Materials & Methods

I have not failed. I've just found 10,000 ways that won't work - Thomas A. Edison

SUBJECTS

The study was approved by Institutional Review Board of the Gujarat Cancer & Research Institute, Ahmedabad, India. Due consent was obtained from all the subjects to participate in the study.

The subjects enrolled for the study consisted of 100 healthy individuals (controls) who had no major illness in the recent past, 50 patients with OPC and 100 histopathologically proven untreated oral cavity cancer patients. The controls were volunteer donors at blood bank of the Institute. The oral cancer patients were enrolled from Outpatient door (OPD) of the Institute. Patients with OPC were collected from Ahmedabad Dental College, Ahmedabad. Out of 50 patients with OPC, 39 patients were with oral sub mucous fibrosis and 11 patients were with oral leukoplakia. Pathological tumor, node and metastasis (TNM) staging of malignant disease was performed as per American Joint Committee on Cancer (AJCC) norms (Green *et al*., 2002). The patients included in the study were followed-up for a period of 45 months after initiation of anticancer therapy. During follow-ups, blood and saliva were collected from 46 post treatment follow-ups. The patient's response to anticancer treatment was assessed on the basis of their clinical and radiological findings during follow-up. Clinical status of the patients during and/or after anticancer treatment was evaluated as suggested by Therasse et al. (Therasse *et al.*, 2000) The follow-up samples were divided into samples from complete responders (CR) (N=38) patients, those who showed good response to anticancer treatment and samples from non-responders (NR) (N=8), the patients with stable progressive disease or with no response to anticancer treatment. The correlation of the molecular markers under the study with overall survival was also analyzed.

The socio-demographic details of the subjects are as mentioned in **Table 4.1**. 84% of oral cancer patients were male. All the patients with OPC and majority (81%) of the oral cancer patients were tobacco habituates. Chewing was the most prevalent habit of tobacco consumption. Various clinico-pathological characteristics including disease site, histopathology, stage, tumor differentiation and lymph node metastasis are as documented in **Table 4.2**. The most prevalent oral cancer site was buccal mucosa

(45%) followed by tongue (21%), with major histopathology as squamous cell carcinoma (97%). Majority of the patients had advanced disease (62%) with moderately differentiated (57%) tumors.

Table 4.1: Socio-demographic details of the subjects

Variables	Controls (N=100)	Patients with OPC (N=50)	Oral Cancer patients (N=100)
Mean age (years)	38.6	36.3	44.4
Age range (years)	19-56	16-65	19-73
Male	92	47	84
Female	08	03	16
Tobacco and / or alcohol habits			
Yes	50	50	88
No	50	00	12
Chewing only	38	31	58
Smoking only	09	05	09
Chewing + Smoking	03	13	09
Chewing + Alcohol drinking	-	-	02
Smoking + Chewing + Alcohol drinking	-	-	03
Smoking + Alcohol drinking	-	-	03
Snuffing	-	01	03
Snuffing + Chewing	-	-	01

COLLECTION AND PROCESSING OF BLOOD AND SALIVA SAMPLES

Fasting blood and saliva (spit) samples were collected between 9.0 to 11.0 a.m. from the subjects to avoid any possible diurnal variations in the study. Blood was collected by veni puncture in serum and heparinized vacuettes. Serum and plasma fractions from plain and heparinized vacuettes, respectively were separated by centrifugation and were stored in different aliquots at -80°C until analyzed. For collection of saliva, subjects were asked to rinse their mouth well with water and then expectorate the water. Further, were asked to spit unstimulated whole saliva into 50 ml falcon tube. The tube was kept on ice and was processed within 30 minutes of collection. For saliva processing, saliva was centrifuged at 2600g for 15 minutes at 4ºC. The supernatant of saliva was collected in different aliquots and protease inhibitors were added (Hu *et al*., 2007). The aliquots were stored at -80°C until analyzed.

Table 4.2: Clinical details of oral cancer patients

Clinical characteristics	**Oral cancer patients (N=100)**
Disease site	
Buccal mucosa	45
Oral tongue	21
Alveolus	08
Others	18
Multiple sites	08
Histopathology	
Squamous cell carcinoma	97
Others	03
Lymph node metastasis	
No	56
Yes	34
Undefined	10

Stage of disease	
I	16
II	16
Early disease (I + II)	32
III	08
IV	54
Advanced disease (III + IV)	62
Undefined	06
Tumor differentiation	
Well	33
Moderate	57
Poor	05
Undefined	05

Tissue sample collection:

Tissue samples from oral cancer patients were collected on ice from operation theater immediately after surgical resection of the tumors. Adjacent normal tissue samples were selected from the tumor free margins at least 2-3 cm away from the tumor as defined by the pathologist. The tissue specimens were washed with ice-cold phosphate buffer saline (PBS: pH 7.4) and were stored at -80°C until analyzed. For RNA analysis, '*RNA later'* (Qiagen, Valencia, CA, USA) was added to the tissues and were stored -80°C until analyzed.

CRITERIA FOR PATIENT SELECTION

Inclusion Criteria:

- Patient should have routine hematological parameters within normal range.
- Patient should be in fasting condition before taking the blood.

- Oral cancer patients should not have undergone surgery, chemotherapy or radiotherapy in past at the time of enrollment.

Exclusion Criteria:

- Patient suffering from diabetes and/or blood pressure or any other major disease in past
- Patient with HIV or HBsAg positive.

REAGENTS, KITS AND INSTRUMENTATION

All reagents and fine chemicals used in the experiments were of analytical grade/extrapure/biotechnology/electrophoresis grade and were procured from SRL (India), Qualigens (India), Merck (India), Sigma (USA) or Fermentas (Canada). RNA isolation (RNeasy tissue mini kit) kits and RT-PCR kits were procured from Qiagen (USA). EGFR ELISA kit of R & D systems (USA) and Sulpho-NHS biotinylation kit of Thermoscientific (USA) were used. ECL western blotting reagent kit, ECL western blotting detection reagent and ECL hyperfilm of GE healthcare (UK), Hybond ECL nitrocellulose membrane of Amersham Biosciences (Germany) were used for western blot analysis. Silver staining kit (for proteins) of Amersham Biosciences (Germany) was used for performing silver staining after SDS-PAGE.

Gel documentation system (Alpha Innotech Inc. USA) was used for gel scanning and densitometric analysis of the gel. Fluorescence Spectrophotometer F4500 (Hitachi, Japan) was used for fluorometric assay. RT-PCR was performed using thermocycler (Eppendorf master cycler gradient, Germany). Systronics 2201 UV-Visible Spectrophotometer (India) was used for spectrophotometric analysis. Apparatus for performing electrophoresis, native PAGE and SDS-PAGE were procured from Bangalore GENEi (India) and Cleaver Scientific Ltd. (Rugby). Western blotting was performed using semidry blotting unit (Biorad, USA). The ELISA plate reader used was from Labsystem Multiscan Spectrum (Champaign).

The methodologies employed for analysis of the parameters are as mentioned in **Table 4.3**

Table 4.3: Methodologies employed for analysis of parameters

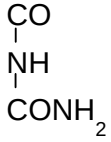
Parameters	Method	Estimation
Total protein (serum)	Biuret	Spectrophotometric
Total protein (Tissues, saliva)	Folin – Lowry's method	Spectrophotometric
Glycoprotein profiling	NATIVE PAGE, glycoprotein staining	Visible Scan, densitometric analysis
Total sialic acid (serum and saliva)	Modified TBA method	Spectrophotometric
Sialidase activity (serum and saliva)	4-MU-NANA substrate	Spectrofluorimetric
α-2,3 and α-2,6 Sialoproteins (serum and saliva)	Linkage Specific Biotinylation (Sulpho-NHS biotinylation kit) Dot blot	Chemiluminescence (capturing on x-ray film)
α-2,3 and α-2,6 Sialyltransferase activity (serum and saliva)	Linkage Specific Biotinylation (Sulpho-NHS biotinylation kit) 96-well plate solid phase assay	ELISA
Fucoproteins (serum and saliva)	Lectin affinity chromatography SDS-PAGE, Silver staining	Visible Scan, densitometric analysis
α-L-Fucosidase activity (serum and saliva)	p-nitrophenyl-α-L-fucopyranoside substrate	Spectrophotometric
ST3GAL1 mRNA	Semi-quantitative Reverse transcriptase (RT) PCR	UV Scan, densitometric analysis
FUT3 mRNA		
FUT5 mRNA		
FUT6 mRNA		
ECAD mRNA		
CJUN mRNA		
MMPs (Plasma and saliva)	Gelatin zymography	Visible scan, densitometric analysis
E-cadherin (tissues)	Western blot	Chemiluminescence capturing on x-ray film
c-Jun (tissues)	Western blot	Chemiluminescence capturing on x-ray film
EGFR (tissues)	ELISA kit method	ELISA

TOTAL PROTEIN ESTIMATION BY BIURET METHOD:

Biuret method was performed for serum protein estimation (Varley *et al*., 1984).

Principle: Substances which contain two $-CONH_2$ groups joined together directly or through a single carbon or nitrogen atom, and those which contain two or more

peptide links, gives a blue to purple colored compound with alkaline copper solutions. One copper atom complexes with four molecules of Biuret, the linkages being central nitrogen atom. The reaction is thus given by proteins.



Biuret complex

Reagents:

- Standard: Bovine serum albumin
- Biuret Reagent: Sodium hydroxide 8.8g, Potassium iodide 5.0 g, Sodium potassium tartarate 9.0 g, Copper sulphate ($CuSO_4.5H_2O$) 3.0 g, was dissolved and made final volume to 1000 ml in DDW.

Procedure: 0.1 ml of serum was mixed with 2.9 ml of DDW and 5.0 ml Biuret reagent. The tubes were vortexed and incubated at 37°C for 10 minutes. Finally the absorbance was read at 540 nm spectrophotometrically.

Calculation:

$$\text{Serum protein g/dL} = \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \text{Amount of standard (1mg)}$$

PROTEIN ESTIMATION BY FOLIN-LOWRY'S METHOD: Lowry's method (Lowry *et al*., 1951) was performed for protein estimation from saliva supernatant and tissue lysates.

Principle: The principle lies in the reactivity of the peptide nitrogen (s) with the copper ions under alkaline conditions and the subsequent reduction of Folin Ciocalteau (phosphomolybdic phosphotungstic acid) to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acid. The Lowry's method is sensitive to low concentration of protein (10µg) of protein. When a Folin reagent (a mixture of Sodium tungstate, Molybdate and Phosphate) together with a Copper sulphate solution is mixed with a protein solution, a blue purple colour is produced which is quantified by its absorbance at 750 nm.

Reagents:

- Reagent A: 2%w/v Sodium carbonate in 0.1 N NaOH
- Reagent B: 1%w/v Sodium potassium tartarate + 0.5%w/v Copper sulphate
- Reagent C: 50 ml of Reagent A + 1 ml of Reagent B
- Folin Ciocalteau Reagent diluted 1:1 with DDW
- Standard: Bovine Serum Albumin

Methodology: 10 µl of saliva supernatant / tissue lysate was mixed with 490 µl of DDW in 10 ml test tubes. All the tubes were vortexed and 2.25 ml of Reagent C was added. The tubes were again mixed properly and incubated at room temperature for 10 minutes. Folin Ciocalteau reagent (1:1 diluted) in final volume of 0.25 ml was added with constant vortexing. All the tubes were incubated in dark at room temperature for 30 minutes. The absorbance was read at 750 nm against reagent blank.

GLYCOPROTEIN PROFILING: Serum glycoprotein profiling was done by performing native PAGE followed by glycoprotein staining.

Reagents:

- 30% w/v Acrylamide-bisacrylamide mixture: 29.18 g Acrylamide, 0.81 g Bisacrylamide. Dissolve and make volume upto 100 ml with DDW
- Running gel buffer pH 8.8: 1.5M Tris base pH 8.8
- Stacking gel buffer: (1 M Tris base pH 6.8)
- 10%w/v Ammonium per sulphate
- 10%w/v SDS
- N N N' N' – TEMED
- Electrode buffer 1X pH 8.3: 25 mM Tris base, 250 mM Glycine
- Sample loading buffer 1X: 50 mM Tris-Cl (pH:6.8), 0.1%w/v Bromophenol blue, 10%v/v Glycerol
- 0.5% w/v Periodic acid in 5%v/v Acetic acid
- 0.5%w/v Sodium arsenite in 5%v/v Acetic acid
- 5%w/v Acetic acid
- 10%w/v Acetic acid
- 0.1% w/v Sodium metabisulphite in 0.01 N HCl

Preparation of Schiff's reagent: 2 g of Basic Fuschin was mixed with prewarmed 400 ml DDW at 60°C and then slowly brought to boiling temperature. After boiling for 3 minutes, it was allowed to cool at 30°C. To this, 8 g Sodium metabisulphite was added and kept overnight in dark in sealed container with stopper. Activated charcoal

was added for complete decolourization. The mixture was vigorously mixed and filtered after 2 hours. Concentrated HCl was added till the filtrate failed to stain the filter paper. The reagent was stored in dark in a sealed container.

Methodology: 1000 µg of serum protein was standardized and separated on 10% native polyacrylamide gel by standardized protocols (Sambrook *et al*., 2001) in non-denaturing and non-reducing conditions at constant voltage (100V) until the dye front reached the bottom of the gels. The gels were separated from the glass plates and kept in fixative consisting of methanol and acetic acid (20:10) for fixation of proteins. This was followed by glycoprotein staining protocol.

Glycoprotein staining (from serum) (Anderson *et al.,* 1974)

For performing Glycoprotein staining, the gels were immersed in 0.5%w/v Periodic acid for 2 hours. The gels were then transferred into 0.5%w/v Sodium arsenite solution prepared in 5%v/v Acetic acid for 1 hour. Further, the gels were transferred and incubated in 0.1%w/v Sodium arsenite solution prepared in 5%v/v Acetic acid for 20 minutes (repeated twice). The gels were kept for fixation in 10%v/v Acetic acid for 30 minutes. After pouring off the acetic acid, Schiff's reagent was added in a tightly sealed box and the gels were dipped completely for 2 hours. Destaining was done with 0.1%w/v Sodium metabisulphite in 0.01 N HCl until the background was clear. The gels were scanned and integrated density value [IDV=∑(each pixel value – background correction was obtained by densitometric analysis.

For molecular weight analysis, native PAGE protein molecular weight standards (Biorad, USA) were run in separate wells along with samples in the same gel and then the gel were cut into 2 portions. The gel portions where the samples were loaded was stained by the glycoprotein staining procedure, and the other half (with the molecular weight marker) was stained with Coomassie Brilliant Blue R-250. Both gel portions were scanned simultaneously to compare the molecular weight of unknown glycoprotein bands.

ELUTION OF ALTERED PROTEIN BAND:

For further characterization of altered protein band, half of the gel was stained with glycoprotein staining and other half was left unstained. The stained gel was aligned

with unstained gel portion and the altered band of interest was excised. The excised band was taken in microcentrifuge tube and was immersed in elution buffer (50 mM Tris-HCl, 150 mM NaCl and 0.1 mM EDTA, pH 7.5). The gel pieces were crushed using a clean pestle and was kept in shaking condition at RT for 6 hours. The tubes were centrifuged at 10,000g for 10 minutes and supernatant was taken into a new microcentrifuge tube. Protein was estimated from the supernatant by Folin-Lowry's method and was subjected to SDS-PAGE (10% gel) (Sambrook *et al*., 2001). The gel was further stained by silver staining protocol (Amersham Biosciences, Germany).

Silver staining (Amersham Biosciences)

Reagents:

Fixing solution:	Methanol	75 ml
	Acetic acid	25 ml
	DDW to 250 ml	
Sensitizing solution:	Methanol	75 ml
	Glutaraldehyde (25%)	1.25 ml
	Sodium thiosulphate (5%)	10 ml
	Sodium acetate	1 pkt (17 g)
	Make upto 250 ml with DDW	
Silver solution:	Silver nitrate (2.5%)	25 ml
	Formaldehyde (37%)	0.1 ml
	Make upto 250 ml with DDW	
Developing solution:	Sodium carbonate	1 pkt (6.25 g)
	Formaldehyde (37%)	0.05 ml
	Make upto 250 ml with DDW	
Stop solution:	$EDTA.Na_2.2H_2O$	1 pkt (3.65 g)
	Make upto 250 ml with DDW	
Washing solution:	DDW	
Preserving solution:	Glycerol (87%)	25 ml
	Make upto 250 ml with DDW	

Silver staining Procedure:

Fixation: 30 minutes	The gel was soaked in fixing solution for 30 minutes
Sensitizing: 30 minutes	The solution was removed. Sensitizing solution was added and left shaking for 30 minutes
Washing: 3 x 5 minutes	The sensitizing solution was removed. DDW was added and washed 3 times for 5 minutes each time
Silver reaction: 20 minutes	Silver solution was added and left shaking for 20 minutes

Washing: 2x 1 minutes	The silver solution was removed. Rinsed twice in DDW for 1 min each time.
Developing: 2-5 minutes	Developing solution was added and left shaking for 2-5 minutes
Stopping: 10 minutes	The developing solution was removed. Stop solution was added and left shaking for 10 minutes
Washing: 3 x 5minutes	The stop solution was removed. DDW was added and washed 3 times for 5 minutes each time

ESTIMATION OF TOTAL SIALIC ACID:

Serum and salivary TSA levels were determined as suggested by Aminoff (Aminoff *et al.*, 1961), duly modified by Skoza and Mohos (Skoza and Mohos *et al.,* 1976).

Principle: The polysaccharide chains of the glycoprotein are hydrolyzed into monosaccharides by boiling with dilute H_2SO_4. The proteins are separated out by precipitation with TCA followed by centrifugation. Periodic acid oxidized –OH (alcoholic) group of N- acetyl neuraminic acid (NANA) to –CHO (Aldehyde) group. Further Sodium arsenite reacts with excess of Periodic acid and liberates iodine and oxygen to stop the reaction. Thiobarbituric acid (TBA) forms a pink coloured complex with – CHO group of NANA. Dimethyl sulphoxide (DMSO) increases the intensity of pink chromophore, which is measured spectrophotometrically at 549 nm. The colour intensity of the chromophore is directly proportional to sialic acid contents.

Reagents:

- 1 N H_2SO_4
- 10%w/v TCA
- 0.025 M Periodic acid in 0.125 N H_2SO_4
- 2%w/v Sodium arsenite in 0.5 N HCl
- 6%w/v Thio Barbituric Acid (TBA): 0.6 g of TBA was weighed in a graduated test tube and 5.0 ml of DDW was added. pH of the solution was adjusted to 10.0 with 10 N Sodium hydroxide and volume was made to 10.0 ml with DDW (pH sensitive, dissolve after adding 10 N NaOH) (check pH with pH strip)
- 0.85%w/v NaCl (Normal saline)
- Std solution: N-Acetyl neuraminic acid (NANA)

Procedure:

1) Acid hydrolysis for the liberation of sialic acid:-

0.1 ml serum/saliva supernatant was mixed with 0.1 ml 1N H_2SO_4 and 0.8 ml normal saline in test tube. The mixture was hydrolyzed at 80° for 60 minutes. Thereafter, 1.0 ml 10% TCA was added to every sample and tubes were centrifuged at 2500 RPM for 10 minutes.

2) *TBA assay for sialic acid:-*

0.1 ml (serum) / 0.5 ml (saliva supernatant) of the hydrolysate was mixed with 0.4 ml DDW. This was followed by addition of 0.25 ml Periodic acid. This mixture was then oxidized at 37° for 30 minutes. The oxidation was terminated by the addition of 0.25 ml Sodium arsenite. Further 0.5 ml TBA was added in test tubes. The reaction mixture was heated in boiling water bath for 7.5 minutes to develop the chromophore. The colour was intensified by the addition of 1.5 ml Dimethylsulfoxide. The absorbance was measured at 549nm. The levels were normalized to total protein and TSA/TP ratio was calculated.

Calculation:

$$\text{TSA (mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of standard}} \times \text{Dilution factor*} \quad (*40 \text{ for serum, } 8 \text{ for saliva})$$

SIALIDASE ACTIVITY: Spectrofluorometric method as suggested by Potier *et al.* (Potier *et al*., 1979) was followed for sialidase assay using 4-methyl umbelliferone N-acetyl neuraminic acid (4-MU-NANA) as substrate.

Principle: Under acidic condition (pH: 4.6) sialidase hydrolyze 4-MU-NANA and release 4-Methylumbelliferone (4-MU), which is determined by spectrofluorimeter using excitation maxima at 365 nm and fluorescence emission at 452 nm.

Reaction: 4-MU---NANA $\xrightarrow{\text{Sialidase}}$ 4-MU + NANA

Reagents:

- 0.1M Sodium acetate buffer (pH: 5.0)
- Substrate for sialidase: 10 µM 4MU-NANA sodium salt
- Standard: 4-Methyl umbelliferone
- 0.133M Glycine buffer (pH: 9.5)

Procedure: 10 µl enzyme source (saliva supernatant/ serum) was mixed with 80 µl of 0.1M sodium acetate buffer (pH 5.0) and 10 µl of 10 µM 4-MU-NANA. The reaction mixture was incubated at 37°C for 1 hour. 900 µl of 0.133 M Glycine buffer (pH 9.5) was added to terminate the reaction. Released fluorescent substrate 4-MU was recorded spectrofluorimetrically using exciting light at 365 nm and fluorescence emission at 450 nm. Standard curve was prepared using 4-MU (Sigma, USA) in concentration range of 5 to 150 nM. One unit of enzyme activity was defined as micromoles of 4-MU released/min/mg protein.

BIOTINYLATION OF LECTINS:

α-2,6 and α-2,3 linkage specific lectins, *Sambucus nigra agglutinin* (SNA) and *Macckia amurensis agglutinin* (MAM) probes were used for the detection of α-2,6 and α-2,3 linked sialic acid, respectively. Biotinylation of SNA and MAM was performed according to procedure of sulpho-NHS Biotinylation kit. Biotin conjugated lectins were used for detection of linkage specific sialoproteins and sialyltransferases activity assay from saliva samples.

Biotin labeling reaction: The vial of Biotin from freezer was removed and equilibrated to room temperature before opening. 2 mg of protein was dissolved in 1 ml of phosphate buffer saline (PBS). BupH^{TM} was prepared as directed on the package label. Immediately before use, 10mM sulpho-NHS-Biotin solution was prepared and appropriate volume was added. The reaction was incubated on ice for two hours for 30 minutes. For HABA assay to determine biotin incorporation, the protein was purified by buffer exchange first.

Buffer exchange and removal of excess biotin reagent using desalting column: A Zeba^{TM} desalt spin column was prepared by breaking off the bottom plug and placing

the column into a 15 ml collection tube. The column was centrifuged at 1000 g for 2 minutes, the storage buffer was discarded and column was returned to the same collection tube. The column was equilibrated by adding 2.5 ml of PBS to the top of the resin bed and centrifuged at 1000 g for 2 minutes. The flow-through was discarded and this step was repeated 2-3 minutes. A column was placed into a new 15 ml collection tube and protein sample was directly applied onto the center of the resin bed. The sample was allowed to absorb into the resin. The column was centrifuged at 1000 g for 2 minutes. The collected flow-through solution was the purified protein sample.

HABA assay for measuring the level of biotin incorporation: To estimate biotin label incorporation, a solution containing the biotinylated protein was added to a mixture of HABA and Avidin. Because of its higher affinity for Avidin, Biotin displaced the HABA from its interaction with Avidin and the absorbance at 500nm decreases proportionately **(Figure 4.1)**. An unknown amount of Biotin present in a solution is estimated in single cuvette by measuring the absorbance of the HABA-Avidin solution before and after addition of the biotin-containing sample. The change in absorbance is related to the amount of biotin in the sample.

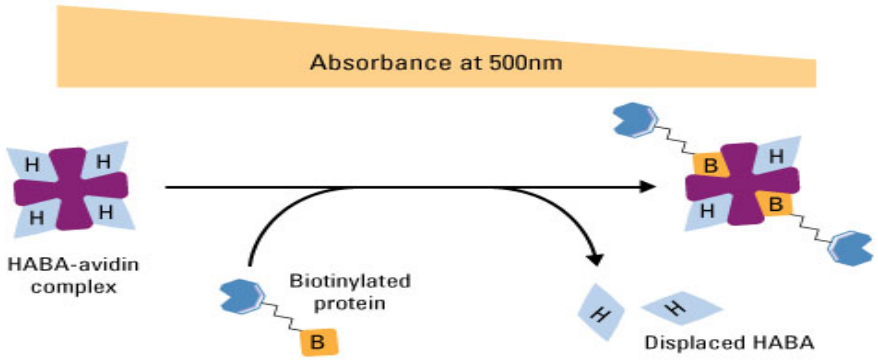


Figure 4.1: Principle of HABA assay. *When a solution containing the biotinylated protein is added to a mixture of HABA and Avidin, due to higher affinity for Avidin, Biotin displaces the HABA from its interaction with Avidin and the absorbance at 500nm decreases proportionately*

Reagents for HABA assay:

- Phosphate buffer saline: 100 mM Sodium phosphate, 150 mM Sodium chloride; pH 7.2
- HABA Avidin solution: Add 1 mg of Avidin and 60 µl of 10 mM HABA to 1.94 ml of PBS. If prepared correctly, the A500 of this solution should be 0.9-1.3 in a 1 cm cuvette.

Procedure for estimating biotin incorporation: 900 µl of HABA-Avidin solution was pipetted into a 1 ml cuvette. The absorbance of the solution in the cuvette at 500 nm was measured and the value as A500 HABA-Avidin was recorded. 100 µl of biotinylated protein sample was added to the cuvette containing HABA-Avidin and mixed well. The absorbance of the solution in the cuvette at 500nm was measured. Once the value remained fairly constant for at least 15 seconds, the values as A500 HABA-Avidin-Biotin sample were recorded. The number of moles of biotin per mole of protein was calculated.

DOT BLOT FOR ESTIMATION OF α-2,6 AND α-2,3 SIALOPROTEINS:

Principle: Dot blot method is based on the immunodetection of sugars using lectins specific for sialic acid linkages. The secondary antibody, Streptavidin-alkaline phosphatase (ALP)-horse radish peroxidase (HRP) reacts with the detection reagent and produces light signal which is captured by exposure to a light sensitive hyperfilm. This is fast and sensitive detection method for sialoproteins.

Reagents:

- Tris buffer saline tween 20 (TBS-T): 100 mM Tris pH 7.5/ 0.1%v/v Tween 20
- Sulpho-NHS-Biotinylation kit
- Biotinylated lectin: SNA (5 µg/ml), MAM (20 µg/ml)
- Secondary antibody: Streptavidin ALP-HRP conjugate (1:1000 dilution)
- ECL western blotting detection reagent kit

Procedure: Saliva supernatant (50 µg) / serum (200 µg) were standardized and spotted onto the membrane and the membrane was allowed to dry for 5 minutes. Thus, proteins were immobilized on the membrane. The membrane was then incubated in blocking solution for 30 minutes followed by 3 rinses with TBS-T. Then

the membrane was incubated overnight with biotinylated linkage specific lectin i.e. SNA-biotin for α-2,6 linkage (5 µg/ml) MAM-biotin for α-2,3 linkage (20 µg/ml). Next day the unbound lectin was removed by washing the membrane thrice with TBS-T for 10 minutes each and further the membrane was incubated with secondary antibody Streptavidin-ALP-HRP conjugate (1:1000) for 2 hours with gentle agitation. The membrane was washed thrice with TBS-T for 10 minutes each to remove unbound secondary antibody and detection was performed using ECL plus detection reagent kit by autoradiography and the image was captured on hyperfilm. The film was scanned and densitometric analysis of sialoprotein dots was done using gel documentation system. The IDV i.e. sum of all the pixel values after background correction were calculated.

ELISA BASED 96-WELL SOLID PHASE ASSAY FOR α-2,6 AND α-2,3 ST ACTIVITY: α-2,6 and α-2,3 ST activities were estimated as described by Hakomori *et al*. (Hakomori *et al*.,1981) and Yeh and cummings (Yeh and cummings, 1996), respectively with minor necessary modifications.

Principle: α-2,6 ST and α-2,3 ST adds N-acetyl neuraminic acid (NANA) from the donor CMP-NANA to an immobilized, desialylated glycoprotein acceptor Asialofetuin. The sialylated product is detected by the binding of sialic acid linkage specific biotin labeled lectins, SNA/MAL. This is followed by probing of secondary antibody Streptavidin-alkaline phosphatase (ALP) and detection by *p*-Nitro phenyl-phosphate, a substrate of ALP **(Figure 4.2)**. The resultant color was read at 410 nm.

Reagents:

- EZ link Sulpho-NHS-Biotinylation kit
- Polystyrene microtiter 96 well plates (Axygen)
- Standard: p-Nitrophenol (PNP)
- Coating buffer: Bicarbonate buffer pH 9.6
- Asialofetuin: Sialic acid acceptor glycoprotein 2mg/ml
- Blocking reagent 2%w/v Bovine serum albumin (BSA), Sigma
- Wash buffer: Phosphate buffer saline, 0.02%w/v Sodium azide, 0.1%v/v Tween 20
- Assay buffer: 50 mM Cacodylate buffer pH 6.5

- Dilution buffer: Phosphate buffer saline, 0.02%w/v Sodium azide, 0.05%v/v Tween 20, 0.1%w/v BSA used for dilution of biotinylated lectins and secondary antibody as follows:

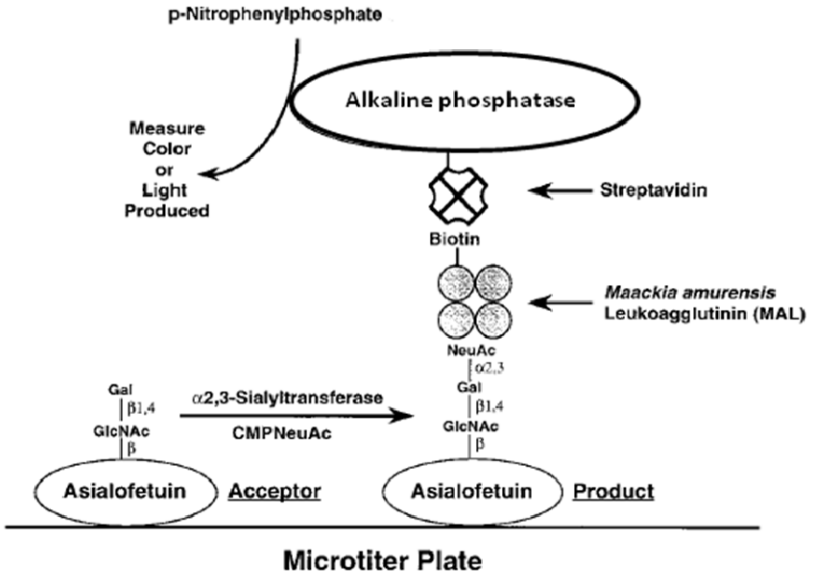


Figure 4.2: ***Principle of Sialyltransferase assay (Yeh et al., 1996)***

- Lectins: SNA – biotin (2 µg/ml)
- Secondary antibody: Streptavidin-ALP (1:500)
- Developing buffer for p-Nitrophenyl-phosphate (PNPP –substrate for ALP): Coating buffer: Bicarbonate buffer pH 9.6 containing 1 mM $MgCl_2$ (Stock: 100 mM)
- 10 mM $MnCl_2$ (stock 200 mM)
- 50 µM Cytidine monophosphate –NANA (CMP-NANA) : Stock 500 µM

Procedure:

- The microtiter plates were coated with 100 µl of desialylated acceptor Asialofetuin (2 mg/ml) diluted in coating buffer (bicarbonate pH 9.6) by overnight incubation at room temperature (RT).
- The wells were then washed three times with wash buffer and then blocked for 2 hours using 2%w/v BSA in wash buffer. After washing the wells, the assay conditions were provided in a final volume of 100µl which contained 50 mM Cacodylated buffer (pH 6.5), 50 µM CMP-N-acetylneuraminic acid (CMP-NeuAc), 10 mM $MnCl_2$ and serum as the enzyme source.

- The reaction mixtures were incubated at 37°C for 3 hours followed by three washes with wash buffer.
- The products of α-2,6 and α-2,3 ST were detected by adding 100 µl of biotinylated lectins SNA (2 µg/ml) or MAM (10 µg/ml), prepared in wash buffer/ 0.1%w/v BSA followed by 1 hour incubation at RT.
- Unbound lectin was removed and washed three times with wash buffer. The bound biotinlated lectins were then incubated with 100 µl of Streptavidin-ALP conjugate (1:500 dilution) for 1 hour at RT.
- The wells were washed with wash buffer followed by DDW and the assay was developed by incubation with freshly prepared Strepatavidin-ALP substrate p-Nitrophenyl phosphate (1mg/ml) [prepared in bicarbonate buffer/ 1 mM $MgCl_2$] at 37°C for 2 hours. The absorption was read at 405 nm using an automated microplate reader (Labsystem Multiscan Spectrum). p-Nitro phenol (PNP) was used as a standard for calibration curve. The enzyme activity (specific activity) was expressed as µmoles of PNP liberated /min/mg protein.

FUCOPROTEINS: For Fucoprotein analysis, lectin affinity chromatography was followed by SDS-PAGE and silver staining (Thomson and Turner, 1987).

Reagents:

- 1 mM Hydrochloric acid
- CNBr activated Sepharose beads
- Fucose specific lectin: *Lotus tetragonolobus* (LTA)
- 0.1 M Bicarbonate buffer (pH 8.3) containing 0.5 M NaCl
- Blocking buffer: 0.2 M Glycine -carbonate buffer (pH 8.0) containing 0.5 M NaCl
- Blocking buffer : 0.1 M Acetate buffer (pH 4.0) containing 0.5 M NaCl
- Wash buffer: 0.05 M Tris buffer (pH 7.4) containing 25 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5%v/v Nonidet P40 (NP-40)
- Elution buffer: 125 mM Tris buffer (pH 6.8) containing 0.35 M SDS.
- 1X sample loading buffer: 50 mM Tris-HCl (pH 6.8), 100 mM Dithiothreitol, 2%w/v SDS, 0.1%w/v Bromophenol blue, 10%v/v Glycerol. Dithiothreitol was added just prior to use.
- Running gel buffer: 1.5 M Tris HCl (pH 8.8)
- Stacking gel buffer: 1 M Tris HCl (pH 6.8)
- Electrode buffer: Tris glycine (pH 8.3)
- 10%w/v SDS

- 10%w/v Ammonium per sulphate
- N, N, N', N' Tetra methylene diamine (TEMED)

Procedure: *Day*-1:

- *Activation of Sepharose beads:* 1 g of CNBR activated Sepharose beads were mixed with 200 ml of 1 mM HCl for 30 minutes at room temperature. The HCl was poured off and this step was repeated twice. The beads were then washed with 0.1 M bicarbonate buffer (pH 8.3).
- *Coupling of lectin [Lotus tetragonolobus-(LTA)] with Sepharose 5.0 mg of lectin:* LTA was dissolved in 1ml of Bicarbonate buffer. The Sepharose beads prepared as above were mixed with lectin and incubated overnight at 4°C on a shaker.

Day -2:

- *Blocking of remaining proteins:* The supernatant was removed and the coupled beads were suspended in 0.2 M Glycine carbonate buffer (pH 8.0) and kept for 2 hours at room temperature with gentle shaking.
- *Removal of excessive proteins:* Alternate washing of coupled beads with Bicarbonate (pH 8.3) and Acetate buffer (pH 4.0) was done to remove excess proteins
- *Binding of serum proteins with lectin sepharose beads:* The coupled lectin sepharose beads were washed three times with Tris buffer (pH 7.4) and equal amount of proteins from saliva supernatant (500 μg) and serum (7 mg) was mixed with equal volume of coupled beads and incubated for 2 hours at 4°C with gentle shaking
- *Removal of unbound fucoproteins:* The unbound serum proteins were removed by 5-6 washes with Tris buffer (pH 7.4)
- *Elution of bound fucoproteins:* The bound fucoproteins were released in 50 μl elution buffer
- *Electrophoresis:* Eluted fucoproteins equal volume of 15 μl serum / 25 μl saliva supernatant were electrophoresed on 8.0 % SDS PAGE using standard protocols (Sambrook *et al.,*2001). Fucoproteins were visualized using silver

staining method (Amersham Biosciences, Germany) by procedure as described earlier.

ESTIMATION OF α-L-FUCOSIDASE ACTIVITY: α-L fucosidase activity was estimated using p-Nitrophenyl fucopyranoside as substrate (Wiederschain *et al*., 1971).

Principle: Under acidic condition (pH: 5.5) fucosidase hydrolyses substrate PNP-α-L-fucopyranoside and release p-Nitrophenol (PNP). Glycine-NaOH (pH: 10.5) is added after termination of reaction and PNP forms yellow colour under alkaline condition, which is measured at 410 nm spectrophotometrically.

Reagents:

- 10 mM PNP α-L-fucopyranoside
- 0.4 M Glycine – NaOH buffer
- 2.5%w/v Zinc sulphate
- 0.15 M NaOH
- Acetate Buffer pH 5.5 (0.05 M)
- p-Nitro phenol (standard)

Procedure: 20 µL enzyme source (serum/saliva supernatant) was mixed with 205 µL of 0.05 M acetate buffer (pH: 5.5) and 25 µL of 10 mM PNP α-L-fucopyranoside (substrate). The reaction mixture was incubated at 37° for 1 hour. To this, 100 µL of 2.5%w/v $ZnSO_4$ and 100 µL of 0.15 M NaOH were added to terminate the reaction. After centrifugation for 5 minutes, 300 µL of supernatant was transferred to a fresh tube and mixed with 300 µL of 0.4 M Glycine-NaOH buffer (pH:10.5). Absorbance of liberated PNP was measured at 410 nm spectrophotometrically.

SEMIQUANTITATIVE RT-PCR:

Principle: Reverse Transcription Polymerase Chain Reaction (RT-PCR) is highly specific and sensitive technique which provides the possibility to assess gene transcription in the cells or tissues. In RT-PCR, cDNA is synthesized from RNA and gene expression is analyzed through PCR enzymatic amplification of a specific cDNA sequence *in vitro*. This process uses multiple cycles of template denaturation, primer

annealing and primer elongation to amplify cDNA sequence. It is an exponential process since the amplified products from each previous cycles serve as templates for the next cycles of amplification, thus making it a highly sensitive techniques for the detection of specific nucleic acid sequences, Enough amplified products is generated after 20-40 cycles of PCR, so that it can be visualized on an Ethidium bromide stained gel, RT-PCR is used to detect or quantify the expression of mRNA, often from a small concentration of target RNA.

Steps:

1. Isolation of RNA from tissue samples
2. Spectrophotometric quantification of RNA
3. One step RT-PCR
4. Agarose gel electrophoresis of amplified products

Isolation of RNA from tissue sample: RNA was isolated from malignant and adjacent normal tissues of oral cancer patients using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction and stored at -80^{0}C. RNA was suspended in RNase free water and stored at -80 ºC until use.

Principle: RNA isolation technique combines the selective binding properties of a silica based membrane with the speed of micro spin technology. A specialized high salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing Thiocyanate containing buffer, which immediately inactivates RNAases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions and the samples is then applied to an RNeasy mini spin column, where contaminant are efficiently washed away. High quality RNA is then eluted in 30-100 µl RNAase free water.

Procedure: Protocol as per instructions provided in the kit was followed:

All steps were performed at room temperature. All the reagents were prepared in DEPC treated distilled water and all plastic wares and glasswares were washed with DEPC treated H_2O and autoclaved. The amount of tissue (not more than 30 mg (25-30 mg) were weighed; it was followed by disruption and homogenization of tissue using

mortar and pestle with liquid nitrogen). 600 µl of RLT Buffer (supplied in kit) was added and centrifuged for 5 minutes at 10000 rpm. The supernatant was transferred to a micro centrifuge tube. 600 µl of 70%v/v of ethanol was added to the clean lysate and mixed immediately. 700 µl of the sample was added, including any precipitation to a RNeasy mini Column placed in 2 ml of collection tube. The tube was closed gently and centrifuged for 15 second at 10,000 rpm. The flow through was discarded. The collection tube was reused for further step.700 µl of buffer RW was added to RNeasy column. The tube was closed gently and centrifuged for 15 second at 10,000 rpm to wash the column. The flow through was discarded. The RNeasy column was transferred into a 2 ml collection tube. 500 µl buffer RPE was added onto RNeasy column, centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded. 500 µl buffer RPE was added to RNeasy column and centrifuged for 2 minutes at 10,000 rpm. The flow through was discarded and the collection tube was reused for next step. The RNeasy column was transferred into a new 2 ml collection tube. The RNeasy column was placed in new 2.0 ml collection tube and centrifuged for 1 minute at 10,000 rpm, to dry the silica gel membrane. To elute, RNeasy column was transferred to new 1.5 ml collection tube. 50 µl of RNase free water was added and centrifuged for 1 minute at 10,000 rpm. This step was repeated once more. The RNeasy column was separated and the collection tube that contains isolated RNA was stored at – 80ºC**.**

Spectrophotometric quantitation of RNA: The RNA concentration was determined by measuring the absorbance at 260 nm (A 260) to check the quality and quantity of RNA samples. An absorbance of 1 unit of 260 nm corresponds to 40 µg of RNA per ml. The ratio between the absorbance values at 260 nm and 280 nm gave an estimate of RNA purity (pure RNA has an A 260/ A 280 ratio of 1.9 – 2.1).

Calculations:

1 unit of 260 nm corresponds to 40 µg of RNA per ml

Concentration of RNA sample = 40 µg/ml × A 260 × dilution factor (100)

Semi-quantitative RT-PCR:

Semi-quantitative RT-PCR for transcripts *ST3GAL1, FUT3*, *FUT5, FUT6, ECAD* and *CJUN* was carried out using specific primers with sequences as depicted in **Table 4.4**. *β-ACTIN* was used as internal control in all the reactions and RT-PCR was carried out using one-step RT-PCR kit (Qiagen, Valencia, CA, USA).

Table 4.4: Primer sequence and amplicon size of genes

Genes	Primer sequence	Amplicon size
ST3GAL1	F5'-ATGAGGTGGACTTGTACGGC-3' R5'-AACGGCTCCAGCAAGATG- 3'	253 bp
FUT6	F5'-CTCAAGACGATCCCACTGTGTAC-3' R5'-CAGCCAGCCGTAGGGCGTGAAGATGTCGGA-3'	404 bp
FUT3 *FUT5*	F5'-CTGCTGGTGGCTGTGTGTTTCTTCTCCTAC-3' R5'-CAGCCAGCCGTAGGGCGTGAAGATGTCGGA-3'	447 bp 486 bp
β-ACTIN	F5'-GGTCACCCACACTGTGCCCAT-3' R5'-GGATGCCACAGGACTCCATGC-3'	350 bp
ECAD	F5'-GTA ACC GAT CAG AAT GAC AA-3' R5'- CGT GGT GGG ATT GAA GAT C-3'	420 bp
CJUN	F5'- AAG TAA GAG TGC GGG AGG CA- 3' F5'- GGG CAT CGT CAT AGA AGG TCG -3'	409 bp

The amplifications were performed using Thermal Cycler. Reactions contained 500 ng of RNA, 0.6 μM of primers for the target genes and 0.3 μM of primers for the house-keeping gene (*β-ACTIN*) in 50 μl RT-PCR reaction volume. The reaction conditions are shown in **Table 4.5**.

The reaction products were electrophoresed on 1.5% agarose gels containing ethidium bromide and gels were analyzed densitometrically using gel documentation system.

For semiquantitative analysis of *ST3GAL1, FUT3*, *FUT5*, *FUT6, ECAD and CJUN* transcripts, the IDV of each sample were compared with the IDV of *β-ACTIN* coamplified in the same tube and relative expression (IDV of the transcripts/ IDV of *β-ACTIN*) was measured.

Table 4.5: Reaction conditions for RT-PCR analysis

	Reverse transcription at 50°C for 30 minutes, initial PCR activation at 95°C for 15 minutes				
	Cycling conditions				
Genes	Denaturation	Annealing	Extension	No. of Cycles	Final extension
ST3GAL1	94°C for 1 min.	55°C for 1 min.	72°C for 1 min.	35	72°C for 10 min.
FUT3 and *FUT5*	94°C for 1 min.	68°C for 30 seconds	72°C for 1 min.	30	72°C for 10 min.
FUT6	94°C for 1 min.	58°C for 1 min.	72°C for 1 min.	35	72°C for 10 min.
CJUN	94°C for 1 min.	63°C for 1 min.	72°C for 1 min.	30	72°C for 10 min.
ECAD	94°C for 1 min.	63°C for 1 min.	72°C for 1 min.	30	72°C for 10 min.

AGAROSE GEL ELECTROPHORESIS

Principle: Agarose gel electrophoresis is one of the methods used to separate DNA/RNA by size. This is achieved by migration of negatively charged nucleic acid molecules on an agarose matrix with an electric field (electrophoresis) towards the positively charged cathode. Shorter molecules moves faster and migrate further than longer ones.

Reagents:

- 1.5%w/v Agarose in 1X Tris Borate EDTA (TBE)
- 1X TBE buffer (pH 8.0)
- Loading dye: Bromophenol blue
- Ethidium bromide (10 mg/ml), (Final concentration 0.5 µg/ml)

Preparation of agarose gel: A pre-weighed Agarose powder was added to 1x TBE buffer. For the experiment a 1.5 w/v% Agarose in 1x TBE was prepared in a flask. The flask was covered and the mixture was swirled. The mixture was heated in a microwave to boil. Heating was continued until a clear solution was obtained. It was allowed to cool to 50-60°C at room temperature. The ends of a gel tray were sealed

(taped) to prevent leakage of agarose gel from the tray. 10 µl of Ethidium bromide solution was added to the flask when temperature achieved was 50-60ºC. The comb was placed onto the tray and the agarose gel solution (50-60°C) was poured into the tray. The gel was allowed to solidify (about 45 minutes). The tape of the tray was removed and the tank was filled with 1x TBE electrophoresis buffer. The tray was placed in the electrophoresis tank, the comb was carefully removed. RNA samples were mixed with a loading dye containing marker dye Bromophenol blue and Glycerol to increase the density of the solution. 10.0 µl of samples were loaded into wells of the gel along with DNA ladder in one lane. It was allowed to run at 100V until dye front reached the bottom.

GELATIN ZYMOGRAPHY: Latent and active MMP-2 and MMP-9 were studied by performing gelatin zymography (Lorenzo *et al*., 1992).

Principle: Zymography involves electrophoretic separation of proteins from the sample under denaturing conditions, but non reducing condition through a polyacrylamide gel containing gelatin as a substrate. After electrophoresis, denatured proteins are renatured by exchange of SDS with non-ionic detergent (Triton X-100). After this, the gel is incubated in an appropriate activation buffer for particular Proteinase expression under study. During this incubation, the concentration renatured MMPs in gel digest the substrate. The gel is stained with CBB proteolytic activities are detected as clear band against a blue background. The area of digestion appears as clean bands against a darkly stained background where the substrate has been degraded by the enzyme.

Reagents:

- 30%w/v Acrylamide: 29.19% w/v Acrylamide, 0.81% w/v Bisacrylamide in DDW. Store in brown bottle at 4ºC
- Running Gel Buffer: 1.5 M Tris Cl (pH 8.8), 0.4%w/v SDS.
- Stacking gel Buffer: 1 M Tris Cl (pH 6.8), 0.8%w/v SDS.
- Gelatin Solution: 5 mg/ml (0.5%w/v) prepare fresh in DDW
- 10%w/v Ammonium per sulfate
- TEMED
- 2x Sample Buffer: 0.125 M Tris Cl (pH: 6.8), 0.004%w/v BPB, 20%v/v Glycerol, 4%w/v SDS

- 1X Electrode buffer/ Running Buffer (pH: 8.3): 0.025 M Tris base, 0.192 M of Glycine, adjust the pH 8.3, 0.2%w/v SDS, Store at 4ºC
- Activation/ Incubation Buffer: 50 mM Tris Cl (pH 7.5), 10 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02%w/v NaN_3, 1%v/v Triton X-100
- Washing solution: 0.25%v/v Triton X-100 in DDW
- Staining Solution: 0.1%w/v Coomasie Brilliant Blue R-250 in 40%v/v Propanol
- Destaining solution: 7%v/v Acetic acid

Processing of plasma samples: Plasma sample equivalent to 0.1 ml was mixed with 0.9 ml of chilled DDW. To this 40 µl of 1%v/v Acetic acid was added and incubated at 0°C for 1 hour. Further the solution was centrifuged at 5000 RPM for 10 minutes in cooling centrifuge. The supernatant was discarded. Further the pellet (euglobulin fraction) was dissolved in 0.1 ml phosphate buffer saline. Protein estimation of the dissolved fraction was estimated using Folin Lowry's Method (Lowry *et al.*, 1951).

Procedure: Zymography was performed using SDS-PAGE (containing 0.5 mg/ml Gelatin) electrophoresis as described by Lorenzo *et al*. (Lorenzo *et al*., 1992). 10 µg saliva supernatant was standardized and mixed with sample buffer dye without reducing agent and electrophoresed on 7.5% polyacrylamide gel at 4°C. For zymography from plasma, plasma euglobulin fraction equivalent to 100 µg was mixed with sample buffer and loaded on 7.5% polyacrylamide gel and were electrophoresed at 120V until dye front reaches the bottom of gel. The gels were then washed with washing solution for 30 minutes. Further the gels were incubated overnight in activation buffer containing 50 mM Tris HCl pH 7.5; containing 10 mM $CaCl_2$, 1 uM $ZnCl_2$, 0.02%w/v NaN_3 and 1%v/v Triton X-100. Next day, the gels were stained with 0.1% w/v Coomassie Brilliant Blue R-250 and destained in 7%v/v Acetic acid. These zymograms were quantitated using gel documentation system. The IDV was determined for each proteinase activity. The reproducibility of the samples was checked by running the samples in same gels as well as in different gels. Standards of pro MMP-9 (92 kDa) and active MMP-9 (83 kDa) (Calbiochem) and molecular weight markers were run for molecular weight analysis.

WESTERN BLOTTING:

Principle: (1) The proteins are separated on SDS-PAGE under denaturing and reducing conditions and transferred onto the hybond nitrocellulose membrane. The immobilized proteins are then probed with primary antibody specific for the sample protein followed by secondary antibody specific for general class of primary antibodies e.g. goat anti-mouse antibody conjugated with peroxidase. The detection of the proteins is done with highly sensitive chemiluminescence method.

(2) Detection of proteins using ECL western blotting chemiluminescence detection kit: The secondary antibody is tagged with HRP (Horse radish peroxidase) enzyme. HRP/ hydrogen peroxide catalyzes oxidation of luminol in alkaline conditions. Enhanced chemiluminescence is achieved by performing the oxidation of luminol by HRP in the presence of chemical enhancers. This has effect on increasing the light output, which is then captured on light sensitive hyperfilms **(Figure 4.3)**.

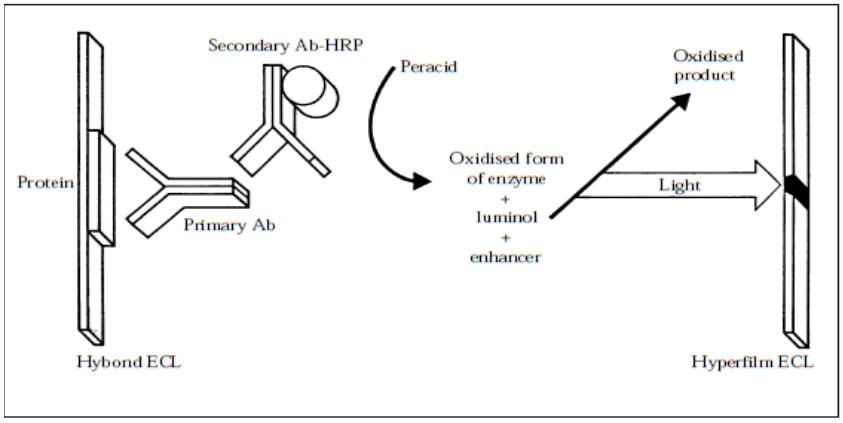


Figure 4.3: ***Principle of Western blot***

Reagents and requirements:

- 30%w/v Acrylamide-bisacrylamide mixture (stock): 29.18g Acrylamide, 0.81g Bisacrylamide. Dissolve and make volume upto 100 ml with DDW
- Running gel buffer pH 8.8 (1.5 M Tris HCl pH 8.8, SDS 0.4%w/v)
- Stacking gel buffer (1 M Tris HCl pH 6.8)
- 10%w/v Ammonium per sulphate
- 10%w/v SDS
- TEMED
- Electrode buffer 5X pH 8.3: 25 mM Tris base, 250 mM Glycine, 0.1%w/v SDS

- 6X sample loading buffer: 300 mM Tris HCl (pH 6.8), 600 mM Dithithreitol, 12%w/v SDS, 0.6%w/v Bromophenol blue, 60% glycerol
- Blotting (Transfer) buffer: Towbins buffer (pH 8.2-8.4) containing 25 mM Tris base, 192 mM Glycine and 20%v/v Methanol
- Ponceau S stain
- Blot washing buffer (TBST): 100 mM Tris pH 7.5/ 0.1%v/v Tween 20
- Mouse monoclonal Anti E-cadherin and c-Jun antibody
- ECL prime western blotting kit containing 5%w/v blocking reagent prepared in TBST, HRP conjugated 2° antibody in appropriate dilution. Mix detection reagent A and B in 1:1 ratio containing chemiluminescence substrate for HRP.
- Lysis buffer: 0.05 M Tris pH 7.5 containing 2 mM EDTA , 5 mM Sodium fluoride, 5 mM EDTA, 150 mM Sodium chloride , 1.0%v/v NP-40, 1 mM PMSF, 10 mM Aprotinin, 1 mM Sodium orthovanadate, 0.3 mM Leupeptin.

Tissue lysate preparation: 50 mg of tissue was weighed and taken in an microcentrifuge tube. The tube was immediately kept on ice. For 50 mg tissue, around 400 µl of lysis buffer was added and the tissues were crushed gently until complete breakdown of tissue. Along with tissue pieces, the lysate was taken in an microcentrifuge tube and centrifuged for 20 minutes at 12000 rpm at 4°C. The lysate supernatant was taken in another fresh microcentrifuge tube and its total protein content was estimated by **Folin-Lowry's method**.

Tissue proteins separation on SDS-PAGE: The running gel of 12% and 10% were respectively prepared for c-Jun and E-cadherin protein. The tissue lysates equivalent to 100 µg proteins were mixed with 6X sample loading buffer, and then denatured by boiling for 3 minutes. The samples were kept at room temperature for 5 minutes and then loaded. The proteins were separated on SDS-PAGE under denaturing and reducing conditions at constant voltage (100 V) until the dye front reached the bottom of the gel.

Electro-transfer of proteins from gel to membrane using semi dry method: Biorad Trans-Blot semi-dry transfer unit was used for electro-transfer of proteins from gel to membrane as follows. The gel and nitrocellulose membrane were equilibrated in Towbin's buffer for 5-10 minutes. Stack consisting of filter paper, nitrocellulose membrane and gel, was prepared as described in **Figure 4.4**. Electrotransfer was done at 300 mA constant current for 3 hours for E-cadherin and 350 mA for 3 hours for c-Jun protein. The membrane transfer of proteins was checked by staining the

membrane with Ponceau S dye. The membrane was subsequently washed with TBS-T thrice for 5 minutes each and subsequently membrane was processed for immunodetection.

Immunodetection: Membrane was incubated in 5%w/v (in TBS-T) blocking reagent for at least 1 hour at room temperature, which was followed by 3 washes with TBS-T (1x15 minutes and 2x5 minutes). Then the membrane was incubated with primary antibody i.e. anti E-cadherin antibody (1:300 dilution) and anti-c-Jun antibody (1:300 dilution) overnight at room temperature with gentle shaking. The membrane was rinsed thrice with TBS-T (1x15 minutes and 2x5 min) and then incubated with appropriate HRP conjugated anti-mouse secondary antibody (1:1000 dilution in TBS-T) for 2 hours at room temperature with gentle shaking on rocker. This was followed by three washes with TBS-T (1 x 15 minutes and 2x5min). The membrane was further processed for detection by ECL chemiluminescence detection kit.

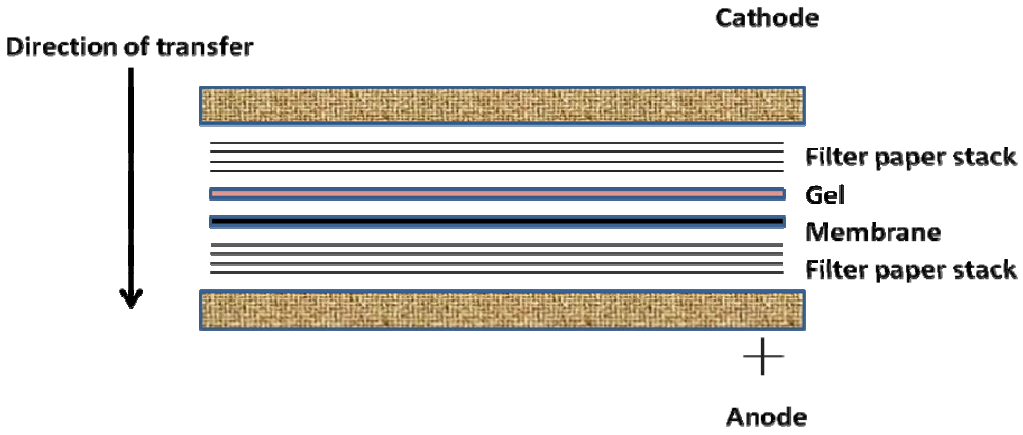


Figure 4.4*: Electro-transfer of proteins from gel to membrane using semi dry method*

The luminescensce was captured on light sensitive hyperfilm and bands were visualized on hyperfilm by developing after 1-5 minutes exposure. The film was then scanned and densitometric analysis of protein band was performed using gel documentation system and IDV were noted.

ESTIMATION OF PHOSPHO-EGFR: Phospho-EGFR expression was estimated using DuoSet IC human phospho-EGFR kit (R& D systems, USA).

Principle: The kit measures tyrosine-phosphorylated epidermal growth factor receptor (phospho-EGFR) in cell lysates. An immobilized capture antibody specific for the human EGFR binds both phosphorylated and unphoshorylated EGFR. After washing away unbound material, an HRP-conjugated monoclonal antibody specific for phosphorylated tyrosine is used to detect only phosphorylated protein, utilizing a a standard HRP format.

Reagents:

- Phosphate buffer saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4
- Wash buffer: 0.05%v/v Tween 20 in PBS, pH 7.2-7.4
- Block buffer: 1%w/v BSA, 0.05%w/v NaN_3 in PBS, pH 7.2-7.4
- IC Diluent 12: 1%v/v NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10%v/v glycerol, 2 mM EDTA, 1 mM activated Sodium orthovanadate
- IC Diluent 14: 20 mM Tris, 137 mM NaCl, 0.05%v/v Tween 20, 0.1%w/v BSA, pH 7.2-7.4
- Substrate solution: 1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine)
- Stop solution: 2 N H_2SO_4
- Human phospho-EGFR capture antibody: Each vial contains 1440 µg/ml of rat anti-human EGFR antibody when reconstituted with 200 µl of PBS.
- Anti-phospho tyrosine HRP: Dilute the anti-phospho tyrosine HRP to the working concentration specified on vial label using IC Diluent 14 (to be prepared freshly). Each vial contains 50 µl mouse anti phosph-tyrosine antibody conjugated to HRP
- Human phospho-EGFR control: Each vial contains 90 ng/ml of recombinant human phosphorylated EGFR when reconstituted with 500 µl of IC Diluent.

Plate preparation: The capture antibody was diluted to a working concentration of 8.0 µg/ml in PBS, without carrier protein. A 96 well microplate was immediately coated with 100µl per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature. Each well was aspirated and washed with wash buffer, repeating the process two times for a total of 3 washes. Each well was washed by filling with wash buffer (400 µl). After the last wash, any remaining wash buffer was removed by aspirating or by inverting the plate and blotting it against clean tissue paper. Blocking of plates was performed by addition of 300 µl of block buffer

to each well. The plate was then incubated at room temperature for 1-2 hours. The aspiration/wash was repeated as in step 2. The plates were then ready for sample addition.

Assay procedure:

- 100 µl of sample or control in IC Diluent 12 was added per well. IC Diluent 12 was used as blank. The plate was covered with a plate sealer and incubated for 2 hours at room temperature.
- The aspiration/wash was repeated as in Step 2 of plate preparation. Immediately before use, the Anti-phospho-Tyrosine-HRP was diluted to the working concentration specified on the vial label using IC Diluent 14. 100 µl of the diluted Anti-phospho-Tyrosine-HRP was added to each well. The plate was covered with a new plate sealer and incubated for 2 hours at room temperature. The aspiration/wash was repeated as in step 2 of plate preparation. 100 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature in dark.
- 50 µl of stop solution was added to each well. The plate was gently tapped to ensure thorough mixing. The optical density of each well was read immediately, using a microplate reader, set to 450 nm and 540 nm. The readings at 540 nm were subtracted from the readings at 450 nm. The final readings were subtracted from the blank optical density after wavelength correction at 540 nm. The standard curve was prepared using phospho EGFR control using concentration range of 10 ng/ml to 40 ng/ml.

STATISTICAL ANALYSIS: Statistical analysis of the data was performed using statistical package for social science (SPSS) software version 17.0.

- Student's paired '*t*' test was used to compare the levels between adjacent normal and malignant tissues of the oral cancer patients.
- Student's independent '*t*' test was performed to assess the levels of significance of markers with various clinico-pathological parameters.
- Pearson's correlation analysis was performed to assess the correlation between various markers.
- Receiver's operating characteristic's (ROC) curves were constructed to analyze the distinguishing capability of the markers and also to obtain optimal cut-off point for survival analysis.
- Kaplan-Meier's survival analysis was used to analyze correlation of the markers with overall survival and significance of differences in survival rates was analyzed by Log rank test.
- Multivariate analysis was performed to correlate the markers with various clinico-pathological parameters.
- The values were expressed as the Mean ± Standard Error of Mean (SEM). 'p' values less than 0.05 was considered to be statistically significant.
- The reproducibility of the samples was checked by running the samples in the same batch as well as in different batches.