expansion (Jiao *et al*., 2010). As we have observed no change in *CJUN* mRNA, we postulated that an increase in c-Jun protein observed in present study might be due to IRES mediated translation.

We observed significant positive correlation of truncated E-cadherin protein with plasma pro MMP-2, plasma active MMP-2, plasma active MMP-9, plasma total MMP-2 and activation ratio MMP-2. Similarly previous studies have simultaneously depicted reduced E-cadherin and increased MMP-9 in SCC of head and neck (You *et al*., 2012). Cavallaro *et al*., (2004) demonstrated that overexpression of E-cadherin in invasive bronchial tumour cell lines is responsible for decreased invasiveness and reduced expression of MMP-1, MMP-3, MMP-9 and MT1-MMP. Hence, the results overall suggested that MMPs play an important role in disruption of cell-cell adhesion by loss of E-cadherin protein, which is subsequently responsible for increase in c-Jun protein in oral cancer.

The present study exhibited that pEGFR expression was positively correlated with truncated E-cadherin protein. From literature search we found that reduction of E-cadherin results in upregulation of EGFR transcriptionally in head and neck cancer (Pidone *et al*., 2014). Supporting this data, we hypothesize that increase in truncation of E-cadherin might be upregulating pEGFR expression, as observed in the present study. It has been documented 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). Mutation in E-cadherin has been reported to cause increased EGFR activation and reduced E-cadherin- EGFR association (Mateus *et al*., 2007; Bremm *et al*., 2008). In contrast, formation of E-cadherin–mediated cell-cell adhesion has been shown to activate EGFR in various experimental settings (Pece *et al*., 2000; Reddy *et al*., 2005; Gavard *et al*., 2008). Earlier reports have suggested several classes of receptor tyrosine kinases, including EGFR, Her2-neu, insulin-like growth factor 1 receptor (IGF-1R) and c-Met, can inhibit E-cadherin–dependent adhesion when they induce epithelial-mesenchymal transition (Fujita *et al*., 2002; Lu *et al*. 2003; Thiery *et al*., 2003). The regulation is

bidirectional, as E-cadherin can in turn inhibit activation of EGFR, Her2-neu, IGF-1R and c-Met (Andl *et al*., 2005). It was observed that *CJUN* mRNA levels were increased in early disease as compared to advanced stage of disease. Moreover, pEGFR and *CJUN* mRNA was observed to be significantly positively correlated. We assume that during early stage of disease, EGFR–JNK/ERK pathway might be involved in increased in c-Jun protein as well as mRNA levels. By screening from available data (Splanger *et al*., 2011; Blau *et al*., 2012), we believe that during advanced stage of disease, the increase in c-Jun protein might be accomplished by IRES mediated translation due to loss of E-cadherin protein. Hence, overall the *CJUN* mRNA levels were comparable between malignant and adjacent normal tissues, while c-Jun protein levels were increased in malignant tissues. Knirsh *et al*., (2009) have observed up-regulation of c-Jun protein with no corresponding increase in *CJUN* mRNA, which might be due to IRES mediated translation.

The present investigation revealed no significant association of pEGFR with plasma MMPs. Thiery *et al*., (2003) have shown that EGFR activation down-modulates E-cadherin, and broad spectrum MMP inhibition ameliorates EGF-stimulated junctional disruption and loss of E-cadherin protein. It has been reported that activation of EGFR promotes SCC cell migration and invasion via inducing EMT-like phenotype change and MMP-9 mediated degradation of E-cadherin via ERK1/2 and PI3K signaling pathway (Zuo *et al*., 2011; Bae *et al*., 2013). Our study depicted simultaneous elevations of truncated E-cadherin protein, pEGFR, MMP-2 and MMP-9 in oral cancer. Similarly previous studies in human ovarian tumors and paired peritoneal metastases have shown that the immunohistochemical staining for activated pEGFR and MMP-9 were colocalized with regions of reduced E-cadherin (Dahl *et al*., 2008). However, as per available data from literature there is lack of reports on simultaneous evaluation of pEGFR, truncated E-cadherin protein and MMPs in oral cancer. Studies have depicted that loss of E-cadherin activates EGFR-MEK/ERK signaling promotes invasion via the ZEB1/MMP-2 axis in non-small cell lung cancer (Bae *et al*., 2013). Moreover, the ERK1/2 blockade prevents EMT in lung cancer cells and promotes sensitivity to EGFR inhibition (Buonato *et al*., 2014).

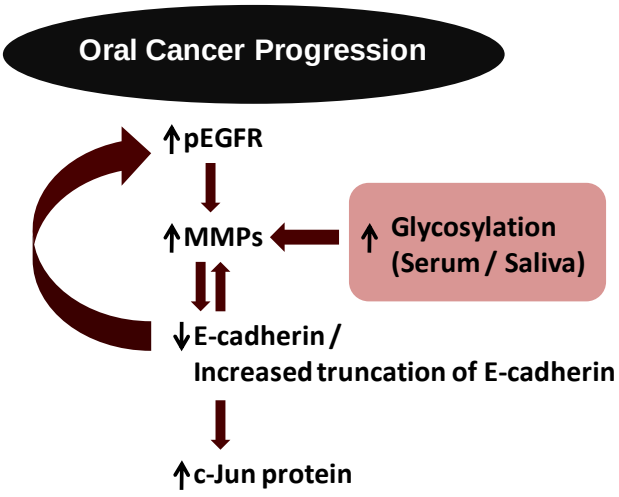

Figure 6.1: Role of glycosylation, pEGFR, E-cadherin, MMPs and c-Jun in oral cancer progression

Thus, based on these studies it is our assumption that decrease in E-cadherin might be involved in upregulation of pEGFR, which is further involved in upregulating MMPs. Moreover, increase in MMPs causes decrease in E-cadherin levels by disruption of cell-cell adhesion **(Figure 6.1)**.

Overall the results indicated that increased expression of pEGFR might cause decrease in E-cadherin levels by up regulation of MMPs. Increased glycosylation is also involved in elevated MMPs expression. Moreover, decrease in E-cadherin might be involved in EGFR activation **(Figure 6.1)**. Also, loss of E-cadherin might be involved in elevated expression of c-Jun protein.

CLINICAL UTILITY OF THE STUDY AS DRUG TARGETS:

The present study revealed increased expression of sialidase and sialyltransferase activity in patients with OPC and oral cancer patients. Earlier studies have reported Alkyne-hinged 3-fluorosialyl fluoride (DFSA) containing an alkyne group as target-specific irreversible inhibitor of sialidases (Tsai *et al*., 2012). Oseltavimir and Zanamivir are drugs frequently used for targeting sialidase enzyme (O'Shea *et al*., 2014). There is much advancement in development of various glycan antagonists and inhibitors of ST and FUCT (Whalen *et al*., 2003; Wang *et al*., 2003; Skropeta *et al*.,

2004; Brown *et al*., 2007; Rillahan *et al*., 2012; Okeley *et al*., 2013). Recently, sialyltransferase inhibitor, 3-FNeuAc and P-3F(ax)-Neu5AC has been suggested to be a global inhibitor involved in systemic blockade of sialylation (Bull *et al*., 2013; Macauley *et al*., 2014) which can be used for anticancer therapy. A novel sialytransferase inhibitor Lith-O-Asp has also been developed that suppresses Fak/Paxillin signaling and cancer angiogenesis and metastatic pathways (Chen *et al*., 2011). Specific inhibition by antisense or small hairpin (Sh) RNA has also been utilized to analyze the role of ST in cancer (Zeng *et al*., 2000; Ko *et al*., 2006; Birkle *et al*., 2000; Zhu *et al*., 2001; Gu *et al*., 2008). The present study depicted increased expression of *ST3GAL1* in malignant tissues as compared to adjacent normal tissues. Soyasaponin I, a ST inhibitor isolated from soybean saponin, has been shown to be CMP-Neu5Ac competitive inhibitor of *ST3GAL1 in vivo* (Wu *et al*., 2001). Lithocholic acid analogues which have similar steroid-related structure as Soyasaponin I, have been found to be noncompetitive inhibitors of Galβ1-3GalNAc α-2,3 STs (Chang *et al*., 2006). AL10, a lithocholic acid derivative was earlier shown to inhibit adhesion, migration and invasion of *ST3GALI* over-expressing A549 and CL1.5 human lung cancer cells (Chiang *et al*., 2010).

The results revealed that increased glycosylation is also involved in increasing MMPs. Increased expression of MMPs observed in the current investigation, might serve as important drug targets. Earlier studies have reported MMP inhibitors which are effective in controlling metastasis (Shi *et al*., 2012; Hadler-Oslen *et al*., 2013). Moreover, elevated levels of MMPs are responsible for decreased expression of E-cadherin protein which is further involved in up-regulation of c-Jun protein. Earlier studies have employed DNAzymes (catalytic DNA molecules) targeting c-Jun as well as MMPs to inhibit c-Jun expression in SCC cells (Zhang *et al*., 2006). Literature search revealed that decreased E-cadherin might be involved in increased expression of EGFR. We have observed elevated pEGFR expression in malignant tissues as compared to adjacent normal. Nowadays much advancement in EGFR based inhibitors and novel strategies in targeting EGFR have been also documented (Spano *et al*. 2005; Dahl *et al*., 2008; Pidone *et al*. 2014; Buonato *et al*., 2014). Recently,

therapeutic strategies to circumvent resistance of EGFR targeted therapies have also been developed (Chong *et al*., 2013).

Thus, several combination therapies targeting different molecular markers like sialylation, fucosylation, EGFR, MMPs and c-Jun might serve as new potent anti-inflammatory, immunosuppressive and anti-metastatic agents for future therapeutic applications which might be effective in controlling the disease. The present study also opened the way for development of newer combination therapies to combat oral cancer.