

3.0 MATERIALS AND METHODS

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3.0 MATERIALS AND METHODS

3.1 SUBJECTS:

The present study included three populations: **(i)** age matched healthy individuals to serve as controls (different groups to compare with two different groups of cancer patients), **(ii)** patients with benign breast diseases (BBD) and patients with oral precancerous conditions (OPC) and **(iii)** cancer survivors. An informed consent to participate in the study was obtained from all the subjects. Pregnant or lactating females were not included in the study. Blood samples were collected between 9.0 and 11.0 A.M to avoid diurnal variations. Sera were separated and stored at -80° till analysed.

3.1.1 Controls:

Two hundred age and sex matched healthy individuals (100 per group) without any symptoms of disease as well as no major illness in recent past were included in study. They served as controls because disease can be illuminated by its contrast to the normal. As the development of cancer in various tissues is readily quickened by an increase in carcinogen stimulus, clinical characteristics that have direct causal association with cancer were gathered from the subjects.

3.1.2 Pathological Controls

To assess the diagnostic specificity of the markers, individuals who have increased vulnerability to cancer should be distinguished from those having cancer. Individuals having BBD or OPC have increased vulnerability to cancer and hence, were included in the study, to serve as pathological controls. The study included 100 patients with BBD and 100 patients with OPC. Among the patients with BBD, 60 females were having fibrocystic disease, 5 females had

benign ductal disease and 35 females had tumours, which was negative for malignancy. The patients with OPC included: **(i)** 46 patients with oral submucous fibrosis and **(ii)** 54 patients with oral leukoplakia. The clinical details of pathological controls are provided in **tables 2-3**.

Table – 2

CLINICAL DETAILS OF PATIENTS WITH BENIGN BREAST DISEASES

Age :	Range	-	17 – 67 years.
	Median	-	45.5 years
	< 45 years	-	68
	≥ 45 years	-	32
Site:	Left	-	64
	Right	-	32
	Bilateral	-	04
HPE:	Fibrocystic disease	-	60
	Benign ductal disease	-	05
	Negative for malignancy	-	35

Table - 3

**CLINICAL DETAILS OF PATIENTS WITH ORAL
PRECANCEROUS CONDITIONS**

<u>Group</u>	<u>Number of subjects</u>
Submucous fibrosis	46
Oral leukoplakia	54
Total	100

Age : Range - 17-67 years Median - 37.75 years

3.1.3 Cancer patients:

Four hundred twenty five histopathologically proven cancer patients were included in the study. Patients were not included in the study if they had previously received any anticancer therapy. Out of these, 225 patients were suffering from breast carcinoma and 200 patients were bearing tumours in oral cavity. Clinical stage of the disease were determined as per UICC norms (UICC, 1980). Blood samples were drawn from the patients prior to initiation of anticancer treatment. Clinical details of the cancer patients are provided in tables 4-5

Table 4

CLINICAL DETAILS OF BREAST CANCER PATIENTS

TNM Stage		I	II	III	IV	Total
No. of patients		2	48	115	60	225
Site :	Left	0	26	67	37	130
	Right	2	22	48	23	95
Menopausal status :	Pre	0	24	53	24	101
	Post	2	23	58	33	116
	Peri	0	1	4	3	8
Histology: Invasive/Infil. Ductal		2	43	100	51	196
Invasive infiltrating lobular		0	0	9	5	14
Others		0	5	6	4	15
Lymphnode Involvement:	Absent	0	25	82	35	142
	Present	2	23	33	2	60
	Unknown	0	0	0	23	23

Age : Range -17 – 74 years

Median – 46 years

Table 5

CLINICAL DETAIL OF ORAL CAVITY CANCER PATIENTS

		Number of subjects
TNM stage:	stage I	11
	stage II	47
	stage III	56
	stage IV	95
Histology:	Epidermoid carcinoma	194
	Others	16
Age Range:	14– 80 years	(Median age: 44.5 years)

3.1.2 Follow-up blood samples:

Successful implementation of a long-term disease free survival depends on the ability to identify those asymptomatic cancer patients who are at increased risk for development of recurrent/ metastatic disease. It is also important to identify patients with occult malignant disease. The prediction of response to the anticancer therapy, in cancer patients is very difficult. Therefore, identification of non-invasive and reproducible treatment monitors to evaluate disease status remains a key goal for neoplastic therapy. Hence, the patients were followed-up after initiation of anticancer therapy. First follow-up blood sample was collected at least one month after initiation of anticancer therapy. Thereafter, 1218 follow-up blood samples were collected during the patients' visit to the hospital. However, minimum one-month interval was kept between two follow-ups. Patients' response to therapy was evaluated on the basis of their clinical and radiological findings during follow-up period. Out of 1218 follow-up blood samples, 824 were from breast cancer patients (table-6) and **394** were from oral cavity cancer patients (table-7).

Table - 6
CLINICAL STATUS AND DURATION OF TREATMENT INITIATION IN
BREAST CANCER PATIENTS AT THE TIME OF FOLLOW-UP

Duration in months	Complete Responders	Non-responders	Total
1-6	240	95	335
6-12	138	67	205
12-24	119	75	194
24-36	34	11	45
36-48	15	09	24
48-60	10	05	15
60-72	03	03	06
Total	559	265	824

Table – 7
CLINICAL STATUS AND DURATION AFTER TREATMENT INITIATION
IN ORAL CAVITY CANCER PATIENTS AT THE TIME OF FOLLOW-UP

Duration in months	Complete Responders	Non-responders	Total
1-6	176	26	202
6-12	64	30	94
12-24	88	10	98
Total	328	66	394

Clinical status of the patients during/after anticancer treatment was evaluated as suggested by Miller et al, 1981. The patients were grouped as mentioned below:

Responders: Patients with disease free survival at-least for one month.

Partial Responders: Patients with decrease in tumour size by more than 50%.

Stable Disease: Patients with no objective treatment response i.e. no changes in tumour size for 3 months.

Progressive Disease: Patients having increase in tumour size and/or appearance of new malignant lesions.

The patients with partial response, stable disease and progressive disease were grouped together as **Non-responders** to simplify the comparisons of marker levels with disease status.

3.1.5 TISSUE COLLECTION

Tissue samples were collected on ice from operation theatre during surgical removal of the tumours. The normal tissues were selected by a pathologist from the free margin of the excised surgical specimens keeping 2-3 centimetres away from tumour margins, histopathological examinations were carried out from all the samples. The normal and tumour tissues were kept frozen at -80° after washing with normal saline.

3.2 CHEMICALS

All the standards and other fine chemicals were purchased either from Sigma Chemical Company, USA, Amersham Pharmacia Biotech, UK. Remaining chemicals, solvents and acids were of Guaranteed Grade or Analytical Grade obtained either from Glaxo, Merck, Sarabhai Chemicals or S.D. fine chemicals, India.

3.3 ASSAYS

3.3.1 Estimation of Sialic acid Sialic acid levels were analysed using method of Skoza and Mohos (1976) as modified by Warren (1978).

Oxidation of N-acetylneuraminic acid (NANA - sialic acid) was carried out by incubation of the samples with 0.25 ml 0.025N Periodic acid at 37°C for 30 minutes. Formylpyruvate was formed due to oxidation of N-acetyl neuraminic acid. The reaction was stopped by addition of 0.2 ml of 2% sodium arsenite to react with remaining periodic acid molecules. 1.5 ml of thiobarbituric acid reagent was added and mixture was kept in boiling water bath for 7.5 minutes. Salmon pink thio barbituric acid chromophore was obtained due to its reaction with formyl pyruvate. The stability of chromophore was increased by addition of 1.5 ml. dimethyl sulphoxide. Intensity of the chromophore was read at 549 nm.

To correct potential interference of 2-deoxy ~~de~~ribose, reaction mixture was also measured at 532 nm which is an absorption maxima of chromophore produced due to 2-deoxy deribose. To calculate sialic acid contents, calibration curves were constructed using NANA and 2-deoxy deribose separately as well as the mixture of both.

3.3.2 Estimation of Total Sialic acid (TSA)

To release bound sialic acid, serum samples were hydrolysed with equal volume of 1M H₂SO₄ at 80°C. Before hydrolysis, sera were diluted 9 time with normal saline. After hydrolysis, proteins were removed by addition of 2.0 ml 10% Trichloroacetic acid. Supernatant was used for determination of sialic acid contents.

3.3.2 Estimation of Protein-bound Sialic acid (PSA)

Perchloric acid soluble and phosphotungstic acid precipitable fractions were obtained as described by Winzler (1955). The sialic acid contents of the seromucoid fractions were estimated after hydrolysis at 80°C in the presence

of 0.1 N H₂SO₄. Sialic acid contents from the hydrolysate were estimated as per the method of TSA estimation.

Reagents:

- (1) 0.85gm % Sodium chloride (NaCl)
- (2) 1 N Sulphuric acid (H₂SO₄)
- (3) 10gm% Trichloro acetic acid.
- (4) 0.025M Periodic acid in 0.025 N H₂SO₄
- (5) 2.0gm% Sodium arsenite in 0.5 N HCl
- (6) 0.6gm % Thiobarbituric acid at pH 10.0
- (7) 2.0gm % N-acetyl Neuraminic acid(NANA)

3.3.4 Estimation of Protein bound Fucose

Protein bound fucose was estimated by employing the method of Dische and Shetles (1948) as modified by Winzler (1955). Bound fucose was released from complex carbohydrates and then converted into furfural derivatives by adding concentrated H₂SO₄. It was followed by boiling for 3 minutes. Free fucose on reaction with cystein^e produced yellow colour chromophore having optimum absorbance at 396 nm. Reaction mixture was also read at 430 nm simultaneously in order to correct the interference due to chromophores of other sugars at absorption maxima at 430 nm. Prior to fucose estimation, serum proteins were precipitated out by addition of 5.0 ml, 95% ethanol to 0.1 ml of serum followed by washing with 2.0 ml 95% ethanol. After centrifugation, the pellet obtained was resuspended into 2.0 ml of 0.2 N NaOH. 0.5 ml of aliquots was used for fucose estimation.

Reagents:

- (1) 95% Ethanol.
- (2) 0.2N Sodium hydroxide
- (3) 85.6% H₂SO₄
- (4) 3% Cystine HCl reagent
- (5) 0.5 mg % L(-) Fucose

3.3.5 Separation of Seromuroid Fraction

Seromuroid fraction was separated as described by Winzler (1955). 0.5 ml serum was diluted with 9 volume of normal saline. Proteins were then precipitated out by drop wise addition of 2.5 ml 1.8 M perchloric acid. It was filtered through Whatman filter paper (no. 44) and further precipitated out by

1.0 ml 5% phosphotungstic acid. The pellets obtained by centrifugation were washed with 95% ethanol and then dissolved in 0.1 N NaOH. It was used for estimation of protein (mucoïd proteins) and hexoses contents.

3.3.6 Estimation of Mucoïd Proteins

The protein content was analysed from seromucoïd fraction using the method of Lowry et al, (1951). In 0.1ml aliquot of seromucoïd fraction, 0.9 ml of distilled water and 5.0 ml of reagent mixture were added. The contents were vortexed and incubated at room temperature for 10 minutes. Then to each tube, 0.5 ml of Folin-phenol reagent was added. After mixing well, it was incubated for 30 minutes. The blue coloured complex formed due to the reaction of peptide bond with Cu^{++} under alkaline conditions, were read at 660 nm. In blank tube, 0.1 ml of 0.2 N NaOH was added instead of sample. The mucoïd protein concentrations were calculated by performing calibration curve using bovine serum albumin as standard.

Reagents:

- (1) Folins A : 2% Sodium carbonate in 0.1N NaOH.
- (2) Folins B : 0.5% CuSO_4 in 1% Na-K tartarate.
- (3) Mixture : 50 volume of folin A was mixed with 1 volume of folin B.
- (4) Folin phenol reagent 1:1 diluted.
- (5) 1% Bovine Serum Albumin

3.3.7 Estimation of Hexoses

Hexoses content of seromucoïd fraction were estimated according to the method of Winzler (1955). 0.1 ml of seromucoïd fraction was used for estimation of hexose contents. Keto-hexoses on treatment with hydrochloric acid formed furfural derivatives which on condensation with resorcinol yielded red coloured compound. The red coloured chromophore obtained due to the reaction with freshly prepared orcinol reagent at boiling temperature were measured spectrophotometrically at 540 nm. The hexoses contents were calculated by plotting calibration curve using mixture of galactose and

mannose as standards.

Reagents:

- (1) Orcinol reagent - 1.8 mg% orcinol in 45% HCl.

3.3.8 Estimation of Sialidase

Sialidase activity was measured as suggested by Potier et al. (1979). The free 4-methylumbeliferon_a^(Mu) released due to the enzyme reaction was determined spectrophotometrically using excitation at 365nm and fluorescence emission at 450 nm. The standard curve of 4-methylumbeliferon was built using concentration of 10^{-6} to 10^{-5} .

Assay system consisted of 0.01ml enzyme source and 0.08 ml 0.1 M Na-Acetate buffer pH 4.0. The reaction was initiated by addition of 0.01 ml of 0.1 mM Mu-N-acetyl neuraminic acid. After incubation at 37°C for 1 hour, reaction was terminated by addition of 0.9 ml 0.133 M glycine carbonate buffer containing 0.06 M NaCl. In controls, substrate was added after termination of the reaction. Enzyme activity was calculated in unit i.e. μg product liberated/mg protein/hour.

Reagents:

- (1) 0.1 M Acetate buffer pH 4.0
- (2) 0.133 M Glycine carbonate buffer
- (3) 1 mM 4-Methylumbelliferyl-N-acetyl neuraminic acid

3.3.9 Estimation of Fucosidase

Activity of fucosidase was assayed by measuring the amount of released p-nitrophenol (PNP) from PNP-fucopyranoside by using the method of Widerschain et al, (1971).

The assay mixture (0.2 ml) consisted of enzyme source and 0.4 M acetate buffer pH 5.5. The reaction was started by addition of freshly prepared substrate, PNP-fucopyranoside. Substrate addition was omitted in control tube. The assay mixture was incubated at 37°C for 2 hours and reaction was

stopped by addition of 0.1 ml, 0.15 N NaOH followed by addition of 0.5 ml 0.4M Glycine carbonate buffer (pH 10.5). The yellow colour obtained was read at 410 nm.

Enzyme activity was expressed as units/mg protein. 1 unit was the amount of enzyme required to release 1 μ mole PNP per minute. Standard curve was performed using PNP.

Reagents:

- (1) 10 mM PNP Fucopyranoside
- (2) 0.4 M Acetate buffer pH 5.5
- (3) 0.15 N sodium hydroxide (NaOH)
- (4) 0.4 M Glycine-carbonate buffer pH 10.5
- (5) 0.5% Para Nitro Phenol (PNP)

3.3.10 Estimation of Sialyl Transferase

Activity of sialyl transferase was measured using the method of (Kessal and Allen, 1975) with necessary modifications.

0.1 ml assay system for sialyl transferase consisted of 20 mM Tris-malate buffer pH 6.8, 10 mM $MnCl_2$, 2 nmole C^{14} -labelled 5'-cytidine monophosphate N-acetyl neuraminic acid (CMP C^{14} NANA) and 4 mg of asialo fetuin as acceptor and the enzyme source. In control tube, addition of acceptor was omitted. Reaction was started by addition of glycoprotein acceptor after equilibration for 5 minutes at 37°C. Termination of reaction was done by addition of ice cold acid mixture. Precipitates were washed twice with the same acid mixture as well as once with 95% ethanol. Washed precipitates were then solubilised in 1.0 ml of NCS tissue solubiliser. After addition of scintillation cocktail, quantitation of incorporated n-acetyl neuraminic acid was done by counting the radio activity of reaction mixture in β counter. The non-specific binding was nullified by deduction of control readings. Enzyme activities were expressed as CPM/mg protein/hour.

Reagents:

- (1) 200 mM Tris malate buffer pH 6.8
- (2) 100 mM manganese chloride (MnCl₂)
- (3) Acid mixture: 0.5 N HCl containing 5% trichloro acetic acid and 2% phosphotungstic acid
- (4) 95% ethanol
- (5) NCS tissue solubiliser
- (6) Scintillation cocktail containing 0.55% POP and 0.016%POPOP

3.3.11 Estimation of Fucosyl Transferase

Previously described assay of Lowe et al. (1991) was used for the detection of fucosyl transferase activity.

0.1 ml fucosyl transferase assay reaction mixture consisted of 20 mM Tris-acetate buffer and 10 mM MgCl₂. Four mg of Ovomuroid was added as a sugar acceptor, 20 µl enzyme source was used. After equilibration at 37°C reaction was initiated by addition of ¹⁴C labelled nucleotide sugar GDP fucose. Control assay for each sample was run in absence of acceptor substrate. Reaction was stopped by addition of ice-cold mixture containing 5% TCA, 2% phospho-tungstic Acid and 0.5 N HCl. Resultant pellet was washed twice with same acid mixture following washing with 95% ethanol. Protein precipitates were solubilised in 1.0 ml of NCS tissue solubiliser and placed in 10.0ml scintillation cocktail. Enrichment of glycoproteins with fucose was measured by measuring radio activity using β counter. Enzyme activity was expressed as CPM/mg protein/hour.

Reagents:

- (1) The reagents were same as the reagents used for sialyl transferase estimation except the glycoprotein acceptor.

3.3.12 Isolation of Glycoproteins using lectin affinity chromatography:

The lectin affinity chromatography for isolation of glycoproteins was performed as described by Thompson and Turner (1987).

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(a) Activation of Sepharose beads:

To remove preservatives, 200.0 ml of 1 mM HCl were added to 1.0 gm of CNBr activated sepharose beads and left for 30 minutes. After this acid was discarded. This step was repeated twice. Immediately prior to coupling with proteins, sepharose bead were washed with 0.1 M bicarbonate buffer pH 8.3.

(b) Coupling of Lectins with Sepharose:

5 mg of lectins were dissolved in 1.0 ml of bicarbonate buffer pH 8.3. Buffer was precipitated and lectins were added to washed sepharose beads in a ratio of 2mg lectin per 1.0 ml of sepharose beads. It was left to rock over night at 4°C.

(c) Blocking of remaining active groups:

Supernatant was removed and coupled beads were resuspended into 0.2M glycine/bicarbonate buffer and incubated overnight at 4°C to block remaining active groups.

(d) Removal of excessive proteins:

Coupled beads were washed alternatively with bicarbonate buffer (pH 8.3) and 0.1 M acetate buffer (pH 4.0). Excessive blocking agents were removed by two final washes with bicarbonate buffer.

(e) Binding of proteins with lectin-Sepharose beads:

Immediately prior to use, lectin sepharose were washed 3 times with Tris buffer (pH 7.4). Serum was mixed with equal volume of coupled beads. Proteins were allowed to bind with lectins for 2 hours at 4°C with gentle agitation.

(f) Removal of unbound serum proteins:

Unbound serum proteins were washed out with 5-6 washes with 2.5 ml Tris buffer (pH 7.4) at 4°C.

(g) Release of bound proteins:

Bound glycoproteins were released by solubilisation in solubilising buffer (pH 6.8) and boiled for 5 minutes. Resultant elute was loaded on 7.5% SDS-polyacrylamide gel and electrophoresis was performed.

Reagents:

- (1) 1 mM HCl
- (2) 0.1 M Sodium bicarbonate buffer containing 0.5 M sodium chloride pH 8.3
- (3) 0.2 M Glycine in bicarbonate buffer
- (4) 0.1 M Acetate buffer pH 4.0
- (5) 0.1 M Tris-HCl buffer pH 8.0
- (6) Washing buffer: 0.05 M Tris buffer (pH 7.4) containing 25 mM KCl, 5 mM MgCl₂, 5mM CaCl₂ and 0.5% Non-idet P40.
- (7) 0.125M Tris HCl buffer pH 6.8

3.3.13 Polyacrylamide gel electrophoresis (PAGE):

Poly acrylamide gel electrophoresis was performed as described by Laemmli (1970).

7.5% Crosslinked polyacrylamide gels were prepared by polymerisation of acrylamide containing methyl bis-acrylamide (0.2%) as crosslinking agent. Reaction was initiated with addition of 10% TEMED and 10% freshly prepared ammonium persulphate solution. 1.5 M Tris-HCl buffer pH 8.8 was used as gel buffer. Gels were overlaid with water saturated butanol to avoid reaction with oxygen of air. Gels were washed with distilled water followed by electrode buffer. The separating gels were covered with a layer of 6% polyacrylamide gel having pH 6.8. Stack gel was used to concentrate protein into thin bands. Approximately 8mg proteins were loaded on cross linked gel. 0.01% Bromophenol blue in 40% sucrose was used as tracking dye. Molecular weight markers were also loaded simultaneously. Electrophoretic separation was performed with a constant current of 3 mA per tube/well at 15°C using Tris Glycine buffer pH 8.3 as electrode buffer. After run, gels were removed and immersed in 5 volumes of fixing solution. The proteins were

stained using different staining techniques.

SDS-PAGE:

For separation of dissociated proteins, sodium dodecyl sulphate (SDS) was added in a final concentration of 0.1% into both gel mixture as well as in the electrode buffer. For SDS-PAGE, proteins were first dissociated by placing into boiling water bath for 3 minutes in 10 volumes of sample buffer. Disulphide linkages of proteins were cleaved by addition of β -mercaptoethanol in a concentration of 0.72 mole per litre. Before denaturation of proteins, serum was diluted with nine volumes of normal saline.

Reagents:

- (1) 30% Acrylamide with 0.8% methyl bisacrylamide
- (2) Gel buffer: 1.5 M Tris-HCL buffer pH 8.8
- (3) 10% Ammonium persulphate
- (4) 10% N-N,N'-N' - Tetramethyl ethylene diamide
- (5) Electrode Buffer: Tris-Glycine buffer pH 8.3
- (6) Bromophenol blue: 0.01% in 40% Sucrose
- (7) Sample buffer: 0.125 M Tris buffer pH 6.8 containing 20% glycerol and 10% SDS.
- (8) 10% SDS solution.

Silver Staining:

To visualise protein bands the gels were stained using ~~using~~ silver nitrate staining method as discribed by Thompson and Turner, (1987). For this, protein bands were fixed in 40% methanol containing 10% acetic acid. Gels were washed with water followed by 2 hours incubation each in Dithioth~~eritol~~ri~~ptol~~ and 0.1% silver nitrate. Bands were developed by pouring carbonate developing solution and staining were stopped by addition of 2.3M citric acid.

Reagents:

- (1) 40% Methanol in 10% acetic acid
- (2) 7.5% Ethanol in 10% acetic acid
- (3) 0.5 mg % Dithiotheriotol
- (4) 0.1% Silver nitrate
- (5) 3% Sodium carbonate containing 0.01% Formaldehyde

- (6) 2.3 M Citric acid

Commasie Brilliant Blue Staining:

After overnight fixation with 40% methanol and 10% acetic acid, gels were immersed in at least 5 volumes of staining solution overnight in Coomassie Brilliant Blue R-250. To carry out destaining the gels were placed into 40% methanol and 10% acetic acid. This step was repeated 3-4 times to bleach out unbound dye from the gel.

Reagents:

(CBB)

- (1) 0.2% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid.
- (2) 40% Methanol in 10% acetic acid.

Glycoprotein staining:

Glycoproteins were stained using periodic acid schiff (PAS) staining procedure as described by Fairbanks et al. (1971). The gels were incubated in 0.5% periodic acid for 2 hour for oxidation of carbohydrate moiety. Extra periodic acid was removed by incubation with 0.5% sodium arsenite in 5% acetic acid for 1 hour. The gels were incubated for 40 minutes with 0.1% sodium arsenite in 5% acetic acid. It was placed into 10% acetic acid for 20 minutes twice. The gels were then incubated with Schiff's reagent for 1 to 2 hours. Stained gels were then placed in to 0.1% sodium metabisulphite/ 0.01 N HCl. Stained proteins of gels were quantitated by densitometric scanning at 520nm. To identify various regions of serum proteins gels were simultaneously stained with CBB.

Reagents:

- (1) 10% Acetic acid
- (2) 0.5% Periodic acid
- (3) 0.5% Sodium arsenite in 5% acetic acid
- (4) 0.1% Sodium arsenite in 5% acetic acid
- (5) 0.4% Basic fuchsin in 0.2N HCL (Schiff's reagent)
- (6) 0.1% Sodium metabisulphite in 0.01N HCl

3.4 Statistical Methods:

Data were statistically analysed using the SPSS statistical software. (Norusis, 1996)

Cut off value: 95th percentile value of reference group was used as cut off level for each marker.

Significance: Level of significance was assessed by computing Fisher's two tailed exact test and paired 't' test. The alterations in marker levels between two groups with "p"values less than or equal to 0.05 were considered statistically significant.

Receiver's Operating Characteristic (ROC) Curves: To analyse diagnostic ability of the markers, ROC curves were constructed as described by Feinstein (1985). Various sensitivity levels were plotted against different specificity levels.

Regression analysis: To study association of the marker levels with clinical stage of malignant diseases, Spermán's correlation coefficient method was used.

Survival Curves: Log survival was calculated using life table analysis (Kaplan and Meier, 1958). Log-Rank statistic was used to assess significance of survival. Mean values of the markers in patients were considered as cut-off for constructing survival curves.

Multivariate analysis: The multivariate analysis to correlate response to anticancer treatment with clinico-pathological parameters and levels of the biomarkers, Cox's (1972) forward Logistic Regression step wise model was used.