Materials & Methods

First, have a definite, clear practical idea; a goal, an objective. Second, have the necessary means to achieve your ends; wisdom, materials and methods. Third, adjust all your means to that end."

- Aristotle

3. MATERIALS AND METHODS

3.1: SUBJECTS

Due consent to participate in the study was obtained from all the subjects included in following three groups:

1. **Controls:** One hundred healthy individuals without any major illness in recent past were included in the study.
2. **Pathological Controls:** To assess the specificity of markers, 75 patients with OPC were included in the study. The patients with OPC included 50 patients with oral submucous fibrosis (OSMF), 23 patients with oral leukoplakia (OLP), one patient with dysplasia and one patient with acanthosis.
3. **Oral Cancer Patients:** One hundred and thirty histopathologically proven untreated patients with oral squamous cell carcinoma were enrolled from out patients' department of The Gujarat Cancer & Research Institute, Civil Hospital, Ahmedabad.

Table-3.1: Proforma used to gather details of the subjects

Name:	**Registration No:**	**Sr. No.:**	**Age:**
Sex: M/F	**Height:**	**Weight:**	
Religion:	**Education:**	**Occupation:**	
Household income:	**Family history of cancer:**	**Any other major illness:**	
Diagnosis and other clinical details:			
Drug/Nutrient supplementation:		**Cleansing habits:**	
Habits	**Tobacco consumption: Smoking/chewing/others** **Alcohol consumption: Others:** **(Frequency/Type/Brand/Duration/Comments)**		
Controls OPC Cancer patients **Reports from GCRI:**			
Outside Reports:			
Histopathological Examination:			
Follow-up History:			

All the subjects were interviewed using specified proforma (**Table-3.1**). The details regarding their age, height, weight, occupation, area of residence, income, education, family history of cancer etc. were gathered from the subjects. The subjects were inquired for the detailed history of their tobacco and alcohol consumption habits. Clinical and histopathological details of the patients were also recorded. The patients were followed after initiation of anticancer treatment.

The analysis of the details gathered from the subjects is summarized in **table-3.2**. It revealed following noteworthy observations: (i) OPC were commonly seen in younger age group, whereas, oral cancer was common in elder age group population. (ii) OPC as well as oral cancer was more prevalent in males as compared to females. (iii) More than 97% of the patients were tobacco habitués. (iv) Habit of tobacco chewing in the form of gutkha, pan masala etc. was commonly seen in male patients, whereas, snuffing habit was seen in female patients.

Table-3.2: Details of Controls and Patients

	Controls (n=100)	**Patients with OPC (n=75)**	**Cancer Patients (n=130)**
Age			
Median (years)	33	28.5	42
Range (years)	20-71	14-80	18-77
Gender			
Males	88%	92%	94%
Females	12%	08%	06%
Male:Female	7.6:1	11.7:1	15.7:1
Tobacco habits			
Yes	61%	97.4%	97.7%
No	39%	02.6%	02.3%
Type of habits			
Chewing	62%	67.5%	40%
Smoking	19%	13.5%	24%
Chewing+Smoking	15.5%	19%	33%
Snuff	03.5%	-	03%

The clinical information of oral cancer patients is provided in **table-3.3.** Clinical stage of malignant disease was determined as per UICC norms (1989).

Table-3.3: Clinical Details of Oral Cancer Patients

Oral Cancer Patients	**130**
Sites: Buccal mucosa	45.4%
Oral tongue	20.8%
Retromolar trigon (RMT)	07.7%
Alveolus	10.7%
Lip	04.7%
Others (Gum, gingival sulcus, Hard palate)	10.7%
Histopathology	
Squamous cell carcinoma	100%
Tumour Size: T1	12.2%
T2	34.0%
T3	15.1%
T4	38.7%
Lymphnode Involvement: No	49.4%
Yes	50.6%
Stage: Stage I	10.6%
Stage II	17.0%
Early Stage (I+II)	**(27.6%)**
Stage III	17.9%
Stage IV	54.5%
Advanced Stage (III+IV)	**(72.4%)**
Tumour Differentiation: Well	30.8%
Moderate	57.7%
Poor	11.5%
Nuclear Grade: I	27.0%
II	61.5%
III	11.5%
Follow-Ups of Oral Cancer Patients	**75**
Responders (CR)	52
Non-Responders (NR)	23

As documented in the table, buccal mucosa was the leading site (45.4%) in oral cancer patients. Histopathology reports revealed that all the patients were diagnosed with squamous cell carcinoma. 72.4% of the patients had advanced (stage III and stage IV) disease. 50.6% of the oral cancer patients

showed lymphnode (LN) metastasis. 57.7% of the patients had moderately differentiated tumours and 61.5% of the patients were having nuclear grade II of the tumours.

3.2: SAMPLE COLLECTION

Blood Samples

Blood samples were collected from all the subjects by venipuncture. Sera were separated and stored at –80°C until analysis. The study also included post-treatment follow-up blood samples (n=75) of the oral cancer patients. Clinical status of the patients at the time of each follow-up was assessed on the basis of their clinical and radiological findings. Clinical status of the patients' during/after anticancer treatment was classified into complete responders (CR, n=52) and non-responders (NR, n=23). The patients with no evidence of disease after surgical resection of tumour were classified as CR. The patients with locoregional failure of disease, stable disease/progressive disease, metastasis or recurrence were classified as NR (Miller et al., 1981).

Tissue Samples

Tissue samples from oral cancer patients were collected on ice, immediately after surgical resection of the tumours, from Operation Theater. Adjacent normal tissue samples were selected from the free margins at least 1-2 cm away from the tumour as defined by the pathologist. The histological examinations of the specimens were carried out. The OPC and adjacent normal tissues from the patients with OPC were also collected at the time of biopsy. The tissue specimens were washed with ice-cold phosphate buffer saline (PBS: pH-7.4) and immediately stored at –80°C until analyzed. OPC (n=10) and their adjacent normal tissues (n=10) as well as malignant (n=75) and adjacent tissues (n=75) were collected.

3.3: REAGENTS, KITS AND INSTRUMENTATION

The experiments were performed using fine chemicals and analytical grade reagents obtained from Sigma, Merck, Qualigens and Sisco Research

Laboratories (India). Molecular biology grade and electrophoresis grade reagents required for the study were procured from Sigma (USA). Kits were purchased from Bio-Rad (USA), Pierce (USA) and Amarsham Bioscience (UK). Antibodies were purchased from Calbiochem (USA) and Chemicon (USA). Beckman DU-640 spectrophotometer (USA), Jasco-FP-750 Spectrofluorimeter, Labsystem Multiscan Spectrum (USA) ELISA reader, Bio-Rad (USA) gel documentation system etc. were used for various experiments.

3.4: PROCESSING OF TISSUE SAMPLES

Enzyme Assays

Malignant, precancerous and adjacent normal tissues were homogenized in PBS (pH:7.4) using glass homogenizers (100 mg tissue/300 μl PBS). The whole process was carried out on ice. The cytosols were separated by centrifugation at 15,000 rpm in cooling centrifuge. The supernatants were used for protein estimation, enzyme assays and dot blots. Protein content of the homogenates was analyzed by method of Lowry et al. (1951).

Sialic Acid

Malignant and adjacent normal tissues were homogenized in 0.05M sulfuric acid (H_2SO_4) using glass homogenizers (100 mg tissue/ml H_2SO_4). Homogenates were incubated at 80°C for 1 hour. Then the samples were centrifuged and supernatants were used for protein and sialic acid estimation.

E-Cadherin and Sialyl Lewis-X

Malignant, precancerous and adjacent normal tissues were homogenized in lysis buffer PBS (pH:7.4) containing 1% triton X-100, 25 mM PMSF, 0.3 mM leupeptin, 10 mM aprotinin and 1 mM Sodium orthovanadate (50 mg tissue/250 μl PBS). The whole process was carried out on ice. The lysate was separated by centrifugation at 15,000 rpm in cooling centrifuge and supernatants were used for protein and western blot analysis.

3.5: METHODS

The methods used for the analysis of parameters from serum and tissues are tabulated below:

No.	Parameters	Method	Detection
Serum Analysis			
1	TSA	Modified TBA method	Spectrophotometric
2	α2,6- and α2,3-sialoproteins	Dot Blot	Chemiluminescence
3	α2,6-SiT and α2,3-SiT	96-well plate solid phase assay	ELISA reader
4	Sialidase	4-MU-NANA substrate	Spectrofluorimetric
5	Fucose	Cysteine-Hydrochloride	Spectrophotometric
6	Fucoproteins	Lectin affinity chromatography	SDS-PAGE
7	α-L-Fucosidase	p-nitrophenyl-α-L fucopyranoside substrate	Spectrophotometric
8	Serum Protein profiling	Native-Page, 2D-PAGE, Blotting, SDS-PAGE	CBB, Schiff's and Silver staining
9	Total Proteins	Biuret	Spectrophotometric
10	TSA/TP	Ratio	
11	Fucose/TP	Ratio	
Tissue Analysis (Malignant, Precancerous and Adjacent normal)			
1	TSA	Modified TBA method	Spectrophotometric
2	α2,6-SiT and α2,3-SiT	96-well plate solid phase assay	ELISA reader
3	Sialidase	4-MU-NANA substrate	Spectrofluorimetric
4	α2,6- and α2,3-sialoproteins	Dot Blot	Chemiluminescence
6	α-L-Fucosidase	PNP-α-L fucopyranoside substrate	Spectrophotometric
7	Sialyl Lewis-X	Western Blot	Chemiluminescence
8	E-cadherin	Western Blot	Chemiluminescence
9	Total Proteins	Lowry	Spectrophotometric

Estimation of Total Sialic Acid

Principle: The polysaccharide chains of the glycoprotein are hydrolysed into monosaccharides by boiling with dilute H_2SO_4. The proteins are separated out by precipitation with TCA followed by centrifugation. Periodic acid oxidizes –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. DMSO increases the intensity of pink chromophore, which is measured spectrophotometrically at 549 nm and 532 nm (Skoza and Mohos, 1976 modified by Warren et al., 1978).

Reaction

(1) NANA-O-Oligosaccharide – CONH – R $\xrightarrow{\textbf{hydrolysis}}$ NANA + Oligosaccharide – COOH + NH_2 – R (protein)

(2) TCA (CH_3COOH) + NH_2 – R $\longrightarrow$ CCL_3 – CONH - R

(3) NANA + $2HIO_4$ $\longrightarrow$ aldehyde of NANA + TBA $\longrightarrow$ Pink coloured complex

Reagents

1. 1N Sulfuric acid (H_2SO_4)
2. 10% Trichloro acetic acid (TCA),
3. 0.025 N Periodic acid in 0.125M H_2SO_4 (HIO_4 .$2H_2O$)
4. 2% sodium arsenite in 0.5 N HCl
5. 6% TBA: pH 10.0 with 10 N Sodium hydroxide (NaOH)
6. Standard: N-acetyl neuraminic acid (NANA) (Sigma)
7. Dimethyl sulfoxide (DMSO)

Procedure

0.1 ml of serum was incubated with 0.8 ml normal saline and 0.1 ml 1N H_2SO_4 at 80°C for 1 hour. Then proteins were precipitated by 1.0 ml of 10% TCA. The tubes were then centrifuged at 3000 rpm for 10 minutes. 0.1 ml supernatant (hydrolyzed serum) was mixed with 0.4 ml double distilled water

(DDW) and 0.25 ml periodic acid. The blank tube contained 0.5 ml DDW. Reaction mixture was incubated at 37°C for 30 minutes. To this, 0.2 ml of 2% sodium arsenite was added. 0.5 ml TBA was added after the solution became colourless. Then the reaction mixture was kept in boiling water bath for 7.5 minutes. It was allowed to cool and then 1.5 ml DMSO was added to intensify the pink chromophores. In order to eliminate 2-deoxyribose interference the absorbance of the reaction mixture was measured at two wavelengths: 549 nm and 532 nm spectrophotometrically.

Calculation for serum TSA

OD 549 – OD 532 = X OD

$$\text{TSA (mg) /100 ml serum (dl)} = \frac{\text{Standard Concentration}}{\text{Standard OD}} \text{ X Sample OD X 20,000*}$$

(* Sample dilution factor)

Tissue samples were processed for the estimation of TSA as described previously in "Processing of tissue samples" section. Hydrolysed tissue homogenates were centrifuged after incubation at 80°C for 1 hour and 100 µl supernatants were used for TSA estimation. Further procedure was same as serum analysis of TSA.

Calculation for tissue TSA

OD 549 – OD 532 = X OD

TSA (µg/mg protein) = (X- Y*) X 1000

(*Regression equation derived from standard curve).

Dot Blot Method for 2,6-Sialoproteins and 2,3-Sialoproteins

Principle: Dot blot method is based on the immuno-detection of sugars using lectins specific for sialic acid linkages. The secondary antibody, Streptavidin conjugated alkaline phosphatase (ALP), reacts with CSPD, the chemiluminescent substrate of ALP, in the presence of a chemical enhancer.

This produces a light signal, which is captured by exposure to a light sensitive X-ray film. This is a fast and sensitive detection method for sialoproteins.

Materials and Reagents

1. Hybond ECL nitrocellulose membrane (0.45 μM)
2. Tris buffer saline (TBS) (20mM Tris-base and 250mM NaCl), pH:7.4
3. TBS-T (TBS with 0.1% Tween-20)
4. 5% blocking reagent (skim milk) prepared in TBS/0.1% Tween
5. Biotinylation of lectins was done using Sulfo-NHS-Biotinylation kit [Pierce].
   Sambucus nigra (SNA) for detection of α2,6-sialoproteins (5 μg/ml TBS)
   Maackia amurensis agglutinin (MAL) for detection of α2,3-sialoproteins (20 μg/ml TBS)
6. Streptavidin-ALP (1:1000 dilution in TBS-T)
7. CSPD- Disodium 3-(4-methoxyspiro {1,2-dioxetane3,2'- (5,-chloro) tricyclo [3.3.1.13,7] decan}–4 yl) phenyl phosphate), chemiluminescent substrate for ALP

Procedure

Day-1: Serum/tissue samples (100μg/10μg proteins) were spotted onto the membrane and the membrane was allowed to dry for 5 minutes. Thus, proteins were immobilized on the membrane. Then the membrane was incubated in blocking solution for 30 minutes followed by 3 rinses with TBS-T. Then the membrane was incubated with biotinylated lectin (SNA-biotin/MAL-biotin) overnight with gentle agitation at room temperature.

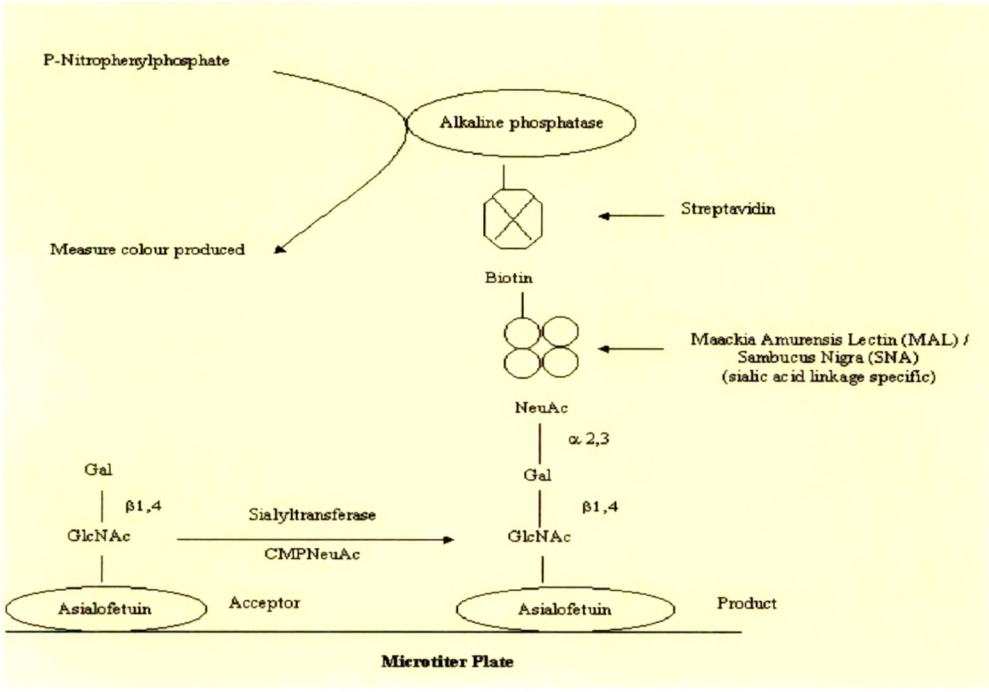
Day-2

Secondary Antibody Binding: To remove unbound lectin, the membrane was washed thrice with TBS-T for 10 minutes each and then the membrane was incubated with secondary antibody streptavidin-ALP 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.

Detection: The washed membrane was placed on a transparent sheet with the protein side facing up. The detection solution (CSPD) was layered evenly to the protein side of the membrane and incubated for at least 5 minutes at room temperature. The excess detection reagent was drained off and the membrane was wrapped with another transparent sheet. The blot with the protein face up was placed in the film cassette and exposed to the light sensitive X-ray film for 2 hours in dark and then the film was developed. The film was scanned and densitometric analysis of sialoprotein dots was done using Gel documentation system (Bio-Rad).

α2,6-Sialyltransferase and α2,3-Sialyltransferase Activities Using 96-Well Plate Solid-Phase Assay

Principle: α2,6-SiT and α2,3-SiT 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*-nitrophenyl-phosphate as substrate of ALP (Yeh and Cummings, 1996). The resultant colour was read at 410 nm.



Reagents

1. EZ-link Sulfo-NHS-Biotinylation Kit (Pierce, USA) for biotinylation of lectin
2. Polystyrene microtiter 96-well plates (Axygen)
3. Standard: *p*-Nitrophenol (PNP)
4. Coating buffer: Bicarbonate buffer pH: 9.6
5. Asialofetuin: Sialic acid acceptor glycoprotein
6. Blocking reagent: 2% Bovine serum albumin (BSA), Sigma
7. Wash buffer: Phosphate buffer saline + 0.02% Sodium azide + 0.1%Tween 20 (PBS+NaN_3+Tween-20) pH: 7.4
8. Assay Buffer: 50 mM Cacodylate buffer pH: 6.5
9. Dilution buffer: PBS + 0.02% NaN_3 + 0.05% Tween-20 + 0.1% BSA
   Used for dilution of biotinylated lectins and secondary antibody as follows:
   Lectins: SNA-biotin (2 μg/ml; stock: 280 μg/ml),
   MAL-biotin (10 μg/ml; stock: 415 μg/ml),
   Secondary antibody: Streptavidin-ALP (1:500)
10. Developing buffer for *p*-nitrophenyl-phosphate (PNPP- substrate for ALP): Coating buffer: Bicarbonate buffer pH: 9.6 containing 1 mM $MgCl_2$ (stock: 100mM)
    PNPP 1 mg/ml
11. 10 mM $MnCl_2$ (stock: 200 mM)
12. 50 μM Cytidine monophosphate-NANA (CMP-NANA [stock: 500 μM])

Procedure

Day 1

(A) Biotinylation of Lectins

2 mg of lectin (SNA/ MAL) was dissolved in 1.0 ml PBS. 2 mg Sulfo-NHS-Biotin was dissolved in 100 μl ultra pure water and 12-fold molar excess Sulfo-NHS-Biotin was added to 2 mg/ml lectin. The reaction mixture was incubated for 45 minutes at room temperature. Simultaneously, the dextran column was equilibrated with 30 ml PBS. Then the lectin- Sulfo-NHS-Biotin mixture was passed through the gel and 1 ml fractions were collected. Protein concentration of each fraction was measured at 280 nm and then the fractions containing protein were pooled. Biotinylated lectin solution was

stored at –20°C until used. Protein stabilizer sodium azide was mixed with lectins.

HABA Assay for Determining Biotin Incorporation with Lectins

1 mg avidin and 60 µl of 10 mm HABA were mixed with 1.94 ml sample buffer. Absorbance of this mixture was read at 500 nm and then 100 µl of biotinylated lectin was added with HABA-Avidin mixture and absorbance was read again at 500 nm to check the biotinylation of lectin. Proportionate decrease in absorbance indicates lectins are biotinylated.

(B) Coating of Microtiter Plate Wells

Asialofetuin, desialylated acceptor of sialic acid, was diluted in coating buffer. The wells of polystyrene mictotiter plates were coated with 100 µl bicarbonate coating buffer containing asialofetuin. The plates were incubated overnight at room temperature for proper coating.

Day 2

(C) Assay of Sialyltransferases using Streptavidin-ALP

The asialofetuin-coated wells were washed with PBS+NaN_3+Tween-20 (3 washes of 350 µl each). 250 µl of 2% BSA was added in each well as blocking reagent and incubated at room temperature for 2 hours. After aspiration of blocking reagent the wells were washed thrice with washing buffer. Then, 100 µl of standard assay conditions were provided containing,

- 50 mM cacodylate buffer: pH 6.5 - 50 µl
- 50 µM CMP-NANA - 10 µl
- 10 mM $MnCl_2$ - 05 µl
- Enzyme source (serum/tissue homogenate) - 35 µl
- Assay blank: without CMP-NANA

The reaction mixture was incubated at 37°C for 2 hours followed by 5 washes with washing buffer. Then, 100µl of biotinylated lectin (SNA: 2 µg/ml or MAL: 10 µg/ml) prepared in PBS+NaN3+Tween-20 containing 0.1% BSA was applied in each well and allowed to react for 1 hour at room temperature.

Lectin solution was removed by aspiration and wells were washed five times with PBS+NaN3+Tween-20. 100 µl of Streptavidin-ALP conjugate (1:1000) was then added and allowed to incubate at room temperature for 1 hour following five washes with buffer and three washes with deionized water. 100 µl of freshly prepared ALP substrate PNPP (1 mg/ml) in bicarbonate coating buffer containing 1 mM $MgCl_2$ was added to each well and incubated at 37°C for 1 hour. O.D. at 405 nm of each well was recorded using Labsystem Multiscan Spectrum ELISA reader.

Specific enzyme activity was defined as µmoles of PNP liberated/min/mg protein.

Calculation: Specific enzyme activity

$$\mu\text{moles/ mg protein/ min} = \frac{\text{OD of sample/min X (Conc. of PNP/OD of PNP)}}{\text{mg protein}}$$

Sialidase Activities using 4-Methyllumbelliferyl-α-D-N-acetyl-neuraminic Acid as Substrate

Principle: Under acidic condition (pH: 4.6) sialidase hydrolyze 4-MU-NANA and release 4-methy-lumbelliferone, which is determined by spectrofluorimeter using excitation maxima at 365 nm and fluorescence emission at 452 nm. (Potier et al., 1979)

Reaction

Reagents

1. Standard: 4-Methyl-umbeliferrone (4-MU) (Conc. $5X10^{-6}$ to 10^{-8}) in glacial acetic acid
2. Substrate for Sialidase: 10 µM 4-Methyllumbelliferyl-α-D-N-acetyl-neuraminic acid (4-MU-NANA) sodium salt
3. 0.1 M Sodium-Acetate buffer (pH: 5.0)

4. 0.133M Glycine-Carbonate buffer (pH: 9.5)

Procedure

10 μl enzyme source (serum/tissue homogenate) was mixed with 80 μl of 0.1M Na-acetate buffer (pH: 5.0) and 10 μl of 10 μM 4-MUNANA. The reaction mixture was incubated at 37°C for 1 hour. 900 μl of 0.133M glycine buffer (pH: 9.5) was added to terminate the reaction. Released fluorescent substance 4-MU was recorded spectrofluorimetrically using excitation light at 365 nm and fluorescence emission at 450 nm.

The enzyme activity was defined as nmoles of 4-MU released/mg protein/hour.

Calculation

The regression equation was derived from standard curve of 4-MU and Specific activity (nmoles/mg protein) was calculated.

Estimation of Fucose

Principle: Fucose on reaction with cysteine-hydrochloride produces yellow coloured chromophore having optimum absorbance at 396 nm. Reaction product is also read at 430 nm in order to correct the interference due to chromophores of other sugars at absorption maxima at 430 nm (Dische and Shettles, 1948 modified by Winzler, 1955).

Reagents

1. 95% Ethanol
2. 0.2N NaOH
3. H_2SO_4: H_2O (6:1)
4. Cysteine hydrochloride – 0.3 gm/10 ml DDW
5. Standard: α-L-fucose (Sigma)

Procedure

0.1 ml serum was mixed with 5.0 ml of 95% ethanol for protein precipitation followed by centrifugation at 2500 rpm for 15 minutes. Supernatant was

discarded and the precipitates were dissolved in 3 ml of 95% ethanol. The pellet obtained after centrifugation at 2500 rpm was dissolved in 2.0 ml of 0.2 N NaOH. 0.5 ml aliquots in duplicates were used for fucose estimation (Blank: 0.5 ml NaOH). The aliquots mixed with 4.5 ml of H_2SO_4: H_2O (6:1) reagent mixture followed by boiling for 3 minutes and allowed it to cool (this reaction releases bound fucose from complex carbohydrate and converts it into furfural derivatives). 1.0 ml of cysteine-hydrochloride reagent was added and incubated for 1 hour at room temperature (forms yellow coloured chromophore with fucose). The absorbance was read at 396 nm and 430 nm (to correct the interference due to chromophores of other sugars absorption maxima at 430 nm).

Calculation

[OD (396+) – OD (430+)] - [OD (396-) – OD (430-)]

+ = with cysteine, - = without cysteine

$$\textbf{Fucose (mg/dl)} = \frac{\text{Standard fucose conc. (mg) X OD of sample X 4000*}}{\text{OD of standard fucose}}$$

(* Sample dilution factor)

Lectin Affinity Chromatography for Fucoproteins

Lectin affinity chromatography for isolation of fucoproteins was performed as described by Thompson and Turner (1987).

Reagents:

1. 1 mM Hydrochloric acid (HCl)
2. CNBr-activated sepharose beads (CNBr: Cynogen bromide)
3. Fucose specific lectin- *Lotus tetragonolobus* (LTA)
4. 0.1 M Bicarbonate buffer (pH: 8.3) containing 0.5 M NaCl
5. Blocking buffer: 0.2 M Glycine-carbonate buffer (pH: 8.0) containing 0.5 M NaCl
6. Blocking buffer: 0.1 M Acetate buffer (pH: 4.0) containing 0.5 M NaCl

7. Wash buffer: 0.05 M Tris buffer (pH: 7.4) containing 25 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% Nonidet P40 (NP-40)
8. Elution buffer: 125 mM Tris buffer (pH: 6.8) containing 0.35 M SDS
9. 6x Sample loading buffer: 125 mM Tris (pH: 6.8), 2.7 M glycerol, 1 mM EDTA, 2.9 mM bromophenol blue, 0.6 M Dithiothreitol (DTT)
10. 30% acrylamide-bis acrylamide mixture (Stock)
11. Running gel buffer: 1.5 M Tris-HCl (pH: 8.8)
12. Stacking gel buffer: 1 M Tris-HCl (pH: 6.8)
13. Electrode buffer- Tris-glycine (pH: 8.3)
14. 10% Sodiumdodecyl sulphate (SDS) (stock)
15. 10% Ammonium per sulphate (APS) (stock)
16. N,N,N',N'-Tetra methylethylene diamine (TEMED)
17. Silver stain-plus kit (Bio-Rad)

Procedure

Day-1

(1) Activation of Sepharose Beads

1 gm CNBr-activated sepharose beads were mixed with 200 ml of 1 mM HCl for 30 minutes at room temperature. Then HCl was poured off and this step was repeated twice. The beads were then washed with 0.1 M bicarbonate buffer (pH: 8.3).

(2) Coupling of Lectin (*Lotus tetragonolobus*) with Sepharose

5.0 mg of lectin – LTA was dissolved in 1 ml of bicarbonate buffer. The sepharose beads prepared as above were mixed with lectin and incubated overnight at 4°C on a shaker.

Day-2

(3) Blocking of Remaining Proteins

The supernatant was removed and the coupled beads were resuspended in 0.2 M glycine-carbonate buffer (pH: 8.0) and kept for 2 hours at room temperature with gentle shaking.

(4) Removal of Excessive Proteins

Alternate washing of coupled beads with bicarbonate (pH: 8.3) and acetate buffers (pH: 4.0) was done to remove the excess proteins.

(5) Binding of Serum Proteins with Lectin-sepharose Beads

The coupled lectin-sepharose beads were washed three times with Tris buffer (pH: 7.4) and then mixed with equal amount of serum proteins (95-100μl equivalent to 7 mg) with equal volume of coupled beads (~100 μl) and incubated for 2 hours at 4°C with gentle shaking.

(6) Removal of Unbound Serum Proteins

The unbound serum proteins were removed by 5–6 washes with Tris buffer (pH: 7.4).

(7) Elution of Bound Fucoproteins

The bound fucoproteins were released in elution buffer (Tris buffer with SDS- pH: 6.8).

(8) Eluted serum fucoproteins (equal volume- 30 μl) were electrophoresed on 7.5% SDS-PAGE.

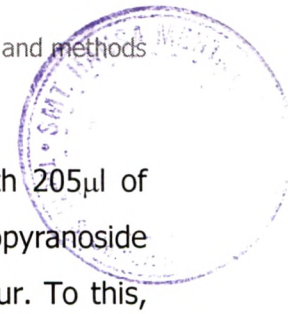
(9) Fucoproteins were visualized using silver staining method (Bio-Rad Kit).

α-L-Fucosidase Activity Using *p*-nitrophenyl-fucopyranoside as Substrate

Principle: Under acidic conditions (pH: 5.5), fucosidase hydrolyzes 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 conditions, which is measured at 410 nm spectrophotometrically (Wiederschain et al, 1971).

Reagents

1. Substrate for fucosidase: 10 mM *p*-Nitrophenyl-α-L-fucopyranoside
2. 0.05 M Acetate buffer (pH: 5.5)
3. 0.4M Glycine-NaOH buffer (pH: 10.5)
4. 2.5% $ZnSO_4$
5. 0.15 M NaOH
6. Standard: *p*-nitrophenol (PNP)



Procedure

20 μl enzyme source (serum/tissue homogenate) was mixed with 205μl of 0.05M acetate buffer (pH: 5.5) and 25 μl of 10 mM PNP-α-L-fucopyranoside (substrate). The reaction mixture was incubated at 37°C for 1 hour. To this, 100 μl of 2.5% $ZnSO_4$ and 100μl of 0.15N NaOH were added to terminate the reaction. After centrifugation for 3 minutes 300 μl supernatant was transferred into 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.

- One unit of enzyme activity in serum was defined as the amount of enzyme that converts 1 nmole *p*-nitrophenol/ml/hour.
- Specific enzyme activity in tissues was defined as nmoles of PNP liberated/mg protein/minutes

Calculation

Serum: Fucosidase activity was calculated as Unit Activity (nmoles/ml/hr) using standard curve.

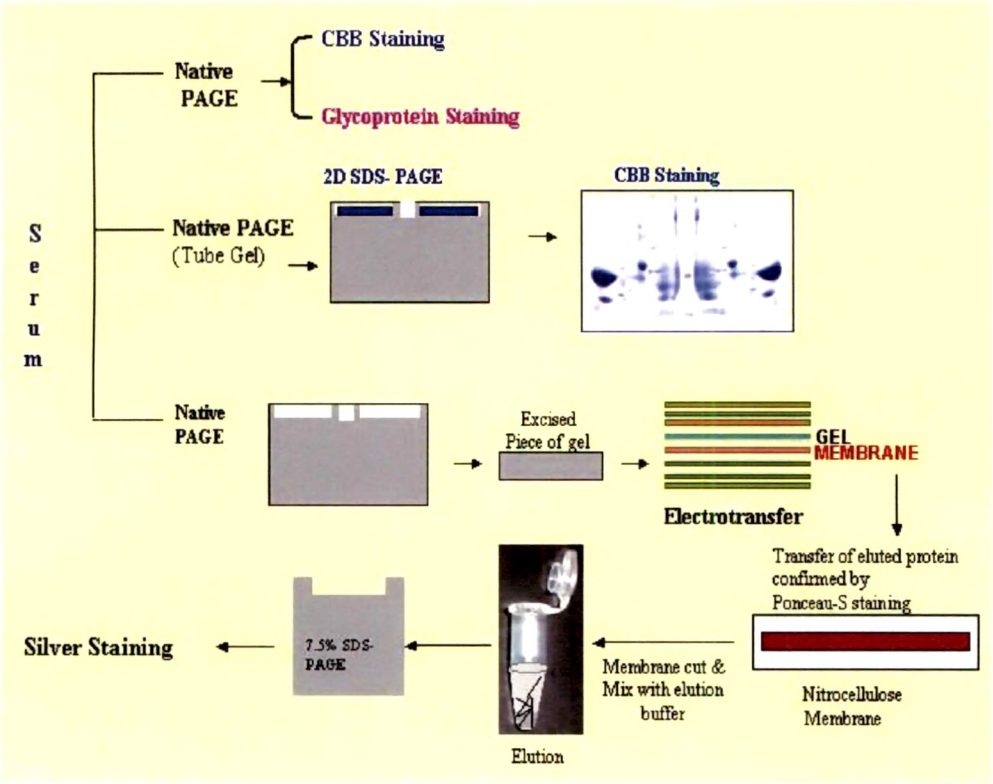
Tissue: Fucosidase activity was calculated as nmoles/mg protein using standard curve.

SERUM PROTEIN PROFILING

The serum protein profiling was performed using three different strategies as follows.

1. Serum protein separation on native-PAGE and CBB as well as Schiff's staining
2. Native PAGE and Two Dimensional (2D-SDS)-PAGE
3. Native-PAGE, Elution of Unusual Serum Protein and SDS- PAGE for Separation of Protein Elute

Schematic representation of working strategies for serum protein profiling is illustrated below:



Strategy-1: Serum Protein Separation on Native-PAGE and CBB as well as Schiff's Staining

Serum proteins were separated on 7.5% native polyacrylamide gel by standard protocols (Hames, 1990) in non-denaturating and non-reducing conditions.

Reagents:

1. Mini slab and tube gel casting unit from Genei (India)
2. 30% acrylamide:bis-acrylamide mixture (stock)
3. Running gel buffer: Tris-HCl pH: 8.8
4. Stacking gel buffer: Tris-HCl pH: 6.8
5. 10% APS (stock)
6. 10% SDS (stock)
7. N,N,N',N'-TEMED

8. 2X Sample loading buffer: 0.002% (w/v) Bromophenol blue in 40% glycerol
9. Electrode buffer Tris-glycine pH: 8.3
10. Fixing solution: 50% methanol and 10% acetic acid in DDW
11. 0.25% Coomassie Brilliant Blue (CBB) R-250 for protein staining
12. Destain for CBB: 10% methanol and 7% acetic acid
13. Schiff's reagent for glycoprotein staining

    2 gm Basic fuschin was mixed with pre-warmed 400 ml DDW at 60°C and then slowly brought to boiling temperature. After boiling for 3 minutes, it was allowed to cool to 30°C. To this, 8 gm sodium metabisulphite was added and kept overnight in dark. 1.2 gm of activated charcoal was then added for decolorization. It was vigorously mixed and filtered after 2 hours. 1N HCl was added till the filtrate failed to stain the filter paper.

Procedure

Mini vertical slab gels were prepared using 7.5% running gel and 5% stacking gel concentration. 100μg of serum proteins were mixed with equal volumes of sample loading buffer and kept at room temperature for 5 minutes. The samples were run under non-denaturing and non-reducing conditions at constant voltage (100 V) until the dye front reached the bottom of the plate. Care was taken to keep the gels cool during the run. The gels were separated from the glass plates and further used either for proteins or glycoproteins staining as described below.

Total Protein Staining

Gels were incubated with fixing solution for 1 hour for the fixation of proteins in the gel matrix. The gels were stained with 0.25% CBB for 1 hour and then destained by frequent changes of fixing solution until a clear background is obtained. Gel scanning and densitometric analysis were done using Bio-Rad gel documentation system (Model GS-700).

Staining for Glycoprotein

The gels were immersed in 0.5% (w/v) periodic acid for 2 hours. They were then transferred into 0.5% (w/v) sodium arsenite solution prepared in 5% acetic acid for 1 hour. The gels were transferred and incubated in 0.1% sodium arsenite (w/v) prepared in 5% acetic acid for 20 minutes (repeated twice). Fixing was performed in 10% acetic acid (v/v) for 30 minutes after pouring off the acetic acid, Schiff's reagent was added in a tightly sealed box till the gels were dipped completely for 2 hours. Destaining was done with 0.1% sodium metabisulphite in 0.01N HCl until the background was clear. The gels were scanned and densitometric analysis was performed.

Strategy-2: Native PAGE and Two Dimensional (2D-SDS)-PAGE

Reagents

1. Equilibration buffer (pH: 6.8): 0.125 M Tris-HCl, 2% SDS, 10% Glycerol, 4.9 mM DTT, bromophenol blue trace
2. 2X Treatment buffer (pH: 6.8): 0.125 M Tris-HCl, 4% SDS, 20% v/v Glycerol, 0.2M DTT

Day-1: Native-PAGE for Serum Proteins in Tube Gel

Native–PAGE was performed for serum proteins separation in first dimension. Method followed for first dimension was similar as described above for Native-PAGE in slab gel. Briefly, 7.5% gels were polymerized in glass columns. 500 μg of serum proteins were mixed with equal volumes of 2X sample loading buffer and incubated at room temperature for 5 minutes. The samples were run under non-denaturing and non-reducing conditions at constant voltage (24mA: 3mA/tube) until the dye front reached the bottom of the tube. The gels were separated from the glass tubes and stored in equilibration buffer at 4°C until processed further.

Day-2: Two Dimensional (2D)-SDS-PAGE

The gels were transferred into treatment buffer before 1 hour of use. Maxi-slab gels were prepared using 7.5% running gel and 5% stacking gel. Three

wells containing comb was inserted in stacking gel. The tube gels with serum proteins separated in first dimension were placed on both sides of the wells and the gap between tube gel and wells was sealed with 0.05% agarose gel. The molecular weight markers were loaded in the middle well. The samples were run in second dimension under denaturing and reducing conditions at constant voltage (250 V) until the dye front reached the bottom of the plate. Care was taken to keep the gels cool during the run. The gels were separated from the glass plates, incubated with fixing solution for 1 hour. Then the gels were stained with 0.25% CBB R-250 for 1 hour and destained. Gels were scanned using Bio-Rad gel documentation system and 2D map of both serum samples were compared.

Strategy-3: Native-PAGE, Elution of Unusual Serum Protein and SDS-PAGE for Separation of Protein Elute

Day-1

7.5% Native-PAGE was performed in slab gel apparatus used with 3 mm spacers and 3 wells comb. The method followed was same as above in **strategy-1**. After completion of protein separation, the gel was separated from the glass plate. A piece of gel from one side in vertical manner was cut, fixed, stained with CBB and destained as described before. Meanwhile, the unstained gels were stored in running buffer at 4°C until processed further.

Day-2: Blotting and Elution

The stained gels were matched with the unstained gel, and the part matched to extra protein band region was cut and the piece of that gel containing extra protein was applied for blotting. The stack was prepared for blotting using sponges/ rough filter papers, 3 mm whatman papers and nitrocellulose membrane presoaked in transfer buffer with a size of separation gel and blotting was carried out at constant 150 mA for 5 hours. The semi-dry transfer apparatus from Amersham Pharmacia was used for blotting.

The transfer of protein on the nitrocellulose membrane was confirmed by staining with Ponceau-S reagent. The nitrocellulose membrane, to which protein was transferred, cut in small pieces and mixed with 250 µl protein elution buffer, Tris-HCl buffer pH: 9, containing SDS and Triton X-100, with constant shaking for 2 hours at 4°C. Protein was estimated from the eluted fraction by Lowry method. The fractions were stored at –20°C until ready to run on SDS-PAGE.

Day-3: SDS-PAGE for protein elute

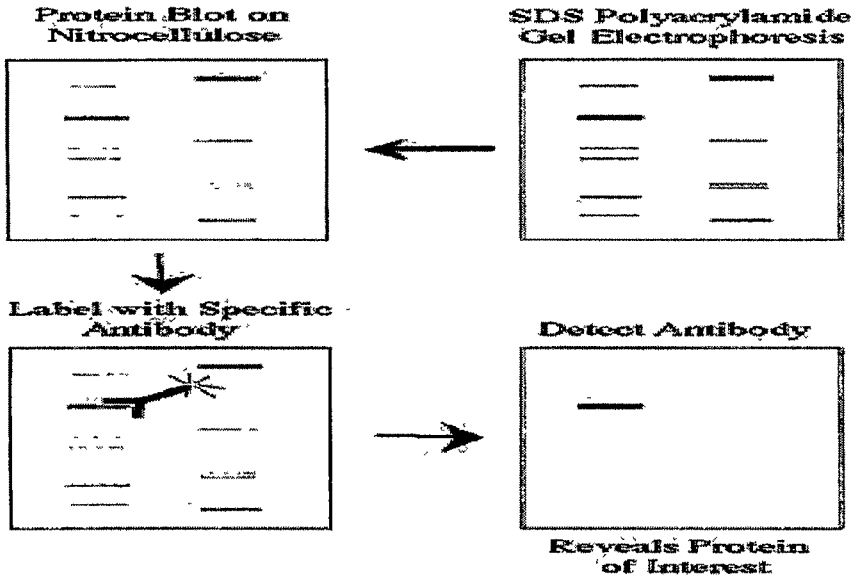
7.5% SDS-PAGE was performed for the protein separation of eluted protein by standard protocols (Laemmli, 1970). 5 µg of protein elute was mixed with 6X sample loading buffer, boiled for 5 minutes and loaded in wells after cooling. The samples were allowed to run at constant voltage (100V). The separated proteins were visualized using silver staining method (Bio-Rad Silver Plus kit) followed by densitometric scanning.

WESTERN BLOT ANALYSIS FOR PROTEINS EXPRESSION

Expressions of Sialyl Lewis-X and E-cadherin were detected using western blot method.

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 mouse primary antibodies specific for the sample protein followed by secondary antibodies specific for the general class of primary antibodies, goat anti-mouse antibody conjugated with peroxidase. The detection of the proteins is done with highly sensitive chemiluminescence method.

Schematic representation of proteins separation, electro-transfer and antibody probing



(2) Detection of proteins using ECL western blotting chemiluminescence detection kit

The secondary antibody is tagged with HRP (horseradish 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 X-ray films.

Antibodies

1. **Sialyl Lewis-X** (Clone KM-93): Mouse (monoclonal) anti-human Sialyl Lewis-X antibody purchased from Chemicon International (USA). Working dilution of antibody used was 1μg/ml in TBS.
2. **E-cadherin** (Clone HECD-1): Mouse (monoclonal) anti-human E-cadherin antibody purchased from Calbiochem (USA). Working dilution of antibody used was 2μg/ml in TBS.
3. **Secondary antibody–HRP** (anti-mouse) conjugated was used with dilution 1:1000 in TBS. Secondary antibody was provided with the ECL kit.

Materials and Reagents

1. 30% Acrylamide - bis acrylamide mixture (stock)
2. Running gel buffer (1.5M Tris-HCl pH: 8.8)
3. Stack gel buffer (1M Tris-HCl pH: 6.8)
4. 10% APS (stock)
5. 10% SDS (stock)
6. N,N,N',N'-TEMED
7. Electrode buffer: Tris-glycine (pH 8.3)
8. 6X sample loading buffer: 50 mM Tris-HCl (pH: 6.8) containing bromo-phenol blue, glycerol, DTT and SDS
9. Molecular weight markers for SDS-PAGE (MW range- 29, 45, 66, 97.4, 116 and 205 kD standard proteins), Sigma
10. Blotting (Transfer) buffer: Towbin buffer (pH: 8.2 to 8.4) containing 25 mM Tris-base, 192 mM glycine, SDS and 20% methanol
11. Whattman filter papers (1 mm)
12. Hybond ECL nitrocellulose membrane (0.45 μm)
13. Ponceau-S stain
14. Blot washing buffer (TBS-T) (100mM Tris, pH: 7.5/ 0.1% Tween-20)
15. Nova-blot semidry transfer unit (Amersham pharmacia)
16. Immunodetection using ECL western blotting kit (Amersham Bioscience, UK) Containing
    - 5% Blocking reagent prepared in TBS-T
    - Horseradish Peroxidase (HRP) conjugated anti-mouse secondary antibody
    - Detection reagents: Solution 1 and 2 mixed in 1:1 ratio containing chemiluminescence substrate for HRP

Procedure

Day-1: Tissue proteins separation on SDS-PAGE

Preparation of running and stacking gels was as follows:

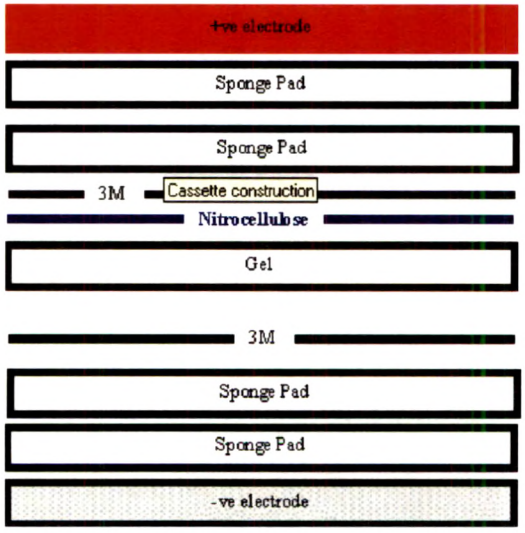
Protein	Running gel	Stacking gel
Sialyl Lewis-X	8%	5%
E-Cadherin	6%	5%

Slab gels were polymerized as mentioned above. The tissue lysates equivalent to 100μg proteins were mixed with 6X sample loading buffer, incubated at room temperature for 5 minutes and then denatured by boiling for 3 minutes. 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

Nova blot Semi-dry transfer unit was used for electrotransfer of proteins from gel to membrane as follows: The gels were separated from the glass plates; stacking gel and sealing gel were removed. The gel and nitrocellulose membrane were equilibrated in Towbin buffer for 5-10 minutes. Stack was prepared as shown in the schematic diagram with serum protein profiling. Electro-transfer was done for 3 hours at 150 mA constant current and membrane was proceed for Immunodetection.

Stack Preparation for Electro-transfer



Immunodetection (ECL Western blotting detection kit)

Membrane was incubated in 5 % blocking reagent (provided with ECL-kit, prepared in TBS/0.1% Tween w/v) for at least 1 hour at room temperature prepared in TBS/0.1% Tween, which was followed by 3 washes with TBS-T (1x15 minutes and 2x5 min). Then the membrane was incubated with primary

antibody (1:1000 dilution in TBS) overnight at room temperature with gentle shaking.

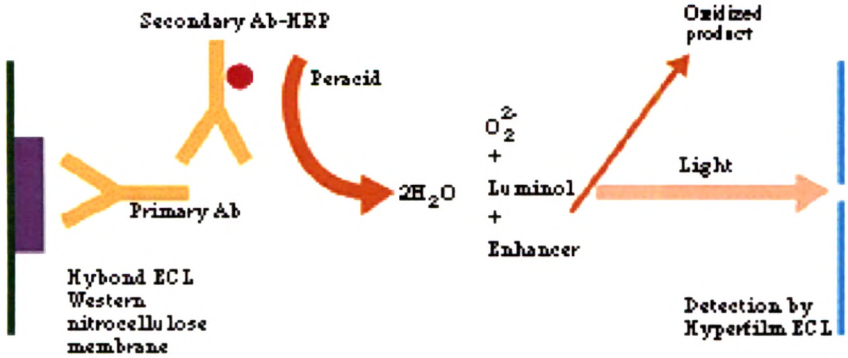
Day-2

The membrane was rinsed thrice with TBS/0.1% Tween (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, which was followed by three washes with TBS/0.1% Tween (1x15 minutes and 2x5 min).

Detection of proteins using ECL western blotting chemiluminescence detection kit

Equal volume of detection reagent-1 and reagent-2 containing chemi-luminescence substrate for HRP provided with the kit were mixed. The nitrocellulose membrane was placed on a transparent sheet with protein side up. Membrane surface was covered with the mixture of detection reagents for at least 1 minute, excess solution was drained off and then the membrane was covered with another transparent sheet. Luminescence captured on light sensitive X-ray film and bands were visualized on film after 2-10 minutes exposure. The film was scanned and densitometric analysis of protein bands was performed using Gel Documentation system and software (Bio-Rad).

Schematic diagram showing principle of ECL detection system for protein detection



3.6: STATISTICAL ANALYSIS

1. Data were statistically analyzed using the SPSS statistical software (Version 10). Results are expressed as means ± S.E.M. unless otherwise stated.
2. Student's unpaired 't' test was performed for comparison of marker levels between subjects in various groups and between malignant/precancerous and adjacent normal tissues.
3. Student's paired 't' test was performed for comparison of marker levels before and after anticancer treatment as well as between malignant and adjacent normal tissues of the same patient.
4. Pearson's correlation coefficient was carried out to assess the association between the markers.
5. Multivariate analysis was performed to correlate markers with various clinico-pathological parameters.
6. Receiver's Operating Characteristic (ROC) curves were constructed to evaluate discriminatory efficacy of parameters between malignant and adjacent normal tissues.
7. 'p' values ≤ 0.05 were considered statistically significant.