Yale University Library

MATERIALS

&

METHODS

3.1 SUBJECTS

Due consent was taken from all the subjects who participated in the study. They were divided into two groups:

Controls: 100 age and sex-matched healthy individuals were included in the study as controls. The controls were further categorized into controls with the habit of tobacco (WHT) and no habit of tobacco (NHT).

Oral cancer patients: 120 clinically and pathologically confirmed cases of oral cancer were recruited for the study from out patients' department of the Gujarat Cancer and Research Institute, Ahmedabad.

Table-3.1: Proforma seeking information of the subject's details

Name:	**Registration No.:**	**Sex:**	**Age:**
Sr.No.# :	**Height:**	**Weight:**	**Religion:**
Education:	**Occupation:**	**Monthly household income:**	
Subject: Control # Cancer patient #	**Family history of cancer:**	**Past / Current history of major illness:**	
Medical /non-medical intervention	Medicinal drugs/Multivitamins/ Nutrients supplementation	Name Duration	
Oral hygiene	Type		
History of habits Current user / Non user	Tobacco: Smoking/Chewing/Snuffing	Type/ Brand/ Frequency/ duration	
	Alcohol Drinking	Type/ Brand/ Frequency/ duration	
Clinical diagnosis			
Chief complaints			
Pathological/radiological reports	Date	Serial no.#	Comments
Clinical pathological reports	GCRI	Private	Other firms
Histological examination Biopsy / Patho-surgical report			
Anti-cancer Treatment	Surgery Date Type Dosage	Radiotherapy Date Type Dosage	Chemotherapy Date Type Dosage
Follow up history			
Follow up's No. #:	Date	Complaints	Reports

The socio-demographic details, detailed history of tobacco habits and clinical details were gathered using a proforma specially designed for the study (**Table-3.1**). The controls with the habit of tobacco consumption were of

younger age (median: 25.0 years). Oral cancer incidence was more prevalent in males and in older age group with median age of 45.0 years (**Table-3.2**). The details including chief complaints, clinical reports at diagnosis, routine investigations, and treatment plan, and clinical status during post-treatment follow-up etc. from oral cancer patients were collected. All the patients were staged according to the TNM classification (AJCC) based on size of tumor, involvement of lymph nodes and presence of distant or occult metastasis. As documented in the **Table-3.3**, cancers of tongue and buccal mucosa were more common than other sites oral cavity cancers. Histologically, all oral cancer patients (100%) had squamous cell carcinoma. 60% of the tumors were

Table-3.2: Details of controls and oral cancer patients

CHARACTERISTICS		**NO. (%)**
Controls (N=100)		
Sex:	Male	65 (65)
	Female	35 (35)
	Male: Female	1.9:1
Age (Years):	Male - Mean (Range)	35 years (25-48 years)
	Female - Mean (Range)	33 years (25-40 years)
Tobacco History:		
	Without tobacco habit (NHT)	70 (70)
	With tobacco habit (WHT)	30 (30)
Oral Cancer Patients (N=120)		
Sex:	Male	84 (70)
	Female	36 (30)
	Male: Female	2.3:1
Age (Years):	Male - Mean (Range)	45 years (25-65 years)
	Female - Mean (Range)	45 years (25-65 years)
Tobacco History:		
	Without tobacco habit (NHT)	19 (15.8)
	With tobacco habit (WHT)	101 (84.2)

Table- 3.3: Clinical details of oral cancer patients

CHARACTERISTICS		NO. (%)
Oral Cancer Patients (N=120)		
Histopathology:	Squamous cell carcinoma (SCC)	120 (100)
Site	Buccal mucosa	41 (34.2)
	Tongue	49 (40.8)
	Others	30 (25)
Tumor size	T1	24 (20.4)
	T2	21 (17.6)
	T3	6 (4.6)
	T4	24 (20.4)
	NA	45 (37.0)
Nuclear Grade	NG-I	21 (17.5)
	NG-II	81 (67.5)
	NG-III	18 (15)
Lymphatic response	Yes	37 (30.8)
	No	8 (6.7)
	NA	56 (46.6)
Tumor infiltration	Yes	56 (46.6)
	No	14 (11.6)
	NA	31 (25.8)
Tumor Differentiation	Well	40 (33.3)
	Moderate	72 (60)
	Poor	08 (6.7)
Lymph Node (LN) involvement	+ LN	44 (36.7)
	- LN	39 (32.5)
	NA	37 (30.8)
Stage of the disease (TNM)		
Early stage	[Stage I + Stage II]	28 (23.3)
Advanced stage	[Stage III+ Stage IV]	92 (76.7)

moderately differentiated while 33.3% of the tumors showed well differentiation. Poorly differentiated tumors were present in only 6.7% of patients. Lymph node metastasis was observed in 44 (36.7%) patients. Majority of the patients (76.7%) presented with advanced disease of cancer. Nuclear grade (NG) of type I, II and III were observed in 17.5%, 67.5% and 15% of patients respectively. 46.6% of patients showed tumor infiltration while 30.8% of oral cancer patients showed lymphatic response.

3.2 SAMPLE COLLECTION:

Blood samples: Blood samples from oral cancer patients were collected at the time of diagnosis (n=120) and during post-treatment follow-ups (n=53). The post-treatment follow-up blood samples were classified into complete responders (CR=44) and non-responders (NR=9) on the basis of their clinical status at the time of blood collection. Patients with no evidence of disease after therapy were classified as CR and patients with loco regional failure of disease, stable/progressive disease, metastasis or recurrence were classified as NR.

Processing of Blood Samples: Blood samples were allowed to form clot at room temperature and centrifuged at 3000 rpm for 10 mins to separate serum. Heparin plasma samples were centrifuged at 3000 rpm for 10 mins to separate plasma. The sera and plasma samples were stored at -80°C until analysis. Red blood cells (erythrocytes) from heparinized samples were washed 3 times with normal saline and packed erythrocytes were stored at -20°C.

Tissue samples: A total of 120 tissue samples (60-paired tissues of malignant and adjacent normal tissues) were collected from oral cancer patients at the time of biopsy/surgery from operation theatre. The tissues were histologically examined and defined by pathologist as malignant and adjacent normal tissue (dissected from free margins atleast 2cm away from tumor). Lymph node positive (LN+) and negative lymphnode (LN-) tissue samples were also collected from the patients. The tissues samples were collected on ice, washed with chilled phosphate buffered saline (PBS), pH 7.4. All the samples were stored at –80°C until analysed.

Processing of oral tissues: The tissue samples were washed thrice with chilled PBS. They were treated differentially for estimation of various proteins from cytosolic extracts and nuclear extracts.

Cytosolic extracts: Briefly, the tissue samples were minced, thawed on ice and homogenized in chilled PBS solution using glass mortar and pastel. The homogenates were placed on ice for 10 mins and then centrifuged at 15,000 rpm for 30 mins at 4°C. The supernatant fractions were stored in prechilled

tubes as tissue cytosol fractions at -80°C until analysis. While for the estimation of proteins for western blot analysis, the pellet was further homogenized in chilled PBS containing 1% Triton-X 100 and PMSF as protease inhibitor on ice. The homogenates were then centrifuged at 15,000 rpm for 10 mins at 4°C and the supernatants were stored in prechilled tubes as tissue cytosols at -80°C until analysis.

Nuclear extracts: In brief, the oral tissues were homogenized in PBS on ice, centrifuged for 1 min at 400g and washed with cold PBS. The homogenized tissues were lysed by adding 400µl of hypotonic buffer and 30µl of 10% NP-40. The mixture was centrifuged at 18,000g for 30secs and the supernatants were collected. While the pellet was homogenized in 220µl of nuclear extraction buffer and centrifuged at 18,000g for 1 min. The supernatant was used as nuclear extract and was stored at -80°C until analyzed.

Reagents, kits and instrumentation:

All reagents used in the experiments were fine chemicals of analytical grade procured from SRL, Qualigens, Merck (Germany), Sisco Research Laboratory (India), Sigma Aldrich (USA), Amersco (USA), or Bangalore Genei (India). Kits for p53 antibodies, NFκB, IL-8, Gelatinases and their tissue inhibitors were purchased from Pharmacell (France), Imgenex (Sandeigo. CA), R&D systems (USA) respectively. Semi dry unit and Kits for western blot analysis were procured from GE health care (USA). Gel Documentation System (Bio-Rad) was used for gel scanning. Spectrophotometric assays were performed using Beckman DU-640 spectrophotometer (USA). ELISA plates were read on ELISA reader (Labsystem Multiscan Spectrum, USA).

Statistical Analysis:

The statistical significance of the data was determined using SPSS software [Version 10.0]. The data was represented as Mean $\pm$ S.E.M and were analyzed using student's 't' test and paired 't' test. ROC curve analysis was done to evaluate the discriminatory efficacy of the biomarker between two groups under study. Univariate and multivariate analysis was performed to

evaluate the association of biomarkers with the clinico-pathological parameters in oral cancer patients. The Bivariate correlations like Spearmans rho and Pearsons coefficient was also done to find correlation between biomarkers. "p" values <0.05 were considered as statistically significant.

3.3 METHODS:

Highly specific and sensitive methods were used for the analysis of parameters from blood and tissues samples of oral cancer patients as tabulated in **Table-3.4.**

3.A. Spectrophotometric methods

3.A.1. Serum/plasma total protein estimation [Wootton IDP, 1964].

Principle: The di-peptide bond or the –CO-NH- groups of proteins form a purple colored complex with copper ions in an alkaline medium. The purple colored complex formed is of varied intensity and depends upon the concentration of the total protein present in the biological sample.

Reagents:

Biuret reagent [Sodium Hydroxide+ Potassium Iodide + Potassium Sodium Tartarate+ Copper Sulphate]

Assay Procedure: 2.9 ml of distilled water and 100 µl of serum/ plasma) were added to the labeled test tubes followed by thorough mixing. 5.0 ml of Biuret reagent was added to the test tubes that were incubated at 37°C for 10 mins. The blue color developed in the solution was read at 540 nm using reagent blank. Bovine serum albumin was used as standard.

Table-3.4 : Methods used for analysis of parameters from blood and tissue samples of oral cancer patients

No.	Parameters	Methods	Detection
Tissue analysis (Malignant And Adjacent Normal)			
1.	NFκB p65	Western Hybridization Sandwich ELISA	Chemiluminescence, ELISA reader
2.	iNOS	Western Hybridization	Chemiluminescence
3.	Bcl-2	Western Hybridization	Chemiluminescence
4.	Bax	Western Hybridization	Chemiluminescence
5.	HSP-70	Western Hybridization	Chemiluminescence
6.	MMP-2	Gelatin substrate	Zymography
7.	MMP-9	Gelatin substrate	Zymography
Blood analysis (serum and plasma)			
8.	TP	Biuret and Lowry	Spectrophotometric
9.	TSA	Modified Thio-Barbituric acid method	Spectrophotometric
10.	LSA	Resorcinol reagent	Spectrophotometric
11.	Hexose	Orcinol reagent	Spectrophotometric
12.	Mucoid Protein	Lowry (FC Reagent)	Spectrophotometric
13.	GST	Kinetic assay	Spectrophotometric
14.	GR	Kinetic assay	Spectrophotometric
15.	SOD	Kinetic assay	Spectrophotometric
16.	CAT	Kinetic assay	Spectrophotometric
17.	Thiol	Ellmen's method	Spectrophotometric
18.	IL-8	Sandwich ELISA	ELISA reader
19.	p53 antibodies	Sandwich ELISA	ELISA reader
20.	MMP-2	Sandwich ELISA	ELISA reader
21.	MMP-9	Sandwich ELISA	ELISA reader
22.	TIMP-1	Sandwich ELISA	ELISA reader
23.	TIMP-2	Sandwich ELISA	ELISA reader

3.A.2. Tissue total protein estimation

Total proteins from tissue cytosols and nuclear extracts were measured using **Lowry OH et al.**, 1951

Principle: Proteins react with Folin – Ciocalteau (FC) Phenol reagent to give a dark blue colored complex. The color so formed is due to the reaction of alkaline copper with the proteins and reduction of phosphomolybdate by aromatic acids. The intensity of color depends on the amount of aromatic amino acids in the sample. O.D. is measured at 750 nm.

Reagents:

1.**Reagent A**: Alkaline Sodium Carbonate (2% Na_2CO_3 in 0.1 N NaOH)

2.**Reagent B:** Freshly prepared (0.5% $CuSO_4$ in 1% Na-K Tartarate)

3.**Reagent C**: 50ml of Reagent A + 1ml Reagent B was mixed with constant stirring avoiding precipitate formation.

4.**Folin Ciocalteau Phenol Reagent** (FC Reagent)**:** It contains solution of Sodium-tungstate and sodium-molybdate in phosphoric and hydrochloric acid solution. Commercially available reagent which is stored at 4^0C was diluted with equal volume of distilled water just before use.

5. **Standard solution:** 2% Bovine serum albumin (BSA) solution.

Procedure: 5µl of tissue extract was incubated for 10 mins with 2.25 ml of reagent-C at room temperature (RT). Then freshly prepared 0.25 ml of FC Reagent was added drop wise on constant stirring and incubated at RT for 30 mins. The resultant blue colored complex formed was read at 750 nm. A standard graph was plotted using a series of standards prepared from known amount of BSA concentrations.

3.A.3. Serum total sialic acid estimation

Principle: The periodate is basically used to determine the extent of branching by oxidatively cleaving the polysaccharides. Hydrolysis of the glycoprotein and release of end groups like sialic acid occurs by boiling with dilute H_2SO_4, which releases monosaccharides from polysaccharide chains. Trichloro acetic acid (TCA) precipitates the proteins that are separated out by centrifugation leaving behind TSA layer in hydrolysed serum. Released TSA reacts with Periodic acid and gets oxidized [–OH (alcoholic) group of Sialic acid to –CHO (aldehyde) group of formyl pyruvic acid]. Addition of thio-barbituric acid (TBA) to this mixture, causes the formation of non-fading pink colored chromophore. The intensity of the pink chromophore is intensified by addition of Dimethyl sulphoxide (DMSO) after cooling which is measured spectrophotometrically at 549 nm [Skoza & Mohos modified by Warren, 1976].

Reagents:

1. 0.85% Saline (NaCl)
2. 1N H_2SO_4 Sulfuric acid
3. 0.025 N Periodic acid in 0.125N H_2SO_4 (HIO_4 $2H_2O$)
4. 10% Trichloroacetic acid (TCA)
5. 2% Sodium arsenite in 0.5N HCl
6. 6% Thiobarbituric acid (TBA), pH 10.0 was adjusted with 10N NaOH
7. Dimethyl sulfoxide (DMSO)

Assay Procedure:

0.1ml of serum samples were incubated with 0.8ml normal saline and 0.1ml 1N H_2SO_4 at 80^0 C for 1 hour to hydrolyze the glycopeptide bonds. After cooling, 10% TCA was added to precipitate the proteins, which were cleared off by centrifugation at 25000 rpm for 10 mins. The resultant supernatant was used for analysis of total sialic acid. 0.1ml supernatant was mixed and incubated at 37^0C for 30 mins with 0.4ml distilled water and 0.25ml Periodic acid to oxidize the sugar components. Reaction was stopped immediately

using 0.2ml 2% sodium arsenite. 0.5ml of thiobarbituric acid (TBA) was added to mixture and kept in boiling water bath for 7.5mins to develop color. The solution was cooled and 1.5ml DMSO was added to intensify the color development. To eliminate the interference of 2-deoxyribose, the absorbance of the sample was read at 549 nm and 532 nm spectrophotometrically. N-acetyl neuraminic acid (NANA) was used as standard.

3.A.4. Serum lipid associated sialic acid (LSA) estimation

Principle: When a sample is treated with the LASA separating agent comprising a polar solvent, various components in the sample such as protein-bound sialic acid and proteinous substances precipitate while the desired LASA remains in the supernatent. Serum Gangliosides were treated with chilled chloroform: methanol (2:1) mixture and extracted in aqueous phase followed by precipitation with phosphotungstic acid (PTA). The treatment of precipitates with resorcinol reagent gives a blue colored complex which is read at 580 nm and gives concentration directly proportional to amount of LASA in the serum samples. This method was followed according to Katopodis and Stock, 1982.

REAGENTS:

1. Chilled Chloroform: methanol (2:1, v/v)
2. 10% Phosphotungstic acid (PTA) 1gm/ml
3. Resorcinol Reagent: 2% resorcinol, 0.1M Copper Sulphate ($CuSO_4$) in conc.HCl
4. Chilled n-Butyl acetate: butanol (85:15)

Procedure:

50µl of serum sample was mixed with chilled distilled water and kept on ice. 5ml of Chilled Chloroform: methanol (2:1, v/v) was added to form admixture and vortexed for 30 secs to extract lipid associated sialic acid (LASA) present in the sample. After addition of chilled distilled water (0.5ml) and centrifugation at 25000 rpm for 10mins, the resultant clear supernatant (upper organic phase containing LASA) is then incubated with 50µl of

phosphotungstic acid for 5 mins at RT to precipitate the proteins. The precipitates were cleared off after centrifugation again at 25000 rpm for 10 mins. The pellet was dissolved in 1ml of distilled water and reacted with freshly prepared 1ml of Resorcinol reagent in boiling water bath (BWB) for 15 mins to give a colored complex. After cooling in ice mug for 10 mins, 3ml of chilled n-butyl acetate: butanol mix was added and vortexed to yield blue colored complex (upper organic phase) whose intensity was read at 580 nm. NANA was used as standard.

3.A.5. Serum Hexose and Mucoid protein content of Seromucoid fraction by Spectrophotometric method:

Serum Hexoses and Mucoid protein content of seromucoid fraction was estimated spectrophotometrically using Orcinol method [Winzler *et al.*, 1955]. Seromucoid, the perchloric acid soluble and phosphotungstic acid precipitable fraction, was isolated according to Winzler method [1955]. Hexose concentrations in the fraction will be estimated using orcinol-sulphuric acid reagent [Winzler, 1955]. Mucoid proteins will be quantitated according to Hartee [1972].

Principle: Seromucoid, the perchloric acid soluble and phosphotungstic acid precipitable fraction, was isolated and Hexose content of SMF is measured by reacting with Orcinol-H_2SO_4 reagent. The intensity of the colored complex formed is directly proportional to hexoses present in the samples and read at 540 nm. The peptide bonds in the mucoid proteins react with Folin – Ciocalteau (FC) Phenol reagent to give a blue colored complex in alkaline conditions and read at 750 nm.

Reagents:

1. 0.85% NaCl
2. 1.8M Perchloric acid ($HClO_4$)
3. 5% Phosphotungstic acid (PTA)
4. 0.1N NaOH
5. 95% Ethanol

6. Orcinol-H_2SO_4 reagent: 1 part of 1.6% Orcinol solution was mixed with 7.5 parts of cold H_2SO_4: H_2O (3:2) solution with constant stirring.

Procedure:

0.5ml of serum sample was mixed with 4.5ml of normal saline solution in a test tube. 2.5ml of perchloric acid was added drop wise on constant stirring. The sample mixture was then filtered through whatmann filter no. 44 within 10 mins. 5ml of the filtrate was then precipitated using 1ml of 5% PTA with an incubation period of 20 mins. The mixture was centrifuged at 3000rpm for 10mins. Resultant white pellet was washed in 1ml of 95% ethanol and finally dissolved in 1ml of 0.1N NaOH solution. The residual solution served as seromucoid fraction, which was then estimated for hexoses and mucoid protein content as follows.

Hexoses estimation: 0.5ml of above solution was incubated with 5ml of freshly prepared Orcinol-H_2SO_4 reagent in BWB for 15 mins. After cooling at RT, the intensity of the colored complex formed was read at 540 nm.

Mucoid protein estimation: 50µl of SMF (diluted in ratio 1:9 with D.D.W) was incubated for 10 mins with 2.25 ml of reagent-C at RT Then freshly prepared 0.25 ml of FC Reagent was added drop wise on constant stirring and incubated at RT for 30 mins. The resultant blue colored complex formed was read at 750 nm. Hexoses (Galactose + Mannose) and BSA were used as standards.

3.A.6 Estimation of plasma Glutathione S- transferase

Principle: GST conjugated with GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) gives a complex, (S-2, 4-dinitrophenyl glutathione) which was spectrophotometrically measured as increase in absorbance at 340 nm [Habig and Jacoby, 1981].

Reagents:

1. Sodium Phosphate buffer (0.1 M), containing 1mM EDTA, pH 6.5
2. 1-Chloro-2,4-dinitrobenzene (CDNB) (40 mM), freshly prepared and dissolved in 95 % ethanol
3. Reduced Glutathine(GSH) (40 mM), freshly prepared in D.W.

Enzyme Unit: One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 µM of S -2, 4-dinitrophenyl glutathione per minute, using 1 mM GSH and CDNB i.e. the amount of product formed was calculated using the molar extinction coefficient of the product at 340 nm and the enzyme activity was expressed as nmole product formed /min/ L (for plasma) or mg of protein (for tissue) or gm of Hb (for whole blood).

Procedure:

	Blank	**Reagent Blank**	**Sample Blank**	**Sample**
Buffer	1 ml	0.95 ml	0.95 ml	0.90 ml
Sample	-	-	0.05 ml	0.05 ml
Incubated at 37^0C for 30 mins.				
GSH	-	0.025 ml	-	0.025 ml
CDNB	-	0.025 ml	-	0.025 ml
Immediately read at 340 nm at every 1 min for 3 mins				
Calculated Δ O.D/min. of test and subtract from reagent blank.				

Calculation: Plasma GST activities were expressed as U/L.

3.A.7 Estimation of plasma Glutathione reductase (GR)

Principle: Glutathione reductase reduces oxidized glutathione (GSSG) into reduced glutathione (GSH) by oxidation of NADPH, which was measured spectrophotometrically by decrease in absorbance at 340 nm [Carlberg and Mannervik, 1985].

Reagents:

1. Potassium phosphate buffer (0.2 M), containing 2 mM EDTA, pH 6.5

2. Oxidized glutathione (GSSG) (40 mM), freshly prepared and dissolved in D.W.
3. Sodium salt of reduced nicotinamide adenine dinuleotide phosphate NADPH-Na_4 (4 mM) in 10 mM Tris-HCl buffer, pH 7.0 freshly prepared.

Enzyme Unit: One unit of enzyme activity was defined as the amount of enzyme that catalysed one micromole of NADPH in one minute by oxidation of NADPH. The decrease in absorbance was measured.

Procedure:

	Blank	**Reagent Blank**	**Sample Blank**	**Sample**
Buffer	1 ml	0.95 ml	0.95 ml	0.90 ml
Sample	-	-	0.05 ml	0.05 ml
Incubated at 37^0C for 30 mins.				
GSSG	-	0.025 ml	-	0.025 ml
NADPH-Na_4	-	0.025 ml	-	0.025 ml
Immediately read O.D. at 340 nm at every 1 min for 3 mins Calculated Δ O.D/min. of test and subtract from reagent blank.				

Calculation: Plasma GR activities were expressed as U/L.

3.A.8 Estimation of RBC Super-oxide dismutase (SOD)

Principle: SOD activity was estimated from erythrocytes as suggested by Kakkar *et al* (1984). The reduction of nitro-blue by NADH was enhanced by PMS indicating that PMS acts as an electron carrier in the system. Ability of SOD to inhibit the PMS mediated aerobic reduction of NBT by monitoring 50% of the maximum inhibition of color development spectrophotometrically at 560 nm

Reagents:

1. 0.052M Sodium phosphate buffer, pH 8.3
2. 186 µM Phenazonium methosulphate (PMS)
3. 300 µM Nitroblue tetrazolium (NBT)
4. 780 µM NADH Disodium salt

Enzyme Unit: One unit of the enzyme activity was defined as enzyme concentration required to inhibit optical density at 560 nm of chromogen produced by 50% in one minute under the assay conditions.

Procedure:

	Blank	Control	Sample Blank	Sample
Buffer	1.5 ml	1.2 ml	1.45 ml	1.15 ml
Sample	-	-	0.05 ml	0.05 ml
186 µM PMS	-	0.05 ml	-	0.05 ml
300 µM NBT	-	0.150 ml	-	0.150 ml
780 µM NADH	-	0.1 ml	-	0.1 ml
Vortexed, Read O.D. at 560 nm after 90 secs.				

Calculation: Erythrocyte SOD activities were expressed as U/gm hemoglobin (Hb).

3.A.9 Estimation of RBC Catalase:

Principle: Catalase activity was measured in terms of decomposition of H_2O_2 into water molecule, which was measured as decrease in absorbance at 240 nm [Aebi, 1984].

Reagents:

1. 50 mM Sodium phosphate buffer, pH 7.0
2. 30 mM H_2O_2, freshly prepared and dissolved in phosphate buffer

Enzyme Unit: 1 µm of H_2O_2 converted into H_2O in one minute was defined as one unit of enzyme activity.

Procedure:

	Blank	Reagent Blank	Sample Blank	Sample
Buffer	1.5 ml	1 ml	1.5 ml	1 ml
Sample	-	-	2 µl	2 µl
30 mM H_2O_2	-	0.5 ml	-	0.5 ml
Read the reagent blank against blank and sample against sample blank. Noted the decrease in O.D. at 240 nm at every 15 secs for 2 mins.				
Calculated Δ O.D./15 secs				

Calculation: Erythrocyte catalase activities were expressed as U/gm Hb.

3.A.10 Estimation of Plasma Thiol:

Principle: 5, 5'-dithiobis-2-nitobenzoic acid (DTNB) also known as Ellemen's reagent reacts with thiol compounds (-SH groups) to give yellow colored complex, which is measured at 520 nm [Ellman, 1959]

Reagents

1. 0.2M Na_2HPO_4 containing 2mM EDTA
2. GSH standard: Stock standard = 0.003 gm / 10 ml (1 mmole)
3. 10 mM 5, 5'-dithiobis-2-nitobenzoic acid (DTNB) in water

Procedure:

	Reagent Blank	**Sample**	**Sample Blank**	**Standard** 0-10 µ mole
0.2M Na_2HPO_4	1 ml	0.975 ml	0.995 ml	0.975 ml
Sample/standard	-	0.025ml	0.025ml	0.025 ml
10 mM DTNB	0.02 ml	0.02 ml	-	0.02 ml
Vortexed and waited for 5 mins. Read at 412 nm against reagent blank				

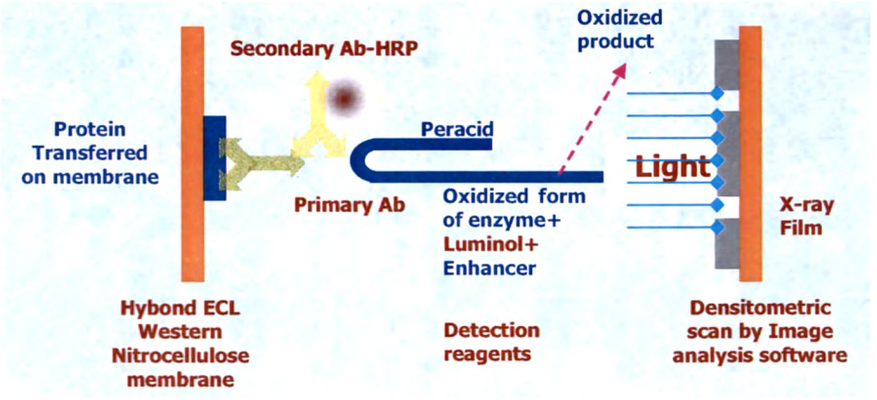
Calculation: Plasma thiol values were expressed as mmole/dl.

3.B. EXPRESSION OF PROTEINS BY WESTERN BLOT METHOD

Expression of NFκB p65, Bcl-2, Bax, iNOS and HSP-70 was studied from malignant and adjacent normal oral tissues using Enhanced Chemiluminescence (ECL) western blotting method [Towbin *et al.*, 1979, Burnette 1981].

Principle: It is a light emitting non-radioactive method used for detection of immobilized specific antigens (proteins), indirectly by Horseradish Peroxidase (HRP) labeled secondary antibodies. HRP/Hydrogen peroxide catalyzes oxidation of luminol under alkaline conditions in presence of chemical enhancers like phenols. Luminol gets excited and emits light at 428 nm during its decay to the ground state which has the effect on increasing the light output several folds with enhanced emission that is then captured on light sensitive X-ray films. The schematic representation of ECL western blot method is depicted in **Figure-3.1.**

Figure-3.1: Principle of ECL Western blot method



Antibodies used for the western blot were as follows: All antibodies were stored in lyophilized form at –20°C and their respective dilutions for working assay are tabulated in **Table-3.5**.

Table-3.5: Antibodies used for the western blot assay

Protein	Antibody	Working Dilution	Company
NFκB	Rabbit (monoclonal) Anti-human NFκB p65	2μg/ml in TBS	Calbiochem
INOS	Mouse (monoclonal) Anti-human iNOS	1μg/ml in TBS	R & D Systems
Bcl-2	Mouse (monoclonal) Anti-human Bcl-2	1μg/ml in TBS	R & D Systems
Bax	Mouse monoclonal anti-Bax antibody	1μg/ml in TBS	R & D Systems
HSP-70	Mouse monoclonal anti-HSP-70	1μg/ml in TBS	R & D Systems

Reagents and Materials:

1. **Acrylamide: Bis-acrylamide**: 29.19gm of Acrylamide and 0.81 gm N-N′ methylene bis-acrylamide was dissolved in double distilled water and made up the volume to 100ml.
2. **Ammonium persulphate**: 0.1 gm/ml freshly prepared solution)
3. **N, N, N′, N′ Tetramethylene diamine** (TEMED): Ready to use
4. **Saturated butanol**
5. **Resolving gel buffer**: [1.5M Tris SDS pH 8.8]; stored at 4^0C
   Prepared 1.5 M Tris and adjusted pH to 8.8 with conc.HCl followed by addition of 0.4% SDS to the buffer.
6. **Stacking gel buffer**: [1.0M Tris SDS pH 6.8]; stored at 4^0C

Prepared 1 M Tris and adjusted pH to 6.8 with conc.HCl followed by addition of 0.8% SDS detergent to the buffer.

7. **5X Electrode buffer:** Dissolved 250 mM of Tris base in double distilled water and adjusted pH to 8.3 by adding 192 mM of Glycine. Made up the volume to 1 litre with distilled water.
8. **5X Sample loading Buffer**: The buffer was prepared as follows:

Tris HCL 0.25 M	0.6057 gm
Glycerol 10%	2.0 ml
Bromophenol Blue (BPB) 0.05 %	0.01 gm
SDS 6%	1.2 gm
Dithiothreitol 20mM	0.06168 gm

9. **Lysis buffer:** (PBS, pH 7.5 containing 1%Triton-X 100) The buffer was prepared as follows:

Na_2HPO_4 80mM	5.844 gm
$NaH_2PO_4.2H_20$ 20mM	3.1202 gm
NaCl 100mM	11.3568 gm
Triton-X 100 1%	1ml
PMSF 200mM	100µl

10. **PMSF**: used as protease inhibitor.
11. **Towbin buffer**: [0.025M Tris-Glycine, Methanol buffer SDS, pH 8.2to 8.4]. Used for transferring the proteins from gel to membrane.
12. **Blotting membrane:** Amersham Hybond™ C extra ECL positively charged Nitrocellulose Membrane (0.45 µm).
13. **Blotter papers**
14. **Ponceau S stain**: 30ml of Ponceau S solution was constituted in double distilled water following kit instructions.
15. **ECL western blotting kit:** This kit was used for Immunodetection of target proteins of interest and was purchased from Amersham Pharmacia Biotech (U.K). It contains blocking reagent, antibodies, detection reagents and all are stored at 4^0C.
16. **Blocking buffer:** 5% powdered non-fat dry milk prepared in PBS-Tween 20, pH 7.4

17. **HRP conjugated whole IgG antibody**: It has two types of Horseradish Peroxidase (HRP) linked secondary antibodies; one is anti-mouse HRP conjugated IgG antibody and the other is anti-rabbit HRP conjugated IgG antibody. Both the secondary antibodies were constituted in 1:1000/2000with PBST.
18. **Detection reagents**: Solutions 1 and 2 were mixed in dark in 1:1 ratio containing chemiluminescence substrate for HRP enzyme.
19. **5X Stripping buffer**: [62.5mM Tris-HCl buffer, β–mercaptoethanol, SDS pH 6.7]; stored at RT stripping buffer was used for stripping primary and secondary antibodies bound to membrane. 62.5mM Tris was prepared and adjust pH to 6.7 with conc.HCl followed by addition of 2% SDS and 100 mM β–mercaptoethanol to the buffer. For working solution of 1X, 4 ml of stripping buffer was diluted to 20 ml of double distilled water.

Following steps were followed for Western blot assay:

1. *Electrophoresis and Transfer* of target proteins. Resolving of different target proteins by subjecting to electrophoresis on appropriate percentage of SDS-PAGE on gel. Electro transfer of separated target proteins from the gel on to a positively charged nitrocellulose membranes by semi-wet blot method.
2. *Incubation with primary and secondary antibodies* of the target proteins to tag them with specific antibodies conjugated with the enzyme.
3. *Immunodetection and visualization* of the signal on the X-ray films generated by labeled enzyme using the ECL kit.
4. *Scanning and quantification* of the blots by gel documentation system (Bio-Rad).
5. *Stripping and Reprobing of membrane* to detect other proteins by removing primary and secondary antibodies for earlier proteins probed on the membrane.

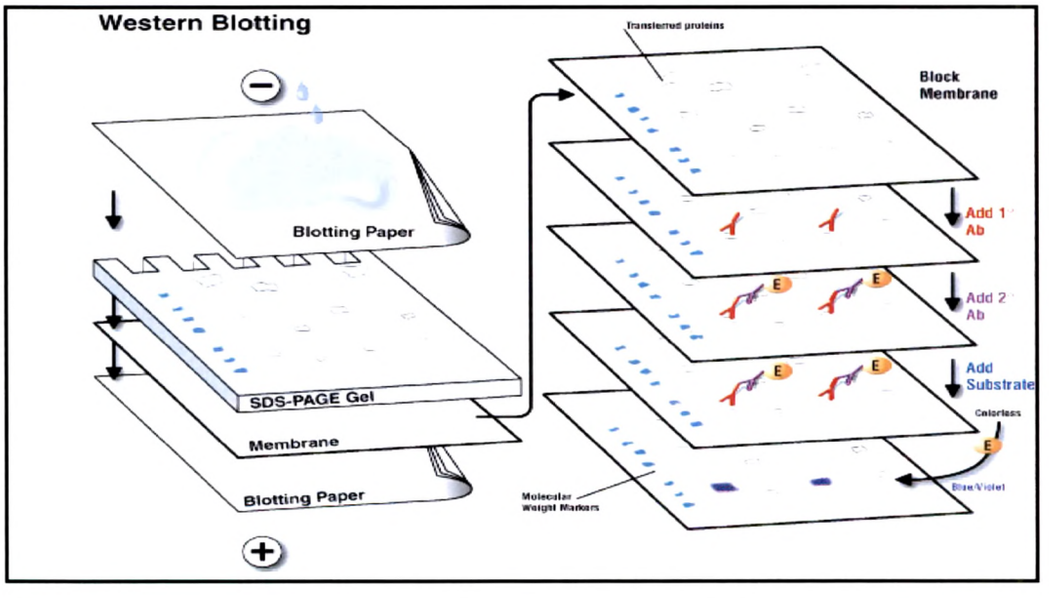
Assay procedure

1ST DAY: 1. Tissue samples were pre-incubated by mixing the microlitre equivalent to 50μg of cytosolic as well as nuclear lysates with 2X SDS-sample buffer for 30 mins at RT and for 5 mins. The proteins of interest were then resolved on SDS-PAGE gel of varied concentrations as summarized below.

Protein	**NFκB**	**iNOS**	**Bcl-2**	**Bax**	**HSP-70**
Resolving gel	8%	8%	12%	12%	8%
Stacking gel	5%	5%	5%	5%	5%

Next the resolved proteins on the gels were then electro-transferred onto a positively charged nitrocellulose membranes by semi-dry transfer method. A stack was prepared by using presoaked gel, nitrocellulose membrane, Whatmann filter papers and rough filter papers after equilibration in ice-cold blotting Towbin buffer for atleast 10 mins as shown in **Figure-3.2**. The stack prepared was placed between the electrodes in the blotting unit and transfer was carried out at constant current (150 mA) for three hours.

Figure-3.2: Diagrammatic representation of western blot procedure



To prevent non-specific background binding of the primary antibody and /or secondary antibodies to the membrane, nitrocellulose membrane was blocked

in 5% blocking reagent in PBS-T, for 1 hr at RT followed with immediate 3 washes in cold blot wash buffer for 5 mins each.

Primary Antibody Incubation: Then the membranes were incubated with the respective primary antibodies (in PBS-T blot wash buffer) overnight at 4^0C on a shaker with gentle shaking as shown in the **Table-3.6.**

Table-3.6 Working dilutions of 1^0 and 2^0 antibodies for different proteins by western blot procedure.

Protein	**Primary antibody** (working dilution)	**HRP-Secondary antibody** (working dilution)
NFκB	0.5µg/ml in PBS-T	0.5µg/ml in PBS-T
INOS	2µg/ml in PBS-T	1µg/ml in PBS-T
Bcl-2	1µg/ml in PBS-T	1µg/ml in PBS-T
Bax	1µg/ml in TBS	0.5µg/ml in TBS
HSP-70	1µg/ml in PBS-T	1µg/ml in PBS-T

2^{ND} Day: Immunodetection of Proteins : The membranes were washed again in wash buffer for 3x5 mins each.

Secondary Antibody Incubation: Membrane was incubated for 1 hr at RT with HRP-Conjugated secondary antibody of appropriate dilution for respective proteins as shown in the **Table-3.6.**

Detection and visualization: After washing the membrane again for 4x5 mins, protein bands were finally visualized using the enhanced chemiluminescence (ECL) detection kits. Detection reagent-1 and reagent-2 (provided in the kit) containing the substrate for HRP were mixed in equal volume in the ratio (1:1) to give the developer solution. The nitrocellulose membrane with the protein side up was placed on a transparent sheet and the developer solution was poured over it and incubated maximum for 1 min at RT in dark. Then the luminescence captured on X-ray film was exposed at different intervals for a period of total 25 secs to 10 mins. The integrated optical density of the resulting bands on the scanned film was determined densitometrically using Bio-Rad gel documentation System. The proteins were quantified and represented as strong, moderate or poor presence on the basis of the band

intensity. The blots were washed and stored in PBS-T buffer at 4^0 C and reused for detection of other proteins after stripping procedure as follows.

Stripping and reprobing of membrane to detect other proteins:

If the proteins of interest can be resolved in the same percentage of gel, then electrophoresis can be omitted and membrane or the blot can be re-used for detecting proteins of other interest. The membrane was stripped by submerging it in 20ml of stripping buffer (1X, pH 6.7) and incubated at 50°C for 30 mins with gentle agitation occasionally for every 5-10 mins. After washing the blot atleast twice for 10 min with PBS-T, it was then blocked again for 1 hr at RT on shaker. This was followed by overnight incubation of blot in primary antibody and the detection procedure was repeated as mentioned above.

3.C. GELATINASES BY ZYMOGRAPHY ANALYSIS

Expression of gelatinases was studied from oral tissues using Gelatin zymography [Lorenzo *et al.*, 1992].

Principle: It is a functional assay that identifies gelatinases by the degradation of their preferred substrate and by their molecular weight. It is based on the ability of SDS denatured proteinases to refold them selves after SDS is removed. During zymography the latent protein unfolds due to SDS and the fully active protein thus formed cleaves the gelatin substrate impregnated in the gels as clear zones of white bands on a blue-stained background of the PAGE-gels.

REAGENTS:

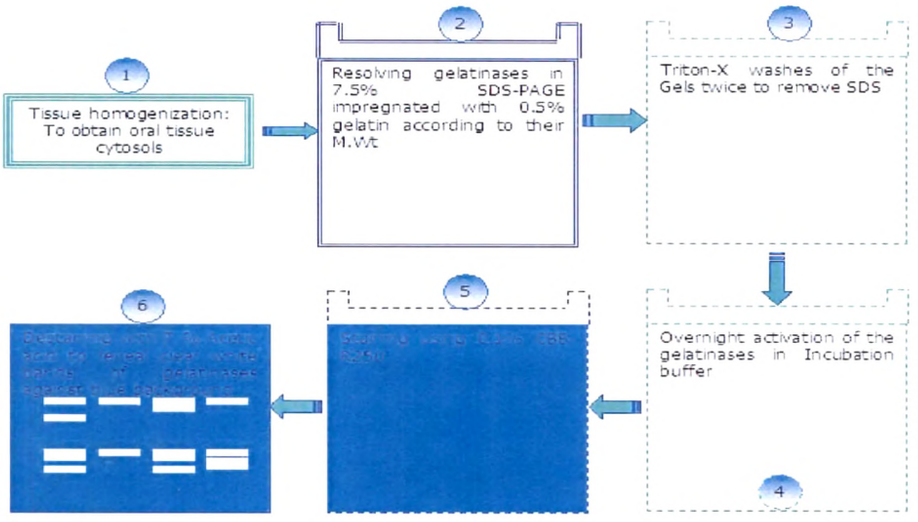
1. Acrylamide: Bis-acrylamide
2. Gelatin solution: 5mg/ml (0.5%) prepared freshly in DW.
3. Ammonium persulphate
4. TEMED
5. Saturated butanol
6. Resolving gel buffer: Prepared 1.5 M Tris (pH 8.8) followed by addition of 0.4% SDS to the buffer.

7. Stacking gel buffer: Prepared 1 M Tris and adjusted pH to 6.8 with conc.HCl followed by addition of 0.8% SDS detergent to the buffer.
8. Electrode buffer: Dissolved 250 mM of Tris base in triple distilled water and adjusted pH to 8.3 by adding 192mM of Glycine. Made up the volume to 1 liter.
9. Staining solution: 0.1% Coomassie Brilliant Blue R-250G
10. Destaining solution: 7% acetic acid in DW.
11. Homogenizing solution**:** Chilled phosphate buffer saline (5X pH7.5).
12. Activation Buffer: 50mM of Tris buffer (Tris-HCl, pH 7.5) containing 10mM $CaCl_2$, 1µM $ZnCl_2$, 1% V/V Triton X-100 and 0.02% NaN_3.

Procedure:

1. **Tissue homogenization**: The malignant and adjacent normal as well lymphnode positive and negative oral tissue samples were thawed on ice and homogenized using glass mortar and pastel. The homogenates were washed thrice in chilled phosphate-buffered saline (pH 7.4) at a concentration of 1 mg tissue/10µL PBS for 15-20 mins. The homogenates were placed on ice for 10 mins and then centrifuged at 15,000 rpm 30 mins at 4^0C. The supernatant was collected and stored in prechilled tubes at -80 °C until zymography assay was done as depicted from **Figure-3.3.**

Figure-3.3: Flow chart depicting the procedure of gelatin zymography



2. **Incubation of samples with loading buffer**: Tissue cytosolic fractions were pre-incubated by mixing 50µg of tissue proteins with an equal volume of 2XSDS sample buffer at RT for 30 mins. Addition of SDS to the samples neutralizes the charge of the gelatinases and also unfolds the proteins in its native primary structure.

3. **Electrophoresis of samples**: The samples were then subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and run at constant voltage (100 volt) for 3 hrs.
4. **Activation** of gelatinases: After electrophoresis, the gels were taken out gently from the glass plates and kept in washing buffer (0.25%Triton X-100) for 2x30 mins to remove the SDS. This was followed by overnight incubation of the gels at RT in activation buffer.
5. **Visualization:** The incubation buffer was decanted and gels were stained with 0.1%(w/v) Coomassie Brilliant Blue R-250G in 40% Propanol for 1 hour. After destaining, the gelatinase activity was detected as unstained white bands on a blue-stained background of the gels, indicating the proteolysis of the gelatin substrate.
6. **Quantitation of gelatinases:** These zymograms were scanned and the intensity of the bands was analyzed densitometrically using gel documentation system (Bio-Rad).

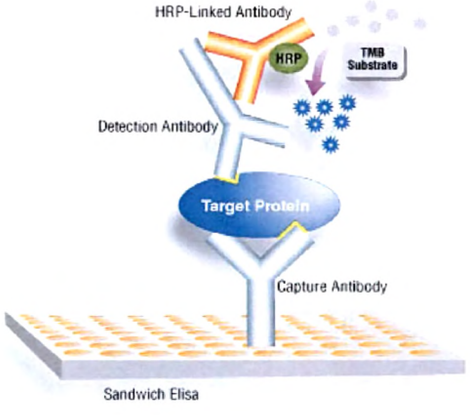
Standards: Quantitation was performed by construction of a standard curve using known concentrations of latent and active forms of purified, recombinant human MMP-2 and human MMP-9 standards.

Calculation: Both the latent and active forms of the gelatinases (MMP-2 and MMP-9) from the oral cancer tissues was calculated from the standard plots and was expressed as ng/50µg of standard protein. Activation ratio of MMP-2 and MMP-9 were calculated as the ratio of active form to that of total form of MMP-2 or MMP-9.

3.D. ELISA METHOD

Sandwich ELISA is used for the estimation of serum p53 Autoantibodies, serum IL-8, tissue NFκB p65, plasma MMP-2, MMP-9, TIMP-1 and TIMP-2 from oral cancer patients. All the kits are based on a solid phase Sandwich enzyme linked immunosorbent assay. A monoclonal antibody (MoAb) specific for target protein of interest (capture Ab) is coated on to the wells of microtitre strips provided for ELISA. Samples and standards of known target protein concentrations are pipetted into wells. During the 1st incubation, the samples with unknown amount of Ag and known standards were incubated. After washing, enzyme labeled usually HRP linked Antibody is added. After incubation, washing is done to remove all unbound enzyme, followed by addition of substrate solution of TMB. The bound enzyme acts and generates a colored reaction product. The intensity of color is directly proportional to amount of Ag present in the biological samples as depicted in **Figure-3.4.**

Figure-3.4
Pictorial presentation of principle of Sandwich ELISA method



3. D.1 SERUM p53 AUTOANTIBODIES

p53 autoantibodies were detected using an anti-p53 ELISA (Pharmacell, France) for the quantitative detection of anti-p53 IgG in human serum, according to the manufacturer's instructions.

Principle

p53 autoantibodies were detected by Enzyme linked Immuno Sorbent Assay using microtitre plates (MTP) coated with recombinant wild-type human p53

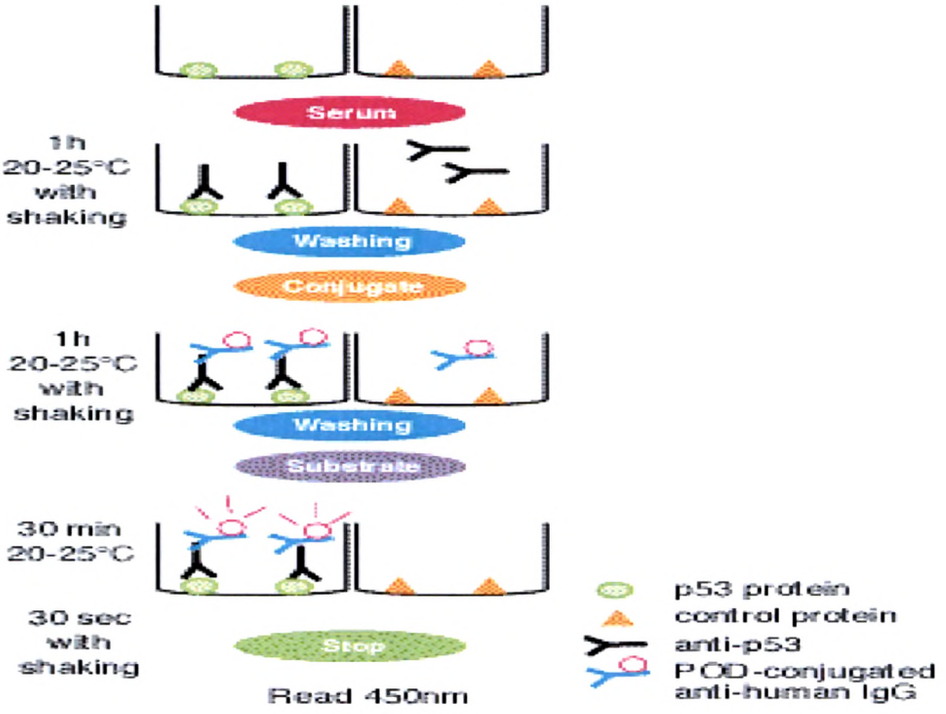
protein (to detect specific anti-p53 antibodies) or with control protein (to determine non-specific interactions). A peroxidase conjugated goat anti-human IgG binds anti-p53 and anti-p53/ conjugate complexes were revealed by addition of a peroxidase substrate (TMB) resulting in a colorimetric reaction which is read at 450 nm.

Reagents: All reagents provided in the kit were either lyophilized, concentrated, or in ready to use form. The kit was stored at 4^0C and the reagents were brought to RT before use and reconstituted following strictly to the manufacturer's instructions.

Procedure

Serum samples were analyzed after dilution of 1:100 with reagent diluent. 100µl of reagent diluent was added to the blank well. All samples and standards were analyzed in duplicate by adding 100 µl each per well and were assayed simultaneously in 2 distinct wells of MTP. The wells of the MTP was covered and incubated for 60 mins at 25°C with constant gentle shaking on a microtitre plate shaker. After washing the MTPs by adding 350µl of wash buffer atleast 4 times and aspirating the wells to remove the residual fluid, 100µl of diluted conjugate was added to each well and incubated for 60 mins at 25°C with gentle shaking. The plates were washed and aspirated again by adding wash buffer. Final color was developed by adding 100µl substrate solution per well, which was incubated for 30 mins in the dark. To stop the reaction, 100µl of stop solution was added onto each well with gentle shaking for 30 secs and absorbance was read within 10 mins at 450 nm with reference filter set at 620 nm. **Figure-3.5** outlines briefly the assay procedure for the detection of serum p53 autoantibodies.

Figure-3.5
Assay for Anti p53 antibodies by Sandwich ELISA.



Calculation: The average absorbance for each blank, standard and sample for wells coated with p53 protein and for wells coated with control proteins was calculated. The net absorbance, specific signal was determined abiding strictly to kit instructions as follows:

p53 Net absorbance = [Average absorbance of Samples / Standards (p53 wells)]– [Average absorbance blank (p53 wells)]

Control protein Net absorbance = [Average absorbance of Samples / Standards (control protein wells)] – [Average absorbance blank (control protein wells)]

Specific Signal = [p53 net absorbance] – [control protein net absorbance]

Unknown amount of p53 antibody levels was then determined using calibration curve plotted from the specific signals of absorbance against the levels of antibodies (1-5-10-15 U/ml) in p53 standard concentrations.

Interpretation of antip53 antibody levels

ANTI -P53 LEVELS	INTERPRETATION
<0.85 U/ml	Absence (Seronegative)
>=0.85 U/ml	Probable presence (Seropositive)
>=0.85 U/ml	Presence (Seropositive)

3.D.2. SERUM IL-8 BY ELISA METHOD

Serum IL-8 was detected using a DuoSet® human CXCL8/IL-8 (R & D Systems, Inc., MN, USA) for the quantitative detection of IL-8 in human serum, according to the manufacturer's instructions.

Principle: It is based on a solid phase Sandwich enzyme linked immunosorbent assay. A monoclonal antibody (MoAb) specific for IL-8 (capture Ab) is coated onto the wells of microtitre strips provided for ELISA. Samples and standards of known IL-8 concentrations are pipetted into wells. During the 1st incubation, the IL-8 Ag and biotinylated Ab are incubated. After washing, enzyme is added. After incubation, washing is done to remove all unbound enzyme, followed by addition of substrate solution, which acts, on a bound enzyme to induce a colored reaction product. The intensity of color at 450 nm is directly proportional to amount of IL-8 present in the samples.

Reagents: All reagents provided in the kit were either lyophilized, concentrated, or in ready to use form. These reagents were brought to RT before use and reconstituted following strictly to the manufacturer's instructions.

Procedure: Day-1: Freshly prepared diluted 100µL of capture antibody without carrier protein was added for coating the 96-well microtitre plate immediately and sealed. The plate was incubated overnight at RT.

Day-2: Assay procedure

All the wells of the coated 96-well microtitre plate were washed and aspirated with 400µL of wash Buffer. This step was repeated twice for a total of three washes. 300µL of block buffer was added to block each well and incubated at RT for 1 hour. After removing the blocking reagent, the microtitre plate wells were washed and aspirated thrice with washing buffer. 100µL of sample and standards prepared in reagent diluent were added and incubated at RT for 2 hrs. This step was repeated thrice and 100µL of the detection antibody diluted was added onto each well and incubated for 2 hrs at RT. After repeating the aspiration/wash step, 100µL of Streptavidin-HRP

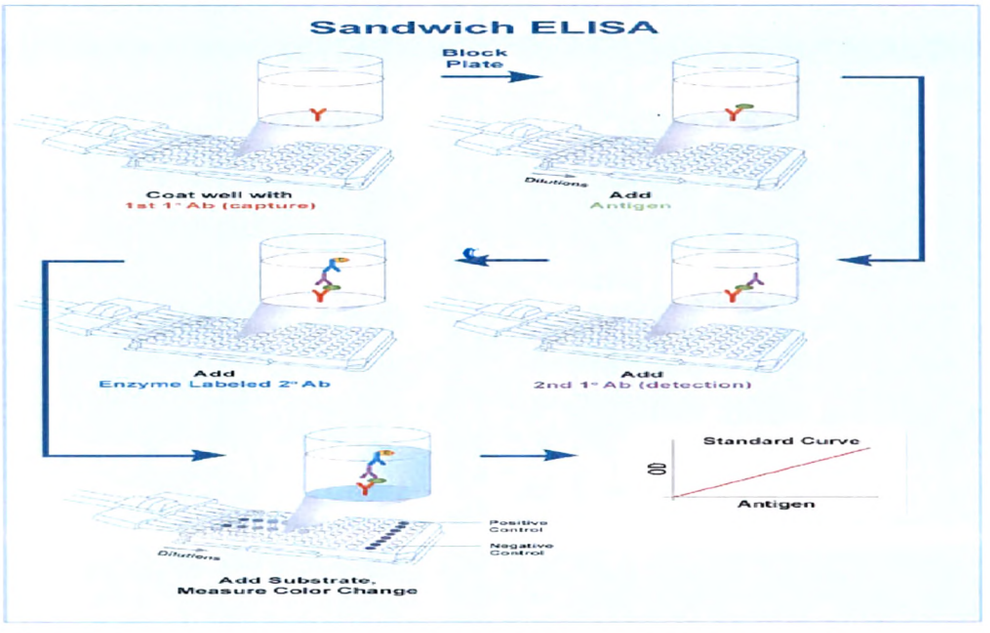
was added onto each well and incubated for 20 mins at RT. 100µL of freshly prepared substrate solution was added onto each well and incubated for 20 mins at RT. Exposure of the plate to direct light was avoided by wrapping it in a black plastic bag. 50µL of stop solution was added to each well and absorbance was read immediately using a microplate reader set to 450 nm.

Calculations: To correct the optical imperfections in the plate, microtitre plate was read at O.D 540 nm/570 nm. A calibration curve for various concentrations of IL-8 standards was plotted and IL-8 activity was calculated.

3.D.3 NFκB FROM TISSUE LYSATES

The NF-κB p65 translocation into the nucleus was measured using p65 ActivELISATM (IMGENEX, San Diego, CA) according to the manufacturer's protocol.

Figure-3.6
Schematic layout of Sandwich ELISA for active NFκB-p65



Principle: The NF-κB/p65 ActivELISATM Kit is based on sandwich ELISA that measures free p65 in the nucleus of cells or tissues. The anti-p65 antibody coated plate captures free p65 present in the nuclear lysates and the amount of bound p65 is detected by adding a second anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody using colorimetric detection in an ELISA plate reader.

Reagents: All reagents were provided in the kit and brought at RT before use.

Procedure: The entire procedure for the assay is shown in **Figure-3.6.**

Day-1: Coating of microtitre plate: Diluted 100µl of capture antibody in 10 ml of coating buffer was added into each well.

Day-2: Blocking of the plates: The coated wells were washed twice with 300µl of 1X wash buffer. 200µl of blocking buffer was added to each well and incubated for 1 hour at RT to block the remaining reactive surface.

Preparation of p65 standards: The standard solution of p65 was prepared and added in duplicate to the wells in the following concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 0.0 (blank) ng/ml.

Test samples: 100µl of positive, negative controls and 100µl of test samples were added into appropriate wells and incubated at 4°C overnight at RT.

Washing: Samples and control lysates were removed and all wells were washed four times with 300µl of 1X wash buffer. Plates were tapped several times upside down to remove residual wash buffer after final wash.

Detecting antibody: Diluted 100µl of detecting antibody in 10 ml of blocking buffer was added into each well and incubated for 1 h at RT. The antibody solution was removed from the wells and washed four times with 300µl of 1X wash Buffer.

Secondary antibody: 100µl of diluted AKP-conjugated secondary Ab was added to each well and incubated for 1 hr at RT. The secondary antibody was removed from the wells and washed thoroughly atleast five times with 300µl of wash Buffer. To ensure a thorough wash and lower background, between each wash the solution was allowed to stand and then discarded. 100µl of **pNPP substrate solution** was added into each well and incubated at RT for 30 min. The optical absorbance was read at 405 nm.

Calculation: A standard curve was plotted for concentration of NFκB p65 standard protein (ng/ml) against the absorbance at 405 nm and NFκB p65 in the samples was calculated.

3.D.4 ELISA FOR MMP-2, MMP-9, TIMP-1 and TIMP-2

Quantikine-Human MMP-2 (total), Quantikine-Human MMP-9 (total), Quantikine-Human TIMP-1 (total), Quantikine-Human TIMP-2 (total) Immunoassay [R & D Systems, Minneapolis, MN, USA] were used for the quantitative detection of total MMP-2, MMP-9, TIMP-1 and TIMP-2 in human plasma, according to the manufacturer's instructions.

Principle:

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for target antigen (MMP-2, MMP-9, TIMP-1 or TIMP-2) has been pre-coated onto a microtitre plate The immobilized antibody binds to specific target antigen present in the standards and samples, which are pipetted into the wells. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for target antigen is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of total (pro and/or active forms of either MMP-2, MMP-9, TIMP-1 or TIMP-2) bound in the initial step. The color development is stopped and the intensity of the color is measured.

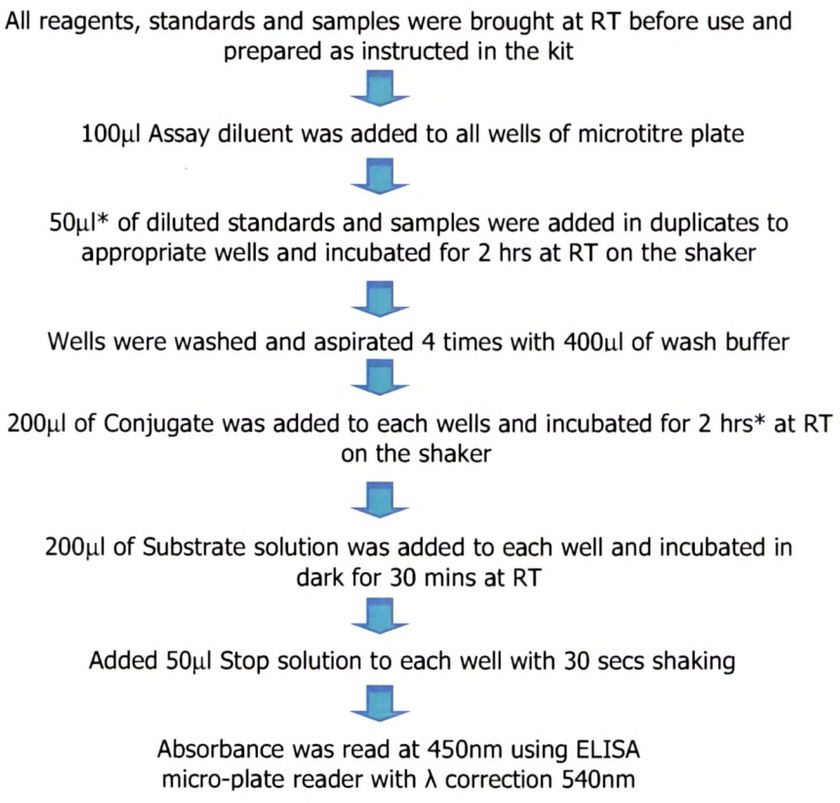
Reagents:

All reagents provided in the kit were either lyophilized or ready to use. Some of the reagents had preservatives and might get contaminated precipitated during storage. Care was taken to dissolve this precipitates by warming them at RT and mixed well before and during use at the time of assay procedure. All reagents, standards and samples were brought to room temperature before use and prepared abiding strictly with manufacturer's instruction. The procedure of the assay for detection of plasma total MMP-2, MMP-9, TIMP-1 and TIMP-2 is outlined briefly in the **Figure-3.8**.

Preparation of the standards for MMP-2, MMP-9, TIMP-1 and TIMP-2: The standards were reconstituted with gentle agitation with 1ml of DW to give a stock solution of 100ng/ml. 450µl of calibrator diluent concentrate was directly put into the tube containing 50µl of standard to give 10ng/ml

concentration. 250µl of calibrator diluent was added into the remaining tubes and 250µl of standard was then added in the first tube to give different dilutions as shown in **Figure-3.8**.

Figure-3.7
Schematic layout for the assay procedure of Quantikine assay of Sandwich ELISA



Addition of samples and standards: 100µl of assay diluent was added to each well, followed by addition of 50µl or 100µl of standard and samples (diluted in specific ratio with calibrator diluent), as shown in **Table- 3.7.**
The microtitre plates were covered with the adhesive strip provided in the kit and incubated for 2 hrs at RT on a horizontal orbital microplate shaker. All wells were aspirated and washed with 400µl of wash buffer. This step of washing was repeated three times for a total of three washes. The plate was inverted and blotted against clean paper towels. 200µl of TIMP-2 conjugate was then added to each well and incubated for 1 or 2 hrs at RT on the shaker. The plate was again aspirated and washed as mentioned earlier.

Figure-3.8: Pictorial presentation for Serial dilutions of MMP-2, MMP-9, TIMP-1 and TMP-2 standards

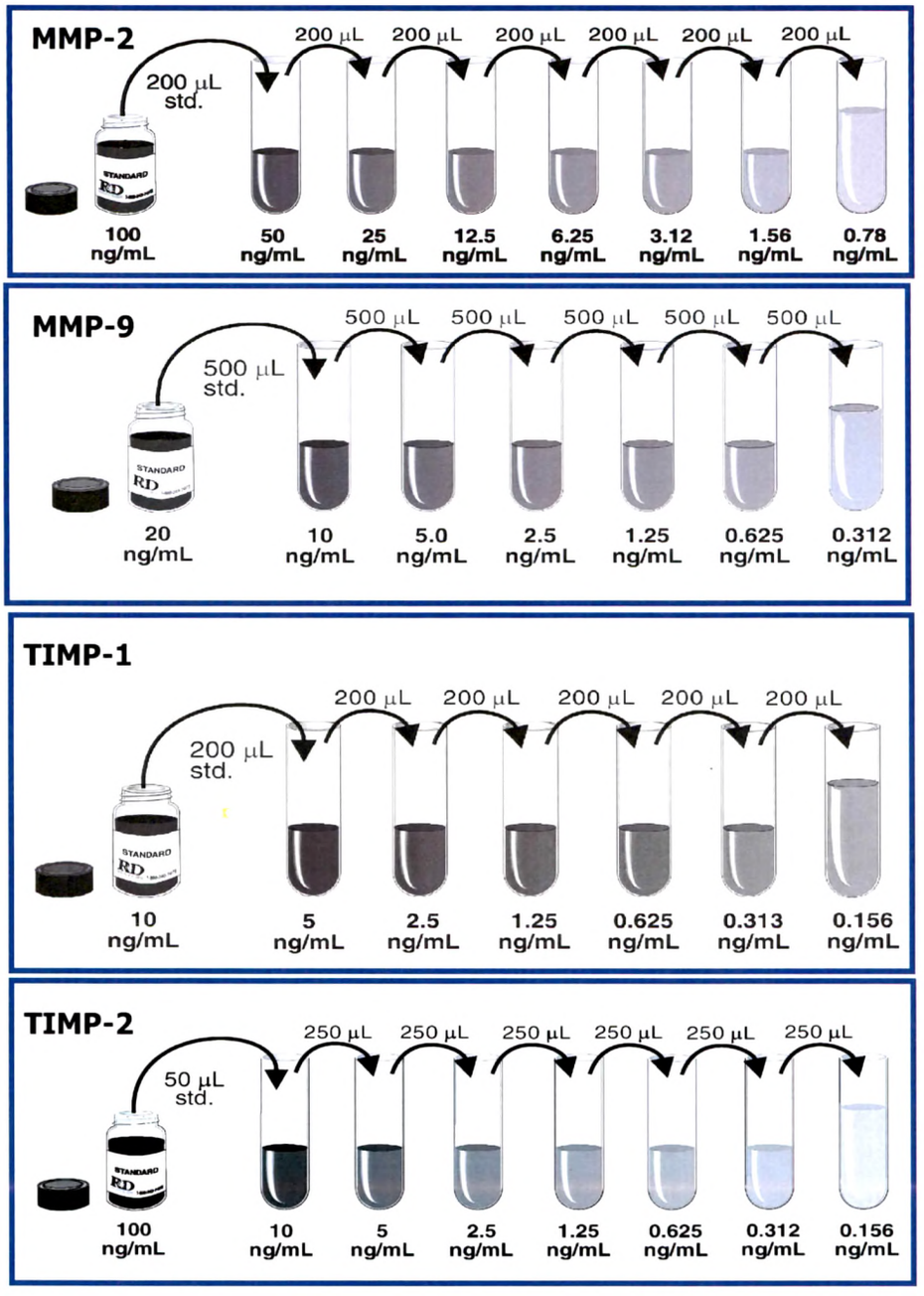


Table-3.7: The specified amount of reagents, standards and samples added in the ELISA

	Standard (Conc.& µl)	Samples (µl & dilution)	Conjugate Solution (µl)	Incubation at RT
MMP-2	50µl 100ng/ml	50µl 1:10	200µl	2 hrs
MMP-9	100µl 20ng/ml	100µl 1:40	200µl	2 hrs
TIMP-1	50µl 10ng/ml	50µl 1:100	200µl	2 hrs
TIMP-2	50µl 100ng/ml	50µl 1:50	200µl	2 hrs

Color reagents A and B were mixed in equal proportions of 1:1 to be used as substrate solution. 200µL of this substrate solution was added to each well and incubated for 30 mins in dark at RT protecting the plates from light. Finally 50µL of stop solution was added to each well and optical density at 450 nm using a microplate reader was taken within 30 mins. To correct the optical imperfections of the plate, the readings at 540 nm or 570 nm were subtracted from the readings at 450 nm.

Calculation

To calculate the plasma total MMP-2 MMP-9, TIMP-1 and TIMP-2, net absorbance for MMP-2, MMP-9, TIMP-1 and TIMP-2 was determined as per kit instructions. A calibration curve was plotted between concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 standards and optical density obtained from the absorbance for the standards.