Results

What we observe is not nature itself, but nature exposed to our method of questioning.

- Werner Heisenberg

4. RESULTS

Alterations in the terminal glycosylation are associated with many signaling pathways during neoplastic transformation. Tumour cell acquire terminal sugars on the cell surface for their survival. The disease development and progression is also linked with altered cell surface characteristics and loss of cell-adhesion. In the present work changes associated with sialylation and fucosylation as well as SLe^X and E-cadherin expressions were studied in oral cancer patients (n=130) and patients with OPC (n=75). The results are presented in following four sections:

4.1: Sialylation changes in serum and tissues

4.2: Fucosylation changes in serum and tissues

4.3: Serum protein profiling

4.4: SLe^X and E-cadherin expressions in malignant/OPC tissues

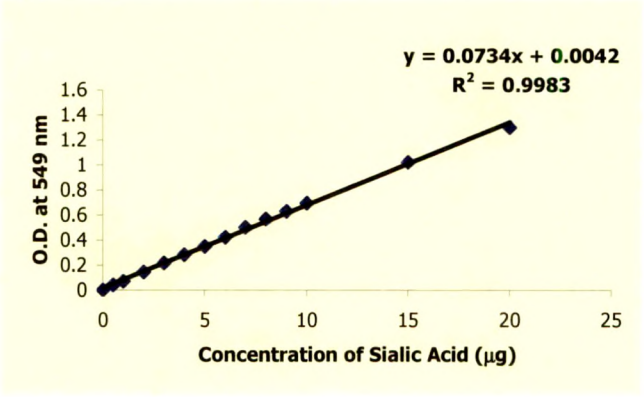
4.1: SIALYLATION CHANGES IN ORAL CANCER AND OPC

SIALYLATION CHANGES IN SERUM

Circulating markers associated with protein sialylation were studied in oral cancer patients. Serum levels were compared with pathological controls (patients with OPC) as well as healthy individuals (controls) to explain their clinical significance.

4.1.1 Serum Total Sialic Acid

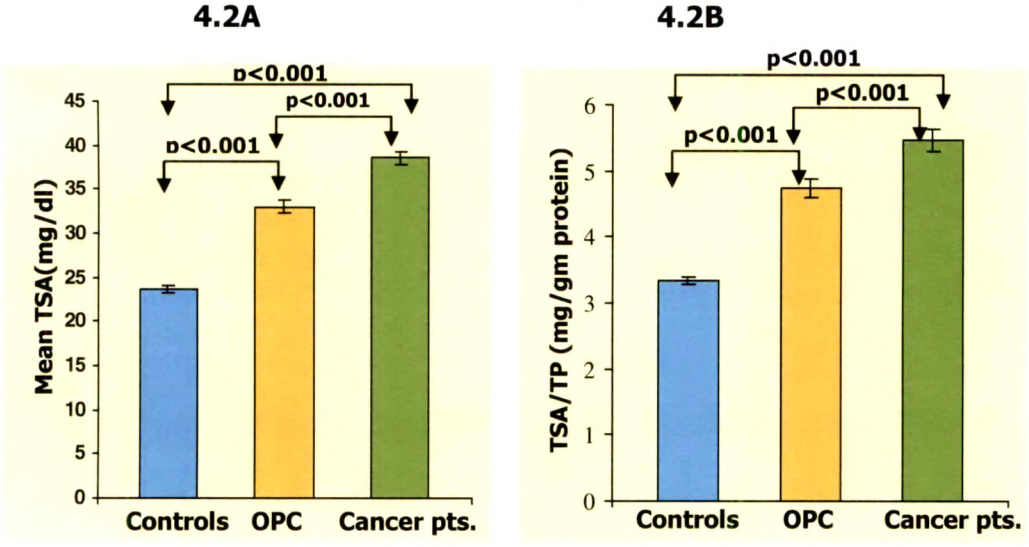
Figure-4.1: Standard Curve for Sialic Acid (N-Acetyl Neuraminic Acid)



As depicted in **figure-4.1**, standard curve for sialic acid (N-acetyl neuraminic acid) was linear (r^2= 0.998) for sialic acid concentrations from 0-20 μg. The intra- and inter-assay coefficients of variations were 5% and 5.5%, respectively.

Serum total sialic acid levels were determined in all the subjects. Also, total sialic acid to total protein ratio (TSA/TP) was calculated to nullify the protein changes. TSA and TSA/TP (**Figure-4.2A, B**) were significantly elevated in untreated oral cancer patients as compared to patients with OPC ($p<0.001$) as well as controls ($p<0.001$). Moreover, serum TSA and TSA/TP in patients with OPC were significantly higher ($p<0.001$) than controls and significantly lower ($p<0.001$) as compared to that in oral cancer patients.

Figure-4.2: Comparison of serum TSA (A) and TSA/TP (B) between the Controls and Patients



4.1.2 Expression of α2,6-Sialoproteins and α2,3-Sialoproteins in serum

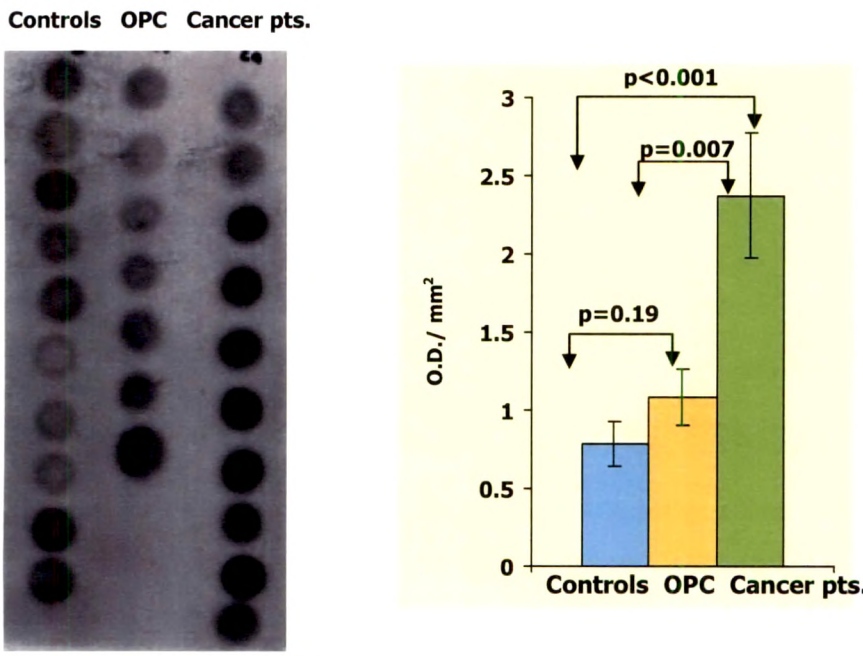
Sambucus nigra (SNA) and *Maackia amurensis* (MAL) are α2,6- and α2,3-sialyl-linkage specific lectins, respectively. These lectins are widely used as probes for the detection of α2,6- and α2,3-linked sialic acid, which are the products of α2,6-SiT and α2,3-SiT, respectively. Representative patterns of SNA and MAL reactive serum sialoproteins were detected by dot-blot method.

α2,6-Sialoproteins

The mean density of α2,6-sialoproteins is presented in the bar graphs as OD/mm^2. As shown in **figure-4.3A**, the reactivity of lectin SNA with serum proteins was markedly increased in untreated oral cancer patients as well as in patients with OPC. **Figure–4.3B** depicts the comparison of mean densities of α2,6-sialoproteins dots (OD/mm^2) in all the three groups. A significant elevation in the α2,6-sialoproteins was observed in cancer patients as compared to controls ($p<0.001$) and patients with OPC ($p=0.007$). Mean α2,6-sialoproteins was also higher in patients with OPC than controls.

Figure-4.3A: Representative blot of α2,6-sialoproteins
Figure-4.3B: Comparison of mean density of α2,6-sialoproteins

4.3A **4.3B**

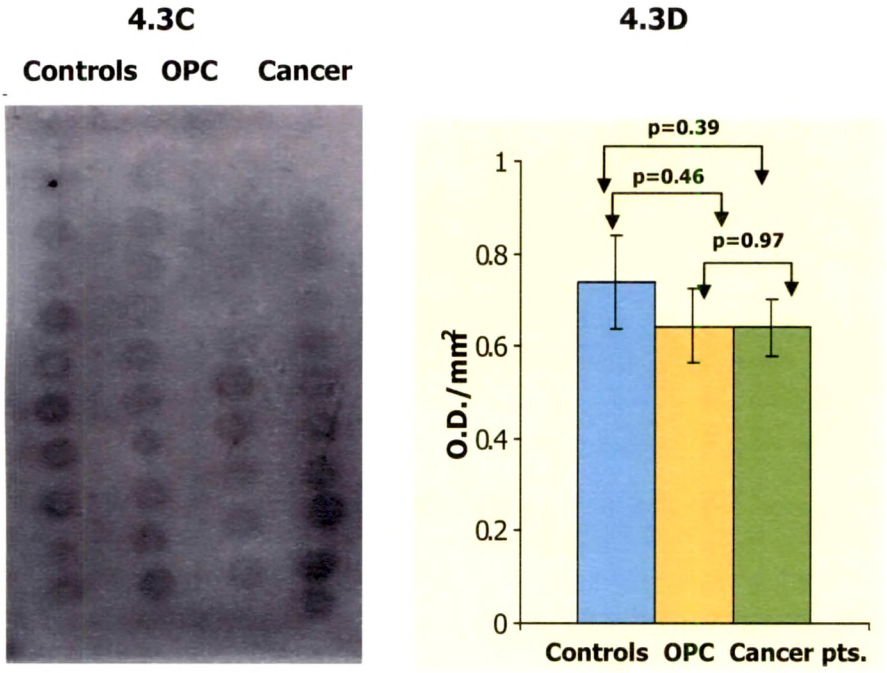


α2,3-sialoproteins

The reactivity of α2,3-linked sialic acid specific lectin MAL with serum proteins is shown in **figure-4.3C**. It is clear from representative blot as well as bar graph that there were no significant changes observed in the MAL- reactivity of

serum proteins in controls and patients with oral cancer as well as patients with OPC. Mean density was higher in controls than the patients; however the difference was not statistically significant. The values were comparable between oral cancer patients and patients with OPC **(Figure-4.3D)**.

Figure-4.3C: Representative blot of α2,3-sialoproteins
Figure-4.3d: Comparison of mean density of α2,3-sialoproteins



4.1.3 Serum Sialyltransferase Activity

Figure-4.4: Standard curve of *p*-Nitrophenol for 96-well plate ELISA based α2,6-SiT and α2,3-SiT assay

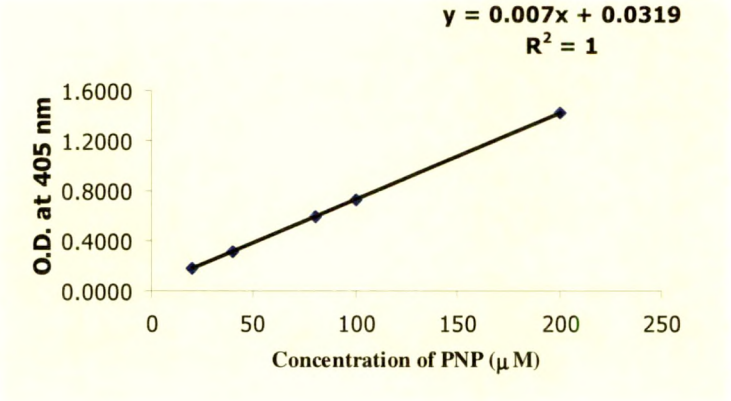


Figure-4.4 represent standard curve for *p*-Nitrophenol, which was linear (r^2= 1.00) for *p*-Nitrophenol (PNP) concentration of 0-200 μM. The intra- and inter-assay coefficients of variations were 2.5.0% and 5.0%, respectively.

Table-4.1: Total sialyltransferase activity in serum (Raval et al., 2003)

Groups	Enzyme activity CPM/mg protein Mean ± S.E.M.	Groups compared	'p' values
Controls	2380.05 ± 399.48	Controls vs OPC	0.10
Patients with OPC	3200.22 ± 484.66	Controls vs Cancer pts.	**0.001**
Cancer Patients	3402.56 ± 329.22	OPC vs Cancer pts.	**0.05**
CR	2489.6 ± 440.26	Cancer pts. vs CR	**0.05**
NR	3380.25 ± 365.38	Cancer pts. vs NR	NS

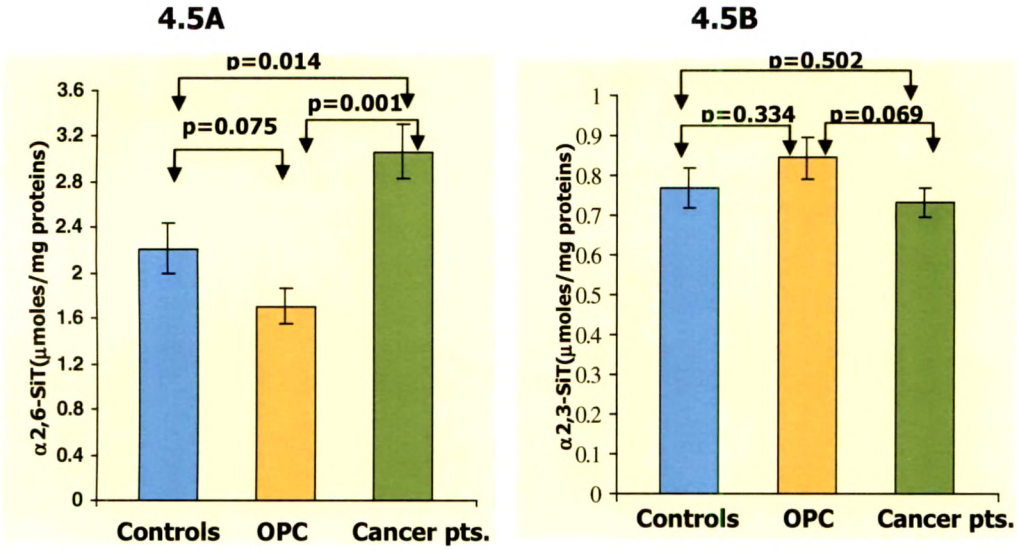
SiT catalyzes incorporation of sialic acid from CMP-NANA to the subterminal sugar residue of glycoproteins either by α2,6-, α2,3- or in case of poly sialylation α2,8-linkages. Previous studies from our laboratory (Raval et al., 2003) reported serum total SiT activity using radioactivity method using [^{14}C]-labeled sialic acid. As detailed in **table-4.1**, serum SiT activity was significantly higher in untreated oral cancer patients as compared to the controls as well as patients with OPC. The complete responders revealed significantly lower activity as compared to untreated oral cancer patients, while the SiT activities were comparable between untreated cancer patients and non-responders.

Serum α2,6-SiT and α2,3-SiT Activity

The Increase in total SiT activities prompted us to study the role of SiT isoforms in protein sialylation in oral cancer. Hence, the present study evaluated activity of α2,6-SiT as well as α2,3-SiT in serum. α2,6-SiT and α2,3-SiT are linkage

specific enzymes that transfer sialic acid in α2,6- and α2,3-linkage to asialofeutin, respectively, which consist galactose as subterminal sugar.

Figure-4.5: Comparison of serum α2,6-SiT and α2,3-SiT activities between the controls and patients

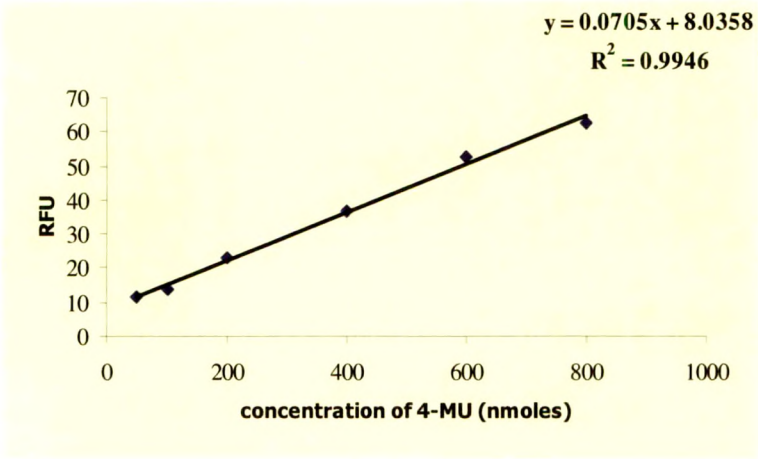


Serum α2,6-SiT activity was significantly elevated in oral cancer patients as compared to patients with OPC (p=0.001) as well as controls (p=0.014). Further, mean α2,6-SiT was lower in patients with OPC as compared to controls (**Figure-4.5A**). Mean serum α2,3-SiT values were higher in patients with OPC as compared to controls as well as oral cancer patients. However, the variations were not statistically significant (**Figure-4.5B**).

4.1.4 Serum Sialidase Activity

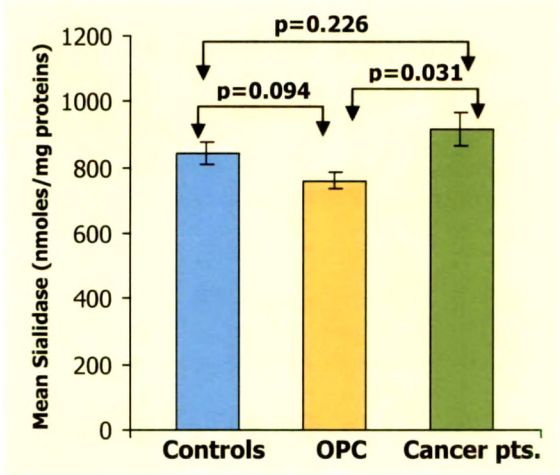
Figure-4.6 show linear standard curve (r^2=0.995) for 4-methyl-umbelliferone (4-MU) and its relative fluorescent unit (RFU). N-acetyl neuraminic acid tagged with 4-methyl-umbelliferone (4-MU-NANA) was used as substrate for sialidase assay. Linearity equation was derived from the standard graphs to calculate activity of sialidase. The intra- and inter-assay coefficients of variations were less than 5%.

Figure-4.6: Standard curve of 4-methyl-umbelliferone (4-MU) for sialidase estimation by spectrofluorimetric method



Sialidase, the important enzyme in sialic acid metabolism, catalyzes hydrolytic cleavage of sialic acid from its sub-terminal sugar. As illustrated in **figure-4.7**, serum sialidase activity was significantly higher in untreated oral cancer patients as compared to patients with OPC (p=0.031). The enzyme activity was declined in patients with OPC as compared to the controls (p=0.094).

Figure-4.7: Comparison of serum sialidase activity between controls and patients



4.1.5 Correlation of Serum Sialylation Markers

SiT catalyze transfer of sialic acid from CMP-NANA to the acceptor sugar of oligosaccharide. Increase in serum sialyltransferase activity leads to increase in sialic acid synthesis, which in turn enhances the sialylation of serum glycoproteins. Increase in the sialylation in the cell requires sialidase to metabolize it. Pearson's correlation analysis was performed to demonstrate the association of serum sialylation markers in oral cancer patients **(Table-4.2)**.

Table-4.2: Pearson's Correlation for serum sialylation markers in oral cancer patients

Correlation of α2,6-SiT with TSA and Sialidase				
Pearson's Correlation	TSA		Sialidase	
Serum α2,6-SiT	r = 0.156	p = 0.08	r = -0.207	p = 0.103
Correlation of α2,6-Sialoproteins with TSA and Sialidase				
	TSA		Sialidase	
Serum α2,6-sialoproteins	r = 0.292	p = 0.08	r=-0.530	**p = 0.012**
Correlation of α2,3-SiT with α2,3-sialoproteins and α2,6-SiT				
	α2,3- sialoproteins		α2,6-SiT	
α2,3-SiT	r=0.136	p=0.324	r=-0.084	p=0.561
Correlation of α2,3-SiT and sialidase				
	Serum sialidase			
α2,3-SiT	r=-0.072		p=0.718	

Serum α2,6-SiT showed positive correlation with TSA (p=0.08) while, negative correlation with sialidase activity (p=0.103). Positive Correlation was also observed between serum TSA and α2,6-sialoproteins (p=0.08), while serum α2,6-sialoproteins and sialidase activities were negatively associated (p=0.012). Likewise, serum α2,3-SiT activity was positively associated with serum α2,3-sialoproteins and negatively associated with α2,6-SiT and sialidase activities, although, it was not statistically significant.

Table-4.3: Correlation between serum sialylation markers in patients with OPC

Correlation of α2,6-SiT with TSA and Sialidase		
Pearson's Correlation	α2,6-SiT	
Serum TSA	r = 0.084	p = 0.596
Serum Sialidase	r = -0.433	**p = 0.014**
Correlation between α2,3-SiT and sialidase		
	Serum sialidase	
α2,3-SiT	r=-0.63	p=0.886

Table-4.3 shows the correlation of serum sialylation markers in patients with OPC. Positive correlation between serum α2,6-SiT and TSA was observed. Serum α2,6-SiT activity was negatively associated with sialidase activity (p=0.014). None of the markers showed correlation with serum α2,3-SiT activity.

4.1.6 Association of Serum Sialylation Markers with Clinicopathologic Parameters

Multivariate analysis was performed to investigate the association of various clinicopathological parameters with serum sialylation markers in the patients. As documented in **table-4.4** serum α2,6-SiT and sialidase activities were found to be associated with nuclear grade (p=0.021, p=0.012, respectively). Significant association was observed between serum TSA, α2,6-SiT activity as well as sialidase activity and tumour differentiation (p=0.027, p=0.026, p=0.001, respectively). Lymphnode involvement showed significant impact on serum levels of α2,3-SiT (p=0.044). Serum α2,6-sialoproteins and sialidase activities were significantly associated with stage of the disease (p=0.021, p=0.038 respectively).

Table-4.4: Multivariate analysis for association of serum markers with clinicopathologic parameters

Variable		TSA	TSA/TP	α2,6-sialo-proteins	α2,3-sialo-proteins	α2,6-SiT	α2,3-SiT	Sialidase
Age	F	3.87	3.97	0.42	1.12	2.24	0.34	0.98
	p	0.055	0.052	0.902	0.337	0.167	1.00	0.542
Gender	F	0.11	0.02	2.8	3.11	2.43	0.46	0.03
	p	0.75	0.892	0.114	0.81	0.139	0.5	0.866
Nuclear Grade	F	2.78	1.46	2.9	0.09	7.86	0.06	6.09
	p	0.094	0.264	0.086	0.916	**0.021**	0.939	**0.012**
Tumour Differentiation	F	4.84	2.27	3.85	0.55	4.88	0.91	12.13
	p	**0.027**	0.143	0.045	0.581	**0.026**	0.41	**0.001**
Lymphnode involvement	F	1.43	0.79	2.00	0.08	0.09	4.29	0.25
	p	0.251	0.389	0.179	0.775	0.768	**0.044**	0.622
Stage of the disease	F	1.34	0.63	3.83	1.48	0.53	1.79	1.92
	p	0.303	0.608	**0.034**	0.232	0.668	0.161	0.172
Early and Advanced Stage	F	0.41	0.13	6.55	1.77	0.01	0.25	5.1
	p	0.53	0.727	**0.021**	0.189	0.948	0.62	**0.038**

4.1.7 Stage of the Disease and serum α2,6-SiT activity

Serum α2,6-SiT activities at different stages of oral cancer are documented in **figure-4.8 and table-4.5**. The enzyme activity increased with the progression of the disease from early to advanced stage. The mean enzyme activity was significantly higher in patients with advanced disease stage as compared to early stage of disease (p=0.05) **(Table-4.5)**. Further, the enzyme activity was significantly higher in stages IV as compared to stage I (p=0.079). Serum sialidase activity was also linearly associated with the stages of the disease but the differences were not significant. Other parameters did not show any linear trend with stage of the disease.

Figure-4.8: Serum α2,6-SiT activities in different stages of oral cancer

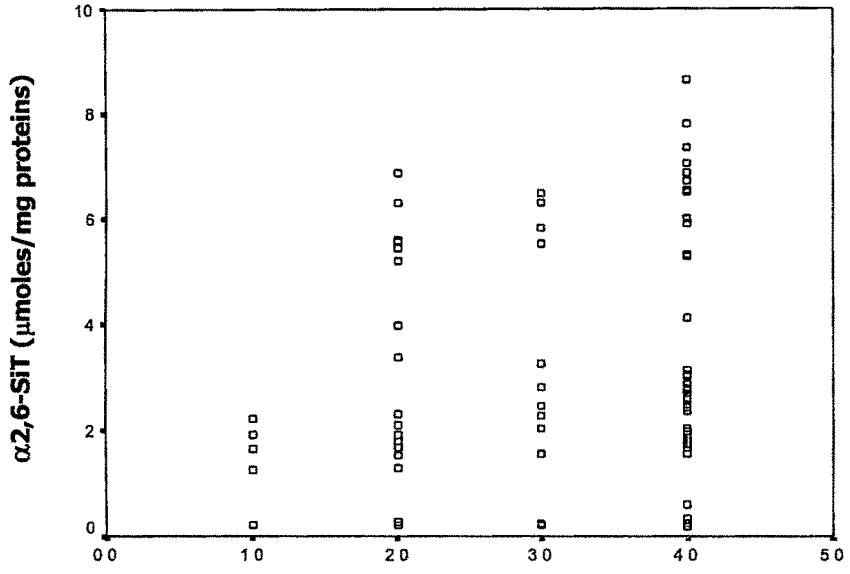


Table-4.5: Comparison of enzyme activity between Early and Advanced stage of the disease

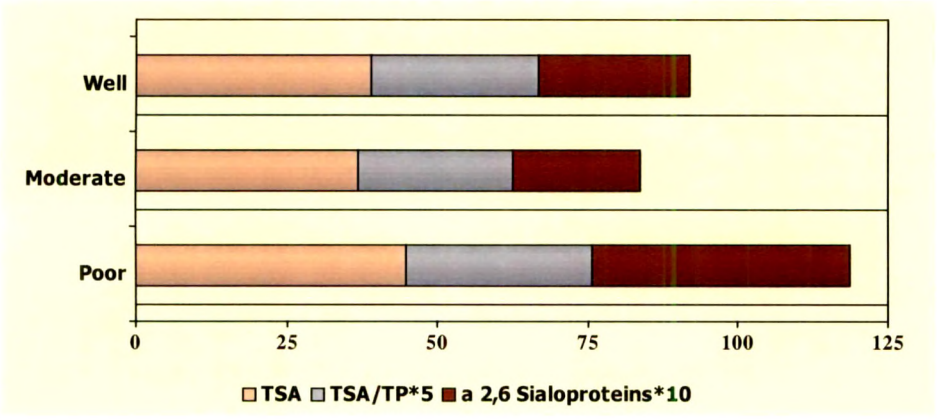
Stage	Mean	± S.E.M.	Significance 'p' value
I	1.46	0.351	I vs. III
II	2.3	0.46	0.228
III	2.82	0.63	I vs. IV
IV	3.28	0.331	0.079
Early (I+II)	2.06	0.352	**0.05**
Advanced (III+IV)	3.17	0.291	

4.1.8 Tumour Differentiation and Serum Markers in Patients

Oral cancer patients were classified into three groups according to the degree of tumour differentiation as well, moderate and poor differentiation. Bar graph (**Figure-4.9**) represents mean levels of TSA, TSA/TP and α2,6-sialoproteins in oral cancer patients in different pathological tumour differentiation. Student 't' test revealed that serum TSA levels were significantly higher in poorly differentiated tumours as compared to well and moderately differentiated

tumours (p=0.077 and p=0.005, respectively). Serum TSA levels were significantly lower in moderately differentiated tumours as compared to well-differentiated tumours (p=0.06). Mean TSA/TP values were also increased in poorly differentiated tumours as compared with moderately differentiated tumours (p=0.012). Mean serum α2,6-sialoproteins was higher in poorly differentiated tumours as compared to moderately as well as well differentiated tumours. None of the other serum markers showed significant association with tumour differentiation.

Figure-4.9: Serum TSA, TSA/TP and α2,6-Sialoproteins levels: association with tumour differentiation



Significance:

TSA: (i) Poor vs Well: p=0.077, (ii) Poor vs. Mod. p=0.005,
(iii) Mod. vs. Well: p=0.066 **TSA/TP :** Poor vs. Mod. p=0.012

SERUM SIALYLATION MARKERS IN PRETREATMENT (PT) AND POST TREATMENT FOLLOW-UPS OF ORAL CANCER PATIENTS

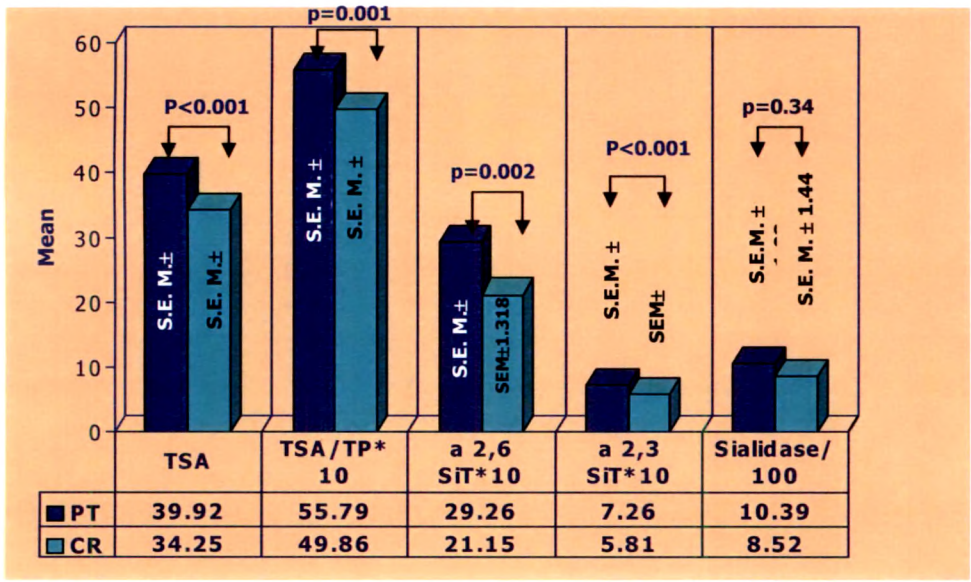
Pretreatment blood samples were collected from the oral cancer patients before initiation of anticancer therapy, which were termed as PT. The post-treatment follow-up samples were collected during/after anticancer treatment. They were classified into complete responders (CR) and non-responders (NR). The

circulating markers were compared between PT, CR and NR to assess their utility in treatment monitoring of oral cancer patients.

4.1.9 Comparison of Serum Markers between PT and CR

Paired 't' test was performed to compare the serum markers between PT and CR. As shown in **figure-4.10,** serum TSA and TSA/TP levels as well as activity of α2,6-SiT and α2,3-SiT were significantly decreased in CR as compared to PT ($p<0.001$, $p=0.001$, $p=0.002$ and $p<0.001$, respectively). Mean serum sialidase activity was lower in CR as compared to their PT activity ($p=0.344$).

Figure-4.10: Comparison of serum TSA, TSA/TP, α2,6-SiT, α2,3-SiT and sialidase between PT and CR

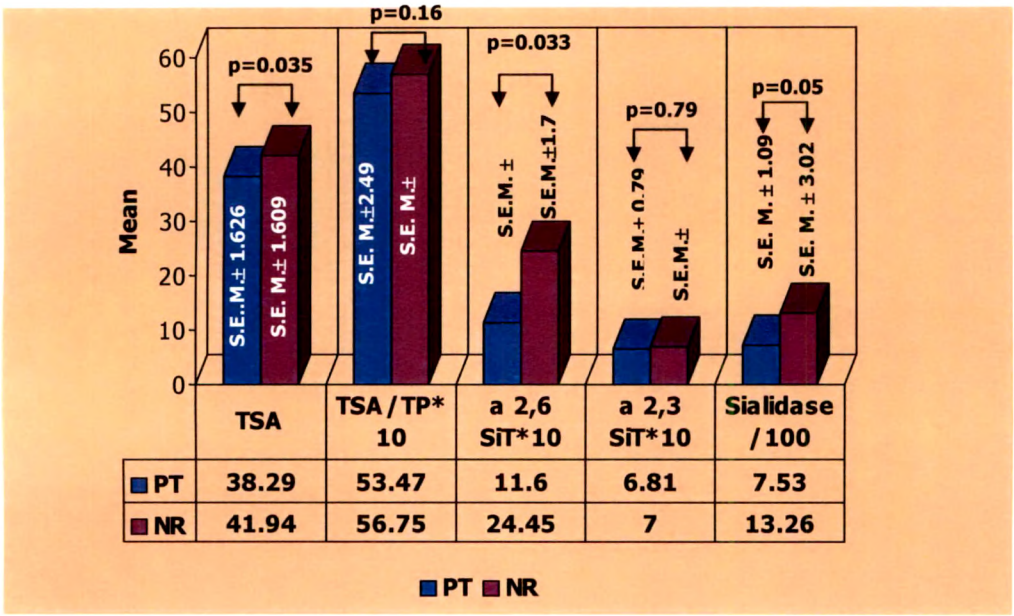


	TSA	TSA/TP* 10	a 2,6 SiT*10	a 2,3 SiT*10	Sialidase/ 100
PT	39.92	55.79	29.26	7.26	10.39
CR	34.25	49.86	21.15	5.81	8.52

4.1.10 Comparison of serum markers between PT and NR

As illustrated in **figure-4.11**, serum TSA levels were significantly elevated in NR as compared to PT ($p=0.035$), while, mean TSA/TP remained comparable between both the groups ($p=0.169$). Serum α2,6-SiT activity was significantly higher in NR as compared to PT ($p=0.033$). Mean α2,3-SiT as well as sialidase activities were higher in NR as compared to PT.

Figure-4.11: Comparison of serum TSA, TSA/TP, α2,6-SiT, α2,3-SiT and sialidase between PT and NR



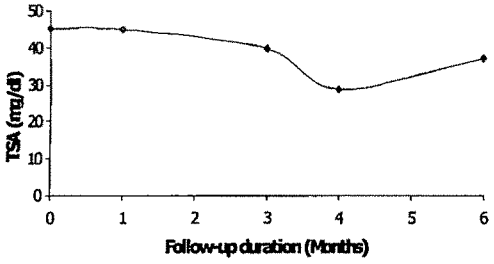
4.1.11 Serum TSA, α2,6-SiT, α2,3-SiT and sialidase levels as treatment monitors for oral cancer patients

Figure-4.12 (A-H) are representative patterns of serum TSA, α2,6-SiT, α2,3-SiT and sialidase levels before and during/after treatment in a CR and a NR cases. **Figure-4.12 (A-D)** shows variations in TSA, α2,6-SiT, α2,3-SiT and sialidase respectively, in a 38 years old male patient with cancer of retromolar trigone (RMT), who clinically showed response to surgery and post surgical radiotherapy. The levels of the four biomarkers were decreased during their anticancer treatment and remained lower throughout the follow-up period as compared to PT levels. The second case is represented in **figure-4.12 (E-H),** a 43 years old patient with cancer of left buccal mucosa. The patient underwent surgery and radiotherapy and till five months he did not have any major complaints. After 5 months, the patient developed lymphnode metastasis and the case of inoperable advanced disease. The patient was then treated with chemotherapy however, he did not respond to the treatment.

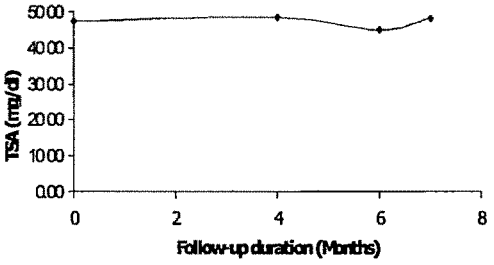
Figure-4.12: Serum TSA, α2,6-SiT, α2,3-SiT and Sialidase in representative cases of a CR (A-D) and a NR (E-H) at each follow-up during anticancer treatment

Complete Responder **Non-Responder**

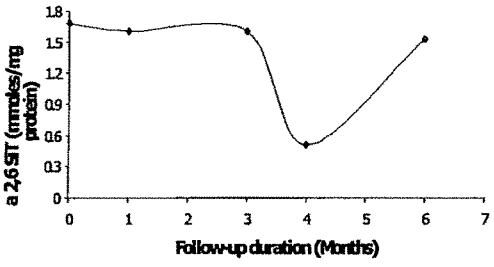
4.12A



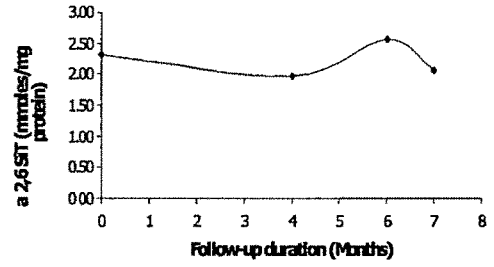
4.12E



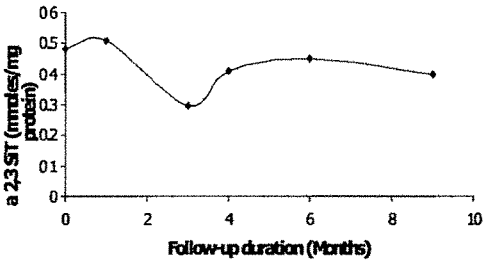
4.12B



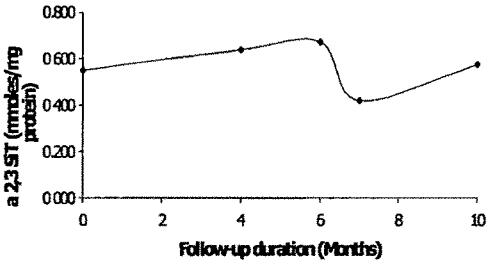
4.12F



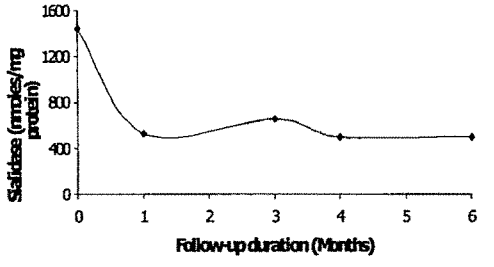
4.12C



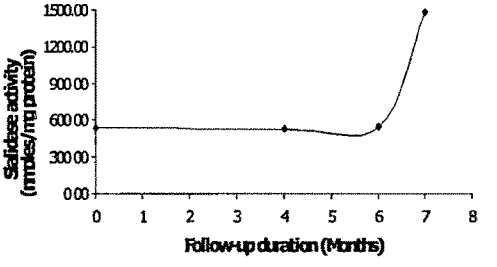
4.12G



4.12D



4.12H



Serum levels of TSA, α2,6-SiT, α2,3-SiT and sialidase (**Figure-4.12 E-H**, respectively) remained increased during the follow-up duration.

SAILYLATION CHANGES IN TISSUES

Sialylation associated marker levels were estimated from malignant/OPC and their adjacent normal tissue specimens obtained from oral cancer patients as well as patients with OPC. The comparison of the tissue markers were performed using students unpaired and paired 't' tests considering adjacent normal tissue as control for malignant as well as OPC tissue of the same patient.

4.1.12 Protein Estimation from Tissue Homogenates

Malignant and adjacent normal tissues were homogenized in phosphate buffer saline (pH: 7.4). Protease inhibitors were added in the homogenates to prevent proteolytic degradation. Protein concentration in cell lysates was estimated spectrophotometrically using Lowry's method (Lowry et al., 1951). The cell lysates were stored at –80°C immediately after homogenization.

4.1.13 Comparison of Sialylation Markers using Unpaired Student's t-Test

As documented in **table-4.6**, TSA levels were significantly higher in malignant tissues as compared to adjacent normal tissues ($p=0.016$). α2,6-sialoproteins were also high in malignant tissue but was not significant ($p=0.097$). Whereas, significant increased α2,3-sialoproteins was observed in malignant tissues ($p<0.001$). The activities of α2,6-SiT, α2,3-SiT and sialidase were significantly higher in malignant tissues than adjacent normal tissues ($p=0.005$, 0.001 and $p=0.05$, respectively).

Table-4.6: Comparison of the markers between malignant and adjacent normal tissue samples of oral cancer patients

Parameters	Adjacent Normal Tissues		Malignant Tissues		'p' value
	Mean ±	S.E.M	Mean ±	S.E.M	
TSA (μg/mg protein)	1.449	0.419	2.922	0.435	**0.016**
α2,6-sialoproteins (OD/mm^2)	2.393	0.378	3.965	0.398	0.097
α2,3-sialoproteins (OD/mm^2)	3.04	0.369	5.55	0.455	**<0.001**
α2,6-SiT activity (μmoles/mg protein	9.978	0.78	13.7714	2.004	**0.005**
α2,3-SiT activity (μmoles/mg protein	14.14	1.281	22.44	2.029	**0.001**
Sialidase activity (nmoles/mg protein)	76156.8	6673.77	102413.8	11615.77	**0.05**

Table-4.7 summarizes the comparison of the markers in precancerous and adjacent normal tissues of patients with OPC. α2,6- and α2,3-sialoproteins as well as sialidase activity were higher in precancerous tissues as compared to their adjacent normal tissues. However, the differences in the marker levels were not statistically significant.

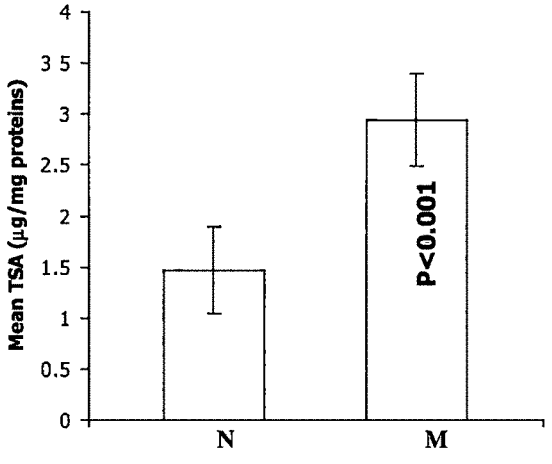
Table-4.7: Comparison of markers between precancerous and adjacent normal tissues in patients with OPC

	Adjacent Normal Tissues		Precancerous Tissues		'p' value
	Mean	± S.E.M	Mean	± S.E.M	
α2,6-sialoproteins (OD/mm^2)	1.027	0.381	1.237	0.156	0.54
α2,3-sialoproteins (OD/mm^2)	1.47	1.00	1.78	0.255	0.66
Sialidase activity (nmoles/mg protein)	80216.0	27905.9	123645.6	13640.4	0.14

4.1.14 Paired t-test for comparison of markers between malignant and adjacent normal tissues in oral cancer patients

The paired 't' test was performed to compare the markers levels between adjacent normal (N) and malignant (M) pairs of tissues. It computed the differences between values of the variables for each case. Mean TSA values were significantly higher in malignant tissues as compared to adjacent normal tissues ($p<0.001$) (**Figure-4.13**).

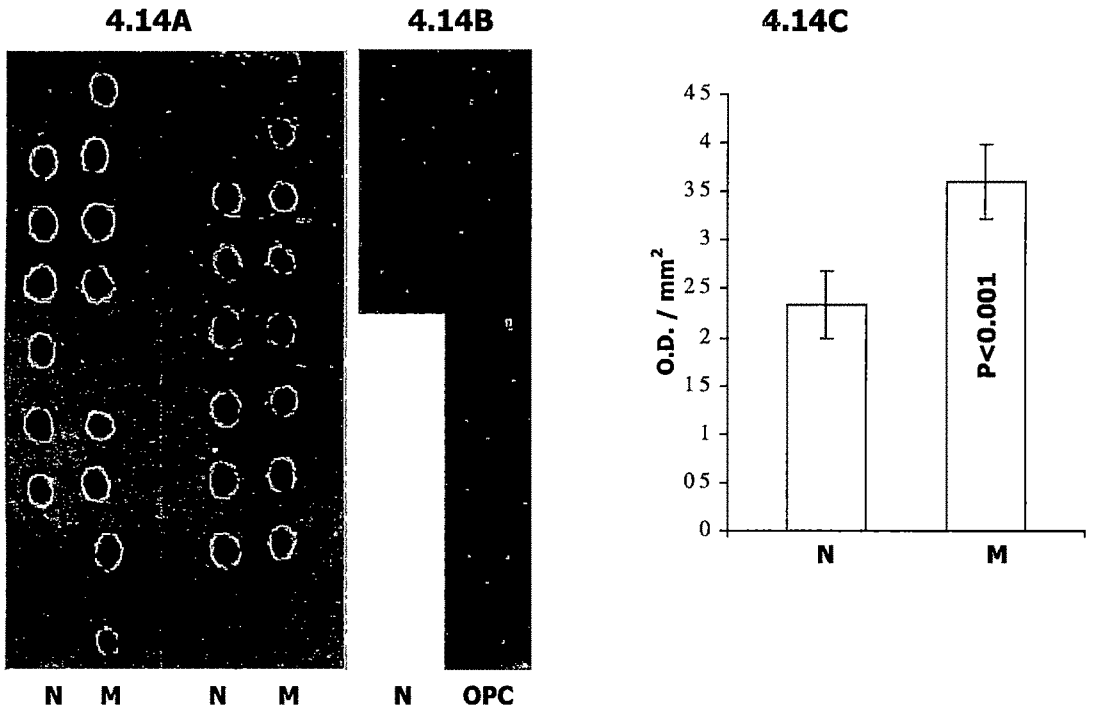
Figure-4.13: Total sialic acid in malignant and adjacent normal tissue



4.1.15 Expression of α2,6-sialoproteins and α2,3-sialoproteins in tissues

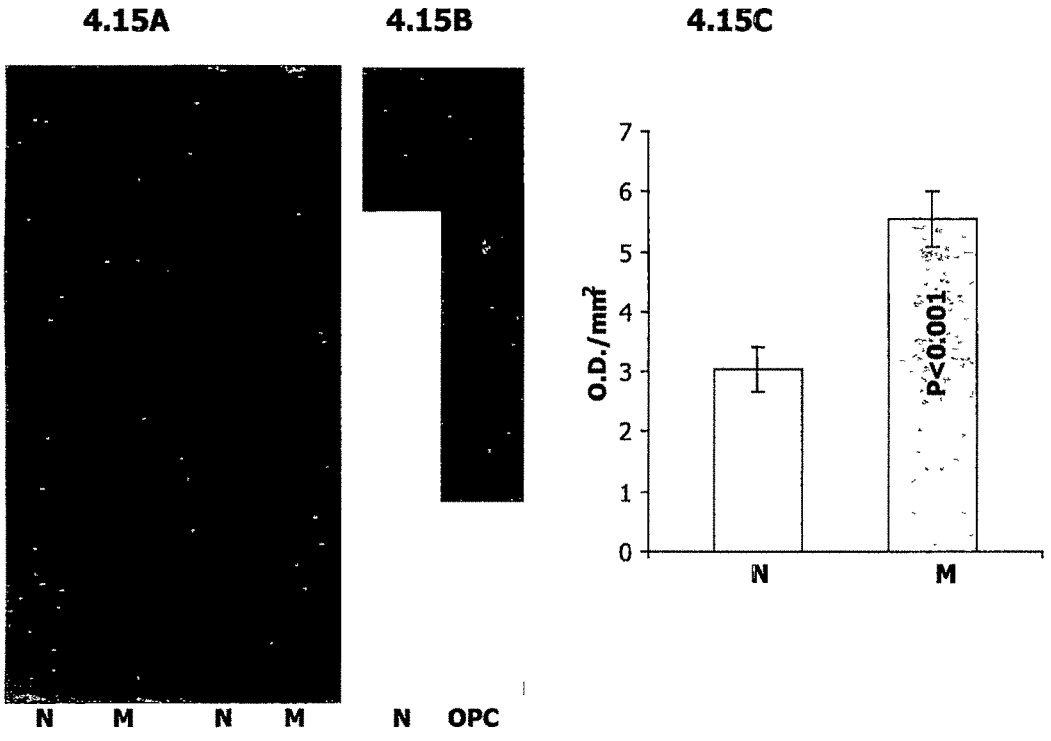
Representative patterns of SNA-dot blot **(Figure-4.14A)** and MAL-dot blot **(Figure-4.15A)** revealed that SNA and MAL reactivity to the proteins in oral malignant tissues was higher as compared to their adjacent normal tissues in majority of the patients. However, it was surprisingly not true in some of the cases for α2,6-linkage specific SNA reactivity. While, few cases showed similar density of the sialoproteins dots in adjacent normal and malignant tissues when quantified. In case of α2,3-sialoproteins, all the patients showed higher MAL-reactivity in the malignant tissues. The density of sialoprotein dots was quantified using molecular analyst software and gel documentation system. **Figure-4.14C and 4.15C** illustrate the results of paired t-test performed between malignant and adjacent normal tissues.

Figure-4.14: Representation of SNA-blot for α2,6-sialoproteins in oral cancer and OPC tissues (4.14A-B). Comparison of density of α2,6-sialoproteins between malignant and adjacent normal tissues (4.14C)



The densities of α2,6- and α2,3-sialoproteins (OD/mm^2) in malignant tissues were significantly higher as compared to their counterparts ($p<0.001$ and $p<0.001$, respectively). Representative blots for SNA and MAL reactivity in adjacent normal and OPC tissues are provided in **figure-4.14B and 4.15B**. The higher density α2,6-sialoproteins and α2,3-sialoproteins was observed in OPC tissues than adjacent normal tissues.

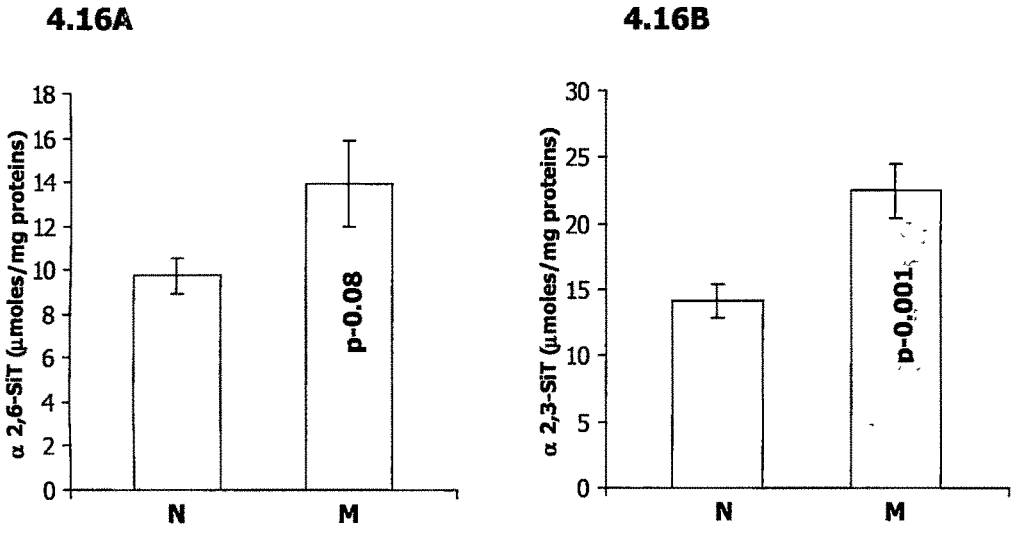
Figure-4.15: Representation of MAL-blot for α2,3-sialoproteins in oral cancer and OPC tissues (4.15A-B). Comparison of density of α2,3-sialoproteins between malignant and adjacent normal tissues (15C)



4.1.16 Sialyltransferase activity in tissues

As represented in the **figure-4.16**, α2,6-SiT activity was significantly higher in malignant tissues as compared to adjacent normal tissues ($p=0.08$) **(4.16A).** Further, significant elevations in α2,3-SiT were also found in oral tumour tissues ($p=0.001$) (**4.16B**).

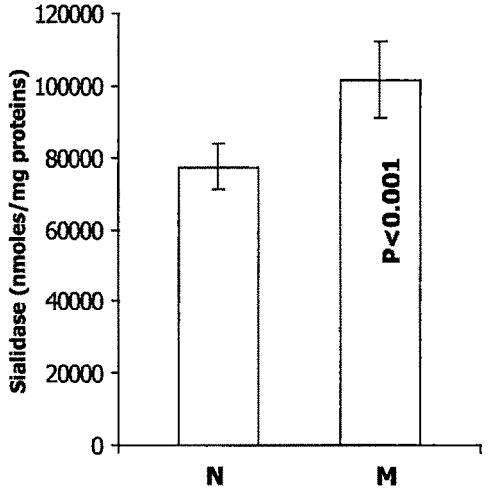
Figure-4.16: Comparison of α2,6-SiT and α2,3-SiT activities between malignant and adjacent normal tissues



4.1.17 Sialidase activity in tissues

Comparison of sialidase activity between malignant and adjacent normal tissues is depicted in the **figure-4.17**. It is clear from the bar graph that sialidase activity was significantly higher in malignant tissues as compared to adjacent normal tissues ($p<0.001$)

Figure-4.17: Sialidase activity in malignant and adjacent normal tissues



4.1.18 Correlation between sialylation markers in malignant tissues

Pearson's two-tailed correlation was performed to study the association between the markers in malignant tissues (**Table-4.8**). A positive correlation of α2,6-SiT activity with TSA levels and sialidase activity in malignant tissues revealed that increased α2,6-SiT activity might be associated with increased synthesis of TSA in the tissues. The positive correlations between α2,6-sialoproteins and TSA exhibit higher sialylation of tissue proteins. α2,3-SiT showed positive correlation with α2,3-sialoproteins and negative correlations with sialidase activity in malignant tissues. The remarkable observation of this analysis was, the positive association between α2,6-SiT and α2,3-SiT. This may indicate that in malignant tissues both the enzymes were simultaneously associated with hypersialylation of the proteins.

Table-4.8: Correlation between (i) α2,6-SiT, TSA and sialidase, (ii) α2,6-sialoproteins and TSA (iii) α2,3-SiT, α2,3-sialoproteins and sialidase (iv) α2,6-SiT and α2,3-SiT activity

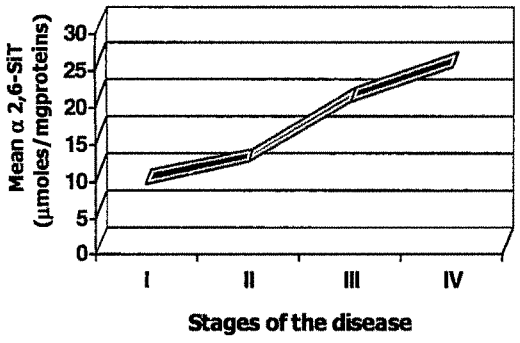
Correlation between α2,6-SiT – TSA and Sialidase				
Pearson's Correlation	Malignant tissue TSA		Malignant tissue sialidase	
Malignant tissue α2,6-SiT	r = 0.015	p = 0.463	r = 0.157	p = 0.265
Correlation between α2,6-sialoproteins and TSA				
Pearson's Correlation	Malignant tissue TSA			
α2,6-sialoproteins	r = 0.097		p = 0.278	
Correlation between α2,3-SiT - α2,3-sialoproteins and Sialidase				
Pearson's Correlation	Malignant tissue α2,3-sialoproteins		Malignant tissue sialidase	
Malignant tissue α2,3-SiT	r = 0.223	p = 0.167	r = -0.122	p = 0.452
Correlation between α2,6-SiT - α2,3-SiT				
Pearson's Correlation	Malignant tissue α2,3-SiT			
Malignant tissue α2,6-SiT	r= 0.242		p = 0.138	

4.1.19 Stage of the Disease and α2,6-SiT, α2,3-SiT and Sialidase Activities in Malignant Tissues

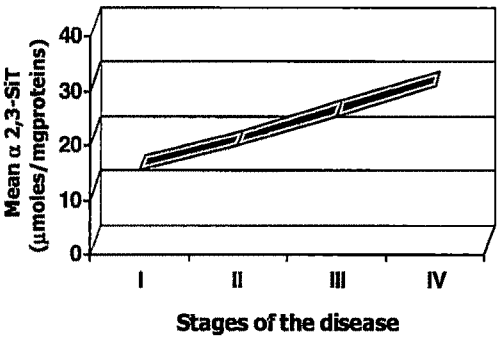
α2,6-SiT, α2,3-SiT and sialidase activities in malignant tissues were compared with the stages of the disease. **Figure-4.18 (A-C)** represents mean levels of α2,6-SiT, α2,3-SiT and sialidase activities at different stage of the disease. As stage of the disease progressed from early (stage I+II) to advance (stage III+IV), serum α2,6-SiT (p=0.015) and α2,3-SiT (p=0.058) were increased, whereas sialidase activity were decreased.

Figure-4.18 (A-C): Mean α 2,6 SiT, α 2,3 SiT and sialidase activities in malignant tissues in different stages of the disease

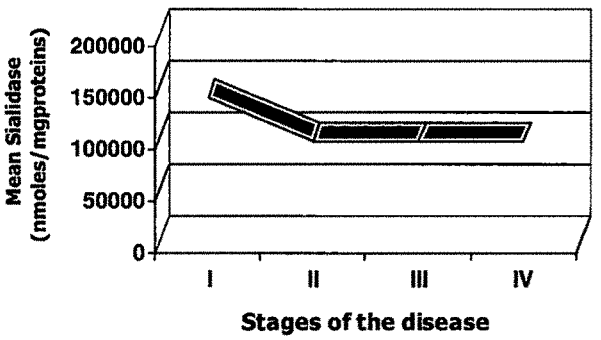
4.18A: α2,6-SiT



4.18B: α2,3-SiT



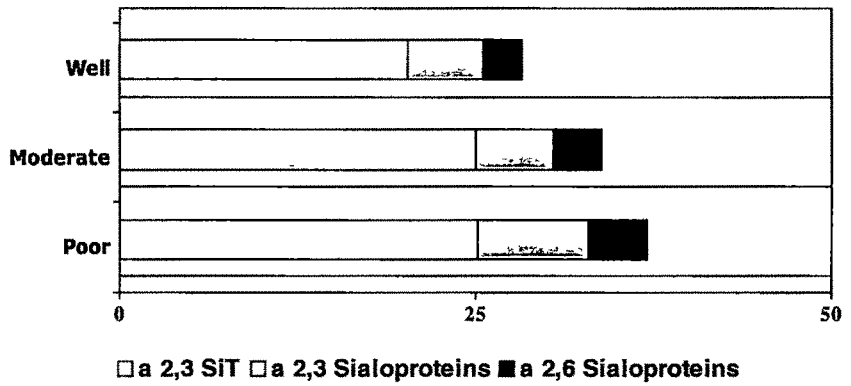
4.18C: Sialidase



4.1.20 Tumour differentiation and α2,3-SiT, α2,3-sialoproteins and α2,6-sialoproteins levels in malignant tissues

Figure-4.19 represents comparison of mean levels of α2,3-SiT as well as α2,3- and α2,6-sialoproteins in malignant oral tissues in patients with different pathological tumour differentiation. Student 't' test revealed that α2,3-SiT activities were significantly higher in poorly differentiated tumours as compared to well differentiated tumours (p=0.048). Whereas, α2,3-sialoproteins (p=0.06 and p=0.042, respectively) and α2,6-sialoproteins (p=0.03 and p=0.078, respectively) were significantly higher in poorly diffenentiated tumours as compred with well and moderately differentiated tumours.

Figure-4.19: Mean tissue α2,3-SiT, α2,3-sialoproteins and α2,6-sialoproteins in different pathological tumour differentiation



4.2: FUCOSYLATION CHANGES IN ORAL CANCER AND ORAL PRECANCEROUS CONDITIONS

SERUM FUCOSYLATION CHANGES

Circulating markers associated with protein fucosylation were studied in oral cancer patients. The serum levels were compared with pathological controls (patients with OPC) as well as controls (healthy individuals).

4.2.1 Alterations in serum Fucose

Figure-4.20: Standard curve for Fucose

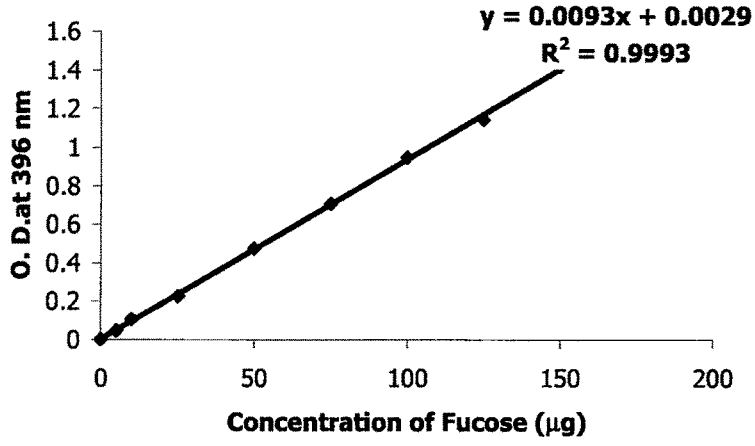
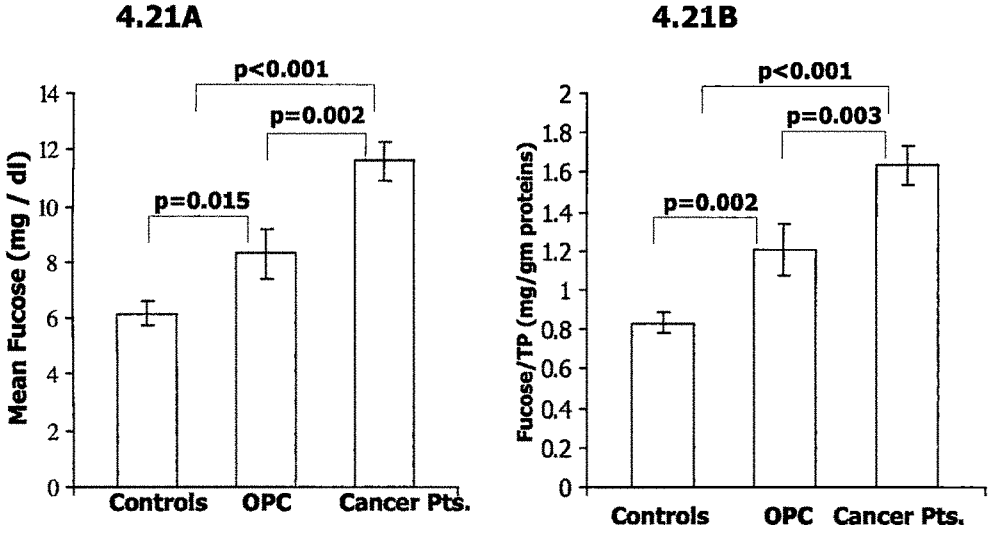


Figure-4.20 depict linear standard graph of fucose plotted between fucose concentration and O.D. (r^2=0.999). Linearity equation was derived from the standard graph to calculate concentrations of fucose. The intra- and inter-assay coefficients of variations were 6% and 5%, respectively.

Figure-4.21: Comparison of serum Fucose and Fucose/TP in the subjects



Alterations in the serum fucose levels were determined in the subjects. Fucose concentration was also normalized with the total proteins variations. The comparison of mean serum fucose and ratio of fucose/TP between the groups is shown in **figure-4.21 (A, B)**. The fucose and fucose/TP were significantly elevated in oral cancer patients as compared to patients with OPC ($p=0.002$ and $p=0.003$, respectively) as well as controls ($p<0.001$). The fucose and fucose/TP levels were significantly elevated in patients with OPC as compared to the controls ($p=0.015$ and $p=0.002$, respectively).

4.2.2 Expression of fucosylated proteins in the subjects and serum levels of fucoproteins as treatment monitors in oral cancer patients

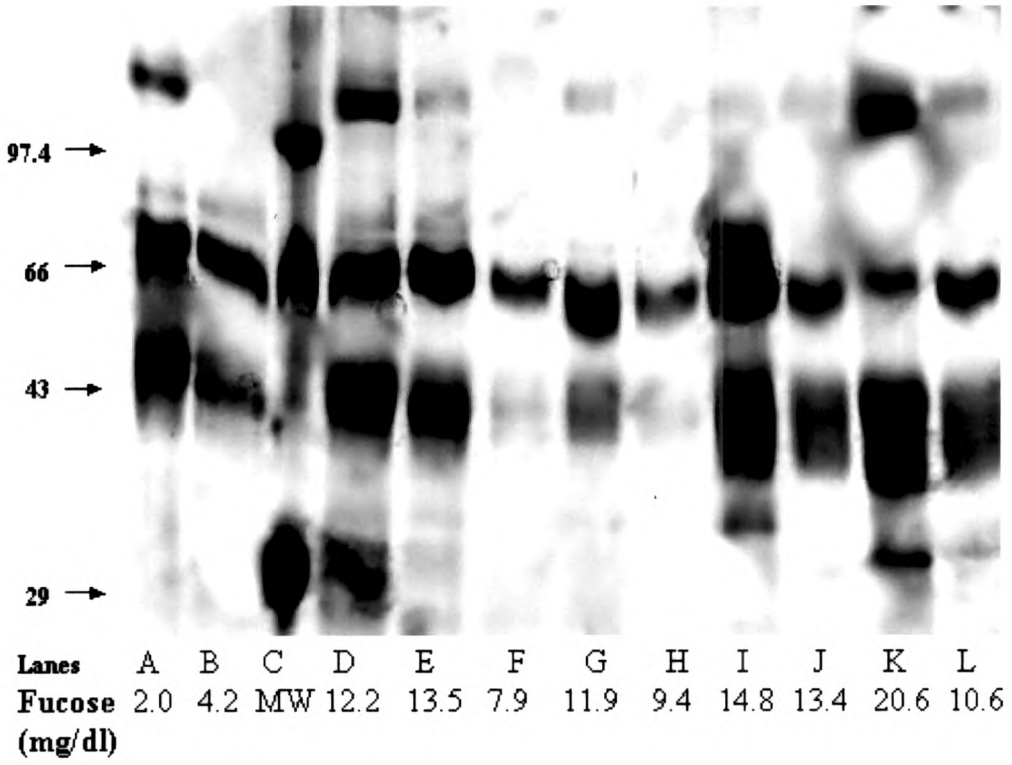
The serum fucoproteins were isolated using fucose specific lectin from Lotus tetragonolobus (LTA). As shown in materials and methods, lectin affinity chromatography was performed for fucoproteins. Representative patterns of electrophoretic separation of serum fucoproteins in controls, patients with OPC and three untreated oral cancer patients with their follow-ups during/after anticancer therapy are shown as below. The fucoproteins band density was quantified using molecular analyst software of gel documentation system.

As shown in the representative electrophoretic patterns of fucoproteins (**Figure-4.22, 4.24**), LTA-reactivity alterations were observed especially in ~43 kD and ~66 kD proteins. Lane A and B (**Figure-4.22**) represent fucoproteins in controls and patients with OPC, respectively. The patterns represent fucoprotein variations in responders, partial responders and non-responders during follow-ups. All the three oral cancer patients showed higher fucoprotein levels as compared to controls and patients with OPC.

As represented in **figure-4.22** (Lanes D-H) and **Figure-4.23A**, lanes D-H represents fucoprotein patterns in a case of 42 years old male patient having

SCC of lateral border of tongue with stage-II disease, who underwent surgery as primary treatment. The fucoproteins levels were compared at PT and during follow-up period. The patient responded well to the surgery. Fucoproteins were lower throughout the follow-up period (lanes E, F, G and H; Follow-up period: 1, 3, 7 and 10 months, respectively) as compared to PT (lane D). This reflected that the decrease in serum fucoproteins was associated with the response to anticancer treatment.

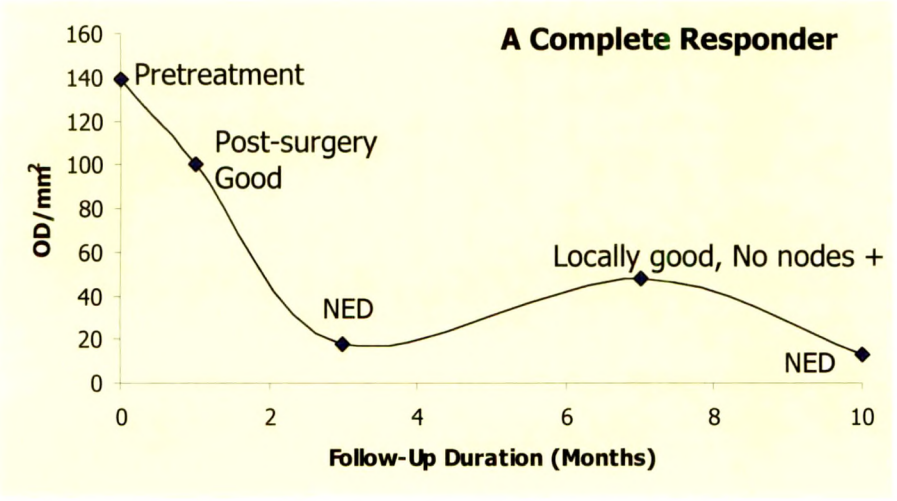
Figure- 4.22: Representative Pattern-1 for fucoproteins



The band density of total serum fucoproteins was quantified and line graphs were plotted for density of fucoproteins in PT as well as at each follow-up of the same patient, which is represented in **figure-4.23 (A-B).** Fucoproteins

alterations in a 54 years old male patient diagnosed with carcinoma of buccal mucosa who underwent surgery as primary treatment and later on received radiotherapy are shown in shown in **figure–4.22** (Lanes I-L) and **figure-4.23B**. Lane I represent PT serum fucoproteins pattern in a partial responder, lanes J-L exhibits serum levels during follow-up period of the same patient.

Figure-4.23 A: Serum Fucoproteins Levels in a CR Before and After Anticancer Treatment (case#41)



NED: No evidence of the disease

Graphical presentation of densities (**Figure-4.23B**) clearly indicated that after 1 month of surgery fucoproteins levels were decreased (lane J) as compared to PT. During the second follow-up (4 months), the patient developed local recurrence where increased fucoproteins levels were observed (lane K). The patient underwent second surgery for recurrent disease. Serum fucoproteins were again decreased upon second surgery (lane L; 7 months follow-up) as compared to PT as well as previous follow-up values.

Figure-4.23B: Serum fucoproteins levels in a PR before and after anticancer treatment (Case#18)

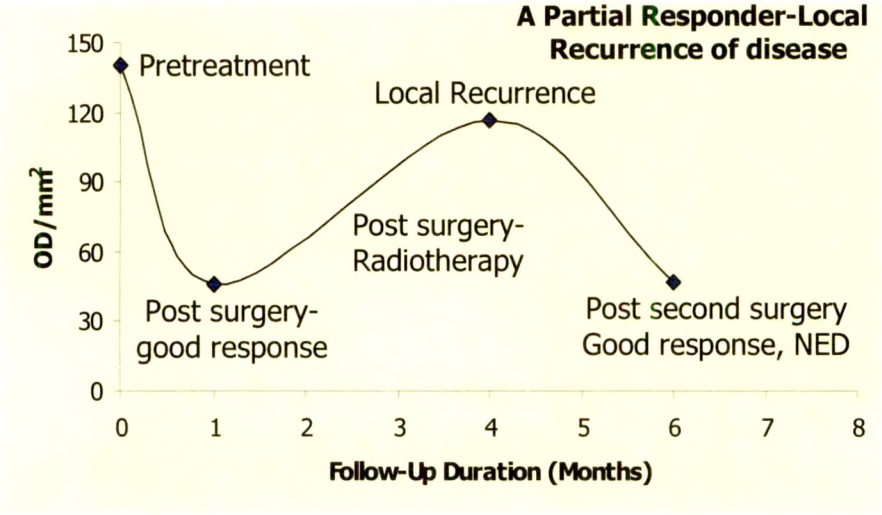
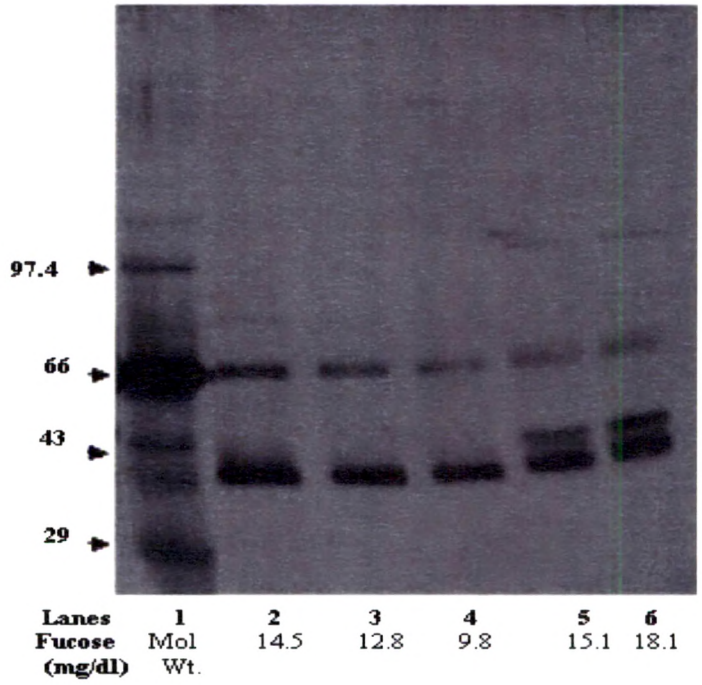


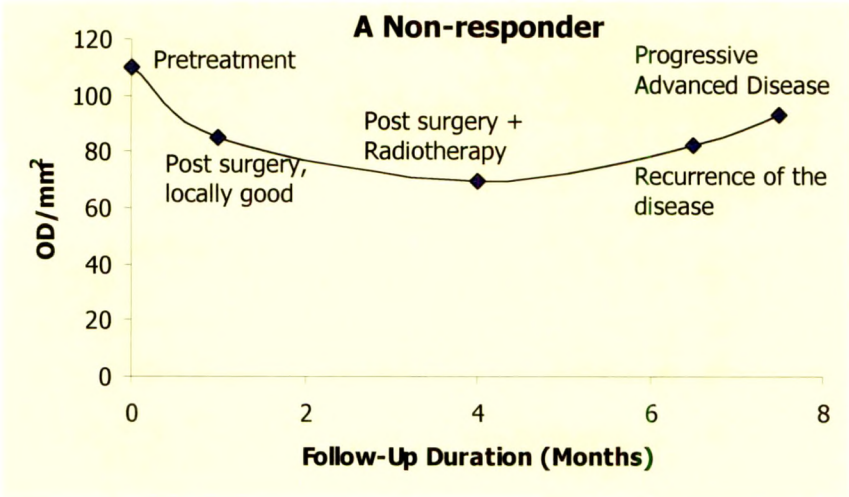
Figure-4.24: Representative Pattern-2 for fucoproteins



Serum fucoproteins levels in a 37 years old male patient having stage IV disease of buccal mucosa, who was clinically evaluated as NR, are shown in

representative pattern-2 (**Figure-4.24**). Lane 2 represents serum fucoproteins in the patient before initiation of anticancer treatment (PT), while lanes 3-6 exhibit post-treatment serum fucoproteins patterns. The band density of serum fucoproteins was higher at the time of PT, whereas, decreased band density was observed after 1 month (post-surgery) and 4 months (post-radiotherapy), respectively (lanes 3,4). Lane 5 represents the follow-up after 6.5 months when the patient developed recurrence. Serum fucoproteins remain elevated after removal of recurrent tumour (lane-6). As depicted in **figure-4.25**, band density of fucoproteins remained increased during the progressive disease.

Figure-25: serum fucoproteins levels in a NR before and after anticancer treatment (Case#40)



Also, significant difference in serum fucose levels in these patients was observed along with fucoproteins before and after anticancer treatment.

4.2.3 Comparison of serum fucoproteins between controls, patients with OPC, PT, CR and NR

Mean fucoproteins band density (OD/mm^2) in controls, patients with OPC and oral cancer patients as well as post-treatment follow ups of oral cancer patients are provided in **Table-4.9**. Serum fucoproteins levels were significantly higher in

oral cancer patients as compared to controls and patients with OPC (p=0.006 and p=0.05, respectively). LTA reactivity was also increased in patients with OPC (p=0.074) as compared to the controls.

Table-4.9: Comparison of serum fucoproteins levels in controls and patients

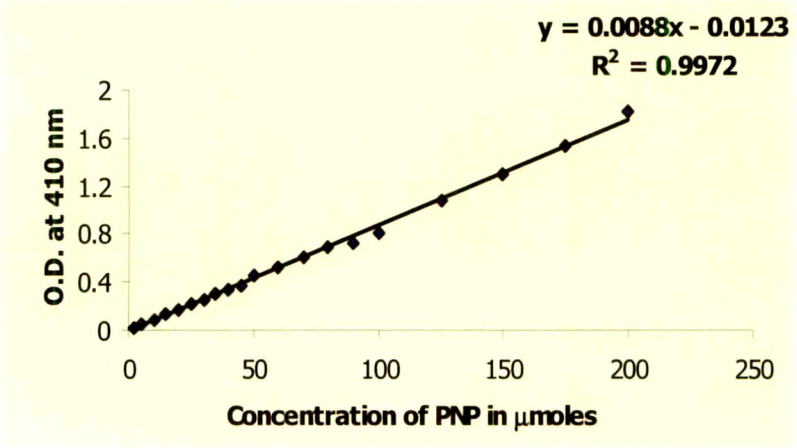
Groups	Fucoproteins (OD/mm^2) Mean ± S.E.M	Significance 'p'
Controls	29.4 ± 7.45	Control vs. OPC: p= 0.074
Patients with OPC	38.2 ± 4.12	Control vs. PT : p=0.006
PT	72.7 ± 1 3.30	OPC vs. PT : p=0.05
CR	44.1 ± 5.04	PT vs. CR : p=0.01
NR	86.2 ± 9.41	PT vs. NR : p=0.083
PT: Untreated cancer patients CR: Complete Responders NR: Non-responders		

The mean fucoproteins density in PT was compared with CR and NR. The fucoproteins were significantly lower in CR (p=0.01), while it was slightly higher in NR than PT (p=0.083). It is evident from the results that fucosylation changes reflected the response to anticancer therapy.

4.2.4 Serum α-L-fucosidase activity in the subjects

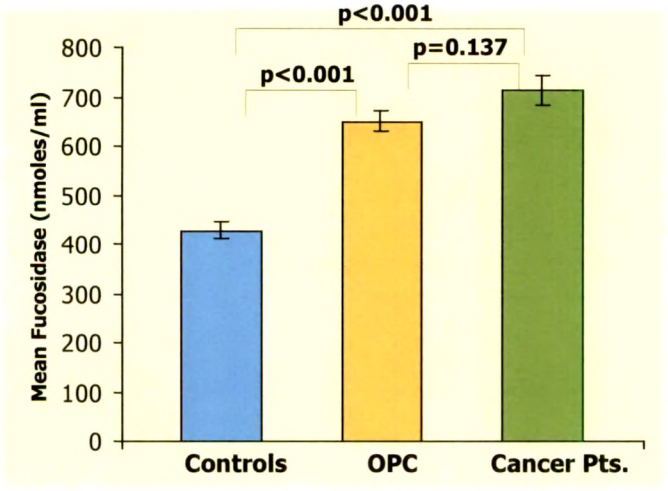
The standard curve of PNP plotted between the concentration and O.D. was found to be linear (**Figure-4.26**). α-L-fucopyranoside tagged with PNP was used as substrate for α-L-fucosidase assay. Linearity equation was derived from the standard curve (r^2=0.997) to calculate activity of α-L-fucosidase. Intra- and inter-assay coefficients of variations were 6.5% and 4.5%, respectively. The sensitivity of the method was in the detection limit up to 2.5 μmoles of PNP liberated during enzymatic reaction in the sample.

Figure-4.26: Standard curve of *p*-Nitrophenol (PNP) for α-L-fucosidase



Fucosidase catalyzes hydrolytic cleavage of fucose from its subterminal sugar residue. As presented in **figure-4.27**, mean serum fucosidase activity was significantly elevated in oral cancer patients as well as in patients with OPC as compared to controls ($p<0.001$). Activity of serum fucosidase was also higher in cancer patients when compared to the patients with OPC.

Figure-4.27: Comparison of serum α-L-fucosidase activity between controls and patients



4.2.5 Association of serum fucosylation markers with clinico-pathological parameters

Multivariate analysis was carried out to study the association of the clinico-pathological parameters with serum fucosylation markers in the cancer patients.

Table-4.10: Multivariate analysis between serum fucosylation markers and clinico-pathological parameters.

Variables		Fucose	Fucose/TP	Fucosidase
Age	F	0.82	0.91	0.93
	P	0.734	0.611	0.585
Gender	F	1.08	1.09	1.18
	P	0.301	0.3	0.28
Nuclear grade	F	0.63	0.55	1.23
	P	0.537	0.578	0.296
Tumour	F	4.11	4.16	0.65
Differentiation	P	**0.02**	**0.019**	0.526
Lymph node	F	1.26	0.91	0.02
Involvement	P	0.265	0.343	0.901
Stage of the	F	1.1	1.04	2.85
Disease	P	0.354	0.377	**0.041**
Early and	F	2.78	1.73	0.65
Advanced Stage	P	0.101	0.192	0.421

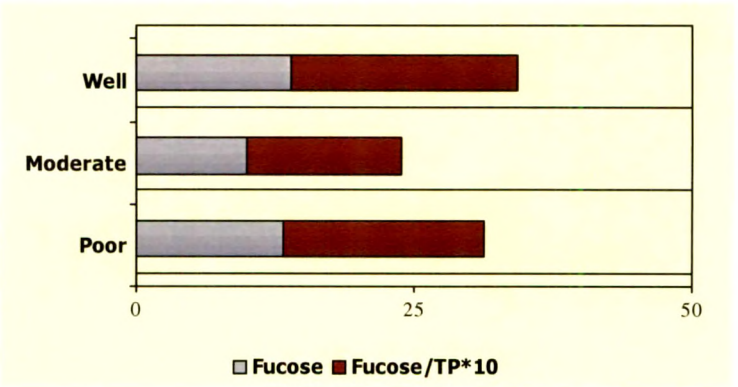
Table-4.10 represents the results of multivariate analysis for serum fucosylation biomarkers with clinicopathological features. Serum fucose and fucose/TP were significantly associated with tumour differentiation ($p=0.02$ and $p=0.019$, respectively). Serum fucosidase activity was significantly associated with stage of the malignant disease ($p=0.041$).

4.2.6 Tumour Differentiation and Serum Fucose, Fucose/TP

Figure-4.28 illustrates mean serum levels of fucose and fucose/TP in oral cancer patients with different grades of tumour differentiation. Student 't' test showed that serum fucose levels were significantly higher in poorly differentiated tumours as compared to moderately differentiated tumours ($p=0.018$). Serum fucose levels were significantly lower in moderately differentiated tumours as

compared to well differentiated tumours (p=0.014). Mean fucose/TP values were also increased in poorly differentiated tumours as compared with moderately differentiated tumours (p=0.031). Serum fucose/TP was significantly lower in moderately differentiated tumours as compared to well differentiated tumours (p=0.008).

Figure-4.28: Comparison of mean serum levels of fucose and fucose/TP with tumour differentiation



Significance: **Fucose:** Poor vs. Mod.: p=0.018, Mod. vs. Well: p=0.014
Fucose/TP: Poor vs. Mod: p=0.031, Mod. vs. Well p=0.008

SERUM FUCOSYLATION MARKERS IN PRETREATMENT (PT) AND POST-TREATMENT FOLLOW-UPS OF ORAL CANCER PATIENTS

The circulating fucosylation markers were compared between PT, CR and NR to assess their significance in treatment monitoring of oral cancer patients.

4.2.7 Comparison of serum markers between PT and CR, NR

Table-4.11: Comparison of serum fucose, fucose/TP and fucosidase between PT and CR

Parameter	PT Mean ± S.E.M	CR Mean ± S.E.M	'p' value (PT vs CR)
Fucose (mg/dl)	15.02 1.673	9.52 0.697	<0.001
Fucose/TP (mg/gm proteins)	2.193 0.235	1.338 0.101	<0.001
Fucosidase (nmoles/ml)	764.8 34.44	498.3 30.84	<0.001

Table-4.12: Comparison of serum fucose, fucose/TP and fucosidase between PT and NR

Parameter	PT Mean ± S.E.M.	NR Mean ± S.E.M.	'p' value (PT vs NR)
Fucose (mg/dl)	11.02 0.703	14.99 1.025	0.001
Fucose/TP (mg/gm proteins)	1.604 0.139	2.091 0.139	0.004
Fucosidase (nmoles/ml)	720.3 62.53	666.8 64.33	0.617

The students paired 't' test was performed to compare the pre-treatment and post-treatment follow-up marker levels in oral cancer patients. As documented in **table-4.11** and **table-4.12**, serum fucose, fucose/TP and fucosidase activity were significantly declined in CR as compared to PT ($p<0.001$, $p<0.001$ and $p<0.001$, respectively). Further, serum levels of fucose and fucose/TP were significantly elevated in NR ($p=0.001$ and $p=0.004$, respectively). Serum fucosidase activity was comparable between PT and NR.

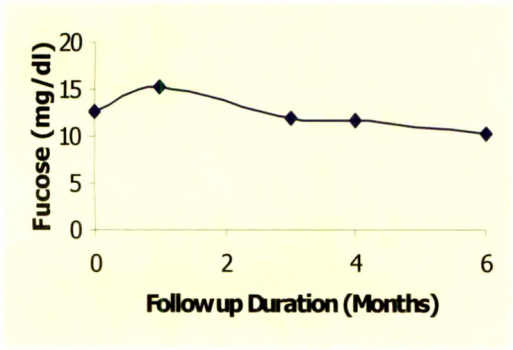
4.2.8 Serum Fucose and Fucosidase as Treatment Monitors of Oral Cancer Patients

To evaluate the role of serum fucose and fucosidase in treatment monitoring, PT levels of the markers were compared with follow-up levels. **Figure-4.29 (A-D)** are representative graphs of serum fucose and fucosidase levels during PT and follow-up in two oral cancer patients; one was CR and the other was NR. **Figure-4.29A-B** depicts fucose and fucosidase, respectively, in a 38 years old male patient diagnosed with carcinoma of retromolar trigone (RMT), who underwent surgery as primary treatment and later on (1 month after surgery) received radiotherapy. The patient clinically responded well to the surgery and post surgical radiotherapy. The levels of both fucose and fucosidases were

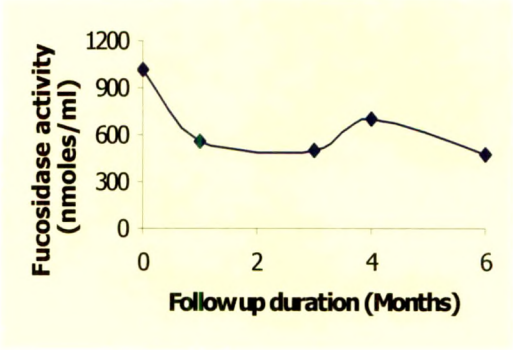
decreased during the anticancer treatment and remained lower throughout the follow-up period as compared to PT level.

Figure-4.29: Representative patterns of serum fucose and fucosidase levels before and after anticancer treatments in a CR (A & B) and a NR (C & D)

4.29A

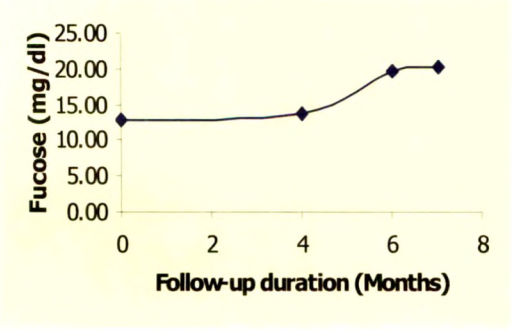


4.29B

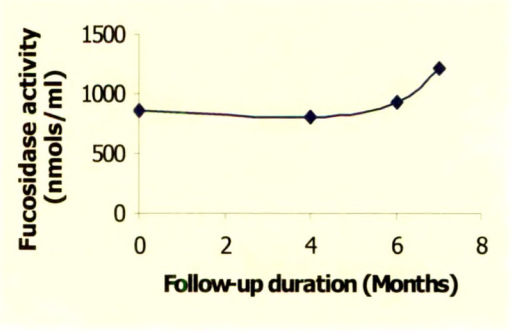


The second case represented in Figure-4.29C-D, was a 43 years old male patient with cancer of left buccal mucosa. He developed lymphnode metastasis after initial post surgical and post radiotherapy response for four months with no any major complaints. After development of lymphnode metastasis the patient was inoperable due to the advanced disease. Hence, he was treated with chemotherapy. But, he did not show response to any anticancer treatments. The serum levels of fucose (4.29C) and fucosidase (4.29D) remained higher than PT during initial follow-up duration of 4 months, which were further elevated during the advanced stage of the disease.

4.29C



4.29D



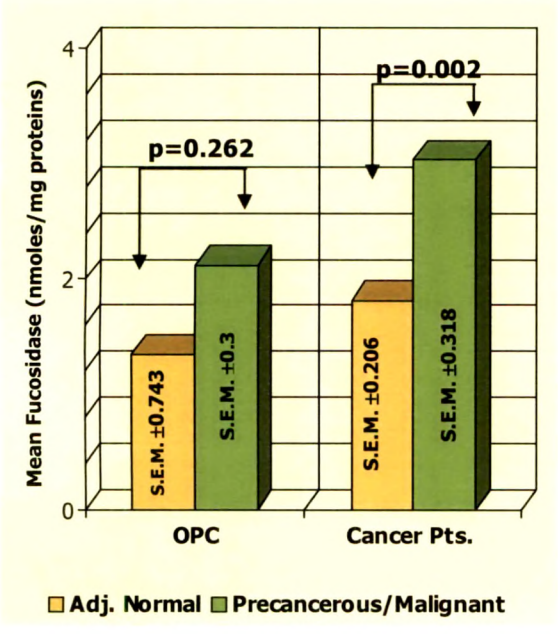
FUCOSYLATION CHANGES IN TISSUES

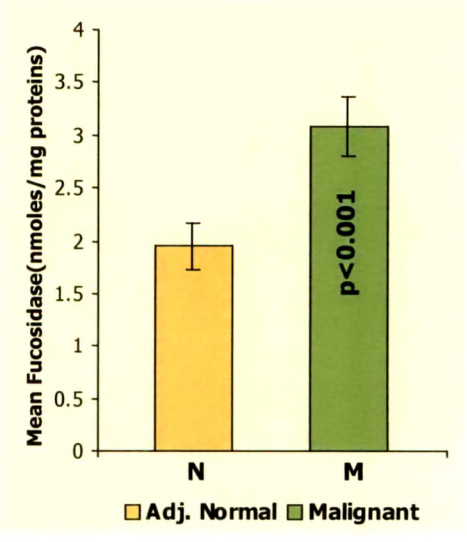
4.2.9 α-L-fucosidase Activity in Malignant/precancerous Tissues

The comparison of the tissue markers were performed using students unpaired 't' and paired 't' test considering adjacent normal tissues as control for malignant as well as OPC tissues of the same patient. Unpaired t-test revealed that α-L-fucosidase activity was significantly higher in malignant tissues than the adjacent normal tissues (p=0.002). α-L-fucosidase activity was also higher in OPC tissues when compared to adjacent normal tissues (**Figure-4.30A**). Student's paired 't' test analysis shown in **figure-4.30B** revealed that α-L-fucosidase activity was significantly higher in malignant tissues than the adjacent normal tissues (p<0.001).

Figure-4.30: Comparison of α-L-fucosidase between malignant/OPC and adjacent normal tissues in the patients

4.30A **4.30B**





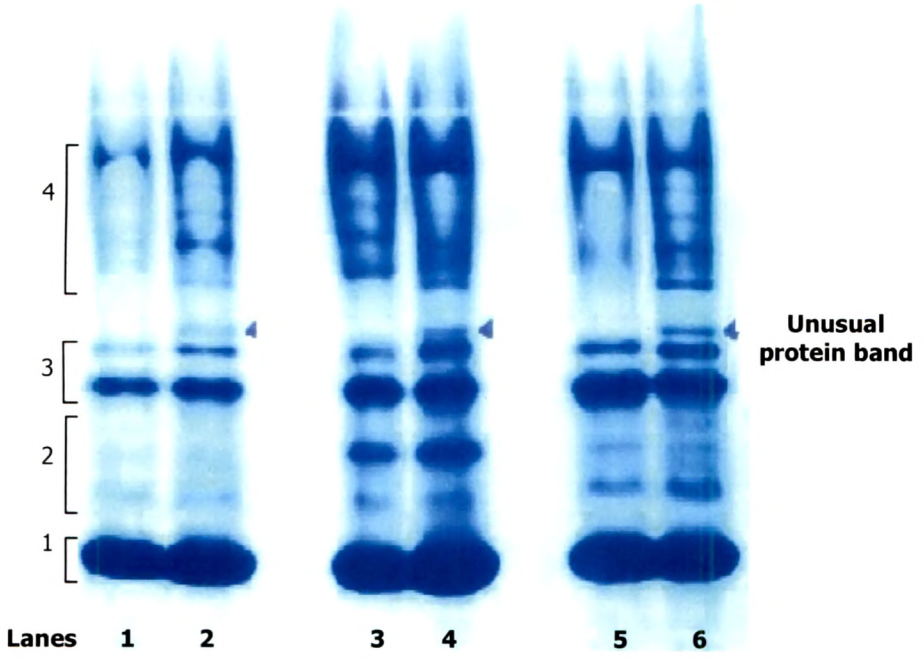
4.3: SERUM PROTEIN PROFILE

Protein and glycoprotein profiling was carried out from blood samples collected from controls, patients with OPC and oral cancer patients.

4.3.1 Serum protein separation on 7.5% Native-PAGE

Serum proteins were separated on 7.5% native polyacrylamide gel electrophoresis (PAGE) using standard protocols under non-denaturing and non-reducing conditions. The proteins were stained using 0.25% coommassie brilliant blue (CBB).

Figure-4.31: Serum protein electrophoretic pattern stained with CBB



Lanes 1, 2: Protein profile in controls with negative and positive unusual protein

Lanes 3, 4: Protein profile in patients with OPC with negative and positive unusual protein

Lanes 5, 6: Protein profile in oral cancer patients with negative and positive unusual protein

Regions 1: Albumin, Globulins- **2**: Alpha, **3**: Beta, **4**: Gamma

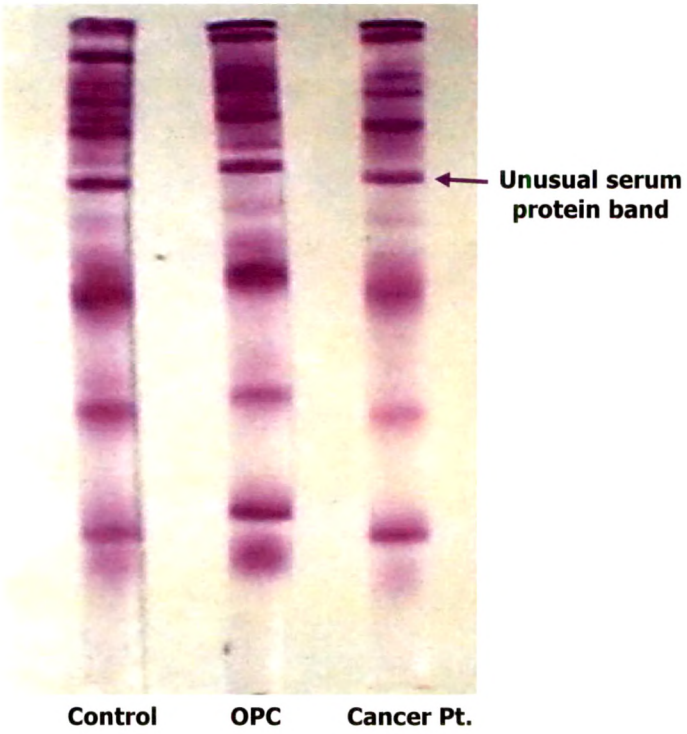
Unusual Protein Band: Post Beta region

Serum protein electrophoretic pattern revealed multiple protein bands (**Figure-4.31**). The remarkable observation of this study was the presence of an unusual (extra) protein band in the post beta region. This band was more prevalent in cancer patients (72%) and patients with OPC (75%). Its presence was also seen in only 24% of the controls. The volume of the protein band was 2 to 4 % of total proteins in the patients. The band volume of the unusual protein (extra) band was higher in patients (1-4%) and pathological controls (OPC) (0.5-2%) as compared to controls.

4.3.2 Serum glycoprotein staining with Periodic Acid Schiff's (PAS) reagent

The representative glycoprotein electrophoretic patterns also revealed multiple bands in pathological controls and cancer patients (**Figure-4.32**). The unusual (extra) protein band was found to be a glycoprotein.

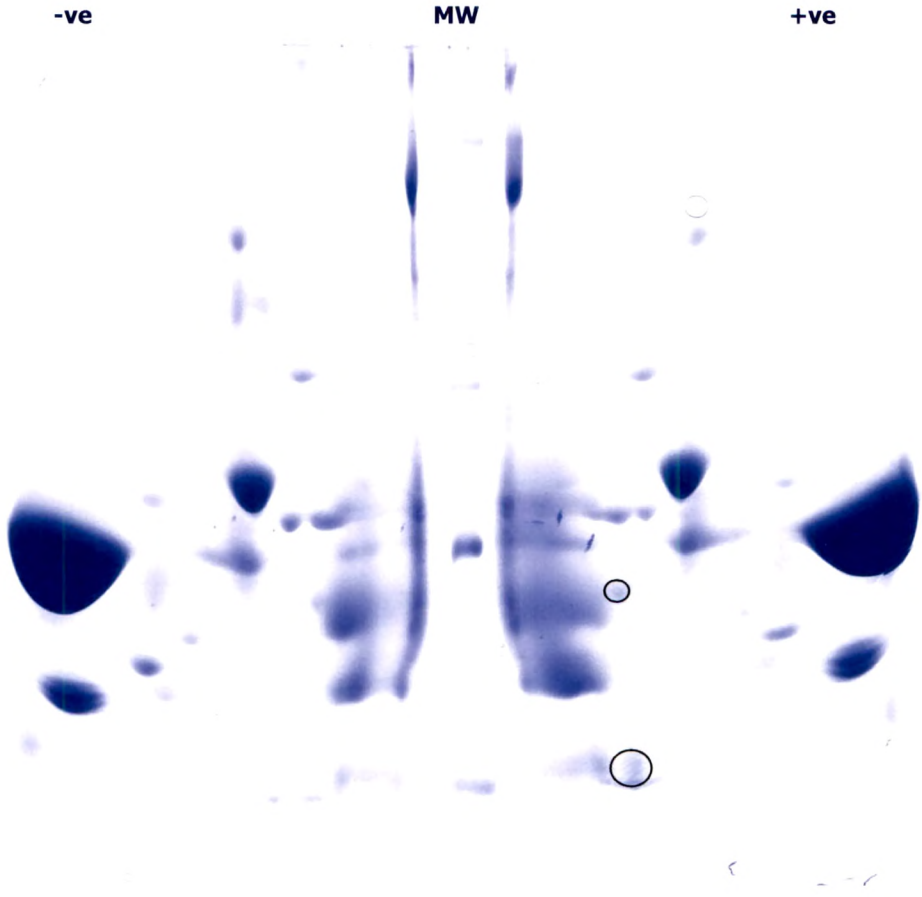
Figure-32: Representative serum glycoprotein electrophoretic pattern stained with PAS



4.3.3 2D-PAGE for serum proteins

In the current era of proteomics, 2D-PAGE is an important tool for recognition of protein expressions in cancer research. Therefore, the unusual (extra) protein observed in the patients was further analyzed using 2D-PAGE. In the first dimension, serum proteins were separated on Native-PAGE and the same gel was further applied for proteins separation in second dimension after necessary processing of the gels. Reducing and denaturing conditions (SDS-PAGE) were applied for the protein separation.

Figure-4.33: Representative 2D map of serum proteins



-ve – Serum sample of a cancer patient with negative unusual (extra) protein band

+ve - Serum sample of a cancer patient with positive unusual (extra) protein band

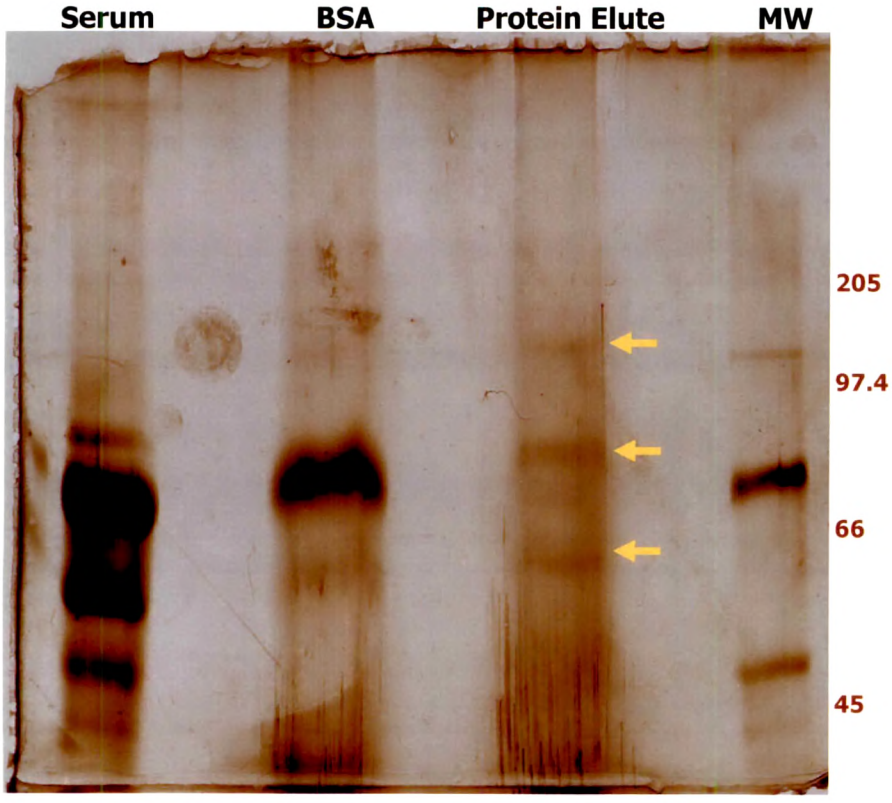
MW – Molecular weight marker

When the protein 2D map of serum samples were compared for unusual (extra) positive and negative serum, 2 distinct spots (circled) were observed in positive serum sample, which were not found in the negative samples (**Figure-4.33**).

4.3.4 Separation of extra protein elutes on SDS-PAGE and Silver staining

The unusual (extra) protein was transferred onto nitrocellulose membrane after separating on 7.5% Native-PAGE. The extra protein was eluted from the membrane and the protein elute was run on SDS-PAGE under reducing conditions. As shown in **figure-4.34**, three distinct bands were observed in the eluted protein fraction. Molecular weights of standard proteins were run simultaneously.

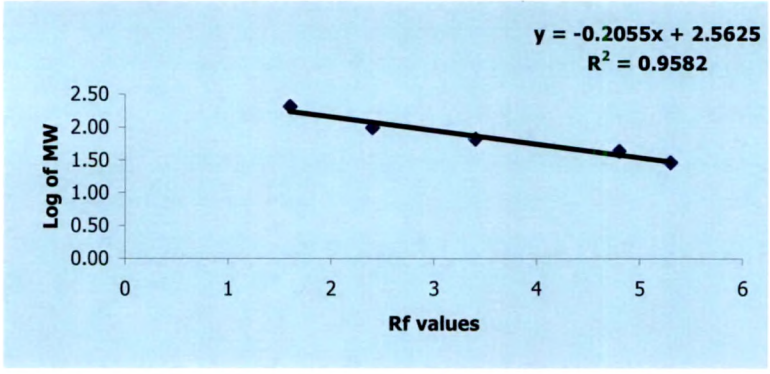
Figure-4.34: Electrophoretic pattern of unusual protein elute



4.3.5 Molecular weight determination of three bands of protein elute

Determination of molecular weight of the unusual (extra) protein band was performed. A graph was plotted between Rf values of standard protein bands of molecular weights and Log of molecular weights of standard proteins, which is shown in **figure-4.35**. As depicted in the figure, linear standard graph for molecular weight markers was obtained. Linearity equation was derived (r^2=0.958) from the standard graph to calculate log values of molecular weight of unknown proteins. These log values were converted into the molecular weight as shown in the **Table-4.13**.

Figure-4.35: Graph representing Rf values vs. Log values of standard protein molecular weight markers



The results confirm that the unusual protein expressed in the serum of the patients consists of three peptide chains and their apparent molecular weights are ~54.95, ~79.43 and ~120 kD respectively (**Table-4.13**). The molecular weights of the protein bands calculated using formula from standard graph as well as obtained by the separation of molecular weight markers on 7.5% SDS-PAGE were identical.

Table-4.13: Apparent molecular weights and their Rf values for unusual (extra) protein band

Standard Mol. Wt.	Rf values	Log of Mol. Wt	Log of MW of unknown proteins calculated using formula (Y=mx+C)	Converted Log values into normal Mol. Wt. (kD)
29	5.3	1.46	1.47	-
43	4.8	1.63	1.58	-
66	3.4	1.82	1.86	-
97.4	2.4	1.99	2.07	-
205	1.6	2.31	2.23	-
Molecular weights of protein elute bands				
-	4	1.72	1.74	**54.95**
-	3.2	1.88	1.90	**79.43**
-	2.35	2.08	2.08	**120**

4.4: MARKERS FOR INVASION AND METASTASIS

Sialyl Lewis-X and E-cadherin

The presence of SLe^X, carbohydrate epitope on cancer cell surface glycoproteins and alterations in E-cadherin expression would help to predict the invasiveness and metastatic potentials of the tumour cells. Western blot analysis was performed for detection of these parameters in oral cancer as well as OPC tissues.

4.4.1 Expression of Sialyl Lewis-X in malignant/OPC tissues

Figure-4.36 represents the blots of SLe^X for (a) OPC and (b) malignant tissues. Reactivity of anti-SLe^X antibody revealed a protein band of ~200 kD molecular weight. It was interesting to note that there was a marked difference in the pattern of SLe^X expressed by cancer cells as compared to their adjacent normal tissues. The patterns clearly indicate that SLe^X expressions were relatively low in OPC tissues than oral malignant tissues.

Figure-4.36: Representative blots of SLe^X in (A) OPC and (B) Oral cancer patients

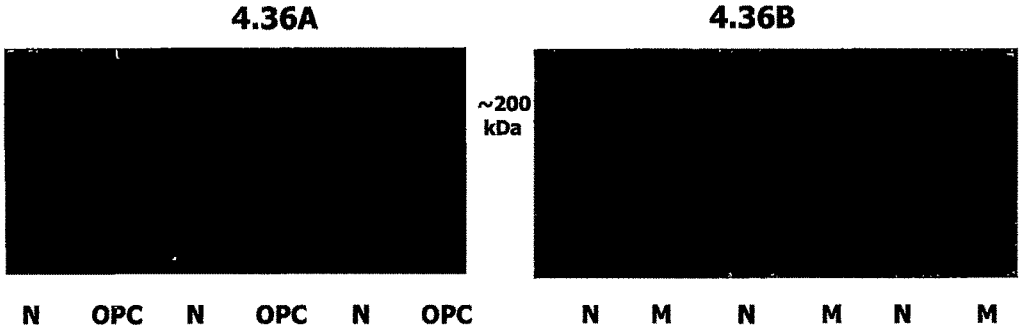


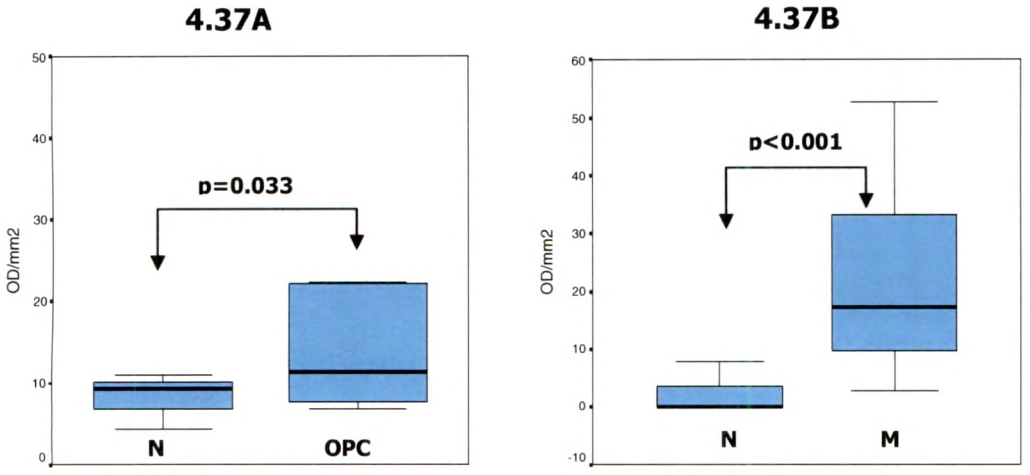
Table-4.14: Comparison of SLe^X band density between malignant/OPC tissues and adjacent normal tissues

	Adjacent normal tissues Mean Density (OD/mm^2)		Malignant/ OPC tissues Mean Density (OD/mm^2)		'p' value
	Mean ±	S.E.M	Mean ±	S.E.M.	
Oral cancer	4.50	2.327	20.05	3.728	**0.001**
OPC	8.21	2.004	16.74	4.59	0.305

The bands obtained were quantified and expressed as density (OD/mm^2). Student's t-test was performed for the comparison of mean density between tumour/OPC tissues and adjacent normal tissues as documented in **table-4.14**. SLe^X expression was significantly higher in malignant tissues as compared to adjacent normal tissues ($p=0.001$). Also, mean band density in OPC tissues was high as compared to their adjacent normal tissues.

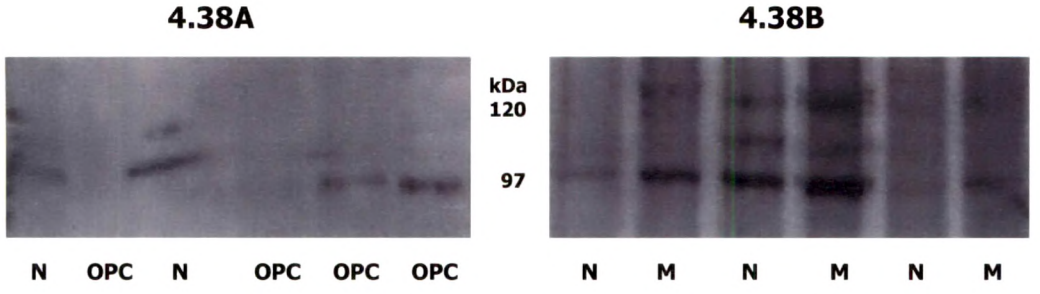
As shown in **Figure-4.37**, significantly higher expression of SLe^X in **(A)** OPC as well as **(B)** malignant tissues was observed as compared to adjacent normal tissue for each pairs as documented by paired 't' test analysis ($p=0.033$ and $p<0.001$, respectively).

Figure-4.37 (A, B): Paired 't'-test analysis for SLe^X in tissues



4.4.2 Expression of E-cadherin in malignant/OPC tissues

Figure-4.38: Representative blots of E-cadherin in (A) OPC and (B) oral cancer patients



The tissue samples showed significant presence of a ~97 kD ($E\text{-}cad^{97}$) protein band reactive with E-cadherin antibody along with 120 KD native E-cadherin ($E\text{-}cad^{120}$). It is clear from **figure-4.38**, that truncated $E\text{-}cad^{97}$ expression was markedly higher as compared to native $E\text{-}cadherin^{120}$ protein in oral malignant tissues. There was significant accumulation of $E\text{-}cad^{97}$ seen in tumour tissues (**4.38B**).

The band density of $E\text{-}cad^{120}$ as well as $E\text{-}cad^{97}$ proteins was quantified for all the tissue specimens and then the ratio of both were also calculated. Results

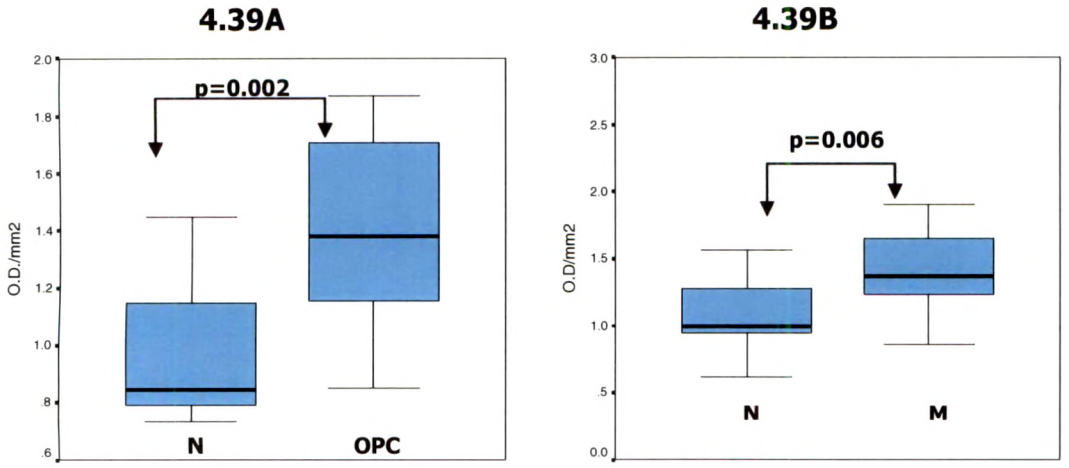
obtained from densitometric analysis and students t-test computation are shown in **Table-4.15**. As documented, mean values of native E-cad^{120} was comparable between malignant/OPC and adjacent normal tissues. While, E-cad^{97} expression was significantly higher in malignant as well as OPC tissues than the adjacent normal tissues (p=0.057, p=0.033, respectively). The ratio of E-cad^{97}:E-cad^{120} was also significantly higher in malignant tissues as compared to adjacent normal tissues (p=0.001).

Table-4.15: Comparison of full-length (120 kD), truncated (97 kD) and (97:120 kD) ratio of E-cadherin between malignant/OPC and adjacent normal tissues

	E-cadherin	Adjacent normal tissues Mean Density (OD/mm^2)		Malignant/OPC tissues Mean Density (OD/mm^2)		'p' value
		Mean ± S.E.M		Mean ± S.E.M		
Oral Cancer	E-cad^{120}	11.83	1.147	11.42	1.232	0.831
	E-cad^{97}	12.56	1.718	18.15	2.241	**0.057**
	E-cad^{97}: E-cad^{120}	1.14	0.095	1.55	0.119	**0.001**
OPC	E-cad^{120}	8.76	1.027	10.39	0.893	0.28
	E-cad^{97}	8.6	1.997	14.5	1.367	**0.033**
	E-cad^{97}: E-cad^{120}	0.99	0.157	1.40	0.156	0.081

Paired 't' test analysis (**Figure-4.39**) also revealed significant higher E-cad^{97}:E-cad^{120} ratio in OPC and malignant tissues as compared to their adjacent normal tissues (p=0.002, and p=0.006, respectively).

Figure-4.39 (A, B): Paired t-test for E-cad^{97}:E-cad^{120} in tissues



4.4.3 Receiver's Operating Characteristic (ROC) curve

Receiver's operating characteristic (ROC) curve is a more meaningful statistical analysis for discrimination between two groups under the study. The analysis simultaneously considers sensitivity and specificity of the parameters. ROC curve analysis revealed that the SLe^X, E-cad^{97} and E-cad^{97}:E-cad^{120} had good discriminatory efficacy between malignant and adjacent normal tissues as documented by their area under the curve (**Figure-4.40**).

Figure-4.40: Receiver's Operating Characteristic (ROC) curve

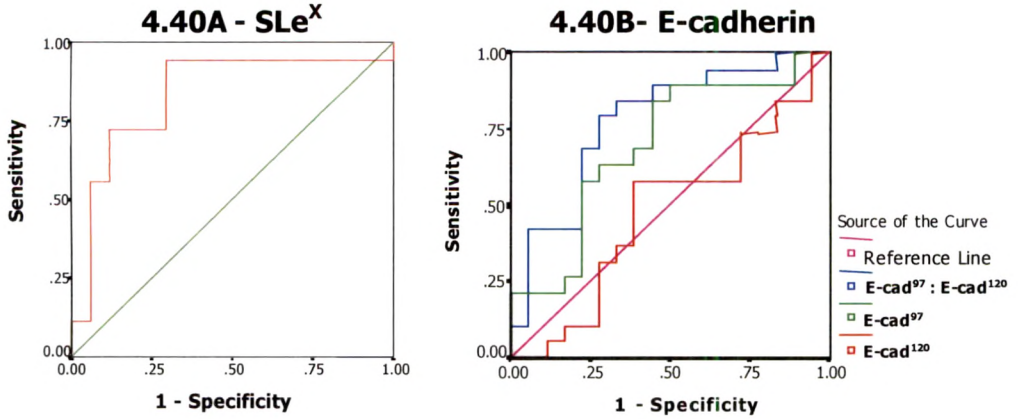


Table-4.16: Statistical data of ROC curve analysis

Parameters	Area under the curve	Significance	95% C. I.	
			Lower	Upper
E-cad^{120}	0.477	0.808	0.285	0.668
E-cad^{97}	0.696	**0.042**	0.522	0.870
E-cad^{97}: E-cad^{120}	0.778	**0.004**	0.624	0.931
SLe^{X}	0.83	**0.001**	0.686	0.981

The area under the curve and significance for SLe^{X}, E-cad^{97} and E-cad^{97}:E-cad^{120} are documented in **table-4.16.** The statistical analysis also revealed high sensitivity and specificity for SLe^{X}, E-cad^{97} and E-cad^{97}:E-cad^{120} expression as markers for discrimination between malignant and adjacent normal tissues in oral cancer patients.

4.4.4 Association of markers with clinicopathological characteristics

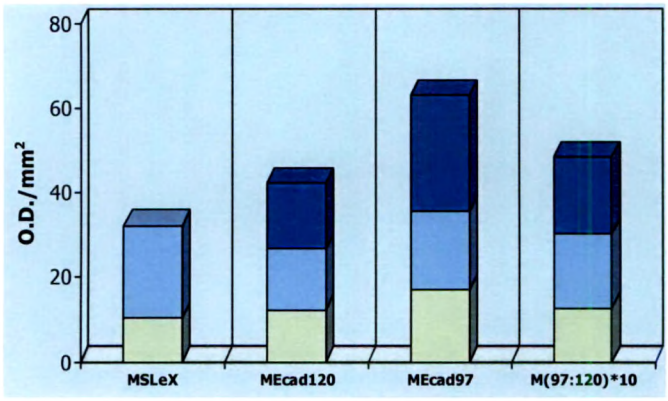
Effect of various clinicopathological parameters on the expressions of SLe^{X} and E-cadherin in malignant tissues were determined. Students 't' test was performed to correlate the association of SLe^{X} and E-cadherin expression with nuclear grade, tumour differentiation, lymphnode involvement and stage of the disease. The values of band density are shown as mean ± S.E.M.

4.4.4.1 Nuclear grade and markers

The mean OD/mm^2 of SLe^{X} and E-Cadherins in nuclear grade (NG) I, II and III is shown in **figure-4.41.**

SLe^{X} expression was significantly higher in malignant tissues with NG II as compared to that in NG I (p=0.047). The expression of E-cad^{97} was significantly increased from nuclear grade I to III (p=0.05) in malignant tissues. Highest expression of E-cad^{97} was seen in NG III. Likewise, ratio of E-cad^{97}:E-cad^{120} was higher in malignant tissues with NG III. E-cad^{120} expression did not show significant variation between NG I, II and III.

Figure-4.41: Comparison of SLe^X, $E\text{-}cad^{120}$, $E\text{-}cad^{97}$ and $E\text{-}cad^{97}$:$E\text{-}cad^{120}$ in oral malignant tissues in patients with nuclear grade I,



$MSLe^X$-SLe^X in malignant tissues, ME-cad^{120}- E-cad^{120}in malignant tissues, ME-cad^{97}- E-cad^{97}in malignant tissues, M(97/120)- E-cad^{97}:E-cad^{120} in malignant tissues

4.4.4.2 Tumour Differentiation and Markers

Figure-4.42: Comparison of SLe^X, $E\text{-}cad^{120}$, $E\text{-}cad^{97}$ and $E\text{-}cad^{97}$:$E\text{-}cad^{120}$ in oral malignant tissues between well, moderate and poorly differentiated tumour

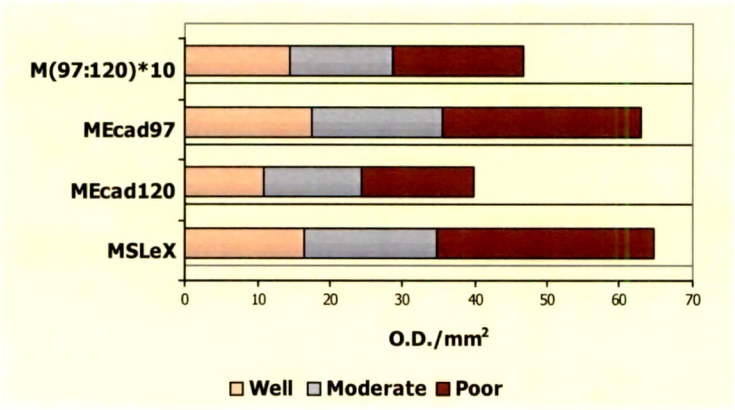


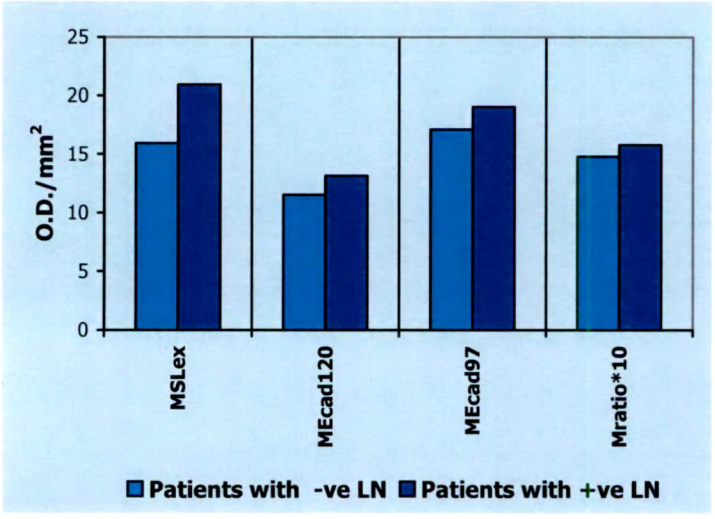
Figure-4.42 represents the comparison of SLe^X and E-cadherins between different grades of tumour differentiation. The poorly differentiated malignant tissues showed significant higher SLe^X expression as compared to well and moderately differentiated tumours (p=0.01 and p=0.028, respectively). $E\text{-}cad^{97}$

expression as well as E-cad^{97}: E-cad^{120} ratio was higher in poorly differentiated tumours than the well (p=0.04 and p=0.015, respectively) and moderately (p=0.036 and p=0.059, respectively) differentiatiated tumours. The E-cad^{120} expression did not reveal significant difference among the groups.

4.4.4.3 Regional Lymphnode Metastasis and Markers

Students 't' test was performed to compare the variations in SLe^X and E-cadherins in oral cancer patients with and without lymphnode (LN) metastasis (Figure-4.43). SLe^X and E-cad^{97} were significantly elevated (p=0.02 and p=0.05, respectively) in malignant tissues with lymphnode metastasis as compared to those without lymphnode metastasis. Malignant tissues showed high E-cad^{97}: E-cad^{120} in patients with lymph node metastasis than without lymphnode metastasis.

Figure-4.43: Comparison of SLe^X, E-cad^{120}, E-cad^{97} and E-cad^{97}: E-cad^{120} expression in oral malignant tissues in patients with and without lymphnode metastasis



-LN= Without Lymphnode metastasis,
+LN= With Lymphnode metastasis

4.4.4.4 Stage of the Disease and Markers

SLe^X and E-cadherins densities were compared in patients with different stages of the disease.

Figure-4.44: Comparison of SLe^X, E-cad^{120}, E-cad^{97} and E-cad^{97}:E-cad^{120} in oral malignant tissues in patients with early and advanced stage of the disease

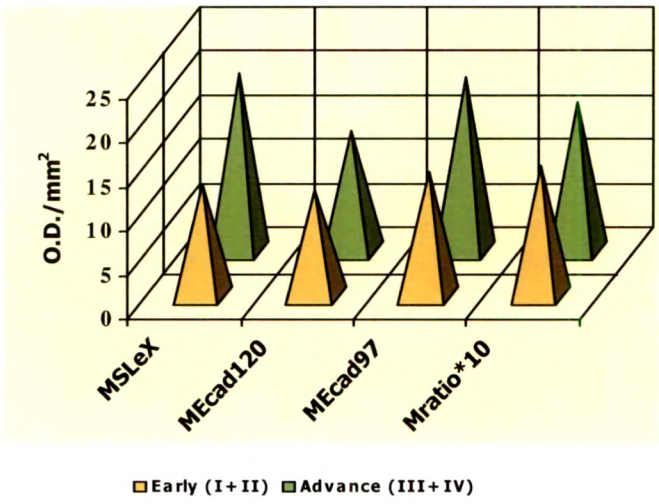


Figure-4.44 represents comparison of the mean SLe^X as well as E-cadherins between early (stage-I and II) and advanced of stages (stage-III and IV) of the disease. As the stage of the disease advanced, E-cad^{97} and E-cad^{97}:E-cad^{120} were increased. Patients with advanced disease showed significantly higher expression of SLe^X, E-cad^{97} and E-cad^{97}:E-cad^{120} as compared to the patients with early disease ($p=0.015$, $p=0.05$ and $p=0.05$, respectively).