Results

Perfection is not attainable, but if we chase perfection we can catch excellence
-Vince Lombardi

OBJECTIVE 1: To study glycoprotein profiling from serum

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ORIGINAL ARTICLE

Glycoprotein electrophoretic patterns have potential to monitor changes associated with neoplastic transformation in oral cancer

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ABSTRACT

Alterations in glycoproteins, important cell surface constituents, have long been associated with various malignancies. The present investigation therefore explored the clinical significance of a glycoproteomics approach in patients with oral pre-cancerous conditions (OPC) and patients with oral cancer. The study included 80 oral cancer patients, 50 patients with OPC, and 84 controls. Native polyacrylamide gel electrophoresis followed by Schiff's staining was carried out to study the alterations in glycoproteins. The results showed significant elevation ($p<0.0001$) of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins in oral cancer patients and patients with OPC compared with controls. The odds ratio indicated a significantly higher risk for oral cancer among users and especially chewers of tobacco. The levels of all the glycoprotein bands (192 kDa, 170 kDa, 116 kDa and 44 kDa) were higher in patients with a habit of tobacco use (WHT) than in patients with no habit of tobacco (NHT) and were also higher in WHT controls than in NHT controls. Moreover, a 230 kDa glycoprotein consistently appeared only in individuals with tobacco habits and an increasing trend was observed from WHT controls to patients with OPC to WHT oral cancer patients. In conclusion, the results indicated the potential utility of glycoprotein alterations in monitoring sequential changes occurring due to tobacco consumption during neoplastic transformation.

Key words: Oral cancer, Oral precancerous conditions, Glycoproteins, Polyacrylamide gel electrophoresis

Expression levels of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins in controls, patients with OPC and oral cancer patients

To analyze glycoprotein profile from serum, native PAGE was performed followed by glycoprotein staining procedure by Anderson *et al*. (Anderson *et al.,* 1974).

Figure 5.1 depicts the representative glycoprotein electrophoretic pattern. The molecular weight of glycoprotein bands were determined by position of marker proteins. The glycoprotein electrophoretic patterns showed alterations in controls, patients with OPC and oral cancer patients.

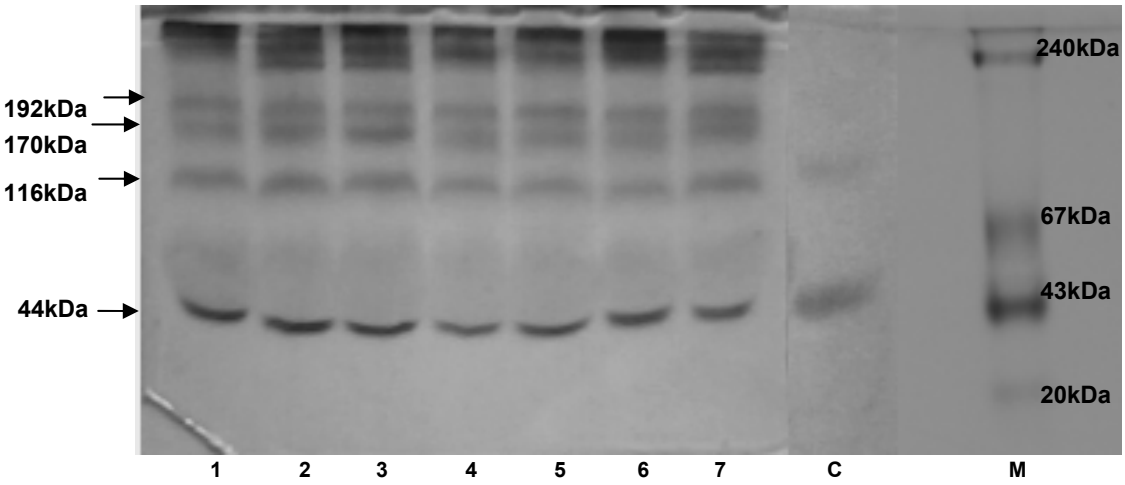


Figure 5.1: ***Representative glycoprotein electrophoretic pattern****. The band density was quantified densitometrically and compared between the subject groups. Lane 1 TH control; Lane 2,3,7 TH oral cancer patients; Lane 4 TNH control; Lane 5,6 Patients with OPC; Lane C- positive control (α_1- acid glycoprotein); Lane M – Molecular weight marker (TH: tobacco habituates, TNH: tobacco non-habituates, OPC: Oral precancerous conditions)*

As documented in **Figure 5.2** a significant increase (p<0.0001) in 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins was observed in patients with OPC and oral cancer patients when compared with the controls. Also, the levels were found to be significantly higher in oral cancer patients as compared to patients with OPC (p<0.0001). Thus, a significant increasing trend from controls to patients with OPC to oral cancer patients was observed.

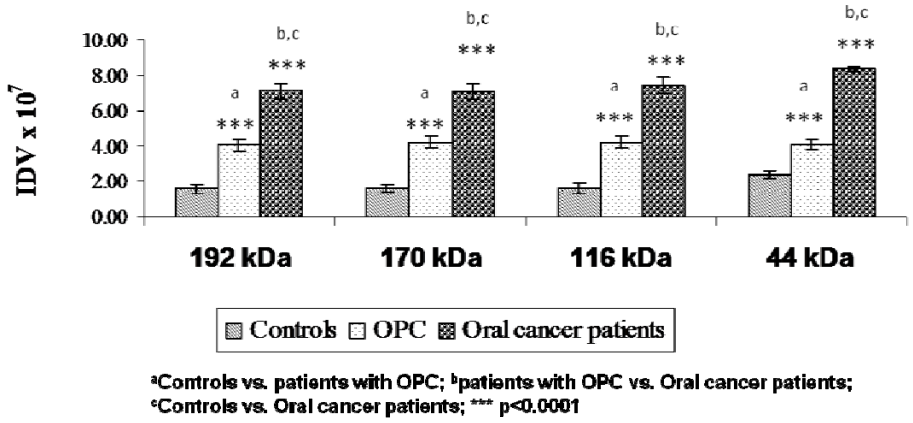


Figure 5.2: ***Comparison of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins between controls, patients with OPC and oral cancer patients.*** *The band densities of glycoproteins were quantified and values (Mean ± SE) are expressed as integrated density values. (OPC: Oral precancerous conditions)*

Expression of 230 kDa glycoprotein among tobacco habituates

Interestingly, we observed that 230 kDa glycoprotein (**Figure 5.3a**) consistently appeared in majority of the tobacco habituates of all the groups. This 230 kDa band was found to be present among tobacco consumers in 57.8% of controls, 50% of patients with OPC and 60% of oral cancer patients. On the other hand 5% of TNH controls and 4% of TNH oral cancer patients also showed the presence of this 230 kDa band.

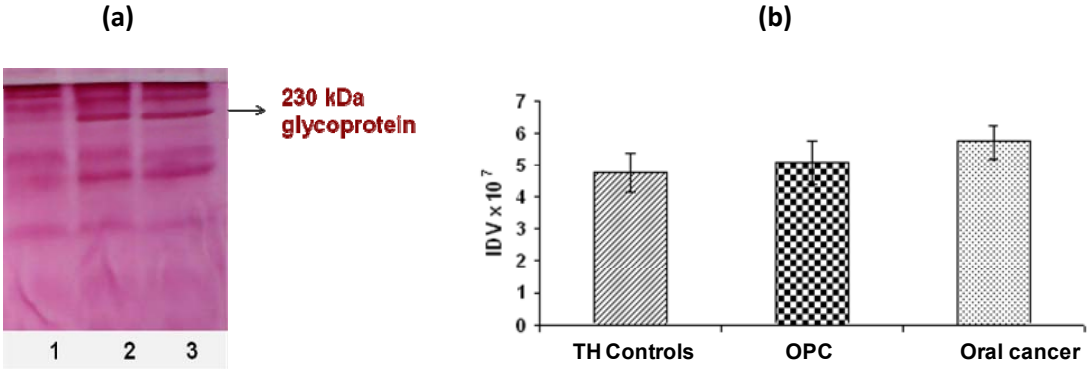


Figure 5.3: (a) ***Glycoprotein electrophoretic pattern showing 230 kDa band.*** *Lane 1 – TNH Controls, Lane 2 – 3 TH Oral cancer patients (TH: tobacco habituates; TNH: tobacco non-habituates).* **(b)** ***Expression of 230 kDa glycoprotein in TH controls, patients with OPC and oral cancer patients*** *(TH: tobacco habituates; OPC: Oral precancerous conditions), TH Controls vs. TH OPC, p=0.531; TH Controls vs. TH Oral cancer, p=0.232, TH OPC vs. TH Oral cancer patients, p=0.818)*

Moreover, an increasing trend in the expression levels of this 230 kDa band from TH controls to OPC to TH oral cancer patients was also observed **(Figure 5.3b)**, however, the alterations were statistically non-significant.

Comparison of glycoprotein levels between TNH and TH groups

The levels were further compared between TNH and TH groups. It was observed that the expression levels of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins were found to significantly higher (p<0.0001) in TNH oral cancer patients than in TNH controls and TH controls (**Figure 5.4** and **Table 5.1**). Also the levels were found to be significantly higher (**Table 5.1**) in TH oral cancer patients as compared to TH controls and TNH controls (p<0.0001). Moreover, all the above mentioned glycoproteins were found to be higher in TH controls as compared to TNH controls.

Table 5.1: Significance of expression levels of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoprotein in TNH and TH Controls with TNH and TH oral cancer patients

Groups compared	*p* value			
	192 kDa	170 kDa	116 kDa	44 kDa
TNH controls vs. TNH oral cancer	<0.0001	<0.0001	<0.0001	<0.0001
TNH controls vs. TH controls	0.070	0.070	0.043	0.093
TNH oral cancer vs. TH controls	0.004	0.006	0.003	0.01
TNH controls vs. TH oral cancer	<0.0001	<0.0001	<0.0001	<0.0001
TH controls vs. TH oral cancer	<0.0001	<0.0001	<0.0001	<0.0001
TNH oral cancer vs. TH oral cancer	0.338	0.223	0.465	0.225

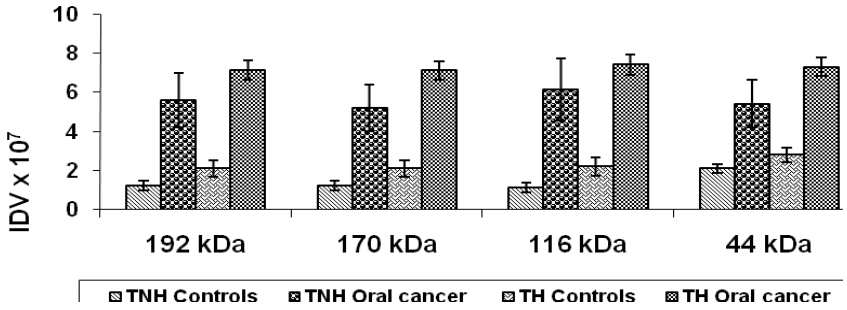


Figure 5.4: ***Expression of 192 kDa, 170 kDa, 116 kDa, and 44 kDa glycoproteins in TH and TNH controls and oral cancer patients.***

Correlation of glycoprotein levels with various clinicopathological parameters

A significant increase in 192 kDa, 170 kDa, 116 kDa and 44 kDa (p<0.0001) glycoproteins was observed in all the stages of disease i.e. stage I to stage IV and also

in various grades (p<0.0001) of tumor differentiation (well, moderate, poor) as compared to controls. While the levels of all 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins were comparable between various stages and different grades of tumor differentiation (well, moderate and poor). Correlation analysis showed no significant association of glycoprotein levels (192 kDa, 170 kDa, 116 kDa and 44 kDa) in oral cancer patients with stage, tumor differentiation and lymph node involvement. **Figure 5.5** shows that the glycoprotein levels of all 192 kDa, 170 kDa, 116 kDa and 44 kDa bands were found to be significantly higher in patients with lymph node metastasis (metastatic) and in patients without lymph node metastasis (non-metastatic) as compared to the controls (p<0.0001 and p<0.0001 respectively). Moreover, the mean levels were higher in metastatic subgroups than in non-metastatic subgroups.

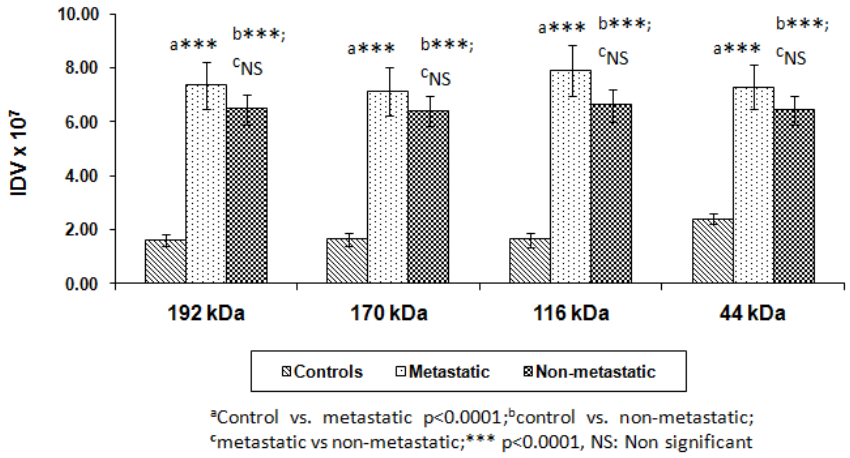
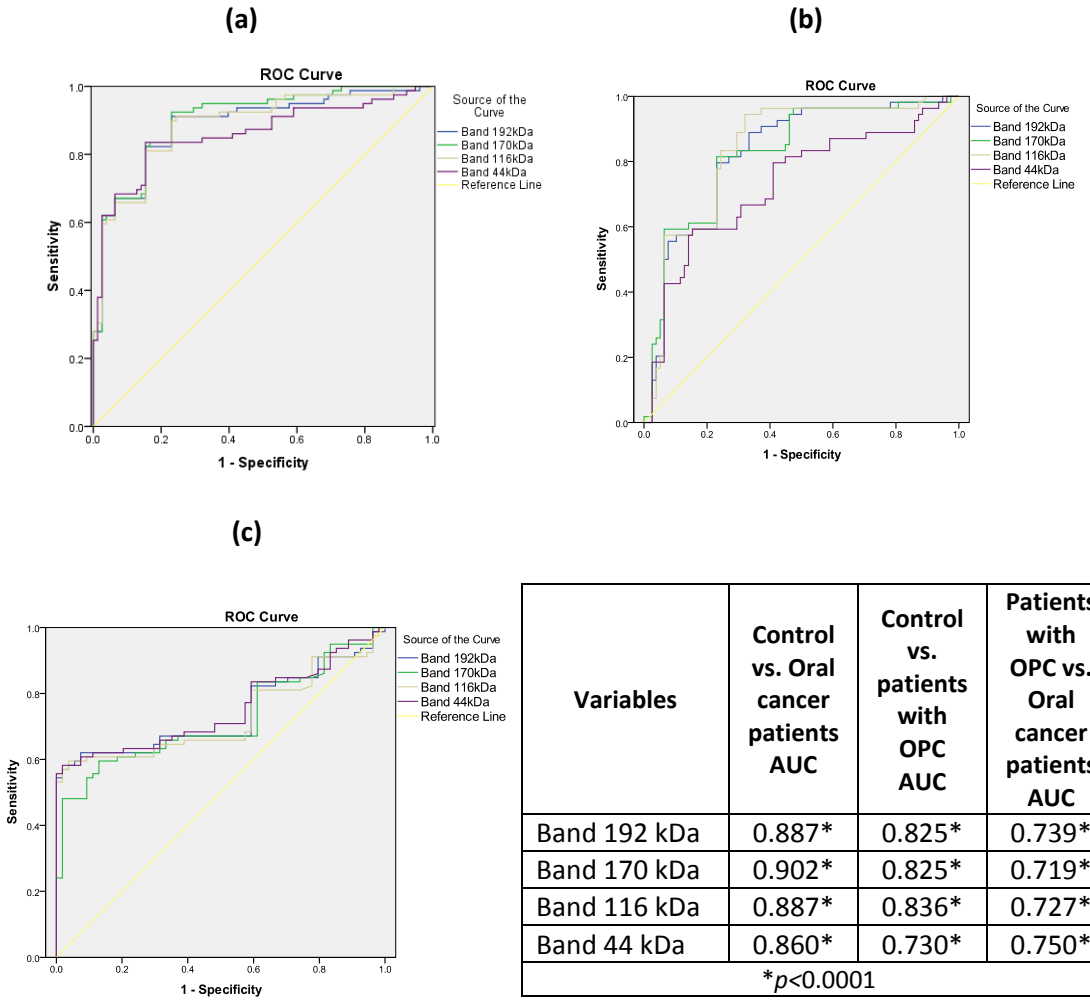


Figure 5.5: ***Comparison of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoprotein expression levels between controls, metastatic and non-metastatic groups.***

ROC curve analysis of glycoproteins

The ROC curves were constructed for 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins. ROC curves (**Figure 5.6**) indicated that 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins could significantly (p<0.0001) discriminate between controls and oral cancer patients, controls and patients with OPC, and also patients with OPC and oral cancer patients.



Variables	Control vs. Oral cancer patients AUC	Control vs. patients with OPC AUC	Patients with OPC vs. Oral cancer patients AUC
Band 192 kDa	0.887*	0.825*	0.739*
Band 170 kDa	0.902*	0.825*	0.719*
Band 116 kDa	0.887*	0.836*	0.727*
Band 44 kDa	0.860*	0.730*	0.750*
*p<0.0001			

Figure 5.6: ***ROC curve to assess the discriminatory efficacy of 192 kDa, 170 kDa, 116 kDa, and 44 kDa glycoproteins between (a) Controls vs. Oral cancer patients, (b) Controls vs. patients with OPC and (c) patients with OPC vs. Oral cancer patients.*** *The values (indicated as line graphs) above the reference line on an ROC curve (middle line in the graphs) suggest a good discriminatory efficacy of the marker that is within acceptable limits. AUC: Area Under curve*

As depicted in the **Figure 5.6**, AUC of 192 kDa, 170 kDa, 116 kDa and 44 kDa glycoproteins significantly discriminated control vs. oral cancer patients, controls vs. patients with OPC and, patients with OPC and oral cancer patients (p<0.0001). The AUC of 192 kDa, 170 kDa, 116 kDa and 44 kDa were found to be highest in controls vs. oral cancer patients, which showed best discriminatory efficacy between controls and oral cancer patients.

For further characterization of 230 kDa band, in-gel digestion approach was employed.

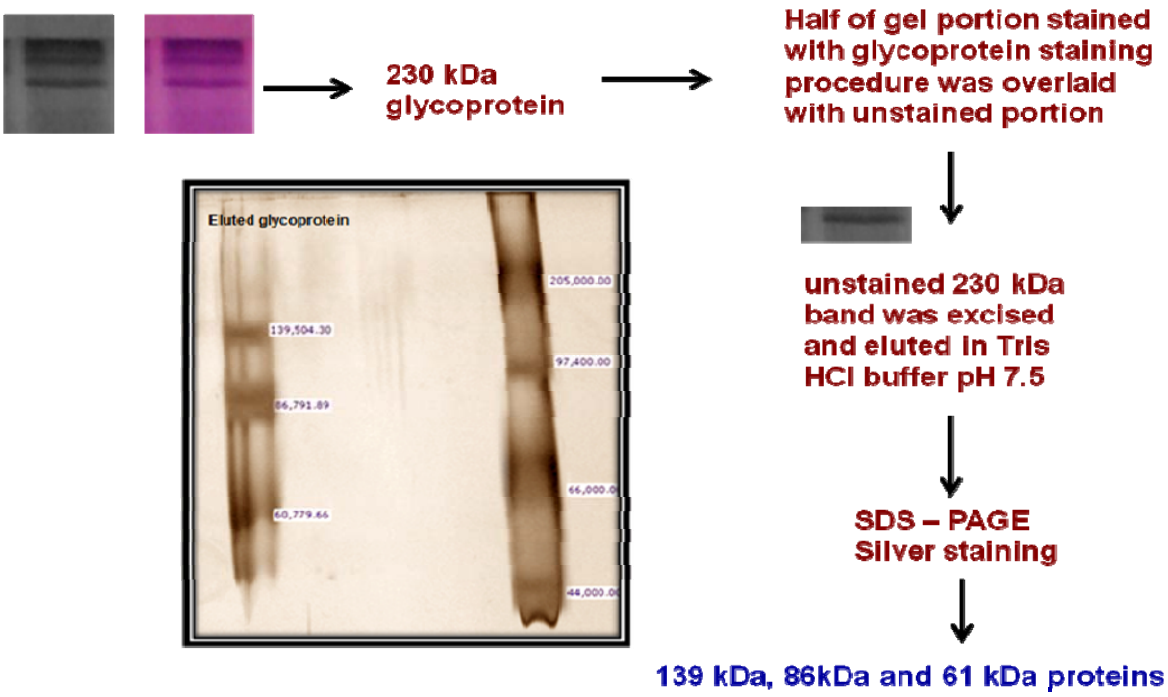


Figure 5.7: In-gel digestion and further characterization of altered 230 kDa band.

Half of the gel portion stained with glycoprotein staining procedure was overlaid with unstained portion as depicted in **Figure 5.7**. The unstained 230 kDa band was excised and eluted in Tris-HCl buffer pH 7.5. This eluted fraction was run on SDS- PAGE and further silver staining was performed. Further molecular weight analysis using densitometer (Alpha Innotech Inc.) revealed three bands of 139 kDa, 86 kDa and 61 kDa proteins.

OBJECTIVE 2: To compare serum and salivary sialylation changes between controls, patients with OPC, oral cancer patients and post treatment follow-ups and to assess the correlation with clinico-pathological parameters.

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Salivary Glyco-sialylation changes monitors oral carcinogenesis

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Abstract Alterations in cell membrane glycosylation play important role in oral carcinogenesis. The present study evaluated salivary sialylation changes *i.e.* total sialic acid (TSA), sialidase activity, linkage specific (α2-3 and α2-6) sialoproteins and sialyl transferase (ST) activity in controls, patients with oral precancerous conditions (OPC) and oral cancer. Subjects enrolled included 100 controls, 50 patients with OPC, 100 oral cancer patients, and 30 post treatment follow-ups. TSA was estimated by spectrophotometric method, sialidase activity by spectrofluorometric assay and linkage specific biotinylated lectins (α2-3: *sambucus nigra* agglutinin and α2-6: *maackia amurensis* agglutinin) were used to detect α-2,3 and α-2,6 STs and sialoproteins by ELISA and dot blot respectively. An increasing trend of salivary TSA/TP ratio, sialidase activity, α2-3 sialoproteins, α-2,3 and α-2,6 ST activities 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. Sialidase activity exhibited significant association with metastasis and infiltration. Sialidase activity, TSA/TP ratio, α-2,3 and α-2,6 ST activities were found to be higher in patients with metastasis as compared to patients without metastasis. A progressive increase in TSA/TP ratio, sialidase activity, α2-3 and α2-6 sialoproteins was observed from controls to early to advanced stage of the disease. Sialidase activity, α2-3 and α2-6 sialoproteins and ST activities were found to be decreased in complete responders; while levels were elevated in non-responders. The results documented utility of salivary sialylation endpoints, a non invasive tool in monitoring of oral carcinogenesis.

Keywords Glycosylation · Oral cáncer · Oral precancerous conditions · Saliva · Sialylation · Sialic acid · Sialidase · Sialyltranferase · Sialoproteins

Abbreviations

AJCC	American Joint Committee on Cancer
AUC	Area under curve
CI	Confidence interval
CR	Complete responders
ELISA	Enzyme linked immunosorbent assay
IDV	Integrated density value
LN	Lymph node
OPC	Oral precancerous conditions
MAM	*Maackia amurensis*
MU	Methyl umbelliferone

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Evaluation of serum and salivary total sialic acid and α-L-fucosidase in patients with oral precancerous conditions and oral cancer

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Objectives. We compared serum and salivary total sialic acid/total protein (TSA/TP) ratios and α-L-fucosidase activity in patients with oral precancerous conditions (OPCs) and oral cancer to better understand the utility of saliva, in monitoring early changes occurring during oral cancer progression.
Study design. A cross-sectional study of 100 oral cancer patients, 50 patients with OPC, and 100 controls was performed.
Results. Serum and salivary TSA/TP ratios and α-L-fucosidase activity were significantly higher in OPC and oral cancer patients compared to the controls. Also, levels were higher in controls and oral cancer patients with tobacco habits as compared to those without tobacco habits.
Conclusion. Salivary TSA/TP ratio and α-L-fucosidase activity were elevated with higher magnitude than serum levels. These results suggest that a larger study may prove the use of these saliva biomarkers as a noninvasive method for detecting early changes occurring during oral carcinogenesis. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:764-771)

4 **Communicated**

Review Manuscript: Sialylation: An Avenue to Target Cancer Cells
Pathology and Oncology Research
Bhairavi N. Vajaria, Kinjal R. Patel, Rasheedunnisa Begum, Prabhudas S. Patel

OBJECTIVE 2.1: Comparison between serum and salivary TSA levels

Serum and salivary TSA was measured by means of TBA method as described by Skoza and Mohos (1976). The levels were normalized with total protein content. It was estimated from 100 controls, 50 patients with OPC and 100 oral cancer patients. From saliva supernatant, 0.5ml of hydrolysate was standardized and taken for TBA assay. For estimation from serum 0.1ml of hydrolysate was taken for TBA assay.

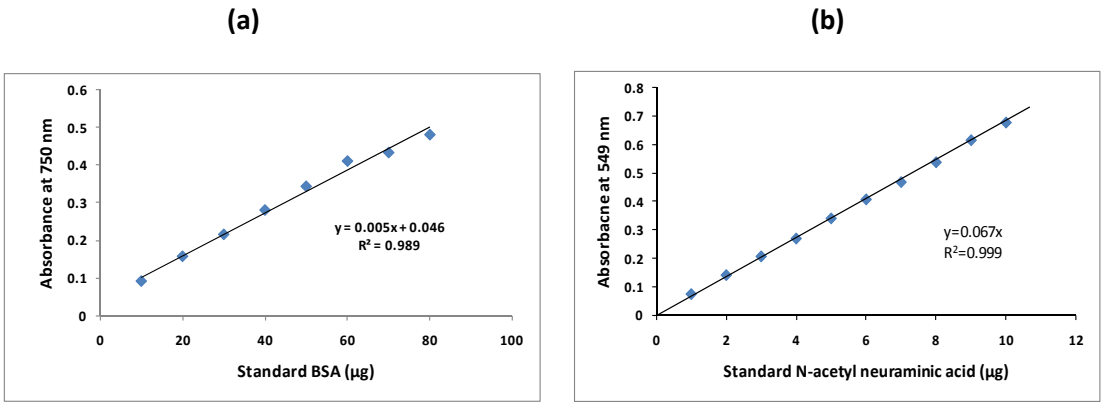


Figure 5.8: ***Standard curve for*** **(a)** ***protein estimation by Folin Lowry's method (b) TSA determination by TBA method***

The standard curve for protein estimation by Folin Lowry's method was prepared using bovine serum albumin (BSA) (**Figure 5.8a)** and TSA determination using N-acetyl neuraminic acid (**Figure 5.8b)** as standard. The standard curves for protein estimation and TSA determination were linear from 10µg to 60µg and 1µg to 10µg, respectively.

Comparison of serum and salivary TSA/TP ratio in controls, patients with OPC and oral cancer patients

As documented in **Figure 5.9**, serum and salivary TSA/TP ratio were significantly higher in patients with OPC and oral cancer patients as compared to controls (p<0.0001 and p=0.005, respectively). An increasing trend in serum and salivary TSA/TP ratio was observed from controls to patients with OPC to oral cancer patients. Moreover, the salivary TSA/TP levels were elevated with higher magnitude than serum levels in the subjects of the same groups.

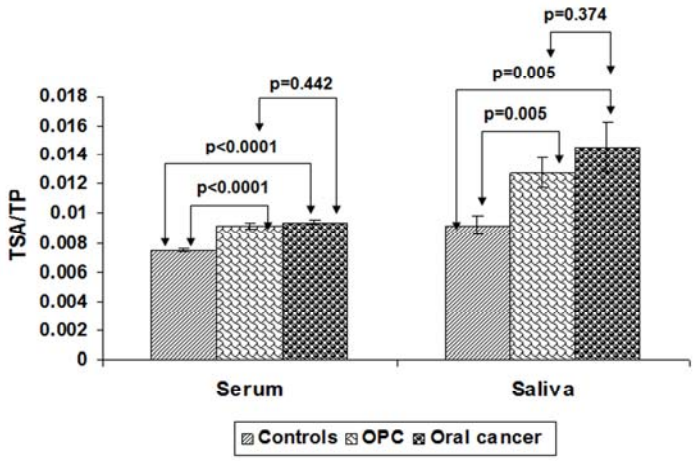


Figure 5.9: ***Comparison of serum and salivary TSA/TP ratio between controls, patients with OPC and oral cancer patients.*** *The values are expressed as Mean ± SEM; OPC: Oral precancerous conditions; TSA/TP: Total sialic acid/Total protein*

Comparison of serum and salivary TSA/TP in TNH and TH groups of subjects

Further, we divided controls and oral cancer patients into tobacco non habituates (TNH) and tobacco habituates (TH) groups and compared serum and salivary TSA/TP ratio. All the patients with OPC were TH.

Table 5.2: Comparison of serum and salivary TSA/TP ratio between TNH and TH groups of subjects

Subjects	Serum TSA/TP Ratio	Salivary TSA/TP ratio
[1]TNH controls (n=50)	0.0071 ± 0.00017	0.0086 ± 0.00065
[2]TH controls (n=50)	0.0080±0.00020 *p*=0.294 (1 vs.2)	0.0113±0.0011 ***p*=0.045** (1 vs.2)
[3]Patients with OPC TH (n=50)	0.0091±0.00021 ***p*<0.0001** (1 vs.3) *p*=0.848(2 vs.3)	0.0126±0.0010 ***p*=0.001** (1 vs.3) *p*=0.411 (2 vs.3)
[4]TNH Oral cancer (n=12)	0.0092±0.00055 ***p*=0.009** (1 vs.4) *p*=0.064 (2 vs.4) *p*=0.922 (3 vs.4)	0.0128±0.0022 ***p*=0.041** (1 vs.4) *p*=0.557 (2 vs.4) *p*=0.932 (3 vs.4)
[5]TH Oral cancer (n=88)	0.0095±0.00018 ***p*<0.0001**(1 vs.5) ***p*<0.0001** (2 vs.5) *p*=0.320 (3 vs.5) *p*=0.655(4 vs.5)	0.0130±0.00 ***p*=0.0021** (1 vs.5) *p*=0.270 (2 vs.5) *p*=0.752(3 vs.5) *p*=0.322(4 vs.5)

As depicted in **Table 5.2**, the mean values of serum and salivary TSA/TP ratio were found to be higher in TH controls as compared to TNH controls but the levels were found to be non-significant except for salivary TSA/TP ratio *(p*=0.045).

The levels of serum and salivary TSA/TP ratio were found to be significantly higher in TNH oral cancer patients as compared to TNH controls (*p*=0.009 and *p*=0.041 respectively). Serum TSA/TP ratio was found to be significantly higher in TH oral cancer patients as compared to TH controls. Also, the mean levels of serum and salivary TSA/TP ratio were observed to be marginally increased from TNH to TH controls to patients with OPC and further to TNH and TH oral cancer patients, however the alterations were statistically non-significant.

Correlation between serum and salivary levels

Pearson's correlation analysis was performed to assess the correlation between serum and salivary levels. The correlation analysis showed significant positive association between serum and salivary TSA/TP ratio (r=0.275, *p*<0.0001).

Correlation of serum and salivary TSA/TP ratio with various clinicopathological parameters

The levels of TSA/TP were compared with various clinicopathological parameters. **Table 5.3** shows serum and salivary TSA/TP ratio in patients with metastasis and without metastasis. Both, serum and salivary levels were found to be higher in patients with metastasis when compared to patients without metastasis. Serum and salivary TSA/TP ratio was found to be significantly higher in early and advanced stage of the disease as compared to the controls (*p*<0.0001) (**Table 5.3**). The Salivary TSA/TP ratio was found to be increased in patients with advanced disease as compared to those with early disease, while serum TSA/TP ratio was comparable. Serum TSA/TP ratio was found to be higher in moderately differentiated tumors as compared to well differentiated tumors (*p*=0.052). The ratio was found to be higher in poorly differentiated tumors as compared to well differentiated tumors (*p*=0.011). However, no significant difference was observed between moderate and poorly differentiated tumors. The levels of salivary TSA/TP ratio were found to be higher in moderately differentiated tumors as compared to well differentiated tumors (*p*=0.033).

Table 5.3: Comparison of serum and salivary TSA/TP ratio according to stage of the disease and metastasis

Groups	Serum TSA/TP ratio	Salivary TSA/TP ratio
	Mean ± SEM	
[1]Controls (n=100)	0.00746 ±0.00013	0.00866±0.00065
[2]Early disease (I +II) (n=32)	0.00978±0.00034 ***p*<0.0001** (1 vs.2)	0.0128±0.0.00101 ***p*<0.0001**(1 vs.2)
[3]Late disease (III+IV) (n=62)	0.00938 ± 0.000227 ***p*<0.0001**(1 vs.3) *p*=0.344(2 vs.3)	0.0156±0.00156 ***p*<0.0001**(1 vs.3) *p*=0.213(2 vs.3)
[4]Patients with Metastasis (n=34)	0.0135±0.00372 ***p*=0.004**(1 vs.4)	0.0135±0.00139 ***p*=0.005**(1 vs.4)
[5]Patients with no metastasis (n=56)	0.00932±0.00022 ***p*<0.0001**(1 vs.5) *p*=0.118(4 vs.5)	0.0133±0.00132 ***p*=0.002**(1 vs.5) *p*=0.942(4 vs.5)

Further multivariate analysis was performed in order to correlate with stage, tumor differentiation and lymph node metastasis. The multivariate analysis revealed no significant correlation of serum and salivary TSA/TP ratio with stage, differentiation and lymph node metastasis.

ROC curve analysis of serum and salivary TSA/TP ratio

ROC curves were constructed to evaluate the discriminatory efficacy of the markers in distinguishing controls vs. oral cancer patients, controls vs. patients with OPC and patients with OPC vs. oral cancer patients. **Table 5.4** shows the discriminatory efficacy of TSA/TP ratio i.e. diagnostic sensitivity and specificity with ideal cut-off and AUC.

As depicted in **Table 5.4**, ROC curve analysis indicated that serum TSA/TP ratio significantly discriminated controls and oral cancer patients ($p<0.0001$), and control and patients with OPC ($p<0.0001$). Moreover, salivary TSA/TP ratio could significantly discriminate between controls and oral cancer patients ($p<0.0001$) as well as controls and patients with OPC ($p=0.004$).

Table 5.4: ROC curve analysis for serum and salivary TSA/TP ratio

Groups compared		Serum TSA/TP ratio	Salivary TSA/TP ratio
Controls vs. Oral cancer patients	Cut-off	0.0087	0.0062
	AUC	0.834	0.664
	Significance	***p*<0.0001**	***p*=0.0001**
	Sensitivity	64.0%	96.6%
	Specificity	90.5%	32.0%
Controls vs. Patients with OPC	Cut-off	0.0087	0.0113
	AUC	0.813	0.658
	Significance	***p*<0.0001**	***p*=0.004**
	Sensitivity	65.2%	57.14
	Specificity	90.5%	71.6
Patients with OPC vs. Oral cancer Patients	Cut-off	0.0112	0.0056
	AUC	0.513	0.522
	Significance	NS	NS
	Sensitivity	-	-
	Specificity	-	-

NS: Non-significant

Levels of serum and salivary TSA/TP ratio in PT, CR and NR

The levels of TSA/TP ratio were compared in follow-up samples between PT (pre-treatment), CR and NR. **Figure 5.10 (a) and (b)** shows the bar chart of serum and salivary TSA/TP ratio in PT, CR and NR. It was observed that serum and salivary TSA/TP ratio were found to be comparable between PT and CR, while the ratio was found to be higher in NR as compared to PT levels.

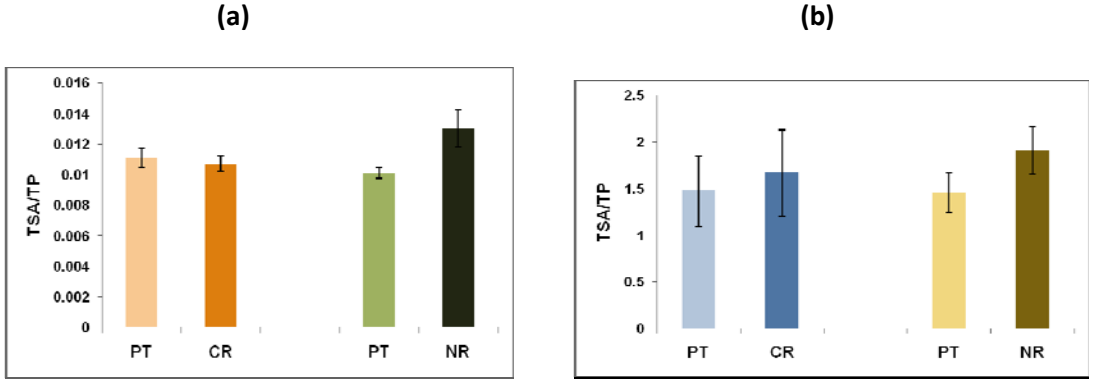


Figure 5.10: ***(a) Serum TSA/TP ratio in PT, CR and NR; (b): Salivary TSA/TP ratio in PT, CR and NR .*** *TSA/TP: Total sialic acid/total protein, PT: Pretreatment, CR: Complete responders, NR: Non-responders*

Survival analysis for serum and salivary TSA/TP ratio

Table 5.5 depicts the Kaplan Meir's survival analysis with ROC cut-off, sensitivity, specificity, AUC, survival in months with values below and above cut-off and Log rank Chi^2 value with significance.

Table 5.5: Survival analysis of serum and salivary TSA/TP ratio

Parameter	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2 Significance
Serum TSA/TP ratio	0.0087 (0.835)	64.0%/ 90.48% *p*<0.0001	44.85±2.585	34.8±2.339	5.102 ***p*=0.024**
Salivary TSA/TP ratio	0.0062 (0.664)	96.63/32.1 *p*=0.0001	44.0±0.000	40.706±1.961	0.245 *p*=0.621

(a) **(b)**

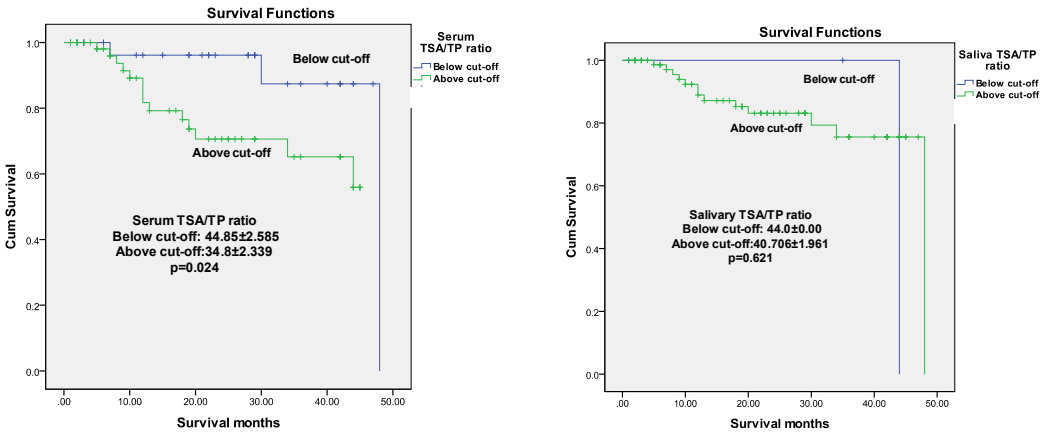


Figure 5.11: Kaplan Meir's survival analysis for (a) serum and (b) salivary TSA/TP ratio. *Kaplan Meier survival curves were compared by Log-rank analysis and (a) serum TSA/TP ratio (b) salivary TSA/TP ratio in patients are depicted as above or below ROC cut-off value (**Table 5.5**). The values above and below cut-off are expressed as survival in months ± SEM.*

It was observed (**Figure 5.11** and **Table 5.5)** that the values above ROC cut-off of serum TSA/TP ratio was significantly associated with lower overall survival (*p*=0.024). Also values above cut-off of salivary TSA/TP was associated with lower overall survival although not statistically significant.

OBJECTIVE 2.2: Comparison of serum and salivary sialidase activities

Sialidase activity was measured spectrofluorometrically as described by Potier *et al.* (Potier *et al*., 1979) from serum as well as saliva supernatant. It was estimated from 30 controls, 30 patients with OPC, 30 oral cancer patients and 15 follow-ups of oral cancer patients.

Standard curve for sialidase activity:

The standard curve for measuring sialidase activity was performed using 4-methylumbelliferone as substrate using a spectrofluorometric method. **Figure 5.12** shows the standard curve for sialidase activity, it was observed to be linear from 5nM to 120nM.

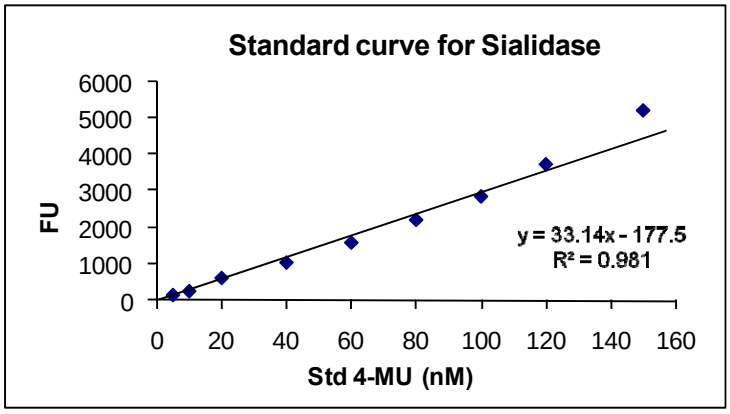


Figure 5.12: ***Standard curve for sialidase activity***

Serum and salivary sialidase activity in controls, patients with OPC and oral cancer patients

An increasing trend of serum and salivary sialidase activity was observed from controls, to patients with OPC to oral cancer patients. Serum and salivary sialidase activity **(Figure 5.13a and Figure 5.13b)** were found to be significantly elevated in oral cancer patients as compared to controls ($p<0.0001$) and were also higher in oral cancer patients as compared to patients with OPC ($p=0.064$, $p=0.0002$, respectively).

The serum and salivary levels of sialidase activity were found to be elevated in patients with OPC as compared to controls. Moreover, salivary sialidase activity was found to be significantly higher than serum activity ($p<0.0001$).

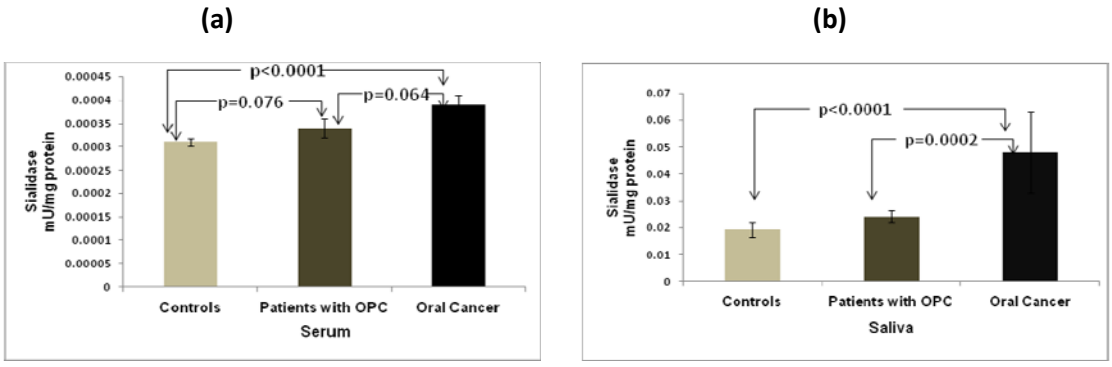


Figure 5.13: (a) ***Serum sialidase activity (b) Salivary sialidase activity in controls, patients with OPC and oral cancer patients***

Serum and salivary sialidase activity with reference to stage, differentiation and metastasis

Serum and salivary sialidase activity were compared in patients with metastasis and patients without metastasis. The results are as shown in **Table 5.6**.

Table 5.6: Serum and salivary sialidase activity in metastasis and various stages of disease

Groups	Serum sialidase activity	Salivary sialidase activity
[1]Controls (n=30)	0.00031 ± 0.0000087	0.019 ± 0.0026
[2]Patients with metastasis (n=10)	0.00044 ± 0.000045 ***p*<0.0001** (1 vs. 2)	0.064 ± 0.0183 ***p*<0.0001** (1 vs. 2)
[3]Patients with no metastasis (n=20)	0.00039 ± 0.000031 ***p*=0.002** (1 vs. 3) *p*=0.405 (2 vs. 3)	0.0367 ± 0.0042 ***p*=0.001**(1 vs. 3) ***p*=0.045** (2 vs. 3)
[4]Stage I (n=7)	0.00034 ± 0.000036 *p*=0.541 (1 vs. 4)	0.0219 ± 0.0033 *p*=0.541 (1 vs. 4)
[5]Stage II (n=6)	0.00036 ± 0.000037 ***p*=0.045** (1 vs. 5) *p*=0.616 (4 vs. 5)	0.0381 ± 0.017 ***p*=0.055**(1 vs. 5) *p*=0.390 (4 vs. 5)
[6]Stage III (n=5)	0.000039 ± 0.000038 ***p*=0.013**(1 vs. 6) *p*=0.381(4 vs. 6) *p*=0.674 (5 vs. 6)	0.0425 ± 0.0089 ***p*=0.004** (1 vs. 6) *p*=0.099(4 vs. 6) *p*=0.825 (5 vs. 6)
[7]Stage IV (n=12)	0.00042 ± 0.000035 ***p*=0.017** (1 vs. 7) *p*=0.124 (4 vs. 7) *p*=0.266 (5 vs. 7) *p*=0.507 (6 vs. 7)	0.0479 ± 0.0170 ***p*<0.0001**(1 vs. 7) ***p*=0.009** (4 vs. 7) *p*=0.612 (5 vs. 7) ***p*=0.051** (6 vs. 7)

The result exhibited higher levels of serum and salivary sialidase activity in patients with metastasis as compared to patients without metastasis. Moreover, when compared between various stages, the results depicted an increasing trend of both serum and salivary sialidase activity from stage I to stage IV of the disease.

An increasing trend of serum sialidase activity was observed from well to moderate to poorly differentiated tumors. The levels of salivary Sialidase activity were comparable between well, moderate and poorly differentiated tumors.

ROC curve analysis of serum and salivary sialidase activities

The results of ROC curve analysis with ideal cut-off, AUC, sensitivity and specificity are mentioned in **Table 5.7**. ROC curve analysis revealed that serum and salivary sialidase activity significantly distinguished controls and oral cancer patients (p=0.0001 and p<0.0001, respectively) and patients with OPC and oral cancer patients (p=0.0528 and p=0.002, respectively). Serum sialidase activity could also distinguish controls and patients with OPC (p=0.0184).

Table 5.7: ROC curve analysis for serum and salivary sialidase activity

Groups compared		Serum sialidase activity	Salivary sialidase activity
Controls vs. Oral cancer patients	Cut-off	0.00034	0.0272
	AUC	0.774	0.833
	Significance	***p*=0.0001**	***p*<0.0001**
	Sensitivity	66.7%	72.41%
	Specificity	89.3%	80.77%
Controls vs. Patients with OPC	Cut-off	0.00031	0.0161
	AUC	0.677	0.640
	Significance	***p*=0.0184**	*p*=0.0764
	Sensitivity	76.0%	80.0%
	Specificity	60.7%	50.0 %
Patients with OPC vs. Oral cancer Patients	Cut-off	0.00034	0.0219
	AUC	0.667	0.749
	Significance	***p*=0.0528**	***p*=0.0002**
	Sensitivity	66.7%	79.3%
	Specificity	84.0%	64.0%

Serum and salivary sialidase activity in PT, CR and NR group of patients

The oral cancer patients were followed after the anticancer treatment, and serum and salivary sialidase activities were compared between their PT and post-treatment follow-up levels by paired *'t'* test. As shown in **Figure 5.14 (a)**, serum Sialidase activity was observed to be significantly decreased (p=0.034) in complete responders as compared to PT levels. While in case of non-responders the levels were found to be comparable (p=0.376). **Figure 5.14 (b)** depicts that salivary sialidase activity was decreased in CR as compared to PT levels (p=0.059) while the activity was elevated in NR as compared to their corresponding PT levels (p=0.193), however the alterations were statistically non-significant.

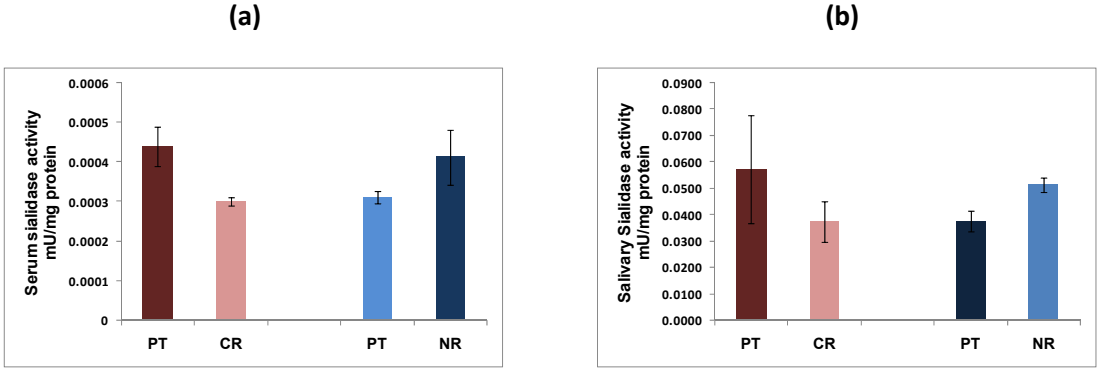


Figure 5.14: ***(a) Levels of serum Sialidase activity in PT, CR and NR. (b) Levels of salivary sialidase activity in PT, CR and NR.*** *PT: Pretreatement, CR: Complete responders, NR: Non-responders*

Kaplan Meir's survival analysis of serum and salivary sialidase activities

Table 5.8 shows the Kaplan Meir's survival analysis of serum and salivary sialidase activities with ROC cut-off, sensitivity, specificity and AUC. The result depicted no significant association of serum and salivary sialidase activity with overall survival.

Table 5.8: Kaplan Meir's survival analysis of serum and salivary sialidase activities with ROC cut-off

Parameter	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2	Signifi-cance
Serum Sialidase activity	0.00034 (0.774)	66.7%/ 89.3% p=0.0001	37.583±7.719	41.697±2.842	0.335	p=0.563
Salivary Sialidase activity	0.0272 (0.833)	72.4/80.8 p<0.0001	41.750±5.099	39.932±3.437	0.204	p=0.652

OBJECTIVE 2.3: Evaluation of serum and salivary expression of α-2,3 and α-2,6 sialoproteins

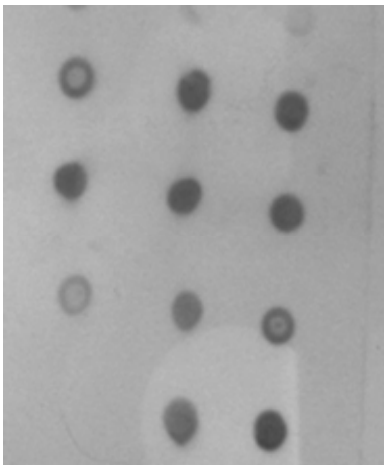
For analysis of α-2,3 and α-2,6 sialoproteins, 200µg of serum protein and 50µg of salivary protein were standardized and spotted onto the nitrocellulose membrane. The biotinylated lectins were used as primary antibody for dot blot analysis of Sialoproteins. The detection was performed by chemiluminescence method and dot blots were captured on x-ray film. The x-ray film was further scanned using densitometer and integrated density value (IDV) was calculated for each spot. The results were noted as IDV/µg. Serum and salivary α-2,3 and α-2,6 Sialoproteins were estimated from 30 controls, 30 patients with OPC, 30 oral cancer patients and 15 follow-ups of oral cancer patients.

Comparison of serum and salivary expression of α-2,3 and α-2,6 sialoproteins between controls, patients with OPC and oral cancer patients

Figure 5.15 (a) and **5.15 (b)** shows the representative patterns of serum and salivary α-2,6 sialoproteins and **Figure 5.15 (c)** and **5.15 (d)** is the representative pattern of serum and salivary α-2,3 sialoproteins in controls, patients with OPC and oral cancer patients.

(a)

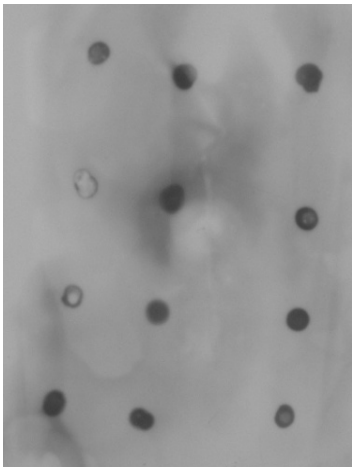
Serum α-2,6 Sialoproteins



1 2 3

(b)

Saliva α-2,6 Sialoproteins



1 2 3

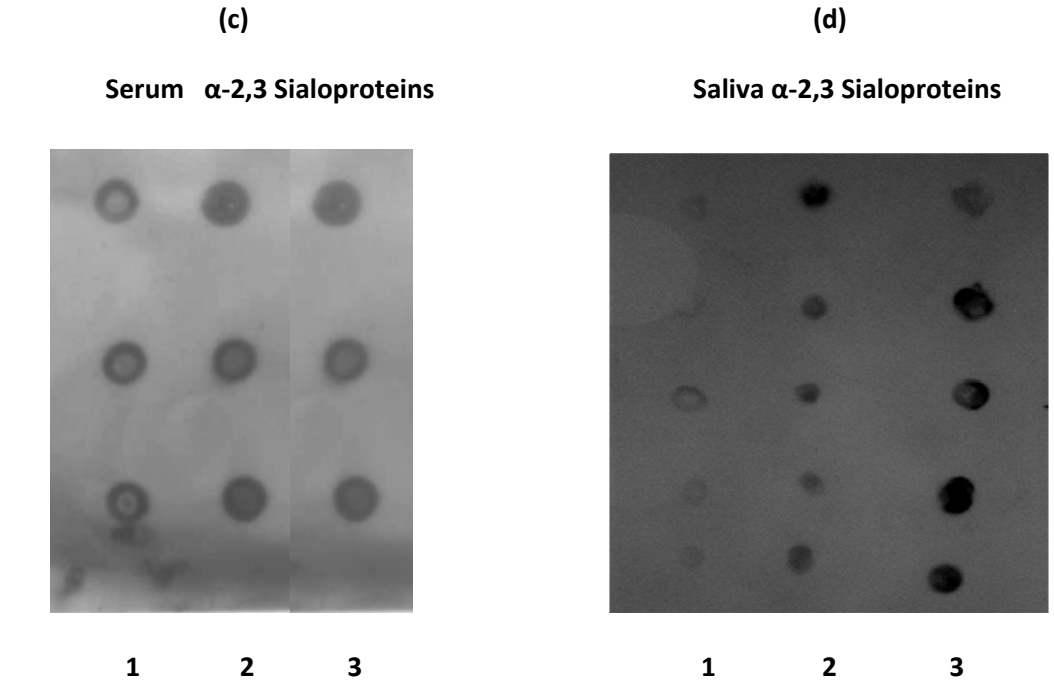


Figure 5.15: ***Representative pattern of (a) Serum α-2,6 sialoproteins and (b) salivary α-2,6 sialoproteins (c) Serum α-2,3 sialoproteins and (d) salivary α-2,3 sialoproteins.*** *1: controls; 2: patients with OPC, 3: oral cancer patients*

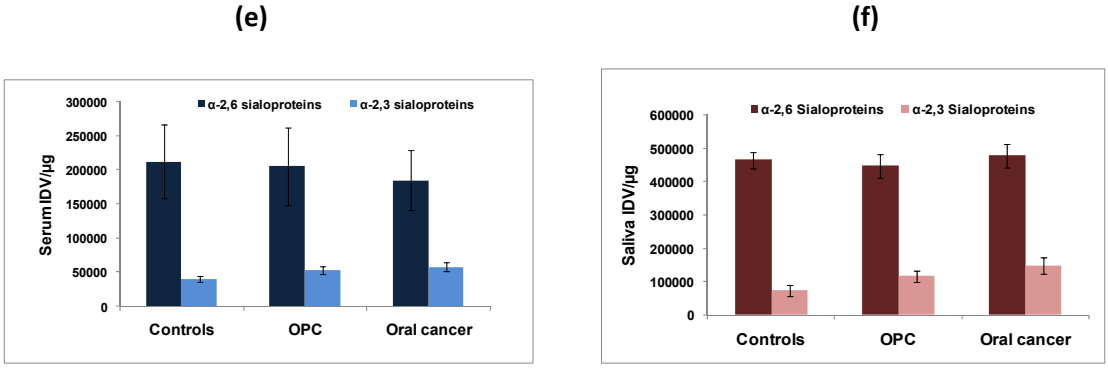


Figure 5.15: ***(e) Serum and (f) salivary expression of α-2,3 and α-2,6 sialoproteins in controls, patients with OPC and oral cancer patients***

Figure 5.15 (e) and **5.15 (f)** documents the levels of serum and salivary α-2,3 and α-2,6 sialoproteins in controls, patients with OPC and oral cancer patients. It was observed that levels of 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. The serum and salivary levels of α-2,3 sialoproteins

were found to be significantly elevated in oral cancer patients as compared to the controls (p=0.022 and p=0.022, respectively)

Comparison of levels of serum and salivary α-2,3 and α-2,6 sialoproteins between various stages, differentiation and metastasis

The serum and salivary α-2,3 and α-2,6 sialoproteins were compared between early and advanced stage of disease as well between patients with metastasis and without metastasis. As depicted in **Table 5.9**, levels of serum and salivary α-2,3 sialoproteins were found to be higher in advanced stage of the disease as compared to early stage, while levels were comparable between patients with and without metastasis. The levels of serum and salivary α-2,6 sialoproteins were comparable between early and advanced stage of disease as well as between patients with metastasis and without metastasis.

Serum α-2,6 sialoproteins levels were found to be higher in moderately differentiated tumors as compared to well differentiated tumors (p=0.337).

Table 5.9: Serum and salivary α-2,3 and α-2,6 sialoproteins in various stages and metastasis

Markers	Serum α-2,6 sialoproteins	Salivary α-2,6 sialoproteins	Serum α-2,3 sialoproteins	Salivary α-2,3 sialoproteins
[1]Controls (n=30)	212318±53754	464046±23874	39894±4438	72379±18020
[2]Early (n=13)	220579±67240 p=0.926 (1 vs. 2)	555398±30825 p=0.198 (1 vs. 2)	35990±7031 p=0.928(1 vs. 2)	133685±34202 p=0.093(1 vs. 2)
[3]Advanced (n=17)	149273±85073 p=0.567 (1vs. 3) p=0.538 (2 vs. 3)	585003±36221 p=0.095 (1vs. 3) p=0.597 (2 vs. 3)	61844±12556 p=0.567 (1vs. 3) p=0.538 (2 vs. 3)	160290±45442 p=0.098 (1vs. 3) p=0.646(2 vs. 3)
[4]Non-metastatic (n=20)	193342±61287 p=0.820 (1vs. 4)	473604±23874 p=0.881 (1vs. 4)	59074±12686 p=0.820(1vs. 4)	151345±39169 p=0.086(1vs. 4)
[5]Metastatic (n=10)	192848±126553 p=0.905 (1vs. 5) p=0.997 (4 vs. 5)	453160±49360 p=0.853 (1vs. 4) p=0.837 (4 vs. 5)	45767±10084 p=0.905 (1vs. 5) p=0.997 (4 vs. 5)	96389±35423 p=0.565 (1vs. 5) p=0.458(4 vs. 5)

Serum α-2,3 sialoproteins levels were found to be comparable between well and moderately differentiated tumors (p=0.922). Levels of salivary α-2,3 and α-2,6 sialoproteins were comparable between well, moderate and poorly differentiated tumors.

Multivariate analysis showed significant association of serum α-2,6 sialoproteins with perineural invasion (F=16.320, *p*=0.027).

Levels of serum and salivary α-2,6 and α-2,3 sialoproteins in PT, CR and NR

The levels of serum and salivary α-2,3 and α-2,6 sialoproteins were compared between PT levels and the levels in CR and NR during post treatment follow-ups.

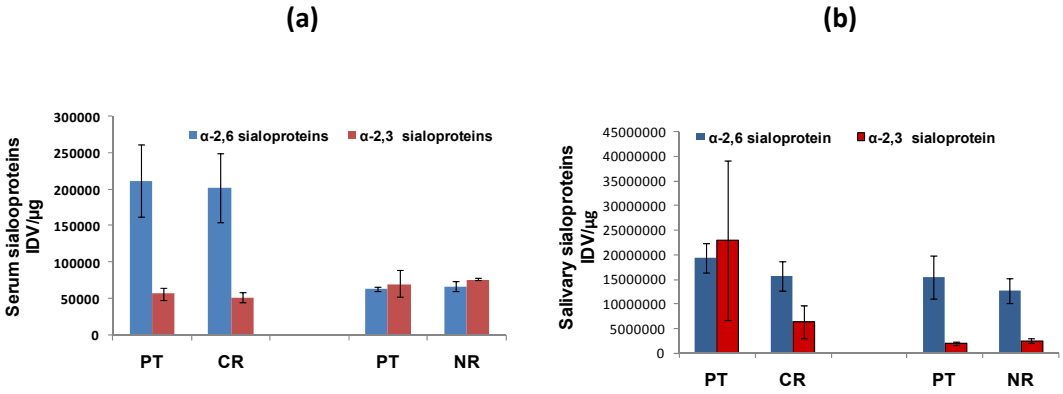


Figure 5.16 ***(a): Levels of serum and (b) salivary α-2,6 and α-2,3 sialoproteins in PT, CR and NR.*** *PT: pre-treatment, CR: complete responders, NR: non-responders*

As depicted in **Figure 5.16 (a),** the serum levels of α-2,3 and α-2,6 sialoproteins were found to be comparable between PT and CR (*p*=0.128 and *p*=0.097, respectively) as well as between PT and NR (*p*=0.052 and *p*=0.558, respectively). **Figure 5.16 (b)** shows that the salivary levels of α-2,3 and α-2,6 sialoproteins were found to be decreased in CR (*p*=0.309 and *p*=0.026, respectively) as compared to PT levels. While salivary levels of α-2,3 and α-2,6 sialoproteins were comparable between PT and NR (*p*=0.083 and *p*=0.354, respectively).

ROC curve analysis of serum and salivary α-2,3 and α-2,6 sialoproteins

ROC curve were constructed to evaluate the diagnostic performance of the test to distinguish between controls, patients with OPC and oral cancer patients. The optimal ROC cut-off, AUC, significance, sensitivity and specificity are mentioned in **Table 5.10**. It was observed that serum and salivary α-2,3 **s**ialoproteins significantly distinguished controls and oral cancer patients (*p*=0.0479 and *p*=0.0015, respectively). Salivary α-2,3 sialoproteins also significantly discriminated controls and patients with

OPC (p=0.0007). Moreover, salivary α-2,6 sialoproteins also significantly distinguished patients with OPC and oral cancer patients.

Table 5.10: ROC curve analysis for serum and salivary α-2,3 and α-2,6 Sialoproteins

Groups compared		Serum α-2,3 sialoproteins	Salivary α-2,3 sialoproteins	Serum α-2,6 sialoproteins	Salivary α-2,6 sialoproteins
Controls vs. Oral cancer	ROC cut-off	8539920	2202816	68415408	25844824
	AUC	0.667	0.754	0.564	0.543
	Significance	***p*=0.0479**	***p*=0.0015**	p=0.6542	p=0.7156
	Sensitivity	81.0%	95.7%	90.9%	61.5%
	Specificity	54.5%	47.1%	50.0%	70.6%
Controls vs. Patients with OPC	ROC cut-off	12991768	3403080	8968896	15793920
	AUC	0.652	0.779	0.556	0.604
	Significance	p=0.0729	***p*=0.0007**	p=0.6916	p=0.2980
	Sensitivity	36.4%	70.0%	100.0%	47.4%
	Specificity	95.5%	76.5%	20.0%	94.1%
Patients with OPC vs. Oral cancer Patients	ROC cut-off	7107552	10030392	68415408	12858624
	AUC	0.515	0.514	0.626	0.704
	Significance	p=0.8687	p=0.8820	p=0.3519	***p*=0.0298**
	Sensitivity	85.7%	34.8%	90.9%	100.0%
	Specificity	31.8%	94.1%	44.4%	36.8%

Survival analysis of serum and salivary α-2,3 and α-2,6 sialoproteins

Kaplan Meir's survival analysis of serum and salivary α-2,3 and α-2,6 sialoproteins was performed to assess overall survival with values below and above ROC cut-off. The Mantel Cox Chi^2 value and significance of serum and salivary α-2,3 sialoproteins are Chi^2=0.887, p=0.346 and Chi^2=0.917, p=0.338, respectively. The Mantel Cox Chi^2 value and significance of serum and salivary α-2,6 sialoproteins were Chi^2=0.667, p=0.414 and Chi^2=0.006, p=0.937, respectively. The results revealed no significant association of serum and salivary α-2,3 and α-2,6 sialoproteins with overall survival.

OBJECTIVE 2.4: Estimation of serum and salivary α-2,3 and α-2,6 ST activities

α-2,6 and α-2,3 ST activities were estimated by means of solid phase ELISA as described by Hakomori *et al*., (1981) and Yeh *et al*., (1996) with minor necessary modifications. Biotinylation of linkage (α-2,6 and α-2,3) specific lectins *(Sambucus*

nigra and *Macckia amurensis,* respectively) were initially done using Sulpho-NHS-Biotinlylation kit and these biotinylated lectins were used in the solid phase assay. It was estimated from 100 controls, 50 patients with OPC and 100 oral cancer patients.

Standard curve for ST activity using p-Nitro phenol

The standard curve for ST activity was prepared using p-Nitro phenol as standard. As depicted in **Figure 5.17**, the standard curve was linear from 20µM to 200 µM concentration.

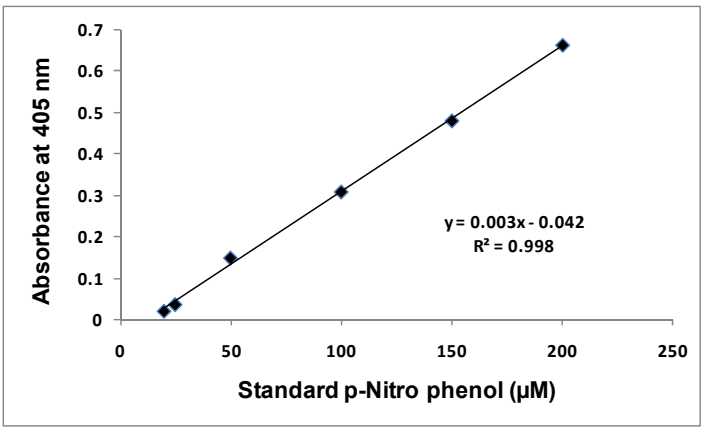
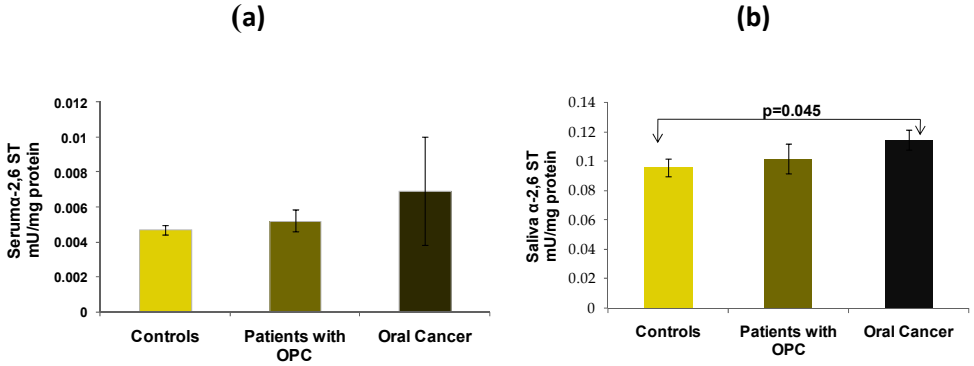


Figure 5.17: ***Standard curve for ST activity using p-Nitro phenol as standard.*** *ST: Sialyl transferase*

Comparison of serum and salivary α-2,3 and α-2,6 ST between controls, patients with OPC and oral cancer patients

Figure 5.18 depicts the comparison of serum and salivary α-2,3 and α-2,6 ST in controls, patients with OPC and oral cancer patients.



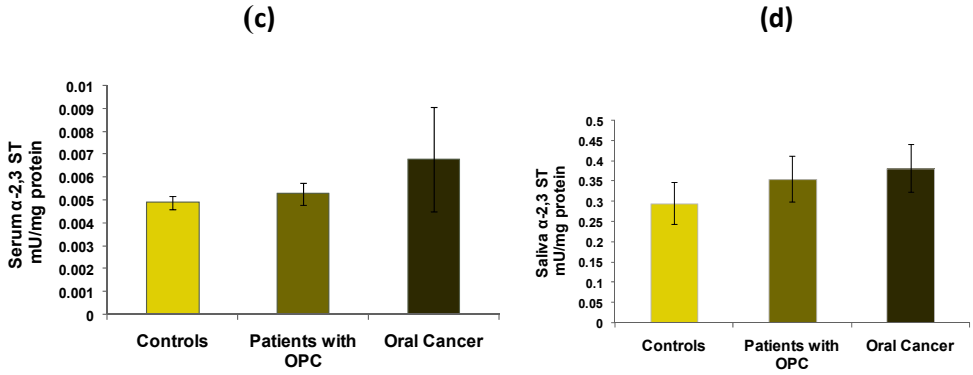


Figure 5.18: ***Comparison of (a) Serum α-2,6 ST (b) Salivary α-2,6 ST (c) Serum α-2,3 ST (d) Salivary α-2,3 ST between controls, patients with OPC and oral cancer patients.*** *ST: Sialyl transferase, OPC: Oral precancerous conditions. Values are expressed as mU/mg protein*

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 of α-2,3 and α-2,6 ST were found to be significantly elevated ($p<0.0001$) in saliva as compared to serum. The levels of salivary α-2,6 ST activity were found to be significantly higher ($p=0.045$) in oral cancer patients as compared to controls (**Figure 5.18**).

Comparison of levels of serum and salivary α-2,3 and α-2,6 ST levels between various clinicopathological parameters

The serum and salivary α-2,3 and α-2,6 ST activities were compared between early and advanced stage of disease as well between patients with metastasis and without metastasis. The results are mentioned in **Table 5.11**. It was observed that the salivary levels of α-2,3 ST were found to be higher in patients with metastasis as compared to the patients without metastasis and were also higher in advanced stage of disease as compared to early stage of disease. The levels of serum and salivary α-2,6 ST were comparable between early and advanced stage of disease, as well as between non metastatic and metastatic tumors. Serum and salivary α-2,6 ST and α-2,3 ST were comparable between well and moderately differentiated tumors.

Table 5.11: Serum and salivary α-2,3 and α-2,6 ST activities with clinico-pathological parameters

Markers	Serum α-2,3 ST	Salivary α-2,3 ST	Serum α-2,6 ST	Salivary α-2,6 ST
[1]Controls	0.0049±0.008	0.2960±0.0052	0.00473±0.0028	0.0963±0.0060
[2]Early Disease	0.0049±0.0008 *p*=0.996 (1 vs. 2)	0.2749±0.046 *p*=0.841 (1 vs. 2)	0.0041±0.0006 *p*=0.249 (1 vs. 2)	0.1170±0.0131 *p*=0.128 (1 vs. 2)
[3]Advanced Disease	0.0038±0.0004 ***p*=0.019** (1 vs.3) *p*=0.177(2 vs. 3)	0.4918±0.1042 *p*=0.100 (1 vs. 3) *p*=0.064 (2 vs. 3)	0.0035±0.0004 ***p*=0.013** (1 vs. 3) *p*=0.397 (2 vs. 3)	0.1231±0.0113 ***p*=0.024** (1 vs. 3) *p*=0.731 (2 vs. 3)
[4]Non-metastatic Disease	0.0045±0.0005 *p*=0.374 (1 vs. 4)	0.3747±0.0909 *p*=422 (1 vs. 4)	0.0040±0.0005 *p*=0.137 (1 vs. 4)	0.1116±0.0083 *p*=0.141 (1 vs. 4)
[5]Metastatic Disease	0.0039±0.0004 *p*=0.374 (1 vs. 5) *p*=0.081 (4 vs. 5)	0.4049±0.1046 *p*=0.339(1 vs. 4) *p*=0.822 (4 vs. 5)	0.0033±0.0003 ***p*=0.015** (1 vs. 4) *p*=0.390 (4 vs. 5)	0.1164±0.0162 *p*=0.176 (1 vs. 4) *p*=0.769 (4 vs. 5)

ROC curve analysis of serum and salivary α-2,3 and α-2,6 ST activities

ROC curves were constructed for serum and salivary α-2,3 and α-2,6 ST to assess diagnostic efficacy of the markers to distinguish controls vs. oral cancer patients, controls vs. Patients with OPC and patients with OPC vs. oral cancer patients. The AUC, ROC cut-off and significance are as mentioned in **Table 5.12**.

Table 5.12: ROC curve analysis for serum and salivary α-2,3 ST and α-2,6 ST

Groups compared		Serum α-2,3 ST	Salivary α-2,3 ST	Serum α-2,6 ST	Salivary α-2,6 ST
Controls vs. Oral cancer	ROC cut-off	0.0027	0.5904	0.0034	0.0708
	AUC	0.589	0.507	0.649	0.588
	Significance	*p*=0.0578	*p*=0.8864	***p*=0.0010**	*p*=0.0608
	Sensitivity	-	-	60.49%	76.32%
	Specificity	-	-	67.16%	41.89%
Controls vs. Patients with OPC	ROC cut-off	0.0042	0.1043	0.0046	0.114
	AUC	0.549	0.594	0.511	0.515
	Significance	*p*=0.3854	*p*=0.1043	*p*=0.8429	*p*=0.7865
	Sensitivity	-	-	-	-
	Specificity	-	-	-	-
Patients with OPC vs. Oral cancer Patients	ROC cut-off	0.0030	0.1922	0.0030	0.112
	AUC	0.577	0.589	0.650	0.602
	Significance	*p*=0.1388	*p*=0.1068	***p*=0.0026**	*p*=0.0599
	Sensitivity	-	-	44.44%	43.4%
	Specificity	-	-	83.72%	79.1%

As depicted in **Table 5.12**, serum α-2,6 ST significantly distinguish controls and oral cancer patients (p=0.0010) with AUC of 0.649 with sensitivity and specificity of 60.49% and 67.16% respectively. Moreover, serum α-2,6 ST significantly also significantly discriminated patients with OPC and oral cancer patients (p=0.0026) with AUC of 0.650.

Comparison of serum and salivary and salivary α-2,3 and α-2,6 ST between PT vs. CR and PT vs. NR

The levels of serum and salivary α-2,3 and α-2,6 ST were compared between PT levels and the levels during post treatment follow-ups in CR and NR. **Figure 5.19** and **Table 5.13** depicts 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 (p=0.012, p=0.001 and p=0.010 respectively) as compared to PT levels.

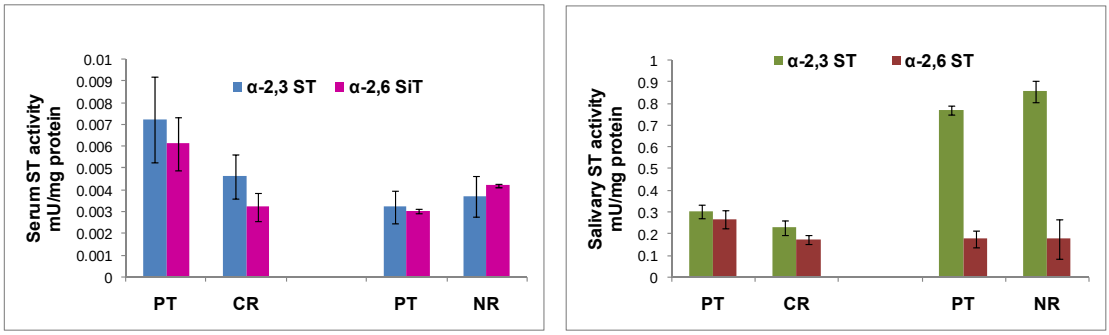


Figure 5.19: ***(a) Levels of serum α-2,3 and α-2,6 ST in PT, CR and NR. (b) and salivary α-2,3 and α-2,6 ST in PT, CR and NR. PT: pre-treatment, CR: Complete responders, NR: Non-responders***

Table 5.13: Significance of serum and salivary α-2,3 and α-2,6 ST in distinguishing PT vs. CR and PT vs. NR

Markers	PT vs. CR	PT vs. NR
α-2,6 ST (serum)	***p*=0.012**	p=0.024
α-2,3 ST (serum)	p=0.107	p=0.812
α-2,6 ST (saliva)	***p*=0.001**	p=0.314
α-2,3 ST (saliva)	***p*=0.010**	p=0.993

The levels of serum α-2,6 ST were found to be significantly increased (p=0.024) in NR as compared to PT levels. The levels of serum α-2,3 ST, serum and salivary α-2,3 ST and α-2,6 ST were also found to be increased in NR as compared to PT levels.

Kaplan Meir's survival analysis of serum and salivary α-2,3 and α-2,6 ST

Kaplan Meir's survival analysis was carried out to assess the correlation of serum and salivary α-2,3 and α-2,6 ST with overall survival. The optimal ROC cut-off, sensitivity, specificity, AUC with survival estimate and Chi^2 value are depicted in **Table 5.14**. Survival analysis showed no significant association of serum and salivary α-2,3 and α-2,6 ST with overall survival.

Table 5.14: Survival analysis of serum and salivary α-2,3 and α-2,6 ST with ROC cut-off

Parameter	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2	p value
Serum α-2,3 ST	0.0027 (0.589)	33.3%/ 90.9% p=0.0578	39.565±3.330	40.2±2.798	0.002	*p*=0.964
Salivary α-2,3 ST	0.5904 (0.507)	21.43%/96.92% p=0.8864	39.0±2.337	40.5±4.209	0.319	*p*=0.572
Serum α-2,6 ST	0.0034 (0.649)	60.5%/67.2% p=0.0010	38.014±2.977	39.155±2.589	0.117	*p*=0.732
Salivary α-2,6 ST	0.0708 (0.588)	76.35%/41.9% p=0.0608	39.465±3.535	39.587±2.332	0.131	*p*=0.718

Correlation analysis between various serum sialylation parameters

Pearson's correlation analysis was performed to assess the correlation between various sialylation changes and the results are as depicted in **Table 5.15**. Serum α-2,6 sialoproteins was observed to be positively correlated with serum α-2,6 ST (r=0.842, *p*=0.0009). Serum α-2,6 ST was observed to be significantly associated with α-2,3 ST (r=0.996, $p<0.0001$).

Serum α-2,3 ST and α-2,6 ST was observed to be negatively associated with sialidase activity (r=-0.269, *p*=0.225 and r=-0.345, *p*=0.137, respectively). Serum TSA/TP ratio was observed to be positively associated with sialidase activity (r=0.150, *p*=0.594) and negatively associated with α-2,3 ST and α-2,6 ST activities (r=-0.093, *p*=0.475 and r=-0.106, *p*=0.410, respectively).

Correlation analysis between salivary sialylation changes

Pearson's correlation analysis was performed to assess the correlation between various salivary sialylation parameters.

Table 5.15: Pearson's correlation analysis between serum sialylation changes

	Serum α-2,6 sialoprotein	Serum α-2,3 sialoprotein	Serum α-2,6 ST	Serum α-2,3 ST	Serum TSA/TP	Serum sialidase
Serum α-2,6 sialoprotein	---	r=-0.531 p=0.469	r=0.842 **p=0.0009**	r=0.570 p=0.109	r=0.135 p=0.728	r=0.2490 p=0.412
Serum α-2,3 sialoprotein	r=-0.531 p=0.469	----	r=0.191 p=0.479	r=-0.114 p=0.675	r=-0.326 p=0.236	r=0.178 p=0.703
Serum α-2,6 ST	r=0.842 p=0.009	r=0.191 p=0.479	-----	r=0.996 **p<0.0001**	r=-0.106 p=0.410	r=-0.269 p=0.225
Serum α-2,3 ST	r=0.570 p=0.109	r=-0.114 p=0.675	r=0.996 **p<0.0001**	-----	r=-0.093 p=0.475	r=-0.345 p=0.137
Serum TSA/TP	r=0.135 p=0.728	r=-0.326 p=0.236	r=-0.106 p=0.410	r=-0.093 p=0.475	-----	r=0.150 p=0.494
Serum sialidase	r=0.249 p=0.412	r=0.178 p=0.703	r=-0.269 p=0.225	r=-0.345 p=0.137	r=0.150 p=0.494	----

Table 5.16: Pearson's correlation analysis between salivary sialylation changes

	Salivary α-2,6 sialoprotein	Salivary α-2,3 sialoprotein	Salivary α-2,6 ST	Salivary α-2,3 ST	Salivary TSA/TP	Salivary sialidase
Salivary α-2,6 sialoprotein	---	r=-0.176 p=0.676	r=0.110 p=0.733	r=0.262 p=0.465	r=0.021 p=0.955	r=-0.203 p=0.870
Salivary α-2,3 sialoprotein	r=-0.176 p=0.676	----	r=-0.018 p=0.943	r=0.030 p=0.907	r=0.247 p=0.309	r=-0.512 p=0.299
Salivary α-2,6 ST	r=0.110 p=0.733	r=0.018 p=0.943	-----	r=0.266 **p=0.037**	r=0.232 p=0.091	r=0.602 **p=0.004**
Salivary α-2,3 ST	r=0.262 p=0.465	r=0.030 p=0.907	r=0.266 **p=0.037**	-----	r=0.086 p=0.554	r=0.257 p=0.304
Salivary TSA/TP	r=0.028 p=0.955	r=0.247 p=0.309	r=0.232 p=0.091	r=0.086 p=0.554	-----	r=0.597 **p=0.003**
Salivary sialidase	r=-0.203 p=0.870	r=-0.512 p=0.299	r=-0.602 **p=0.004**	r=-0.257 p=0.304	r=0.597 **p=0.003**	----

As depicted in **Table 5.16** a significant positive correlation was observed between salivary α-2,6 ST and α-2,3 ST (r=0.266, *p*=0.037). Salivary TSA/TP ratio was significantly positively correlated with sialidase activity (r=0.597, *p*=0.003). Moreover, a significant negative correlation was observed between salivary α-2,6 ST activity and sialidase activity (r=0.602, *p*=0.004), also salivary α-2,3 ST was negatively correlated with sialidase activity (r=-0.257, *p*=0.304).

OBJECTIVE 3: To compare serum and salivary fucosylation changes in controls, patients with OPC, oral cancer patients and post treatment follow-ups and to correlate with clinico-pathological parameters.

3

Evaluation of serum and salivary total sialic acid and α-L-fucosidase in patients with oral precancerous conditions and oral cancer

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Objectives. We compared serum and salivary total sialic acid/total protein (TSA/TP) ratios and α-L-fucosidase activity in patients with oral precancerous conditions (OPCs) and oral cancer to better understand the utility of saliva, in monitoring early changes occurring during oral cancer progression.
Study design. A cross-sectional study of 100 oral cancer patients, 50 patients with OPC, and 100 controls was performed.
Results. Serum and salivary TSA/TP ratios and α-L-fucosidase activity were significantly higher in OPC and oral cancer patients compared to the controls. Also, levels were higher in controls and oral cancer patients with tobacco habits as compared to those without tobacco habits.
Conclusion. Salivary TSA/TP ratio and α-L-fucosidase activity were elevated with higher magnitude than serum levels. These results suggest that a larger study may prove the use of these saliva biomarkers as a noninvasive method for detecting early changes occurring during oral carcinogenesis. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:764-771)

OBJECTIVE 3.1: Evaluation of serum and salivary expression of fucoproteins

For fucoprotein estimation, fucose specific lectin *Lotus tetragonolobus* was used in lectin affinity chromatography, and further SDS-PAGE and silver staining was carried out. The method as described by Thomson and Turner was performed with minor necessary modifications for estimation from saliva supernatant (Thomson and Turner, 1987). It was estimated from 30 controls, 30 patients with OPC and 30 oral cancer patients. The results depicted alterations in 44 kDa fucoprotein in serum and saliva in patients with OPC and oral cancer patients. **Figure 5.20 (a)** and **Figure 5.20 (c)** are representative patterns of serum and salivary fucoproteins, respectively. As depicted in **Figure 5.20 (b)** and **5.20 (d)**, the fucoprotein analysis 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 *(p*=0.035).

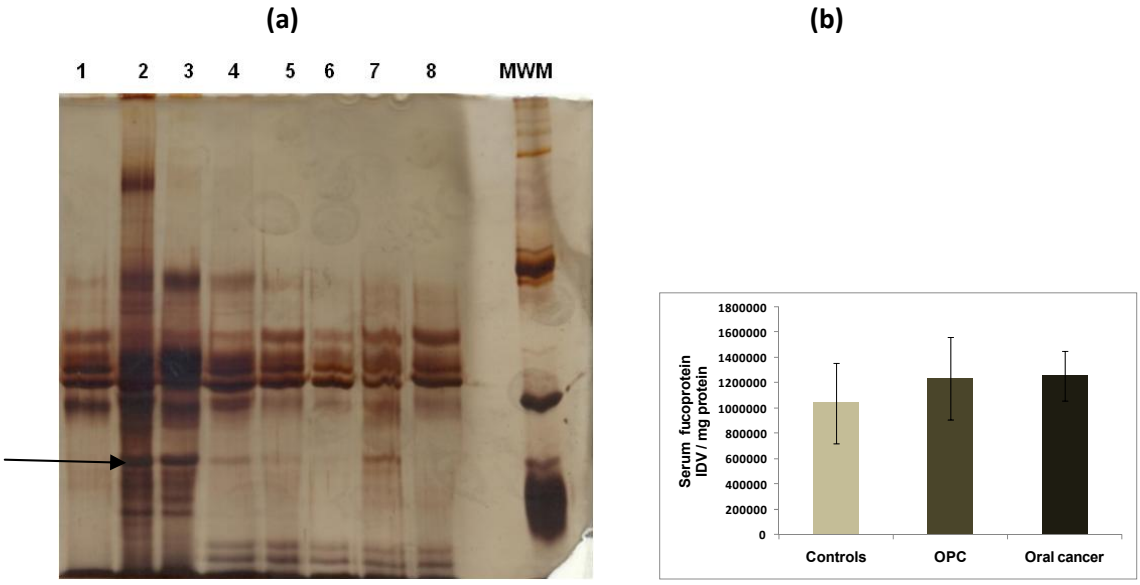

Figure 5.20: ***(a) Representative pattern of fucoproteins from serum and (b) Bar chart representing 44 kDa fucoproteins in controls, Patients with OPC and oral cancer patients.*** *Black pointed arrow depicts 44 kDa fucoprotein. Values are expressed as integrated density value (IDV)/mg protein. Lanes 1,2, 3 represents oral cancer patients, Lanes 4, 5 and 7 represents patients with OPC, Lanes 6, 8 represent controls.*

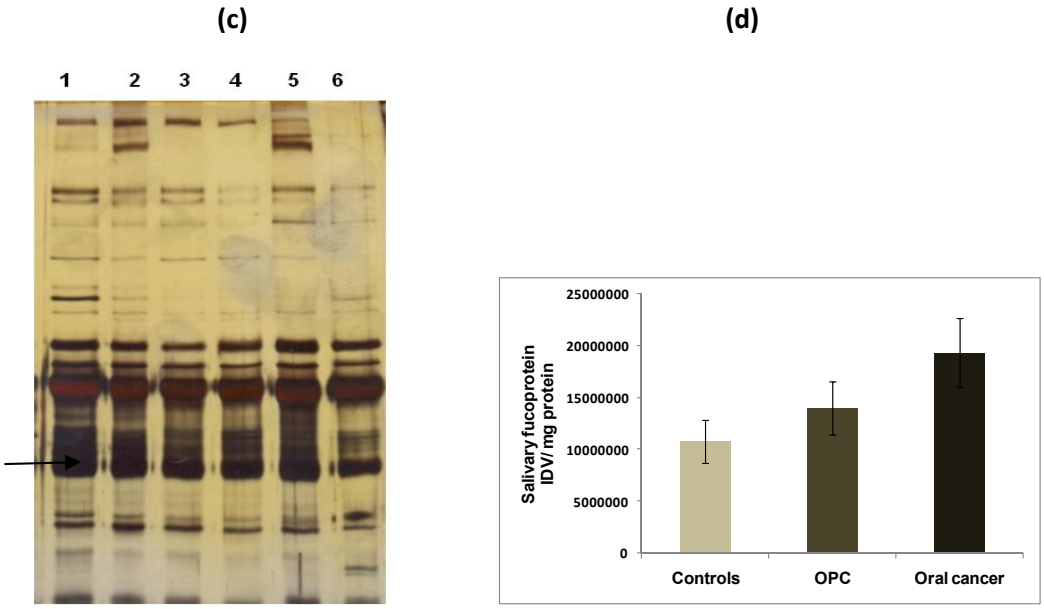

Figure 5.20: ***(c) Representative Salivary fucoprotein profile (d) Salivary fucoprotein levels in controls, patients with OPC, and oral cancer patients.*** *Black pointed arrow depicts 44 kDa fucoprotein. Values are expressed as integrated density value (IDV)/mg protein. Lanes 1,2 represents oral cancer patients, Lanes 3,4 represents patients with OPC, Lanes 5, 6 represents controls.*

Serum and salivary fucoproteins: Correlation with various clinicopathological parameters

The levels of serum and salivary 44 kDa fucoprotein were compared between early (n=13) and advanced (n=17) stage of disease. Both serum and salivary levels were found to be higher in advanced stage of the disease [**Figure 5.21(a) and Figure 5.21(b)**] as compared to early stage (p=0.197 and p=0.038, respectively) and levels were significant for saliva (p=0.038). Moreover, it was observed that serum and salivary levels were marginally decreased in early stage as compared to controls (p=0.785 and p=0.322, respectively).

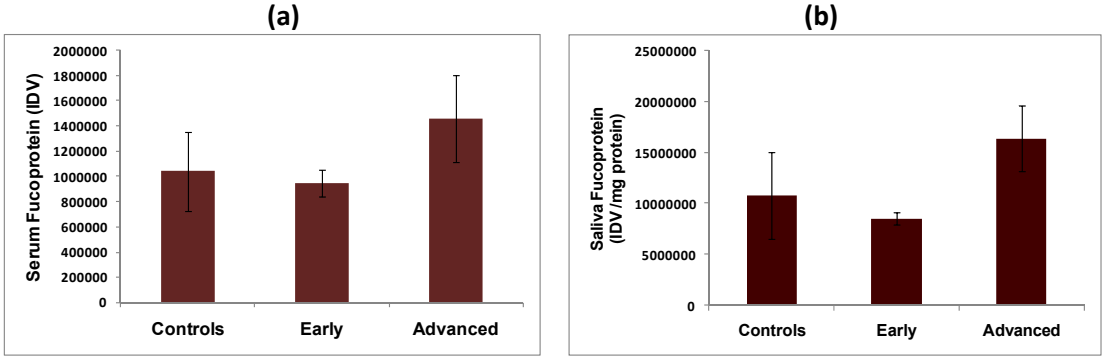

Figure 5.21: ***Levels of (a) serum and (b) salivary fucoproteins in early and advanced disease***

Multivariate analysis revealed significant association of salivary fucoproteins with stage of the disease (p=0.014). Serum and salivary fucoproteins levels were comparable between patients with metastasis (n=10) and patients without metastasis (n=20).

ROC curve analysis of serum and salivary fucoproteins

ROC curves were constructed to study the discriminatory efficacy of serum and salivary fucoproteins. The results indicated that salivary fucoprotein levels significantly discriminated controls and oral cancer patients (p=0.0167) with AUC of 0.771 (**Table 5.17).**

Table 5.17: ROC curve analysis for serum and salivary fucoproteins

Groups compared		Serum fucoprotein	Salivary fucoprotein
Controls vs. Oral cancer patients	Cut-off (IDV/mg protein)	810576	7703424
	AUC	0.625	0.771
	Significance	p=0.3901	***p*=0.0167**
	Sensitivity	69.2%	100%
	Specificity	75.0%	50.0%
Controls vs. Patients with OPC	Cut-off	752400	5154240
	AUC	0.672	0.658
	Significance	p=0.2371	p=0.1930
	Sensitivity	75.0%	100.0%
	Specificity	62.5%	41.7 %
Patients with OPC vs. Oral cancer Patients	Cut-off	869760	8036496
	AUC	0.529	0.712
	Significance	p=0.8455	p=0.1004
	Sensitivity	69.2%	100.0%
	Specificity	62.5%	40.0%

Serum and salivary fucoproteins in post-treatment follow-up samples

The levels of fucoproteins were also assessed in follow-up samples and were compared with the PT values [**Figure 5.22 (a) and (b)]**. The analysis of serum and salivary fucoproteins in follow-up samples revealed decreased expression in CR (p=0.182, p=0.721, respectively) as compared to PT levels while levels were higher in

NR as compared to PT (p=0.072, p=0.507, respectively) levels, however levels were statistically non-significant.

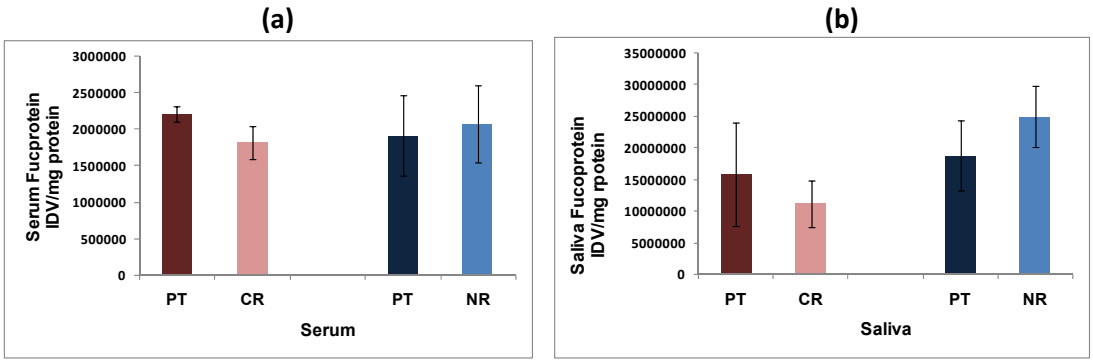

Figure 5.22: ***(a) Levels of serum fucoproteins and (b) salivary fucoproteins in PT, CR and NR.*** *PT: pre-treatment, CR: complete responders, NR: non-responders*

OBJECTIVE 3.2: Estimation of serum and salivary α-L-fucosidase activity

Serum and salivary α-L-fucosidase activity was measured by means of Spectrophotometric method as described by Weiderschain *et al*. (1971). It was estimated from 100 controls, 50 patients with OPC and 100 oral cancer patients. Standard curve was prepared using p-Nitro phenol as standard. **Figure 5.23** depicts the standard curve for α-L-fucosidase activity which was linear from 0.01µg to 7.0 µg.

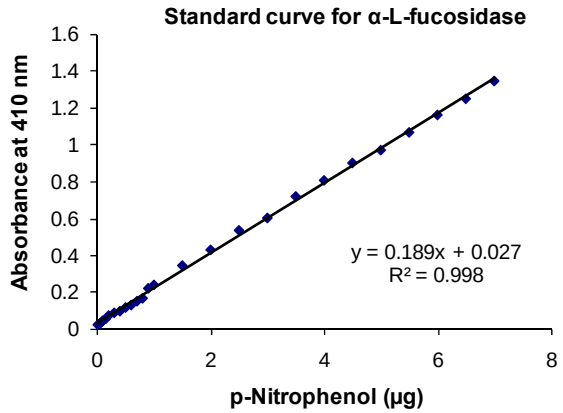

Figure 5.23: ***Standard curve for α-L-fucosidase activity using p-Nitrophenol as standard***

Serum and salivary α-L-fucosidase activity in controls, patients with OPC and oral cancer patients

Figure 5.24 (a) and **(b)** represents serum and salivary α-L-fucosidase activities in controls, patients with OPC and oral cancer patients. Serum α-L-fucosidase activity was found to be significantly higher in oral cancer patients and patients with OPC as

compared to the controls (p<0.0001). Salivary α-L-fucosidase activity was found to be significantly higher in oral cancer patients (p=0.001) 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.

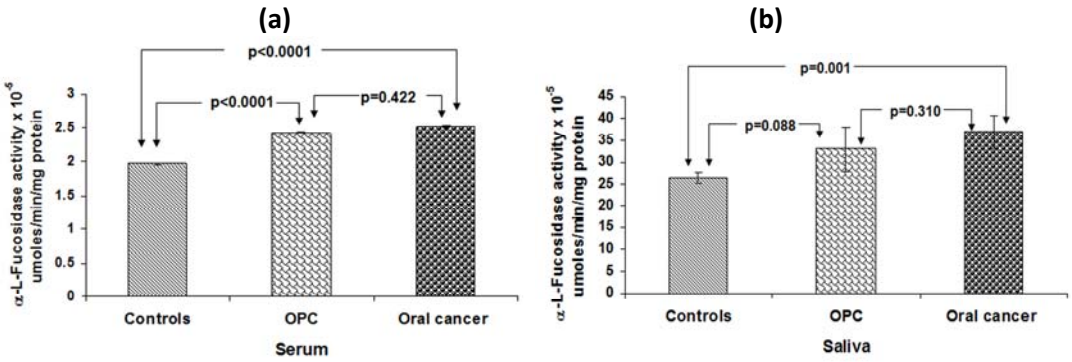

Figure 5.24: ***(a) Serum and (b) salivary α-L-fucosidase activity in controls, patients with OPC and oral cancer patients***

Serum and salivary α-L-fucosidase activity in TNH and TH sub-groups

The serum and salivary α-L-fucosidase activity were compared between TNH and TH sub-groups. The results depicted 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 **Table 5.18**.

Table 5.18: Serum and salivary α-L-fucosidase activity in TNH and TH subgroups

Subjects	Serum α-L-fucosidase activity X 10^{-5} µmoles/min/mg protein	Salivary α-L-fucosidase activity X 10^{-5} µmoles/min/mg protein
[1]TNH controls (n=50)	1.87 ± 0.111	27.04 ± 1.481
[2]TH controls (n=50)	1.92±0.10 *p*=0.695 (1 vs.2)	31.45±2.694 *p*=0.148 (1 vs.2)
[3]Patients with OPC TH (n=50)	2.38±0.0086 ***p*=0.003** (1 vs.3) ***p*<0.0001** (2 vs.3)	33.01±5.113 *p*=0.284 (1 vs.3) *p*=0.785 (2 vs.3)
[4]TNH Oral cancer (n=12)	2.49±0.271 ***p*=0.047** (1 vs.4) ***p*=0.017** (2 vs.4) *p*=0.685 (3 vs.4)	33.48±4.48 *p*=0.071 (1 vs.4) *p*=0.572 (2 vs.4) *p*=0.891 (3 vs.4)
[5]TH Oral cancer (n=88)	2.51±0.0092 ***p*=0.001** (1 vs.5) ***p*<0.0001**(2 vs.5) *p*=0.425 (3 vs.5) *p*=0.961(4 vs.5)	39.97±4.4 ***p*=0.0071** (1 vs.5) *p*=0.102 (2 vs.5) *p*=0.310 (3 vs.5) *p*=0.391(4 vs.5)

Comparison of serum and salivary α-L-fucosidase activity with various clinico-pathological parameters

Table 5.19 documents serum and salivary α-L-fucosidase activity in early and advanced disease, and the activities in patients with metastasis and patients without metastasis. It was observed that serum and salivary α-L-fucosidase activities were higher in advanced disease as compared to early disease and were also higher in patients with metastasis as compared to patients without metastasis. Moreover, both serum and salivary levels were significantly higher in early and late disease as compared to controls ($p<0.0001$). Also, serum and salivary levels were significantly higher in patients with ($p<0.0001$and $p=0.001$, respectively) and without metastasis ($p=0.01$ and $p=0.048$, respectively) as compared to controls.

Table 5.19: Comparison of Serum and salivary α-L-fucosidase activity according to stage and metastasis

Groups	Serum α-L-fucosidase activity x 10^{-5} µmoles/min/mg protein	Salivary α-L-fucosidase activity x 10^{-5} µmoles/min/mg protein
	Mean ± SEM	
[1]Controls (n=100)	1.967±0.0809	27.04±1.48
[2]Early disease (I +II) (n=32)	2.584 ±0.119 ***p*<0.0001**(1 vs.2)	33.83±2.35 ***p*<0.0001**(1 vs.2)
[3]Late disease (III+IV) (n=62)	2.668±0.151 ***p*<0.0001**(1 vs.3) *p*=0.661(2 vs.3)	37.79 ±2.42 ***p*<0.0001**(1 vs.3) *p*=0.244(2 vs.3)
[4]Patients with Metastasis (n=34)	2.726±0.170 ***p*<0.0001**(1 vs.4)	37.54±2.89 ***p*=0.010**(1 vs.4)
[5]Patients with no metastasis (n=56)	2.435±0.108 ***p*=0.001**(1 vs.5) *p*=0.136(4 vs.5)	35.78±6.69 ***p*=0.048**(1 vs.5) *p*=0.620(4 vs.5)

An increasing trend of serum α-L-fucosidase activity was observed from well to moderate to poorly differentiated tumors. The levels were found to be significantly higher in moderately differentiated tumors as compared to well differentiated tumors ($p=0.024$). The levels of salivary α-L-fucosidase activity were comparable between well, moderate and poorly differentiated tumors.

ROC curve analysis of serum and salivary α-L-fucosidase activity

ROC curves were constructed to evaluate the diagnostic performance of the test for serum and salivary α-L-fucosidase activity. The results of ROC curve analysis with cut-off, AUC, sensitivity and specificity are as mentioned in **Table 5.20**. The results revealed that both serum and salivary α-L-fucosidase activity could significantly distinguish controls and oral cancer patients ($p<0.0001$) as well as controls and patients with OPC ($p<0.0001$ and $p=0.014$ respectively). Salivary α-L-fucosidase activity also significantly distinguished patients with OPC and oral cancer patients ($p=0.0025$).

Table 5.20: ROC curve analysis for serum and salivary α-L-fucosidase activity

Groups compared		Serum α-L-fucosidase activity	Salivary α-L-fucosidase activity
Controls vs. Oral cancer patients	ROC cut-off	2.3×10^{-5}	30.0×10^{-5}
	AUC	0.695	0.689
	Significance	***p*<0.0001**	***p*<0.0001**
	Sensitivity	54.0%	62.0%
	Specificity	72.1%	69.4%
Controls vs. Patients with OPC	ROC cut-off	2.3×10^{-5}	20.0×10^{-5}
	AUC	0.703	0.633
	Significance	***p*<0.0001**	***p*=0.0118**
	Sensitivity	64.4%	72.2%
	Specificity	74.4%	46.2%
Patients with OPC vs. Oral cancer Patients	ROC cut-off	3.2×10^{-5}	30.0×10^{-5}
	AUC	0.506	0.652
	Significance	NS	***p*=0.0025**
	Sensitivity	-	64.1%
	Specificity	-	68.5%

NS: Non-significant

Levels of serum and salivary α-L-fucosidase activity in PT, CR and NR

The levels of α-L-fucosidase activity were compared between PT and CR or NR [**Figure 5.25(a) and Figure 5.25(b)].** As depicted in **Figure 5.25 (a)**, serum α-L-fucosidase activity was observed to be significantly decreased in CR ($p=0.034$) as compared to the corresponding PT value. However, levels were comparable between

PT and NR. **Figure 5.25(b)** shows that salivary levels of α-L-fucosidase activity were also comparable between PT and CR as well as PT and NR.

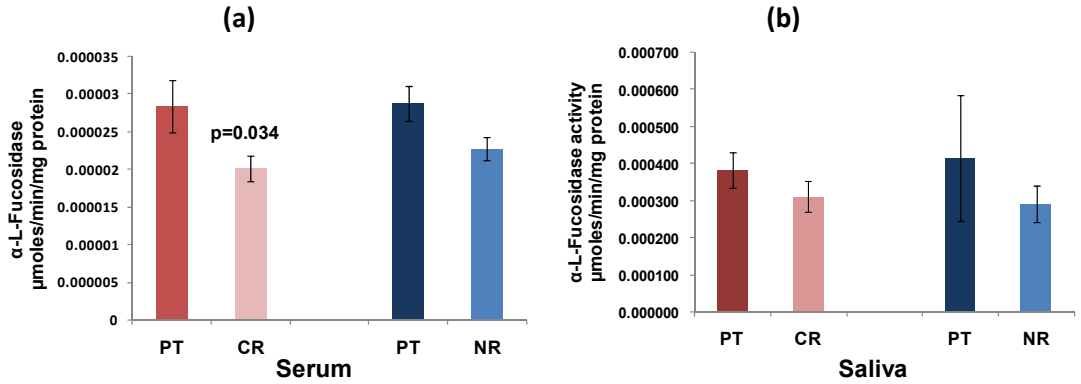

Figure 5.25: ***Levels of (a) serum α-L-fucosidase activity in PT, CR and NR (b) salivary α-L-Fucosidase activity in PT, CR and NR.*** *PT: Pretreatment, CR: Complete responders; NR: Non-responders*

Kaplan Meir's survival analysis of serum and salivary α-L-fucosidase activity with ROC cut-off

Kaplan Meir's survival analysis was carried out to assess the correlation of serum and salivary α-L-fucosidase activity with overall survival **[Figure 5.26 (a)** and **5.26(b)**]. The optimal ROC cut-off, sensitivity, specificity, AUC with survival estimate and Chi^2 value are depicted in **Table 5.21**,

Table 5.21: Survival analysis of serum and salivary α-L-fucosidase activity with ROC cut-off

Parameter	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2 Significance
Serum α-L-fucosidase	$2.3x10^{-5}$ (0.695)	54.0%/ 72.1% *p*<0.0001	41.40±1.72	35.91±2.89	5.190 ***p*=0.023**
Salivary α-L-fucosidase	$30x10^{-5}$ (0.689)	62.0%/ 69.4% *p*<0.0001	38.62±3.73	39.26±2.29	0.089 *p*=0.765

The results depicted that values above ROC cut-off of serum α-L-fucosidase activity was significantly (*p*=0.023) associated with lower overall survival with Chi^2 value of 5.190.

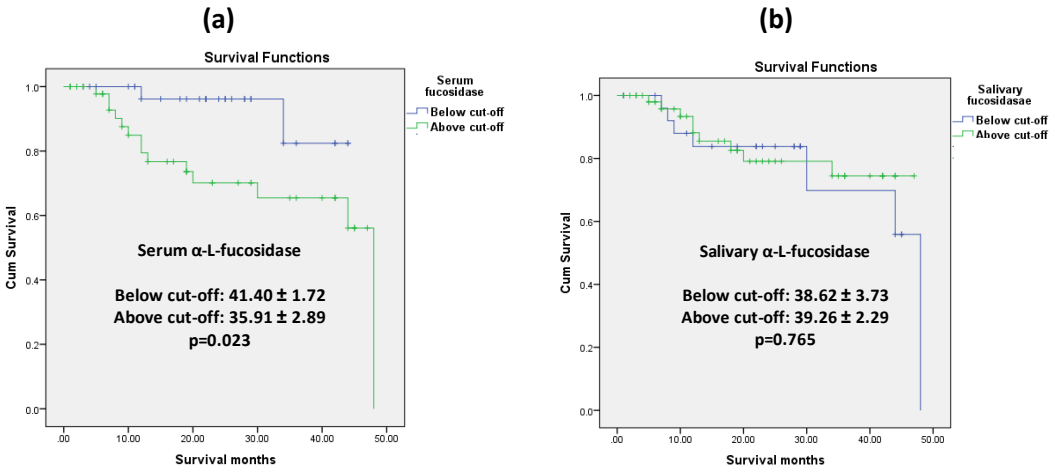

Figure 5.26: ***Kaplan Meir's survival analysis of (a) serum and (b) salivary α-L-fucosidase activity.*** *Kaplan Meier survival curves were compared by Log-rank analysis and the expression levels in patients were depicted as above or below relative to the ROC cut-off value (Table 5.21). The values above and below cut-off are expressed as survival in months ± SEM*

Correlation between fucoproteins and α-L-fucosidase activity

Pearson's correlation analysis was performed to analyze correlation between α-L-fucosidase activity and fucoproteins. The results showed significant negative correlation (r=-0.706, *p*=0.033) between salivary α-L-fucosidase activity and salivary fucoproteins. No significant correlation was observed between serum α-L-fucosidase activity and fucoproteins (r=0.249, *p*=0.412).

OBJECTIVE 4: To study mRNA expression of *ST3GAL1, FUT3, FUT5* and *FUT6* in malignant and adjacent normal tissues of oral cancer patients

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Glycobiology Insights

5

Expression of Glycosyltransferases; *ST3GAL1*, *FUT3*, *FUT5*, and *FUT6* Transcripts in Oral Cancer

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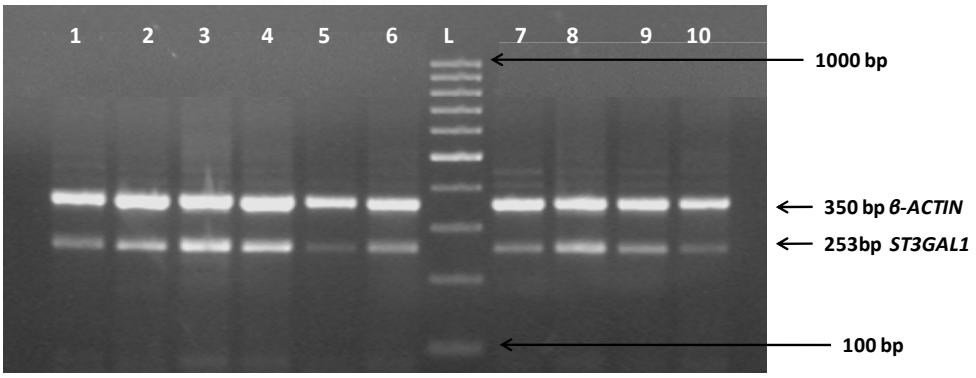
ABSTRACT: Oral carcinogenesis process is frequently accompanied by alterations in glycosylation, regulated by sialyltransferase (ST) and fucosyltransferase (FUT) enzymes. The study aimed to assess *ST3GAL1*, *FUT3*, *FUT5*, and *FUT6* mRNA expression by semi-quantitative reverse transcriptase PCR in 50 oral cancer and 50 adjacent normal tissues. The results indicated increased *ST3GAL1* mRNA levels in malignant tissues as compared to adjacent normal tissues. A significant decrease in *FUT3* and *FUT5* transcripts was observed in malignant tissues as compared to adjacent normal tissues. Survival analysis of *FUT3* transcript levels depicted significant lower survival with values above cutoff. The levels of *ST3GAL1* and *FUT6* were found to be higher in metastatic tissues as compared to the non-metastatic tissues and were also higher in advanced disease as compared to the early disease. The results indicated potential clinical utility of *ST3GAL1*, *FUT3*, *FUT5*, and *FUT6* transcript levels in oral cancer pathogenesis.

KEYWORDS: fucosyltransferase, glycosyltransferase, glycosylation, oral cancer, sialyltransferase

Expression of *ST3GAL1*, *FUT3*, *FUT5* and *FUT6* in malignant and adjacent normal tissues

The expression of ***ST3GAL1, FUT3, FUT5*** and ***FUT6*** transcripts were analyzed from 50 paired malignant and adjacent normal tissues. **Figure 5.27(a)** depicts the representative pattern of *ST3GAL1* expression. **Figure 5.27(b)** shows the graphical representation of the levels of *ST3GAL1* mRNA expression in adjacent normal and malignant tissues. It represents that *ST3GAL1* mRNA expression was found to be higher in malignant tissues as compared to adjacent normal tissue (p=0.535), however levels were not statistically significant.

(a)



(b)

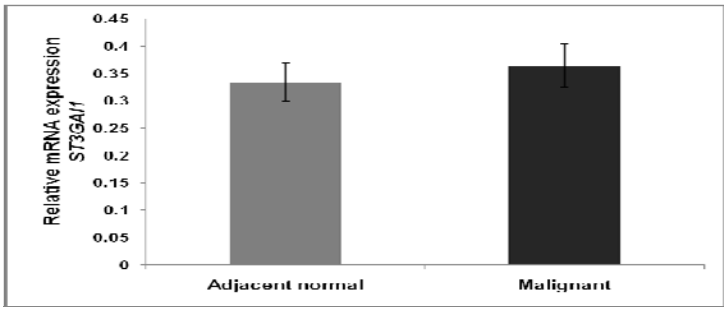


Figure 5.27 (a): ***Representative pattern of ST3GAL1 expression in paired malignant and adjacent normal tissues.*** *Lanes 1, 3, 5, 7, 9 represent the amplicon pairs of ST3GAL1 (253 bp) and β-ACTIN (350 bp) from adjacent normal tissues whereas Lanes 2, 4, 6, 8, 10 represent the amplicon pairs of ST3GAL1 (253 bp) and β-ACTIN (350 bp) from malignant tissues. Lane L represents DNA ladder (100-1000 bp). ST: sialyltransferase*. **(b):** ***Graphical representation of mean expression of ST3GAL1 in paired malignant and adjacent normal tissues.*** *The levels are expressed as ratio of IDV of the glycosyl transferase transcripts and β-ACTIN; levels expressed as Mean±SEM; ST: sialyltransferase*

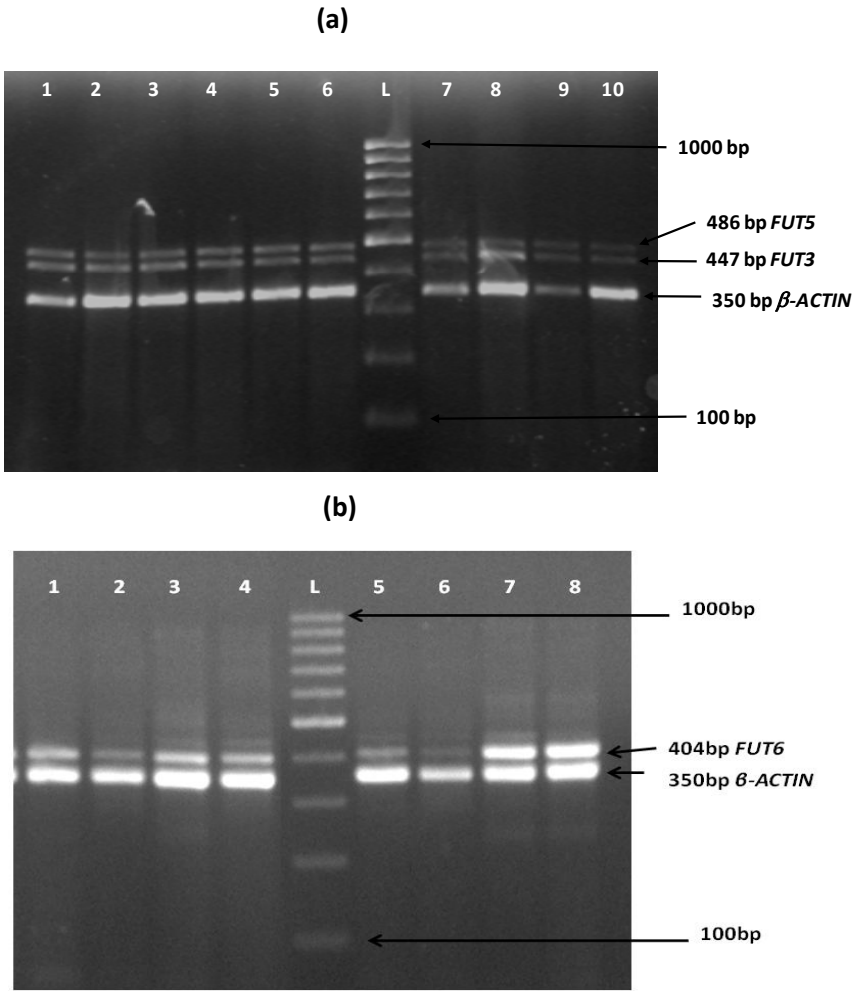


Figure 5.28(a): ***Representative pattern of FUT3, FUT5 expression in paired malignant and adjacent normal tissues***. *Lanes 1, 3, 5, 7, 9 showing the amplicon pairs of FUT3 (447 bp), FUT5 (486 bp) and β-ACTIN (350 bp) from adjacent normal tissues and Lanes 2, 4, 6, 8, 10 showing the amplicon pairs of FUT3 (447 bp), FUT5 (486 bp) and β-ACTIN (350 bp) from malignant tissues. Lane L represents DNA ladder (100-1000 bp).* ***(b): Representative pattern of FUT6 expression in paired malignant and adjacent normal tissues.*** *Lane 1, 3, 5, 7 shows the amplicon pairs of FUT6 (404 bp) and β-ACTIN (350 bp) from adjacent normal tissues, Lane2, 4, 6, 8 show the amplicon pairs of FUT6 (404 bp) and β-ACTIN (350 bp) from malignant tissues, Lane L represents the DNA ladder (100-1000 bp). FUT: fucosyl transferase*

Figure 5.28 (a) is the representative pattern of *FUT3* and *FUT5* mRNA expression, and **Figure 5.28 (b)** is the representative pattern of *FUT6* mRNA expression. *FUT3* and *FUT5* transcripts levels were found to be significantly lower (p=0.008 and p=0.0021, respectively) in malignant tissues (ratio: 0.189 and 0.170, respectively) as compared to the adjacent normal tissues (ratio: 0.381 and 0.363, respectively) (**Figure 5.28(c)**.

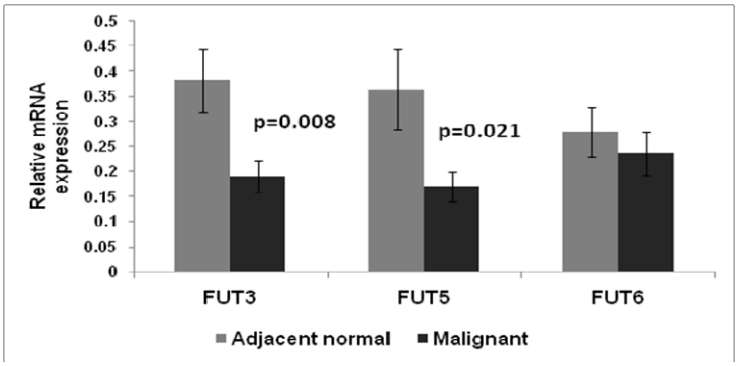


Figure 5.28(c): ***Comparison of FUT3, FUT5 and FUT6 mRNA levels in paired adjacent normal and oral cancer tissues.*** *The levels are expressed as ratio of IDV of the glycosyl transferase transcripts and β-ACTIN* ***;*** *FUT: fucosyltransferase*

The mRNA expression of *FUT6* was comparable (p=0.154) between malignant (ratio: 0.279) and adjacent normal tissues (ratio: 0.236).

Table 5.22: ROC curve analysis of the transcripts showing cut-off, sensitivity, specificity, and AUC

Markers	ROC cut-off	Sensitivity	Specificity	AUC	p value
FUT3	0.164	63.04	65.91	0.675	**p=0.002**
FUT5	0.191	73.47	47.43	0.607	p=0.0727
FUT6	0.307	38.1	85.4	0.561	p=0.4447
ST3GAL1	0.281	65.0	61.1	0.572	p=0.2893

Correlation of *ST3GAL1*, *FUT3*, *FUT5* and *FUT6* transcript levels with overall survival of oral cancer patients

The optimal cut-off point of the transcripts was determined using ROC curve analysis with maximum sensitivity and specificity which can distinguish adjacent normal and malignant tissues. The levels below cut-off and above cut-off were analyzed for overall survival analysis.

Survival analysis depicted significant lower survival (Chi^2=4.76, p=0.029) in patients with expression above ROC cutoff (cutoff: 0.164, sensitivity=63.04; specificity=65.91, AUC=0.675; p=0.002) of *FUT3* transcripts **(Figure 5.29)** in malignant tissues.

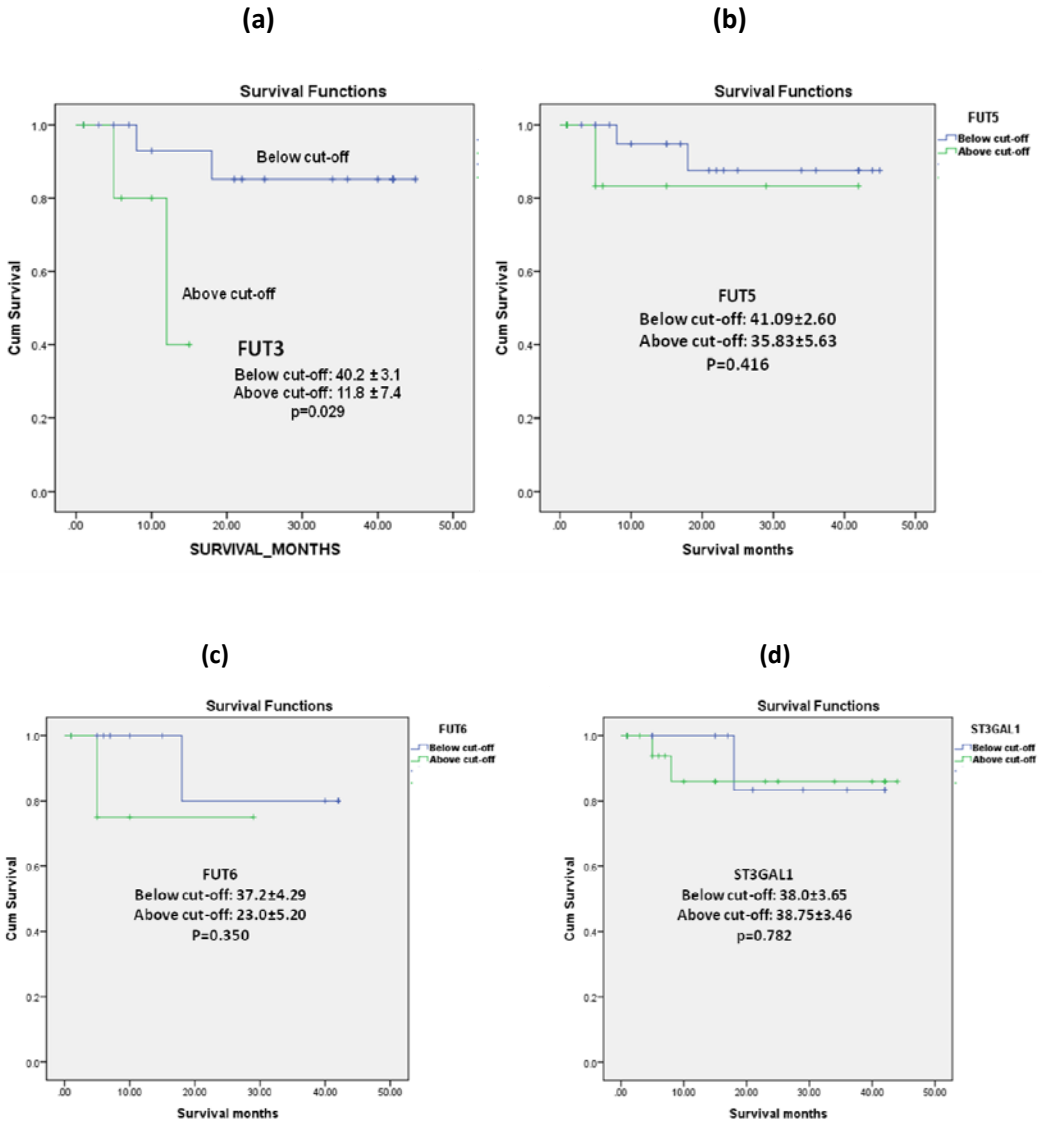


Figure 5.29: ***Kaplan-Meier survival analysis of FUT3, FUT5, FUT6 and ST3GAL1 transcripts***
Kaplan Meier survival curves were compared by Log-rank analysis and ***(a) FUT3, (b) FUT5, (c) FUT6 and (d) ST3GAL1 expression levels*** *in patients were depicted as above or below relative to the ROC cut-off value (Table 4.23). The values above and below cut-off are expressed as survival in months ± SEM; FUT: fucosyltransferase ST: sialyltransferase; AUC: Area under curve*

Also the results of ROC curve analysis depicted that *FUT3* expression could significantly (p=0.002) distinguish malignant and adjacent normal tissues. Moreover, lower overall survival was observed for *FUT5* and *FUT6* transcripts with values above ROC cut-off by Kaplan Meir's survival analysis (Chi^2=0.663, p=0.416; Chi^2=0.874, p=0.350, respectively). The optimal ROC cut off of *FUT5, FUT6,* and *ST3GAL1* are as mentioned in **Table 5.22**.

Correlation of *ST3GAL1, FUT3, FUT5* and *FUT6* expression with various clinico-pathological parameters

The expression levels of *ST3GAL1, FUT3*, *FUT5* and *FUT6* transcripts were compared between lymph-node negative (non-metastatic) (n=29) and lymph-node positive (metastatic) tumors (n=18). **Figure 5.30** documents the graphical representation of the mRNA levels of *ST3GAL1, FUT3*, *FUT5* and *FUT6* in non-metastatic and metastatic tumors of oral cancer patients. It was observed that mean levels of *ST3GAL1* and *FUT6* transcripts were found to be non-significantly higher (p=0.237 and p=0.260, respectively) in metastatic tumors (ratio; 0.433 and 0.348, respectively) as compared to non-metastatic tumors (ratio: 0.315 and 0.228, respectively). The levels of *FUT3* and *FUT5* were comparable (p=0.678 and p=0.795, respectively) between non-metastatic (ratio: 0.189 and 0.138, respectively) and metastatic tumors of the patients (ratio: 0.220 and 0.149, respectively).

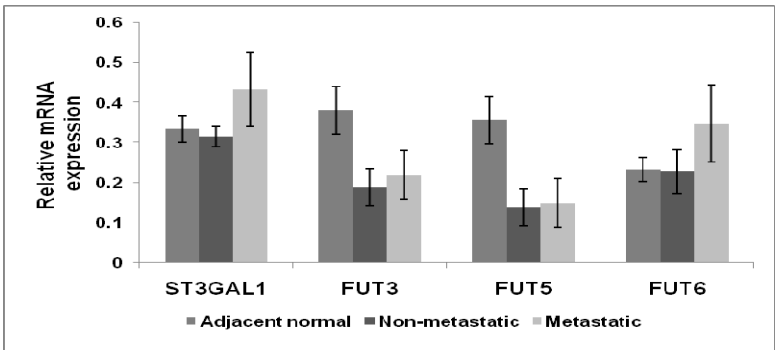


Figure 5.30: ***Comparison of ST3GAL1, FUT3, FUT5 and FUT6 mRNA expression levels in non-metastatic and metastatic oral cancer tissues.*** *The levels are expressed as ratio of IDV of the glycosyl transferase and βACTIN; FUT: fucosyltransferase; ST:*

The expression of *ST3GAL1, FUT3*, *FUT5* and *FUT6* transcripts were compared between early (n=17) and advanced stage (n=31) of the disease. As depicted in **Figure 5.31**, the bar chart represents *ST3GAL1, FUT3*, *FUT5* and *FUT6* mRNA levels in early and advanced disease in oral cancer patients. It was observed that *ST3GAL1* and *FUT6* transcript levels were marginally higher in advanced disease (ratio: 0.380 and 0.315, respectively) as compared to the early disease (p=0.367 and p=0.101, respectively) (ratio: 0.309 and 0.179), whereas the mean mRNA levels of *FUT3* and *FUT5* were comparable (p=0.905 and p=0.455, respectively) between early (ratio: 0.193 and 0.120, respectively) and advanced stage (ratio: 0.203 and 0.151,

respectively) of the disease. *ST3GAL1* and *FUT5* transcripts levels were observed to be increased from well to moderate to poorly differentiated tumors however levels were statistically non-significant (well vs. moderate, *p*=0.097 and *p*=0.372; well vs. poor, *p*=0.136 and *p*=0.015; moderate vs. poor, *p*=0.285 and *p*=0.233 respectively The levels of *FUT3* and *FUT6* transcripts were comparable between well, moderate and poorly differentiated tumors except for *FUT6* transcript which significantly distinguished moderate and poorly differentiated tumors (well vs. moderate, *p*=0.904 and *p*=0.421; well vs. poor, *p*=0.994 and *p*=0.071, moderate vs. poor, *p*=0.944 and *p*=0.001).

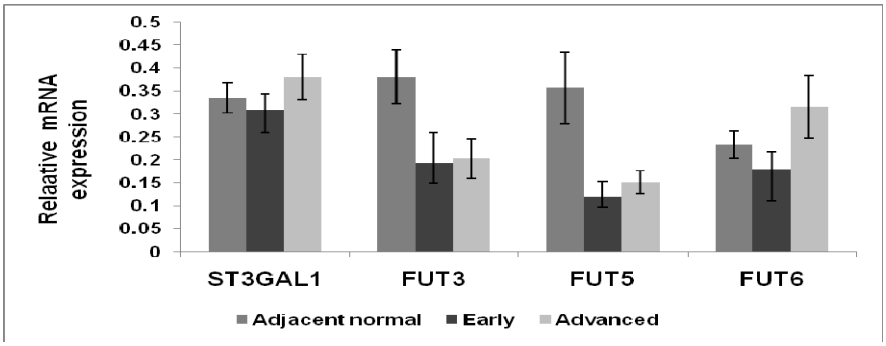


Figure 5.31: ***Comparison of ST3GAL1, FUT3, FUT5 and FUT6 mRNA expression levels in oral cancer tissues with early and advanced disease.*** *The levels are expressed as ratio of IDV of the glycosyl transferase transcripts and βACTIN; FUT: fucosyltransferase; ST: sialyltransferase*

Table 5.23: Multivariate analysis of *ST3GAL1*, *FUT3*, *FUT5* and *FUT6* transcripts with clinico-pathological parameters

Parameters	*ST3GAL1*	*FUT3*	*FUT5*	*FUT6*
Metastasis	F=1.070 *p*=0.319	F=1.135 *p*=0.305	F=1.001 *p*=0.334	F=0.996 *p*=0.333
Differentiation	F=5.778 ***p*=0.016**	F=0.164 *p*=0.850	F=0.233 *p*=0.795	F=1.423 *p*=0.272
Stage	F=0.340 *p*=0.797	F=0.527 *p*=0.671	F=1.310 *p*=0.313	F=1.159 *p*=0.358
Lymphocytic permeation	F=1.264 *p*=0.279	F=0.289 *p*=0.599	F=0.186 *p*=0.673	F=0.299 *p*=0.592
Perineural invasion	F=0.654 *p*=0.431	F=0.087 *p*=0.772	F=0.766 *p*=0.395	F=0.156 *p*=0.698
Infiltration	F=0.127 *p*=0.727	F=0.846 *p*=0.372	F=1.413 *p*=0.253	F=4.321 ***p*=0.054**

Moreover, multivariate analysis revealed significant association of *ST3GAL1*expression with tumor infiltration (F=4.321, *p*=0.054) and *FUT6* expression

with differentiation (F=5.778, p=0.016) (**Table 5.23**). Further, pair wise analysis revealed that *FUT6* expression was significantly different when compared between well and moderately differentiated tumors (p=0.021) and also between well and poorly differentiated tumors (p=0.005).

OBJECTIVE 5: To study glycosylation associated molecular markers in oral cancer and to correlate with clinico-pathological parameters.

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CLINICAL SIGNIFICANCE OF SALIVARY MATRIX METALLOPROTEINASE-9 IN ORAL PRECANCEROUS CONDITIONS AND ORAL CANCER

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ABSTRACT

Background: Oral cancer is a one of the major health burden worldwide and also in India. The most strenuous problem in oral cancer is metastasis, where use of newer non-invasive biomarkers for early detection of metastatic tumors would be greatly helpful. Aim: The aim was to evaluate salivary matrix metalloproteinase-9 in patients with oral precancerous conditions (OPC) and oral cancer to assess its utility in monitoring oral cancer progression. Subjects and Methods: Total 250 subjects including 100 controls, 50 patients with OPC and 100 oral cancer patients were enrolled for the study. Gelatin zymography was performed for the evaluation of matrix metalloproteinase (MMP)-9 and truncated 42 kDa MMP. Statistical Analysis: Statistical analysis like Student's independent't' test, Receiver's Operating Characteristic (ROC) curve, correlation and multivariate analysis were performed to analyse the results. Results: A truncated form of MMP i.e. 42 kDa was observed to have gelatinolytic activity. An increasing trend of salivary pro MMP-9, active MMP-9, MMP-9 activation ratio and truncated 42 kDa MMP was observed from controls to patients with OPC to oral cancer patients. ROC curve analysis revealed that all forms of MMPs could significantly discriminate controls and oral cancer patients; moreover, pro MMP-9 and active MMP-9 significantly distinguished patients with OPC and oral cancer patients. All the levels were found to be higher in patients with advanced disease and with metastasis as compared to patients with early disease and without metastasis, respectively. Conclusion: Elevated levels of salivary MMP-9 and truncated 42 kDa MMP in patients with OPC and oral cancer may be important for assessing early changes occurring during neoplastic transformation of oral cancer.

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Manuscript to be Communicated:

Loss of E-cadherin by Matrix-metalloproteinases modulates cell-cell adhesion and upregulates c-Jun expression in oral carcinogenesis

Authors: Bhairavi N. Vajaria, Kinjal R. Patel, Rasheedunnisa Begum, Jayendra B. Patel, Franky D. Shah[1], Prabhudas S. Patel

8

Manuscript to be communicated:

Significance of Phosphorylated Epidermal Growth Factor Receptor, Matrix Metalloproteinases and E-cadherin in Oral Cancer

Authors: Bhairavi N. Vajaria, Kinjal R. Patel, Rasheedunnisa Begum, Franky D. Shah, Jayendra B. Patel, Prabhudas S. Patel

Objective 5.1: To study plasma and salivary levels of gelatinases (MMPs) in controls, patients with OPC and oral cancer patients.

Comparison of plasma pro, active and total MMP-2 and MMP-9 in controls, patients with OPC and oral cancer patients

Figure 5.32 (a) is the representative pattern of gelatin zymogram of plasma MMPs in controls (n=100), patients with OPC (n=50) and oral cancer patients (n=100). As depicted in **Figure 5.32 (b)** and **5.32 (c)**, the levels of pro, active and total MMP-2 and MMP-9 were significantly higher in patients with OPC (p<0.0001) as compared to controls.

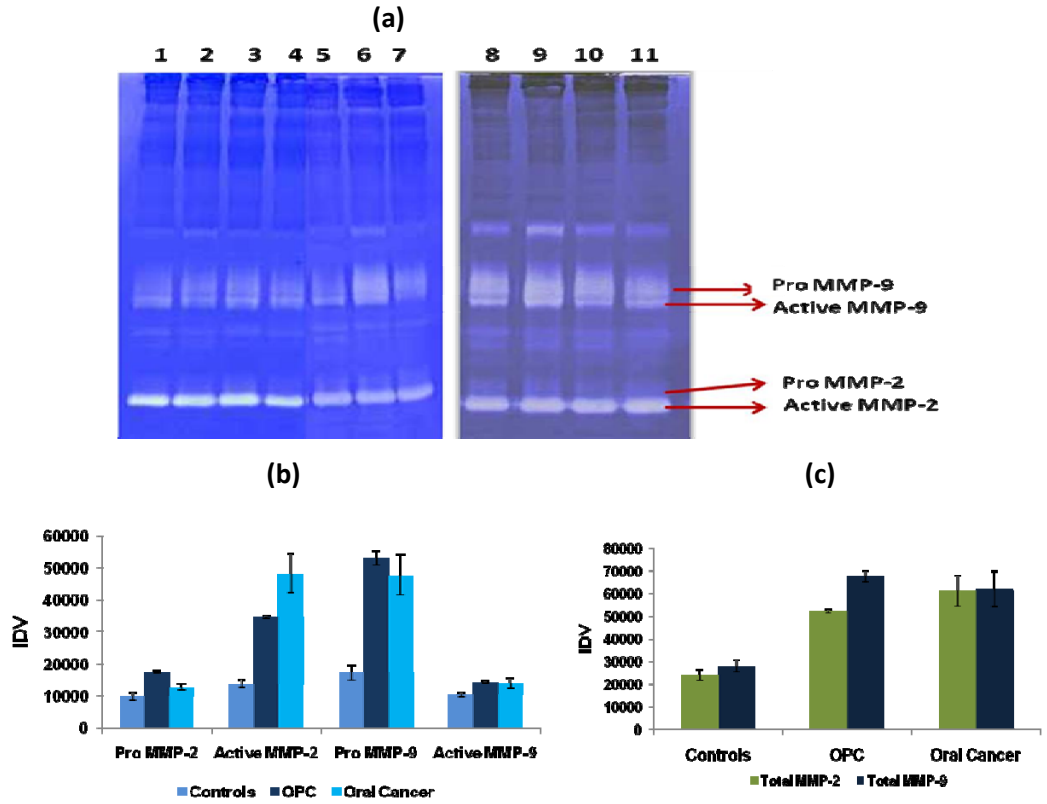


Figure 5.32 (a): ***Representative pattern of gelatin zymography from plasma.*** *Lanes 1, 2, 3 and 4 depict controls; lane 5, 6 and 7 represent patients with OPC; lanes 8, 9, 10 and 11 represent oral cancer patients.* ***(b): Comparison of plasma pro MMP-2, active MMP-2, pro MMP-9, active MMP-9 between controls, patients with OPC and oral cancer patients (c): Comparison of total MMP-2 and total MMP-9 between controls, patients with OPC and oral cancer patients.*** *OPC: Oral precancerous conditions*

Active MMP-2, pro and active MMP-9, total MMP-2 and MMP-9 were significantly higher in oral cancer patients (p<0.0001, p<0.0001, p=0.051, p<0.0001) as compared

to controls (**Table 5.24**). Active MMP-2 was observed to be significantly higher in oral cancer patients as compared to patients with OPC (p=0.025).

Table 5.24: *Comparison of pro, active and total MMP-2 and MMP-9 in controls, patients with OPC and oral cancer patients: 'p' values by student's 't' test*

Groups compared	Pro MMP-2	Active MMP-2	Pro MMP-9	Active MMP-9	Total MMP-2	Total MMP-9
Controls Vs. patients with OPC	***p*<0.0001**	***p*<0.0001**	***p*<0.0001**	***p*<0.0001**	***p*<0.0001**	***p*<0.0001**
Controls vs. Oral cancer	*p*=0.071	***p*<0.0001**	***p*<0.0001**	***p*=0.051**	***p*<0.0001**	***p*<0.0001**
Patients with OPC vs. Oral cancer	***p*<0.0001**	***p*=0.025**	*p=0.427*	*p*=0.868	*p*=0.341	*p*=0.496

Table 5.25: Correlation analysis between different forms of MMP-2 and MMP-9

	Plasma Pro MMP-2	Plasma active MMP-2	Plasma pro MMP-9	Plasma active MMP-9	Plasma Total MMP-2	Plasma Total MMP-9	Activation ratio MMP-2	Activation ratio MMP-9
Plasma Pro MMP-2	---	r=0.559 ***p*<0.0001**	r=0.628 ***p*<0.0001**	r=0.596 ***p*<0.0001**	r=0.662 ***p*<0.0001**	r=0.626 ***p*<0.0001**	r=-0.130 *p*=0.221	r=-0.187 *p*=0.077
Plasma active MMP-2	r=0.559 ***p*<0.0001**	---	r=0.854 ***p*<0.0001**	r=0.823 ***p*<0.0001**	r=0.992 ***p*<0.0001**	r=0.834 *p*=0.221	r=-0.524 *p*=0.077	r=-0.124 *p*=0.246
Plasma Pro MMP-9	r=0.628 ***p*<0.0001**	r=0.854 ***p*<0.0001**	---	r=0.955 ***p*<0.0001**	r=0.871 ***p*<0.0001**	r=0.998 ***p*<0.0001**	r=-0.363 ***p*<0.0001**	r=-0.188 *p*=0.075
Plasma active MMP-9	r=0.596 ***p*<0.0001**	r=0.823 ***p*<0.0001**	r=0.955 ***p*<0.0001**	---	r=0.837 ***p*<0.0001**	r=0.971 ***p*<0.0001**	r=0.429 ***p*<0.0001**	r=-0.017 *p*=0.876
Plasma total MMP-2	r=0.662 ***p*<0.0001**	r=0.992 ***p*<0.0001**	r=0.871 ***p*<0.0001**	r=0.837 ***p*<0.0001**	---	r=0.870 ***p*<0.0001**	r=0.453 ***p*<0.0001**	r=0.141 *p*=0.185
Plasma total MMP-9	r=0.626 ***p*<0.0001**	r=0.854 ***p*<0.0001**	r=0.998 ***p*<0.0001**	r=0.971 ***p*<0.0001**	r=0.870 ***p*<0.0001**	---	r=0.379 ***p*<0.0001**	r=-0.155 *p*=0.144
Activation ratio MMP-2	r=-0.130 *p*=0.221	r=0.524 ***p*<0.0001**	r=0.363 ***p*<0.0001**	r=0.429 ***p*<0.0001**	r=0.453 ***p*<0.0001**	r=0.379 ***p*<0.0001**	---	r=0.253 ***p*=0.016**
Activation ratio MMP-9	r=-0.187 *p*=0.077	r=-0.124 *p*=0.296	r=-0.188 *p*=0.075	r=-0.017 *p*=0.876	r=-0.141 *p*=0.185	r=-0.155 *p*=0.144	r=0.253 ***p*=0.016**	---

Correlation analysis between different forms of plasma MMP-2 and MMP-9

Pearson's correlation analysis was performed to study the inter-correlation between various forms of MMP-2 and MMP-9. The results are as depicted in **Table 5.25**.

As documented in the **Table 5.25**, plasma pro MMP-2 revealed significant positive inter-correlation with active MMP-2, pro and active MMP-9, total MMP-2 and MMP-9 (p<0.0001). Plasma pro and active MMP-9, total MMP-2 and total MMP-9 were also significantly inter-correlated with activation ration of MMP-2 (p<0.0001). Activation ratio of MMP-2 was significantly inter-correlated with activation ratio of MMP-9 (p=0.016).

Levels of different forms of MMPs in early and advanced stage of disease

The levels of different forms of MMPs were further compared between early (n=32) and advanced stage (n=62) of disease. The levels of pro MMP-2, active MMP-2, pro MMP-9, Total MMP-2 and total MMP-9 were observed to be higher in advanced disease as compared to early stage of disease and levels were significant for pro MMP-2 (p=0.003) **(Figure 5.33)**.

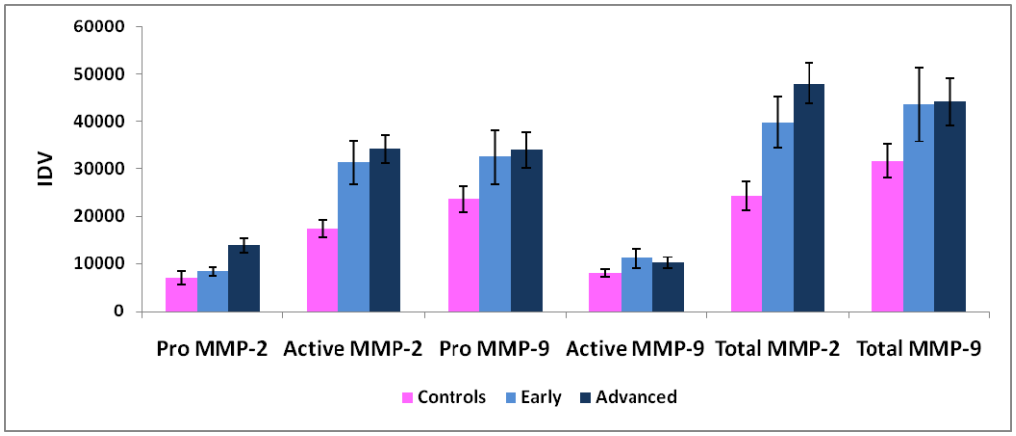


Figure 5.33: ***Levels of different forms of MMPs in early and advanced stage of disease****. IDV: Integrated density value. IDV: Integrated density value*

Multivariate analysis of different forms of plasma MMP-2 and MMP-9 with various clinico-pathological parameters

Multivariate analysis was performed to correlate the markers with various clinico-pathological parameters including stage, differentiation, nuclear grade, metastasis, lymphovascular permeation, lymphocytic infiltration, perineural invasion and

infiltration. The results are as depicted in **Table 5.26**. Multivariate analysis depicted significant correlation of pro MMP-2, pro MMP-9, active MMP-9 and total MMP-9 (p=0.007, p=0.052, p=0.052, p=0.050, respectively) with nuclear grade. Active MMP-2 and total MMP-2 were observed to be significantly associated with lymphovascular permeation (p=0.053 and p=0.052 respectively). Pro MMP-2 was found to be significantly associated with perineural invasion (p=0.021) and infiltration (p=0.053).

An increasing trend of plasma active MMP-2, pro MMP-9, active MMP-9, total MMP-2 and total MMP-9 was observed from well to moderate to poorly differentiated tumors. Plasma pro MMP-2, activation ratio of MMP-2 and MMP-9 were comparable between well, moderate and poorly differentiated tumors.

Table 5.26: Multivariate analysis of different forms of plasma MMP-2 and MMP-9 with various clinico-pathological parameters

	Pro MMP-2	Active MMP-2	Pro MMP-9	Active MMP-9	Total MMP-2	Total MMP-9
Stage	F=1.267 p=0.292	F=0.306 p=0.821	F=0.557 p=0.645	F=0.418 p=0.740	F=0.297 p=0.828	F=0.532 p=0.662
Differentiation	F=1.152 p=0.321	F=0.457 p=0.635	F=2.022 p=0.139	F=2.504 p=0.088	F=0.478 p=0.622	F=2.139 p=0.124
Nuclear grade	F=5.337 ***p*=0.007**	F=1.354 p=0.266	F=3.118 ***p*=0.052**	F=3.116 ***p*=0.052**	F=2.117 p=0.130	F=3.164 ***p*=0.050**
Metastasis	F=0.869 p=0.423	F=0.670 p=0.514	F=0.372 p=0.690	F=0.682 p=0.508	F=0.738 p=0.481	F=0.432 p=0.650
Lymphovascular permeation	F=0.021 p=0.885	F=3.866 ***p*=0.053**	F=0.334 p=0.565	F=0.322 p=0.572	F=3.102 ***p*=0.052**	F=0.336 p=0.563
Lymphocytic stromal response	F=1.174 p=0.282	F=1.420 p=0.237	F=0.398 p=0.530	F=0.196 p=0.659	F=0.846 p=0.360	F=0.357 p=0.552
Perineural invasion	F=5.560 ***p*=0.021**	F=1.748 p=0.190	F=0.286 p=0.594	F=0.208 p=0.650	F=2.411 p=0.124	F=0.274 p=0.602
Infiltration (muscles)	F=3.863 ***p*=0.053**	F=0.125 p=0.724	F=0.664 p=0.418	F=0.056 p=0.813	F=0.001 p=0.971	F=0.495 p=0.483

ROC curve analysis of plasma pro, active and total MMP-2 and MMP-9

ROC curves were constructed for plasma pro, active and total MMP-2 and MMP-9. As depicted in **Table 5.27**, pro MMP-2, active MMP-2, pro MMP-9, active MMP-9, total MMP-2 and total MMP-9 could significantly distinguish controls and patients with OPC ($p<0.0001$, $p<0.0001$, $p<0.0001$, p=0.0002, $p<0.0001$, $p<0.0001$, respectively). Pro MMP-2, active MMP-2, pro MMP-9, total MMP-2 and total MMP-9 could significantly discriminate controls and oral cancer patients ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$, $p=0.006$, respectively). Moreover, pro MMP-2, pro MMP-9, active MMP-9 and total MMP-9 could significantly distinguish patients with OPC and oral cancer patients ($p<0.0001$, $p<0.0001$, $p=0.003$ and $p<0.0001$ respectively).

Table 5.27: ROC curve analysis of plasma pro, active and total MMP-2 and MMP-9

Groups compared		Pro MMP-2	Active MMP-2	Pro MMP-9	Active MMP-9	Total MMP-2	Total MMP-9
Controls vs. patients with OPC	AUC *p* value	0.736 ***p*<0.0001**	0.893 ***p*<0.0001**	0.901 ***p*<0.0001**	0.664 ***p*=0.002**	0.877 ***p*<0.0001**	0.901 ***p*<0.0001**
Controls vs. oral cancer patients	AUC *p* value	0.657 ***p*<0.0001**	0.810 ***p*<0.0001**	0.730 ***p*<0.0001**	0.512 *p*=0.789	0.757 ***p*<0.0001**	0.621 ***p*=0.006**
Patients with OPC vs. oral cancer patients	AUC *p* value	0.267 ***p*<0.0001**	0.452 *p*=0.335	0.305 ***p*<0.0001**	p=0.353 ***p*=0.003**	0.443 *p*=0.225	0.322 ***p*<0.0001**

Kaplan Meir's survival analysis of different forms of plasma MMPs

Kaplan Meir's survival analysis was carried out to estimate overall survival of oral cancer patients. Survival analysis depicted that the oral cancer patients with values above ROC cut-off of active MMP-2, pro MMP-9, total MMP-2 and total MMP-9 **(Table 5.28)** showed lower overall survival as compared to those with values below ROC cut-off. Moreover, for pro MMP-2, activation ratio MMP-2, activation ratio - MMP-9 survival estimate cannot be computed as all the cases were censored.

Table 5.28: Survival analysis of different forms of plasma MMPs using ROC cut-off

Parameters	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2 Significance
Pro MMP-2	3360 (0.657)	96.04%/ 51.35% *p*=0.0009	----	-----	0.134 *p*=0.714
Active MMP-2	13248 (0.810)	77.23%/ 70.27% *p*<0.0001	41.282±3.688	39.359±2.177	0.449 *p*=0.503
Pro MMP-9	9680 (0.730)	82.2%/ 55.4% *p*<0.0001	48.00±0.00	38.248±1.982	2.314 *p*=0.128
Active MMP-9	18360 (0.512)	72.28%/ 4.05% *p*=0.7898	39.269±2.518	39.122±2.585	0.118 *p*=0.731
Total MMP-2	24382 (0.757)	62.38%/ 74.32% *p*<0.0001	43.767±2.639	35.889±2.316	2.826 *p*=0.093
Total MMP-9	39832 (0.621)	45.54%/ 83.78% *p*=0.0041	40.267±2.785	37.732±2.414	0.103 *p*=0.748
Activation ratio MMP-2	0.475 (0.517)	99.01%/ 32.43% *p*=0.7381	-----	-----	0.407 *p*=0.816
Activation ratio MMP-9	0.35 (0.752)	93.07%/ 50.0% *p*<0.0001	-----	------	0.675 *p*=0.411

Expression of salivary pro MMP-9, active MMP-9 and truncated MMP 42 kDa in patients with OPC and oral cancer patients

As depicted in **Figure 5.34** and **Figure 5.35**, the expression of salivary pro MMP-9 and active MMP-9 were found to be significantly higher ($p<0.001$) in oral cancer patients as compared to the controls, also the levels were found to be significantly higher ($p=0.01$ and $p=0.001$, respectively) in oral cancer patients as compared to the patients with OPC. Moreover, an increasing trend was observed from controls to patients with OPC to oral cancer patients. The truncated form of MMP i.e. 42 kDa MMP was observed to have gelatinolytic activity. As illustrated in **Figure 5.35**, the expression of truncated 42kDa MMP was found to be significantly higher in oral cancer patients ($p<0.001$) as compared to the controls. In addition, the levels were found to be higher in patients with OPC as compared to controls and an increasing trend was observed from controls to patients with OPC to oral cancer patients.

Activation ratio i.e. active MMP-9/total MMP-9 was found to be elevated in patients with OPC as compared to the controls (p=0.07). Also the activation ratio was found to be significantly elevated in oral cancer patients as compared to the controls (p<0.001). An increasing trend was observed from controls to patients with OPC to oral cancer patients.

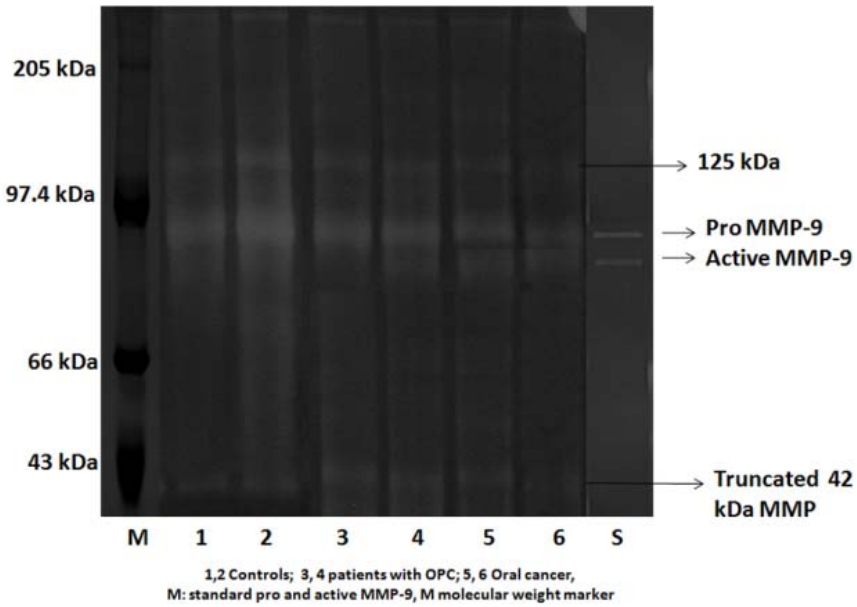
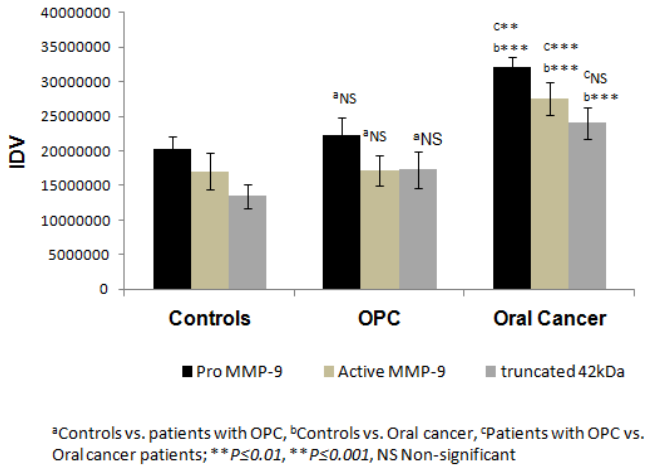


Figure 5.34: ***Representative pattern of gelatin zymogram from saliva.*** *OPC: Oral precancerous conditions*



[a]Controls vs. patients with OPC, [b]Controls vs. Oral cancer, [c]Patients with OPC vs. Oral cancer patients; ***P*≤0.01, ***P*≤0.001, NS Non-significant

Figure 5.35: ***Expression of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in controls, patients with OPC and oral cancer patients.*** *Values are expressed as mean ± SEM. IDV: Integrated density value; OPC: oral precancerous conditions*

When the levels were further compared between TNH and TH, the mean values of pro MMP-9 and active MMP-9 were found to be higher in TH controls as compared to TNH controls, while truncated 42 kDa MMP levels were comparable.

Increased expression of salivary pro MMP-9, active MMP-9 and truncated 42kDa MMP in advanced disease as compared to early disease

As documented in **Figure 5.36**, the levels of pro MMP-9, active MMP-9 and truncated 42 kDa were found to be significantly higher in advanced disease as compared to controls (p=0.007, p=0.010, p<0.001, respectively). The levels of truncated 42 kDa MMP were found to be significantly elevated in early disease as compared to controls (p=0.05). Also, the levels of pro MMP-9, active MMP-9 and truncated 42 kDa MMP were found to be non-significantly higher in advanced disease as compared to early disease (p=0.746, p=0.694 and p=0.086 respectively). The activation ratio of MMP-9 was found to be significantly higher in advanced disease as compared to the controls (p=0.019). The activation ratio was found to be higher in advanced disease as compared to early disease.

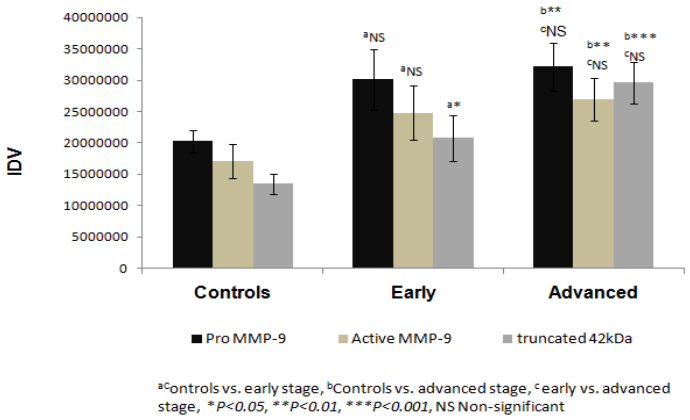
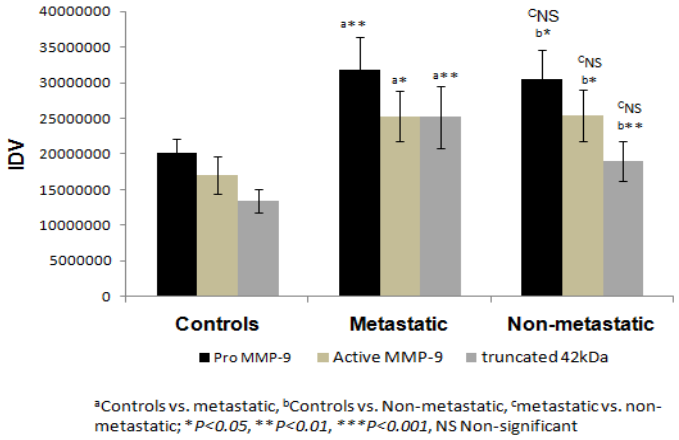


Figure 5.36: ***Expression of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in early and advanced stage of disease.*** *Values are expressed as mean ± SEM. IDV: Integrated density value*

Higher expression of pro MMP-9, active MMP-9 and truncated MMP 42 kDa in patients with lymph-node (LN) metastasis as compared to the patients without LN metastasis

As documented in **Figure 5.37**, the levels of pro MMP-9, active MMP-9 and truncated 42 kDa MMP were found to be significantly higher in patients with LN metastasis (n=34) (p=0.007, p=0.026 and p=0.004 respectively) and without LN metastasis (n=56) (p=0.022, p=0.042 and p=0.085 respectively) as compared to controls. Also, the levels were found to be higher in patients with LN metastasis as compared to patients without LN metastasis. The activation ratio of MMP-9 was found to be higher in patients with metastasis as compared to patients without metastasis. Multivariate analysis depicted significant positive correlation between

truncated 42 kDa MMP with infiltration (F=4.165, p=0.049). Salivary pro MMP-9, active MMP-9, truncated 42 kDa MMP were also comparable between well, moderate and poorly differentiated tumors.



[a]Controls vs. metastatic, [b]Controls vs. Non-metastatic, [c]metastatic vs. non-metastatic; *$P<0.05$, **$P<0.01$, ***$P<0.001$, NS Non-significant

Figure 5.37: ***Expression of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in patients with LN metastasis and without LN metastasis.*** *Values are expressed as Mean ± SEM. MMP: Matrix metalloproteinase; IDV: Integrated density value; LN: Lymph node*

Significant positive correlation between all forms of MMPs

Pearson's correlation analysis was performed to evaluate the correlation between different forms of MMPs. As shown in **Table 5.29**, the results revealed significant positively correlation of salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP (p<0.0001).

Table 5.29: Correlation analysis between all forms of salivary MMPs

	Salivary pro MMP-9	Salivary active MMP-9	Truncated 42 kDa MMP
Salivary pro MMP-9	---	r=0.903 ***p*<0.0001**	r=0.683 *p*<**0.0001**
Salivary active MMP-9	r=0.903 ***P*<0.0001**	----	r=0.723 ***p*<0.0001**
Truncated 42 kDa MMP	r=0.683 *p*<**0.0001**	r=0.723 ***p*<0.0001**	----

ROC curve analysis revealed good discriminatory efficacy of pro MMP-9, active MMP-9 and truncated 42 kDa MMP in discriminating controls, patients with OPC and oral cancer patients

The ROC curves were constructed for pro MMP-9, active MMP-9 and truncated 42 kDa MMP. The analysis showed that salivary pro MMP-9, active MMP-9 and

truncated 42kDa MMP could significantly discriminate between controls and oral cancer patients (p=0.006, p=0.016, p=0.001), respectively **(Table 5.30)**.

Table 5.30: ROC curve analysis of salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP

Groups compared		Pro MMP-9	Active MMP-9	Truncated 42kDa MMP
Controls vs. patients with OPC	AUC Significance	0.478 p=0.735	0.442 p=0.379	0.576 p=0.248
Controls vs. oral cancer patients	AUC Significance	0.617 ***p*=0.0138**	0.609 ***p*=0.0267**	0.699 ***p*=0.001**
Patients with OPC vs. oral cancer patients	AUC Significance	0.694 ***p*=0.004**	0.700 ***p*=0.003**	0.622 p=0.069

Moreover, pro MMP-9 and active MMP-9 significantly distinguished patients with OPC and oral cancer patients (p=0.004 and p=0.003 respectively). The AUC of truncated 42 kDa MMP was 0.622 (p=0.069) in distinguishing patients with OPC and oral cancer. The highest AUC (0.702) was of truncated 42 kDa MMP (p=0.001), which significantly distinguished controls and oral cancer patients.

Survival analysis of different forms of salivary MMPs using ROC cut-off

Kaplan Meir's survival analysis was performed to estimate overall survival of salivary MMPs with markers above and below ROC cut-off. The results are depicted in **Table 5.31**. No significant correlation of salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP was observed with overall survival.

Table 5.31: Survival analysis of different forms of salivary MMPs using ROC cut-off

Parameters	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2 p value
Pro MMP-9	48289696 (0.617)	24.66%/ 98.48% P=0.0138	36.239±2.652	35.444±4.089	0.114 p=0.736
Active MMP-9	33286236 (0.609)	34.29%/ 90.48% p=0.7898	36.523±2.9311	35.774±3.262	0.108 p=0.743
Truncated 42 kDa MMP	30314880 (0.699)	45.1%/ 90.38% P=0.001	38.737±2.684	37.061±3.519	0.094 p=0.759

OBJECTIVE 5.2: To evaluate *ECAD* mRNA and E-cadherin protein levels in malignant and adjacent normal tissues of oral cancer patients

Levels of truncated 97 kDa E-cadherin in malignant and adjacent normal tissues

Figure 5.38(a) is the representative western blot for truncated 97 kDa E-cadherin protein which was analyzed from 25 paired (malignant and adjacent normal) tissues. Western blot analysis of E-cadherin revealed significant increase (p=0.025) of truncated 97 kDa E-cadherin protein in malignant tissues as compared to adjacent normal tissues **[Figure 5.38(a) and (b)]**.

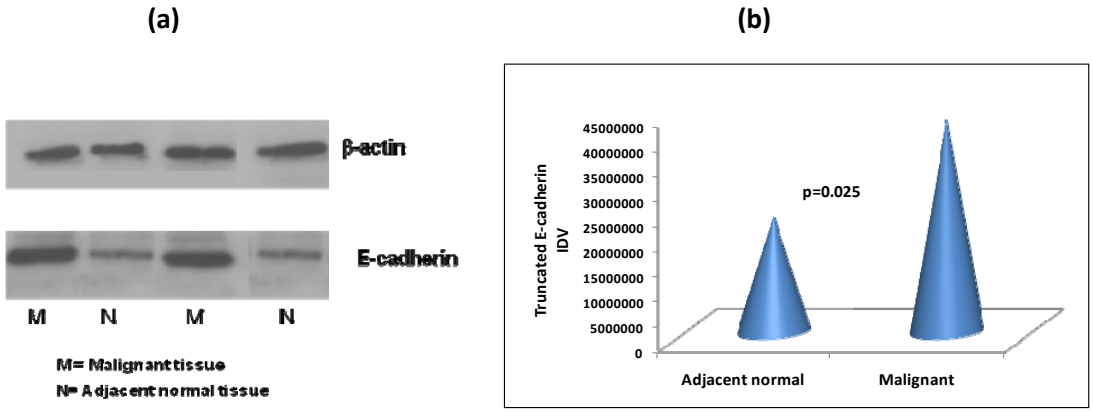


Figure 5.38*(a): Representative pattern of truncated 97 kDa E-cadherin protein in adjacent normal and malignant tissues (b): Graphical representation of truncated E-cadherin expression in paired adjacent normal and malignant tissues*

Figure 5.39 (a) reveals that the levels of truncated E-cadherin protein (97 kDa) were found to be higher (p=0.313) in advanced stage (n=15) as compared to early stage (n=9). As depicted in **Figure 5.39 (b)** the levels of truncated E-cadherin protein were found to be increased in infiltrative tumors (n=8) as compared to non-infiltrative tumors (n=17) (p=0.121) and were also significantly higher in metastatic tumors (n=5) [**Figure 5.39 (c)**] as compared to non-metastatic (n=19) tumors (p<0.0001). Multivariate analysis depicted significant association of truncated E-cadherin protein with metastasis (p<0.0001). Moreover, the levels of E-cadherin protein were comparable between well, moderate and poorly differentiated tumors.

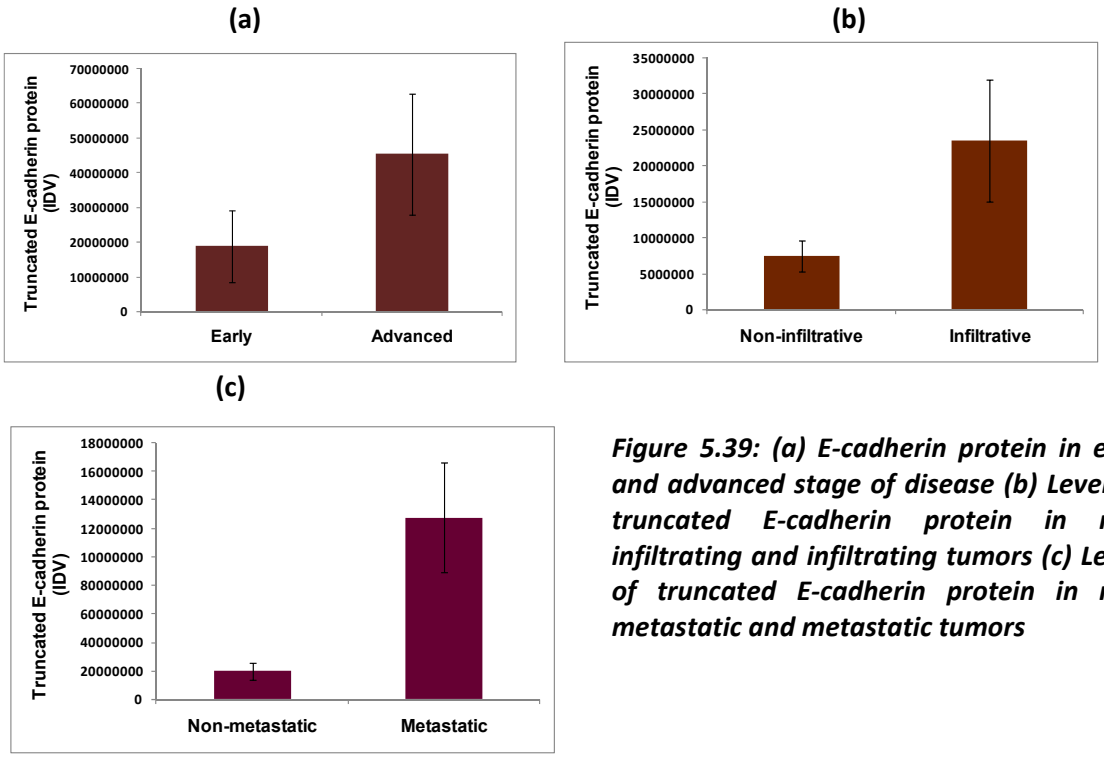


Figure 5.39: (a) E-cadherin protein in early and advanced stage of disease (b) Levels of truncated E-cadherin protein in non-infiltrating and infiltrating tumors (c) Levels of truncated E-cadherin protein in non-metastatic and metastatic tumors

ECAD mRNA expression in adjacent normal and malignant tissues

The levels of *ECAD* mRNA from 50 paired tissues were analyzed by semiquantitative RT-PCR using *ECAD* specific primers and levels were normalized to *βACTIN* expression. **Figure 5.40 (a)** shows the band of 420bp i.e. of *ECAD* mRNA expression. The results revealed that the mRNA levels were comparable between malignant (n=50) and adjacent normal tissues (n=50) **[Figure 5.40 (b)].**

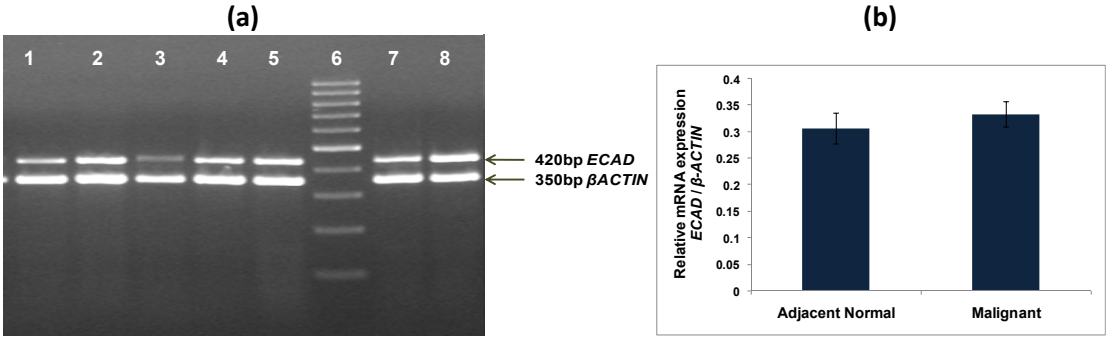


Figure 5.40(a): ***Representative pattern of ECAD mRNA expression in paired malignant and adjacent normal tissues.*** *Lanes 1, 3, 5 and 8 shows the amplicon pairs of ECAD (420bp) and β-ACTIN (350bp) from malignant tissues. Lanes 2, 4 and 7 shows the expression from adjacent normal tissues. Lane 6 represents the DNA ladder (100-1000bp).* **(b): *Graphical representation of ECAD mRNA expression in paired adjacent normal and malignant tissues.*** *The levels are expressed as IDV of ECAD/ IDV of β-ACTIN (Mean±SEM). IDV: Integrated density value*

Moreover, *ECAD* mRNA expression was found to be lower in infiltrating tumors (n=21) as compared to non-infiltrating tumors (n=27) [**Figure 5.41(a)**]. Multivariate analysis showed significant association of *ECAD* mRNA with differentiation and stage of disease (p=0.05 and p=0.048, respectively).

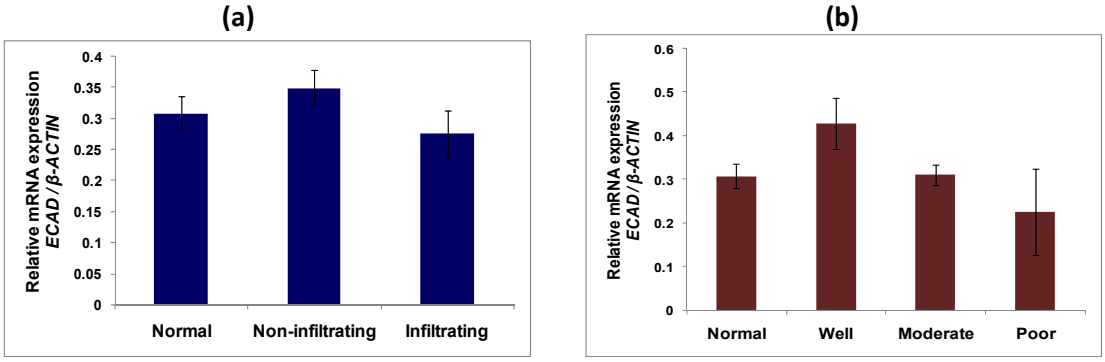


Figure 5.41: ***(a) Graphical representation of ECAD mRNA expression in infiltrating and non-infiltrating tumors. (b) ECAD mRNA expression in well, moderate and poorly differentiated tumors.*** *The levels are expressed as IDV of ECAD and βACTIN*

A decreasing trend of *ECAD* mRNA levels was observed from well to moderate to poor differentiated tumors, however levels were non-significant. The levels were found to be significantly decreased in moderately differentiated tumors as compared to well differentiated tumors (p=0.041).

Kaplan Meir's Survival analysis of truncated E-cadherin protein and *ECAD* mRNA

Table 5.32: Survival analysis of truncated E-cadherin protein and *ECAD* mRNA

Parameter	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2 Significance
Truncated E-cadherin protein	3523968 (0.673)	100%/ 26.8% p=0.2602	-----	------	0.400 p=0.527
***ECAD* mRNA**	0.272 (0.570)	66.7/57.6 p=0.3416	36.0±6.928	40.282±3.139	0.182 p=0.670

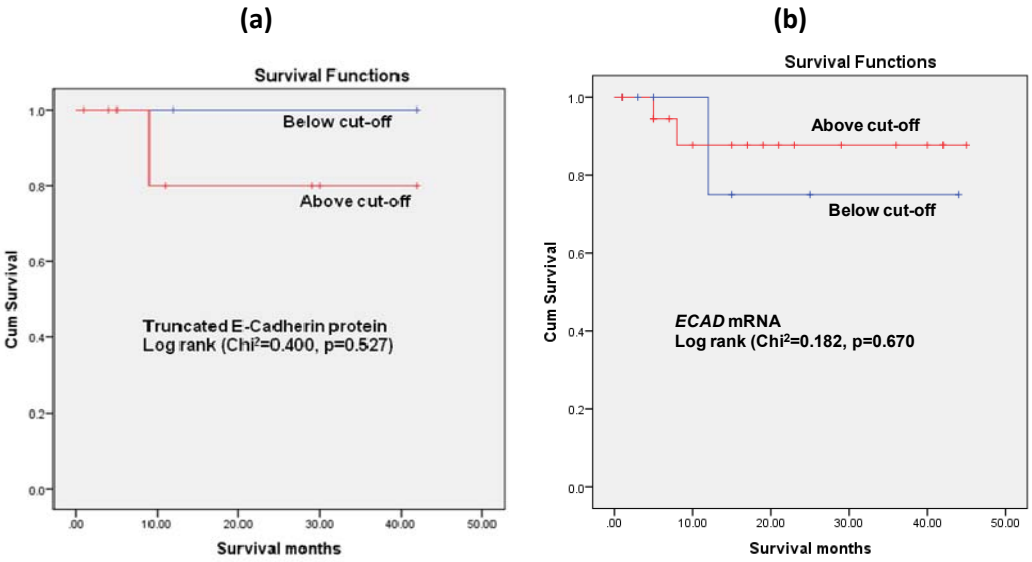


Figure 5.42: ***Overall Survival analysis of (a) truncated E-cadherin protein and (b) ECAD mRNA*** *Kaplan Meier survival curves were compared by Log-rank analysis and levels in patients were depicted as above or below ROC cut-off value.*

Kaplan Meirs's Survival mean estimate of E-cadherin protein cannot be computed as all the cases were censored. Kaplan Meir's survival analysis of *ECAD* mRNA depicted lower survival with values below ROC cut-off [**Figure 5.42(b)**].

The ROC cut-off values with sensitivity, specificity and AUC along with Log Rank Chi^2 value are as mentioned in **Table 5.32**.

OBJECTIVE 5.3: To assess *CJUN* mRNA and protein levels in malignant and adjacent normal tissues of oral cancer patients

CJUN mRNA levels were analyzed from 50 paired (malignant and adjacent normal) tissues by semiquantitative RT-PCR using primers specific for *CJUN* and levels were normalized to *β-ACTIN* expression. **Figure 5.43 (a)** is the representative pattern of *CJUN* mRNA expression. As depicted in **Figure 5.43 (b)**, the levels of *CJUN* mRNA were comparable between paired malignant and adjacent normal tissues.

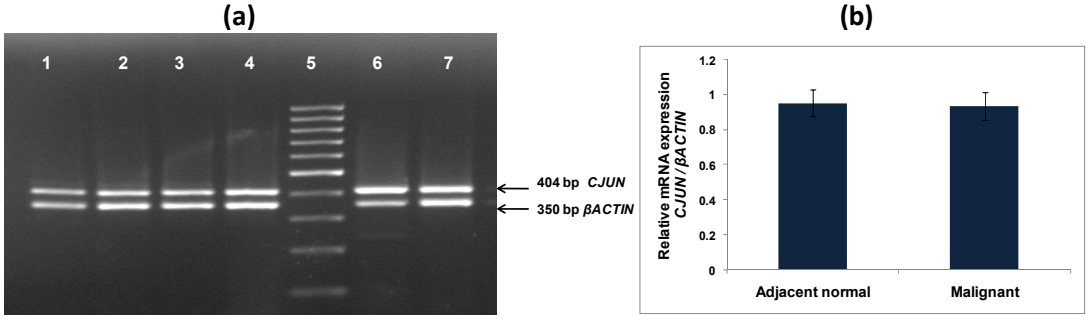


Figure 5.43 (a): ***Representative pattern of CJUN mRNA expression in paired malignant and adjacent normal tissues.*** *Lanes 1, 3, 6 shows the amplicon pairs of CJUN (404bp) and β-ACTIN (350bp) from adjacent normal tissues. Lanes 2, 4, 7 shows the expression from malignant tissues. Lane 5 represents the DNA ladder (100-1000bp).* **(b):** ***Graphical representation of CJUN mRNA levels in paired malignant and adjacent normal tissues of oral cancer patients***

Multivariate analysis depicted significant association of *CJUN* mRNA with the stage of disease (p=0.044). It was observed that *CJUN* mRNA expression levels were comparable between metastatic and non-metastatic tumors **(Table 5.33)**.

Table 5.33: *CJUN* mRNA expression with reference to stage and metastasis

Groups	*CJUN* mRNA
[1]Adjacent normal (n=50)	0.8717±0.0075
[2]Early disease (I +II) (n=17)	1.0510±0.1437 p=0.252 (1 vs. 2)
[3]Late disease (III+IV) (n=31)	0.8658±0.0777 p=0.958 (1 vs. 3) p=0.222 (2 vs. 3)
[4]Patients with no metastasis (n=29)	0.9181±0.0952 p=0.709 (1 vs. 4)
[5]Patients with metastasis (n=18)	0.9517±0.1182 p=0.577 (1 vs. 5) p=0.825 (4vs. 5)

Figure 5.44 depicted that *CJUN* mRNA expression was observed to be higher in early disease (n=17) as compared to advanced disease (n=31) (p=0.222). *CJUN* mRNA expression was comparable between well and moderately differentiated tumors.

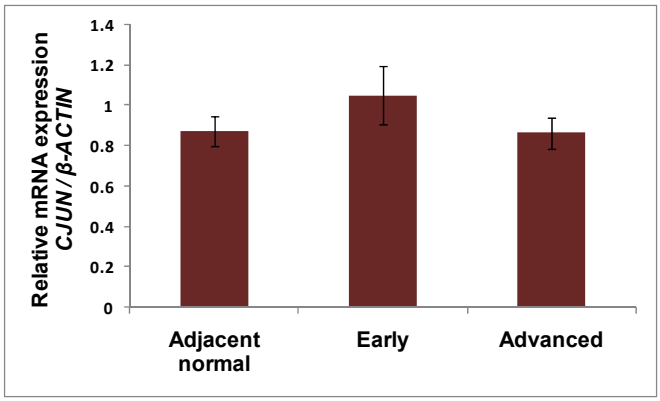


Figure 5.44: ***CJUN mRNA expression in early and advanced stage of disease***

Levels of c-Jun protein levels in malignant and adjacent normal tissues

The levels of c-Jun protein were compared in malignant and adjacent normal tissues (n=25) of oral cancer patients. **Figure 5.45 (a)** is the representative of c-Jun protein in malignant and adjacent normal tissues. The bar chart in **Figure 5.45 (b)** depicts that the levels of c-Jun protein were found to be significantly higher in malignant tissues as compared to adjacent normal tissues (p=0.031).

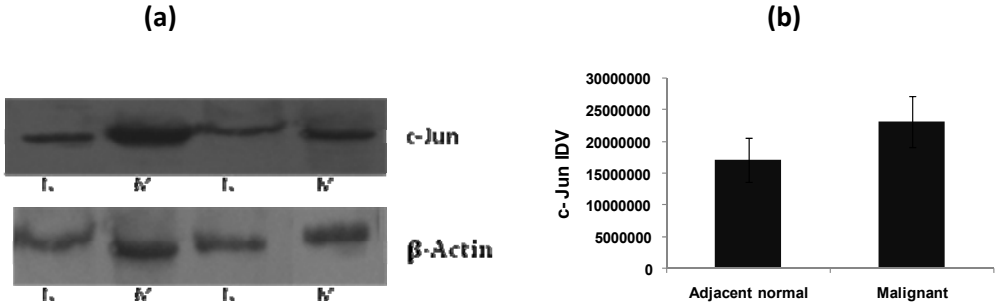


Figure 5.45: (a) ***Representative pattern of c-Jun protein and β-actin in adjacent normal and malignant tissues*** **(b)** ***c-Jun protein expression in malignant and adjacent normal tissues***

Comparison of c-Jun protein expression with various stages, differentiation and metastasis

The c-Jun protein expression levels were compared between early and advanced stage of malignant disease as well as between patients with and without metastasis. It was observed that the levels were comparable between early and advanced stage of disease (p=0.303). The levels were also comparable between patients with and without metastasis (p=0.599) as well as between well and moderately differentiated tumors (p=0.258)

Kaplan Meir's survival analysis of *CJUN* mRNA and protein with ROC cut-off

Kaplan Meir's survival analysis was carried out to assess the correlation of *CJUN* mRNA and protein expression with overall survival (**Figure 5.46 (a) and Figure 5.46 (b)**). The optimal ROC cut-off, sensitivity, specificity, AUC with survival estimate and Chi^2 value are depicted in **Table 5.34.**

Figure 5.46 (a) depicts no significant association of *CJUN* mRNA with overall survival. For c-Jun protein all the cases were censored so survival estimates could not be computed. c-Jun protein expression with values above ROC cut-off depicted significant association with overall survival (Chi^2=7.038, *p*=0.008) **[Figure 5.46 (b)].**

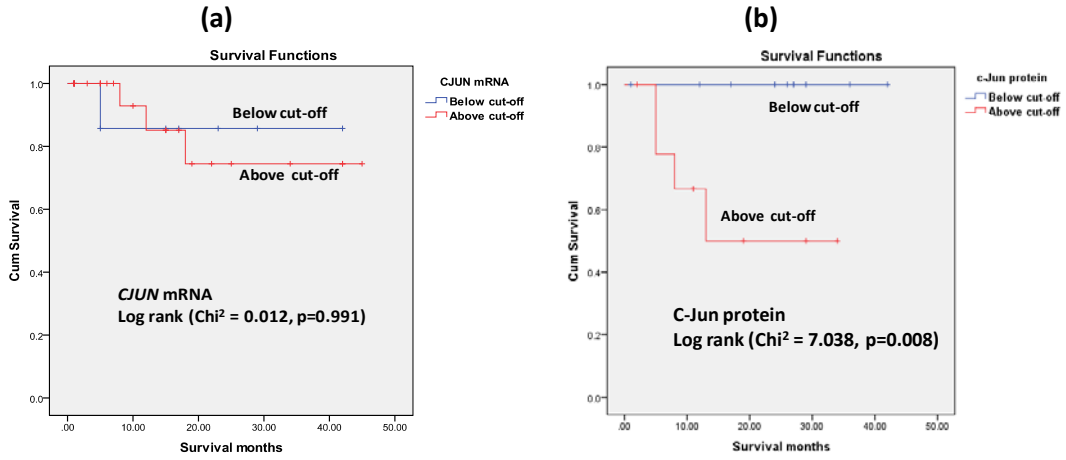


Figure 5.46: ***Kaplan Meir's survival analysis of (a) CJUN mRNA and (b) c-Jun protein with ROC cut-off***

Table 5.34: Survival analysis of *CJUN* mRNA and protein with ROC cut-off

Parameter	ROC cut-off (AUC)	ROC cut-off Sensitivity/ specificity p value	Below cut-off Survival estimate (months) ± SEM	Above cut-off Survival (months) estimate ± SEM	Log rank (Mantel Cox) Chi^2 Significance
***CJUN* mRNA**	0.666 (0.542)	76.2%/40.0% p=0.5178	36.714±4.894	36.931±4.079	0.012 *p*=0.911
c-Jun protein	20169184 (0.548)	40.0%/82.6% p=0.5792	-----	----	7.038 ***p*=0.008**

Correlation between c-Jun mRNA and protein

Pearson's correlation analysis was performed to analyze the correlation between c-Jun mRNA and protein. No significant correlation was observed between *CJUN* mRNA and protein (r=0.021, *p*=0.933).

OBJECTIVE 5.4: To study the expression of pEGFR from malignant and adjacent normal tissues of oral cancer patients.

Standard curve for pEGFR

Standard curve was prepared using pEGFR as control in concentration range of 10ng/ml to 40ng/ml. The standard graph was observed to be linear from 10ng/ml to 40ng/ml **(Figure 5.47)**.

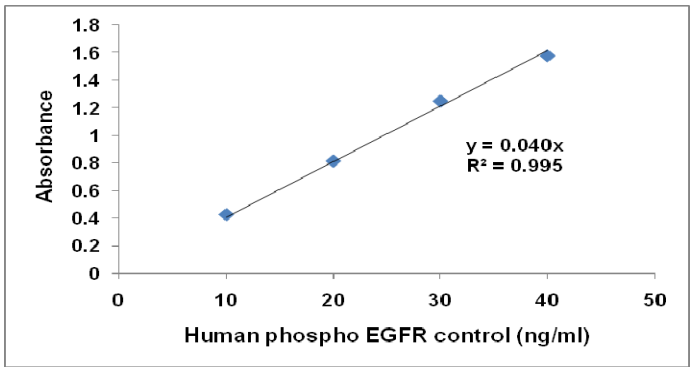


Figure 5.47: ***Standard graph for pEGFR expression by ELISA***

Levels of pEGFR expression in malignant and adjacent normal tissues

The levels of pEGFR expression were compared between malignant and adjacent normal tissues. It was observed that the levels were significantly higher (*p*=0.019) in malignant tissues as compared to adjacent normal tissues (**Figure 5.48**).

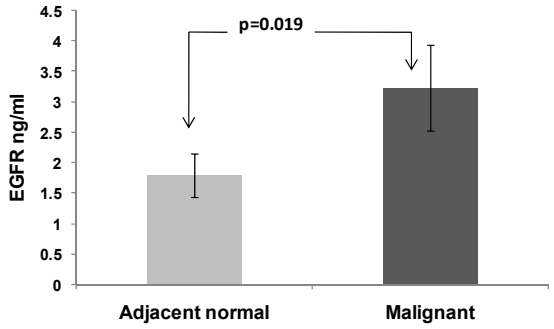


Figure 5.48: ***Comparison of pEGFR expression in adjacent normal and malignant tissues***

ROC curve analysis

ROC curve were constructed to evaluate the distinguishing capacity of the pEGFR expression in discriminating malignant and adjacent normal tissues.

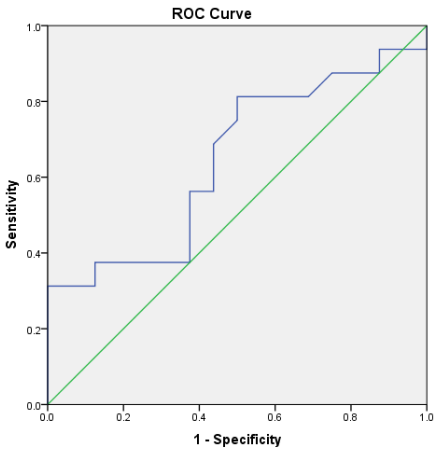


Figure 5.49: ***ROC curve analysis of pEGFR expression to distinguish adjacent normal and malignant tissues.*** *The values (indicated as line graphs) above the reference line on an ROC curve (middle line in the graphs) suggest a good discriminatory efficacy of the marker that is within acceptable limits. AUC: Area under curve; ROC: Receiver's Operating Characteristics.*

As depicted in **Figure 5.49**, ROC curve analysis depicted that pEGFR could distinguish adjacent normal and malignant tissues with AUC of 0.674 (p=0.0620) with sensitivity and specificity of 84.0% and 50.0% respectively.

Levels of pEGFR with various clinico-pathological parameters

The levels of pEGFR when compared between early and advanced stage of disease, the levels were found to be higher in advanced stage as compared to early stage (p=0.723), however levels were non-significant **[Figure 5.50 (a)]**. **Figure 5.50 (b)** depicts that pEGFR levels were found to be higher in metastatic tumors as compared to non-metastatic tumors (p=0.416), however, the levels were statistically non-significant. While the levels of pEGFR expression was comparable between well, moderate and poorly differentiated tumors.

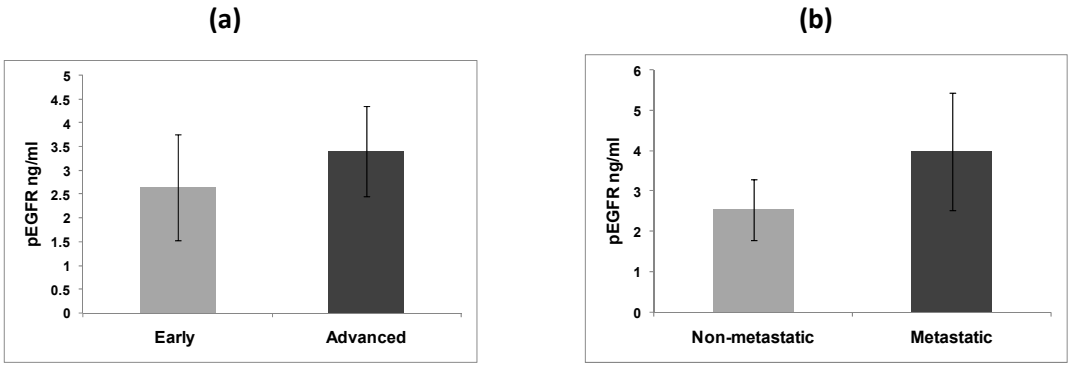


Figure 5.50 (a): p*EGFR expression in early and advanced stage of disease* (b): *Levels of pEGFR expression in metastatic and non-metastatic tumors*

Kaplan Meir's survival analysis

Kaplan Meir survival analysis was performed to evaluate the association of pEGFR expression with overall survival. The ROC cut-off value was 0.9331 ng/ml.

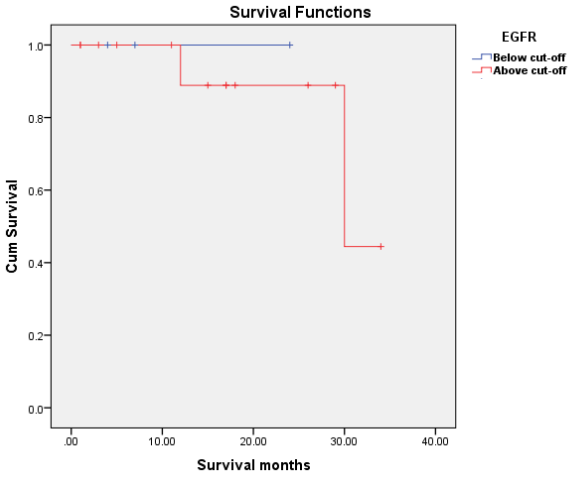


Figure 5.51: ***Overall Survival analysis of pEGFR expression.*** *Kaplan Meier survival curves were compared by Log-rank analysis and EGFR expression in patients were depicted as above or below relative to the ROC cut-off value. The values above and below cut-off are expressed as survival in months ± SEM; EGFR: Epidermal growth factor receptor*

Kaplan Meir's survival analysis in **Figure 5.51** indicated that values above ROC cut-off of pEGFR expression was associated with lower overall survival (Chi^2=0.111, p=0.739). The statistics could not be computed as all the cases were censored.

OBJECTIVE 6: To study the correlation of glycosylation, pEGFR, E-cadherin, MMP-2, MMP-9 and c-Jun to unravel the pathway of oral cancer progression

7 **Manuscript to be communicated:**
Loss of E-cadherin by Matrix-metalloproteinases modulates cell-cell adhesion and upregulates c-Jun expression in oral carcinogenesis

Authors: Bhairavi N. Vajaria, Kinjal R. Patel, Rasheedunnisa Begum, Jayendra B. Patel, Franky D. Shah[1], Prabhudas S. Patel

8 **Manuscript to be communicated:**

Significance of Phosphorylated Epidermal Growth Factor Receptor, Matrix Metalloproteinases and E-cadherin in Oral Cancer

Authors: Bhairavi N. Vajaria, Kinjal R. Patel, Rasheedunnisa Begum, Franky D. Shah, Jayendra B. Patel, Prabhudas S. Patel

Correlation between serum glycosylation changes and plasma MMPs

Pearson's correlation analysis was performed to analyze the correlation between different markers in oral cancer patients.

It is depicted from **Table 5.35** that serum α-2,6 sialoproteins showed significant positive correlation with plasma pro MMP-2 (r=0.903, p=0.001), active MMP-2 (r=0.990, p<0.0001), pro MMP-9 (r=0.890, p=0.001, active MMP-9 (r=0.835, p=0.005), total MMP-2 (r=0.994, p<0.0001), total MMP-9 (r=0.882, p=0.002). Serum α-2,6 ST was significantly positively correlated with plasma pro MMP-2 (r=0.275, p=0.034), plasma pro MMP-9 (r=0.321, p=0.013) and plasma total MMP-9 (r=0.307, p=0.017). Serum α-L-fucosidase activity also depicted significant positive correlation with active MMP-2 (r=0.338, p=0.002), pro MMP-9 (r=0.470, p<0.0001), active MMP-9 (r=0.530, p<0.0001), total MMP-2 (r=0.329, p=0.003) and total MMP-9 (r=0.485, p<0.0001).

Table 5.35: Pearson's correlation between plasma MMPs and serum glycosylation changes

	Plasma pro MMP-2	Plasma active MMP-2	Plasma pro MMP-9	Plasma active MMP-9	Plasma total MMP-2	Plasma total MMP-9	Activation ratio MMP-2	Activation ratio MMP-9
Serum α-2,6 sialoprotein	r=0.903 ***p*=0.001**	r=0.990 ***p*<0.0001**	r=0.890 ***p*=0.001**	r=0.835 ***p*=0.005**	r=0.994 ***p*<0.0001**	r=0.882 ***p*=0.002**	r=0.735 ***p*=0.024**	r=-0.906 ***p*=0.001**
Serum α-2,3 sialoprotein	r=-0.263 *p*= 0.292	r=-0.130 *p*=0.606	r=-0.242 *p*=0.334	r=-0.257 *p*=0.304	r=-0.148 *p*=0.557	r=-0.246 *p*=0.326	r=-0.074 *p*=0.769	r=0.019 *p*=0.939
Serum α-2,6 ST	r=-0.081 *p*= 0.534	r=-0.004 *p*=0.978	r=0.003 *p*=0.984	r=-0.071 *p*=0.587	r=-0.013 *p*=0.923	r=-0.012 *p*=0.924	r=-0.021 *p*=0.875	r=-0.357 ***p*=0.005**
Serum α-2,3 ST	r=0.275 ***p*= 0.034**	r=0.159 *p*=0.225	r=0.321 ***p*=0.013**	r=0.243 *p*=0.062	r=0.178 *p*=0.172	r=0.307 ***p*=0.017**	r=-0.032 *p*=0.807	r=-0.355 ***p*=0.005**
Serum sialidase	r=0.088 *p*= 0.698	r=0.091 *p*=0.686	r=0.072 *p*=0.750	r=0.071 *p*=0.755	r=0.094 *p*=0.679	r=0.072 *p*=0.750	r=-0.032 *p*=0.887	r=0.149 *p*=0.508
Serum TSA/TP	r=-0.119 *p*= 0.283	r=-0.095 *p*=0.395	r=0.019 *p*=0.866	r=0.027 *p*=0.808	r=-0.104 *p*=0.350	r=0.021 *p*=0.854	r=0.008 *p*=0.944	r=-0.058 *p*=0.605
Serum fucoprotein	r=0.313 *p*= 0.298	r=-0.006 *p*=0.985	r=0.090 *p*=0.770	r=0.064 *p*=0.834	r=0.062 *p*=0.841	r=0.086 *p*=0.781	r=-0.256 *p*=0.398	r=0.384 *p*=0.195
Serum α-L-fucosidase	r=0.150 *p*=0.186	r=0.338 ***p*=0.002**	r=0.470 ***p*<0.0001**	r=0.530 ***p*<0.0001**	r=0.329 ***p*=0.003**	r=0.485 ***p*<0.0001**	r=0.141 *p*=0.214	r=-0.022 *p*=0.850

Pearson's correlation between salivary MMPs and salivary glycosylation changes

Table 5.36 shows the correlation analysis between salivary MMPs and salivary glycosylation changes.

Table 5.36: Pearson's correlation between salivary MMPs and salivary glycosylation changes

	Saliva pro MMP-9	Saliva active MMP-9	Saliva truncated 42 kDa MMP
Salivary α-2,6 sialoproteins	r=0.259 *p*=0.392	r=0.261 *p*=0.413	r=0.037 *p*=0.925
Salivary α-2,3 sialoproteins	r=0.420 *p*=0.106	r=0.475 *p*=0.074	r=0.678 ***p*=0.011**
Salivary α-2,6 ST	r=0.190 *p*=0.293	r=0.077 *p*=0.626	r=0.026 *p*=0.877
Salivary α-2,3 ST	r=0.081 *p*=0.617	r=0.056 *p*=0.732	r=-0.193 *p*=0.238
Salivary sialidase	r=0.462 *p*=0.096	r=0.672 ***p*=0.009**	r=0.658 ***p*=0.028**
Salivary TSA/TP	r=-0.044 *p*=0.759	r=-0.022 *p*=0.884	r=0.029 *p*=0.857
Salivary fucoprotein	r=0.327 *p*=0.357	r=0.313 *p*=0.379	r=0.461 *p*=0.180
Salivary α-L-fucosidase	r=0.170 *p*=0.234	r=0.100 *p*=0.495	r=-0.144 *p*=0.369

Salivary α-2,6 sialoproteins were positively correlated with pro MMP-9 (r=0.259, p=0.392), active MMP-9 (r=0.261, *p*=0.413) and truncated 42 kDa MMP (r=0.037, *p*=0.925). Salivary α-2,3 sialoproteins showed significant positive correlation with truncated 42 kDa MMP (r=0.678, p=0.011). Salivary sialidase activity depicted significant positive correlation with active MMP-9 (r=0.672, *p*=0.009) and truncated 42 kDa MMP (r=0.658, *p*=0.028).

Pearson's correlation between glycosylation, *ECAD* mRNA and Protein, *CJUN* mRNA and Protein

As depicted in **Table 5.37**, a significant positive correlation was observed between serum α-2,6 ST and c-Jun protein (r=0.729, *p*=0.007). Serum TSA/TP ratio and salivary α-2,6 ST activity showed significant negative correlation with *ECAD* mRNA (r=-0.902, *p*=0.005 and r=-0.956, *p*=0.044 respectively). Moreover, a significant negative correlation was observed between serum α-L-fucosidase activity and *ECAD* mRNA (r=-0.792, *p*=0.034).

Table 5.37: Pearson's correlation between glycosylation, *ECAD* mRNA and Protein, *CJUN* mRNA and Protein

	E-cadherin Protein	*ECAD* mRNA	*CJUN* mRNA	c-Jun protein
Serum α-2,6 ST	r=-0.077 *p*=0.870	r=-0.035 *p*=0.965	r=0.648 *p*=0.237	r=0.729 **p=0.007**
Serum α-2,3 ST	r=0.385 *p*=0.451	r=0.045 *p*=0.955	r=0.836 *p*=0.164	r=0.350 *p*=0.321
Serum TSA/TP	r=0.054 *p*=0.881	r=-0.902 ***p*=0.005**	r=-0.379 *p*=0.402	r=-0.314 *p*=0.254
Serum α-L-fucosidase	r=0.509 *p*=0.133	r=-0.792 ***p*=0.034**	r=0.288 *p*=0.531	r=-0.254 *p*=0.381
Saliva α-2,6 ST	r=-0.394 *p*=0.439	r=-0.956 ***p*=0.044**	r=-0.439 *p*=0.561	r=-0.301 *p*=0.318
Saliva α-2,3 ST	r=-0.334 *p*=0.464	r=-0.740 *p*=0.153	r=-0.699 *p*=0.301	r=0.039 *p*=0.896
Saliva TSA/TP	r=0.229 *p*=0.553	r=-0.095 *p*=0.858	r=-0.193 *p*=0.714	r=-0.031 *p*= 0.920
Saliva α-L-fucosidase	r=0.433 *p*=0.212	r=0.621 *p*=0.137	r=0.447 *p*=0.315	r=0.257 *p*=0.420

Pearson's correlation between truncated E-cadherin Protein and mRNA, *CJUN* mRNA and Protein

The pearson's correlation analysis between E-cadherin and c-Jun protein expression as well as its mRNA expression depicted **(Table 5.38)** a significant positive correlation between E-cadherin protein and *CJUN* mRNA (r=0.826, *p*=0.006).

Table 5.38: Pearson's correlation between E-cadherin Protein and mRNA, *CJUN* mRNA and Protein

	Truncated E-cadherin Protein	*ECAD* mRNA	*CJUN* mRNA	c-Jun protein
Truncated E-cadherin Protein	----	r=0.110 *p*=0.795	r=0.826 ***p*=0.006**	r=0.261 *p*=0.533
E-cadherin mRNA	r=0.110 *p*=0.795	----	r=0.193 *p*=0.378	r=0.053 *p*=0.858
***CJUN* mRNA**	r=0.826 ***p*=0.006**	r=0.193 *p*=0.378	----	r=0.021 *p*=0.933
c-Jun protein	r=0.261 *p*=0.533	r=0.053 *p*=0.858	r=0.021 *p*=0.933	-----

Correlation between protein and mRNA expression of E-Cadherin and c-Jun

Table 5.38 depicts the Pearson's correlation analysis between E-cadherin and c-Jun protein and mRNA. A significant positive correlation of truncated E-cadherin protein (r=0.826, *p*=0.006) was observed with *CJUN* mRNA. Moreover, *ECAD* mRNA was observed to be positively correlated with *CJUN* mRNA (r=0.193, *p*=0.378). Truncated E-cadherin protein was observed to be positively correlated with c-Jun protein (r=0.261, *p*=0.533).

Correlation analysis of plasma and salivary MMPs with E-cadherin and c-Jun protein and mRNA

As depicted in **Table 5.39**, truncated E-cadherin protein showed positive correlation with salivary pro MMP-9 (r=0.391, *p*=0.609), active MMP-9 (r=0.476, *p*=524), truncated 42 kDa MMP (r=0.164, *p*=0.895).

Table 5.39: Correlation analysis of Plasma and salivary MMPs with E-cadherin and c-Jun protein and mRNA

	***CJUN* mRNA**	**c-Jun protein**	***ECAD* mRNA**	**E-cadherin Protein**
Plasma pro MMP-2	r=0.158 *p*=0.450	r=0.013 *p*=0.958	r=0.277 *p*=0.237	r=0.948 ***p*<0.0001**
Plasma active MMP-2	r=0.197 *p*=0.346	r=0.167 *p*=0.508	r=-0.024 *p*=0.919	r=0.748 ***p*=0.008**
Plasma pro MMP-9	r=0.163 *p*=0.435	r=0.181 *p*=0.472	r=-0.089 *p*=0.708	r=0.210 *p*=0.535
Plasma active MMP-9	r=0.228 *p*=0.273	r=0.372 *p*=0.129	r=-0.040 *p*=0.867	r=0.873 ***p*<0.0001**
Plasma total MMP-2	r=0.200 *p*=0.338	r=0.126 *p*=0.619	r=0.045 *p*=0.851	r=0.778 ***p*=0.005**
Plasma total MMP-9	r=0.182 *p*=0.383	r=0.232 *p*=0.355	r=-0.079 *p*=0.741	r=0.0062 *p*=0.857
Plasma activation ratio MMP-2	r=-0.162 *p*=0.440	r=0.017 *p*=0.947	r=-0.412 *p*=0.071	r=0.647 ***p*=0.031**
Plasma activation ratio MMP-9	r=0.137 *p*=0.515	r=0.176 *p*=0.484	r=0.134 *p*=0.573	r=0.105 *p*=0.773
Salivary pro MMP-9	r=0.042 *p*=0.898	r=0.218 *p*=0.573	r=-0.108 *p*=0.753	r=0.391 *p*=0.609
Salivary active MMP-9	r=0.234 *p*=0.465	r=0.158 *p*=0.684	r=0.111 *p*=0.746	r=0.476 *p*=0.524
Salivary truncated 42 kDa MMP	r=0.571 *p*=0.108	r=0.017 *p*=0.965	r=0.525 *p*=0.146	r=0.164 *p*=0.895

Truncated E-cadherin protein showed significant positive correlation with plasma pro MMP-2 (r=0.948, $p<0.0001$), plasma active MMP-2 (r=0.748, $p=0.008$), plasma active MMP-9 (r=0.873, $p<0.0001$), plasma total MMP-2 (r=0.778, $p=0.005$) and activation ratio MMP-2 (r=0.062, $p=0.857$). Moreover, all the forms of plasma and salivary MMPs were positively correlated with c-Jun protein and mRNA. Plasma active MMP-2, pro MMP-9, active MMP-9, total MMP-9, activation ratio MMP-2, salivary pro MMP-9 was observed to be negatively correlated with *ECAD* mRNA.

Correlation analysis between pEGFR, truncated E-cadherin, plasma MMPs and c-Jun

As illustrated in **Table 5.40**, pEGFR and *CJUN* mRNA expression was significantly positively correlated (r=0.555, $p=0.039$).

Table 5.40: Correlation between pEGFR, truncated E-cadherin protein, plasma MMPs and c-Jun

Correlation	Truncated E-cadherin Protein	pEGFR
Plasma pro MMP-2	r=0.948 ***p*<0.0001**	r=0.247 *p*=0.465
Plasma active MMP-2	r=0.748 ***p*=0.008**	r=0.064 *p*=0.851
Plasma pro MMP-9	r=0.210 *p*=0.535	r=0.057 *p*=0.869
Plasma active MMP-9	r=0.873 ***p*<0.0001**	r=-0.107 *p*=-0.754
Plasma total MMP-2	r=0.778 ***p*=0.005**	r=0.124 *p*=0.717
Plasma total MMP-9	r=0.0062 *p*=0.857	r=0.014 *p*=0.967
Plasma activation ratio MMP-2	r=0.647 ***p*=0.031**	r=-0.265 *p*=0.431
Plasma activation ratio MMP-9	r=0.105 *p*=0.773	r=-0.180 *p*=0.596
Truncated E-cadherin protein	----	r=0.729 *p*=0.163
***ECAD* mRNA**	r=0.110 *p*=0.795	r=0.396 *p*=0.258
***CJUN* mRNA**	r=0.826 ***p*=0.006**	r=0.555 *p*=0.039
c-Jun protein	r=0.261 *p*=0.533	r=0.305, *p*=0.392

The levels of pEGFR were also positively correlated with c-Jun protein (r=0.305, *p*=0.392). Moreover, pEGFR was positively correlated with *ECAD* mRNA and truncated E-cadherin protein (r=0.396, *p*=0.258 and r=0.729, *p*=0.163 respectively).

Overall, MMPs were found to be positively correlated to truncated E-cadherin protein, c-Jun protein and pEGFR. Moreover, α-2,6 sialoproteins and α-L-fucosidase activity were significantly positively correlated with MMPs.