MATERIALS
3. MATERIALS

3.1 PATIENTS

A group of patients suffering from different histological types of breast carcinoma at different clinical stages of the disease and admitted to the Hospital of Chittaranjan National Cancer Institute (CNCI), Calcutta were included in the study. The patients were of both sexes and of age group ranging from 25 to 65 years and received no prior radiotherapy or chemotherapy. They were grouped as pre-operated and post-operated ones. The diagnosis of the diseases were confirmed histologically and clinical stages were recorded according to the TNM classification.

As control, untreated patients suffering from other cancers, such as carcinoma of lung, cervix, stomach and liver and patients with benign breast diseases were also included in this study.

3.2 HEALTHY VOLUNTEERS

Healthy persons of both sexes and ages ranging from 25 to 55 years were volunteers and included in this study as control subjects.

3.3 EXPERIMENTAL ANIMALS

3.3.1 Mice

Inbred colony of C_{3}H/Jax mice were obtained from the Animal Care and Maintenance Department of the CNCI. These mice had a viral etiology and developed mammary tumors spontaneously in female breeders by transmission of Mouse Mammary Tumor Virus (MuMTV) to the offspring through mother's milk. The tumor incidence in the female breeders was 70-75% within 24-28 weeks. Female mice of about 24 weeks with mammary tumors were used as tumor donors and female mice of around 16 weeks without any tumor served as tumor free controls.
3.3.2 Rabbits

Adult Belgian rabbits, usually male, and weighing 1 to 1.5 kg were used for immunization purposes.

3.4 TISSUES

3.4.1 Human Breast Tumors

Human malignant breast tumors (MBT) from the patients suffering from different types of breast carcinoma, were obtained from the Hospital of CNCI. The tumors were collected immediately after surgical removal. The specimens were then washed with cold phosphate buffered saline (PBS), 0.15 M, PH 7.2. Fat and necrotic tissues were removed by dissection. Tumor tissues were then either used immediately or stored at -20°C till used for antigen preparation. Disease states of the tumor tissues were confirmed histologically.

3.4.2 Human Normal Breast Tissues

The normal human breast (NHB) tissues were collected from normal part of the breast undergoing radical mastectomy. The tissues were cleaned in cold PBS, and were either stored frozen at -20°C or used immediately for antigen preparation. Normalcy of the tissues were confirmed histologically.

3.4.3 Human Fetal Tissue

A human fetus of ten weeks of age was obtained from the clinic for Medical Termination of Pregnancy at Chittaranjan Sevasadan, Calcutta. Prescribed ethical norms were followed for collection of the fetus. The fetus was washed thoroughly to remove the blood vessels and was stored at -20°C till used for antigen preparation.

3.4.4 Mouse Mammary Tumors (MMT)

C3H/Jax mice with MuMTV induced spontaneously arising mammary tumor were sacrificed by cervical dislocation and the mammary tumors were excised, washed in cold PBS and stored at -20°C till use.
3.5 SERA

3.5.1 Sera from cancer patients

Blood was collected from a number of female and male patients suffering from different types of breast carcinoma, admitted at the Hospital of CNCI. Pre-operated and post-operated patients suffering from stage I, II, III of the disease and without receiving any prior chemotherapy or radiotherapy were used as blood donors. The blood samples were allowed to clot at room temperature and sera were separated by centrifugation. The serum samples were coded randomly, heat-inactivated at 56°C for 30 minutes and stored at -20°C till use.

Blood was also collected from a number of untreated patients, attending the Hospital of CNCI, suffering from benign breast diseases and carcinoma of lung, cervix, stomach and liver. Sera were prepared from the blood and stored as described earlier.

3.5.2 Sera from healthy individuals

Blood samples were collected from healthy volunteers of both the sexes, who did not suffer from any disease for at least six months prior to the date of collection of blood. The sera separated from the blood were heat inactivated, randomly coded and stored at -20°C until use.

3.5.3 Sera from normal goat

Blood samples were collected from normal goats, sacrificed at slaughter house. The sera separated from blood were heat inactivated and stored at -20°C until use.

3.6 ANTIBODIES

3.6.1 Antisera to murine mammary tumor antigen (anti-MMT)

Antisera to mouse mammary tumor (MMT) were raised in Belgian rabbits by subcutaneous administration of the antigen (10 mg protein)
emulsified in equal volume of complex Freund's adjuvant. The antisera (anti-MMT) were inactivated at 56°C and stored at -20°C until used.

3.6.2 Goat Antisera to Mouse Mammary Tumor Virus (anti-MuMTV)

Anti-MuMTV, the goat polyvalent antisera to triton disrupted MuMTV was obtained from National Institute of Health (NIH), Bethesda, USA, as a generous gift. The murine mammary tumor virus (MuMTV) was propagated in MM5 MT/CA line. Triton X-100 disrupted cells were used as the source of MuMTV.

3.6.3 Anti-Human IgG

Anti-IgG₁, IgG₂ and IgG₃ raised in mouse were obtained from Sigma, St. Louis, MO.

3.7 FINE CHEMICALS

3.7.1 Chemicals for Column Chromatography

3.7.1.1 DEAE (Diethyl Amino Ethyl Cellulose)

DEAE was obtained from Sigma, St. Louis, MO. It is an anion exchanger with fixed positive charges, which binds mobile anions.

3.7.1.2 Sephadex

Sephadex G-100 and G-200 (bead size 10-40 μ) were obtained from Pharmacia fine chemicals, Uppsala, Sweden. Sephadex is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. The large number of hydroxyl groups renders the gel extremely hydrophillic. Sephadex is supplied as a dry powder which is swelled in excess solvent before use.

3.7.1.3 Protein-A-Sepharose

Protein-A-Sepharose was purchased from Sigma, St. Louis, MO. It was made activated by Cyanogen bromide.
3.7.1.4 **Protein PAK 300 SW Column**

The protein PAK 300 SW column of length 7.5 mm (ID) x 30 cm was purchased from Waters, USA, and used for size-exclusion high performance liquid chromatography. The column of choice is for use with larger biological active molecules in the molecular weight range of: Native Globular 10,000 - 400,000 and Random coil 2000 - 150,000. The column was shipped in 0.05% sodium azide in water solution.

3.7.2 **Chemicals for Gel Electrophoresis**

3.7.2.1 **Bis**

N,N' - Methylene-bis-Acrylamide was obtained from Sigma, St. Louis, MO.

3.7.2.2 **Acrylamide**

Acrylamide was obtained from Sigma, St. Louis, MO. For gel electrophoresis, Acrylamide was cross-linked with Bis to form a porous solid matrix. The pore size varied from gel to gel depending on the separable macromolecules according to their sizes and shapes.

3.7.2.3 **TEMED**

N,N,N',N'-Tetramethyl-ethylenediamine was obtained from Sigma, St. Louis, MO. It acts as a catalyst for initiation of crosslinking between Bis and Acrylamide.

3.7.2.4 **Ammonium Persulfate**

Ammonium Persulfate was purchased from Bio-rad, Richmond, CA. It also acts as a catalyst for initiation of cross-linking between Bis and Acrylamide.

3.7.2.5 **Lauryl Sulfate (Sodium Lauryl Sulfate; Dodecyl Sodium Sulfate)**

SDS was obtained from Sigma, St. Louis, MO. It was used as a detergent for breaking the protein polymers.
3.7.2.6 **Pre-Stained-SDS PAGE standard molecular weight markers**

Low range pre-stained SDS-PAGE standard molecular weight markers was obtained from Bio-rad, Richmond, CA. It was composed of six proteins — phosphorylase B (130 Kd), Bovine Serum Albumin, BSA (80 Kd), Ovalbumin (50 Kd), Carbonic anhydrase (39 Kd), Soyabin trypsin inhibitor (27 Kd) and Lysozyme (18 Kd).

3.7.2.7 **B - Mercaptoethanol**

B - Mercaptoethanol was obtained from E.Merck, Darmstadt.

3.7.3 **Stain for Polyacrylamide Gel**

3.7.3.1 **Coomassie Brilliant Blue G**

Coomassie Brilliant Blue G was purchased from Sigma, St. Louis, MO.

3.7.3.2 **Silver Nitrate**

Silver Nitrate was purchased from E.Merck, Darmstadt. It was used to stain the fine bands of polyacrylamide gels.

3.7.4 **Chemicals for Enzyme Linked Immunosorbent Assay (ELISA)**

3.7.4.1 **Anti-Human IgG Peroxidase Conjugate**

Anti-human IgG (γ-chain specific) peroxidase conjugate was obtained from Sigma, St. Louis, MO. The antibody was raised in goat.

3.7.4.2 **Anti-Goat IgG Peroxidase Conjugate**

Anti Goat IgG (whole molecule) peroxidase conjugate was obtained from Sigma, St. Louis, MO. The antibody was raised in rabbit.

3.7.4.3 **Anti-Mouse and anti-Rabbit IgG Peroxidase Conjugate**

Anti-Mouse IgG(γ-chain specific) and anti-Rabbit IgG (whole molecule) peroxidase conjugates were obtained from Sigma, St. Louis, MO. The antibodies were raised in goat.
3.7.4.4. **O-Phenylenediamine dihydrochloride (OPD)**

OPD was purchased from Sigma, St. Louis, MO. It was used as the substrate for the enzyme peroxidase.

3.7.4.5 **Polysorbate 20 (Tween-20)**

Tween-20 was obtained from Sigma, St. Louis, MO. It was used as a detergent for washing the wells of the ELISA plate.

3.7.5 **Other Chemicals**

3.7.5.1 **Bacto-Agar**

Bacto-Agar was purchased from Difco Laboratories, USA. This was used in immunodiffusion studies.

3.7.5.2 **Enzymes**

The enzymes Papain and Neuraminidase were purchased from Sigma, St. Louis, MO and Trypsin was obtained from E. Merck, Darmstadt.

3.8 **BUFFERS**

3.8.1 **Phosphate Buffered Saline (PBS), 0.15M, PH 7.2**

- Sodium Chloride - 8.0 g
- Potassium Chloride - 0.2 g
- Disodium hydrogen phosphate - 1.15 g
- Potassium Dihydrogen phosphate - 0.2 g

Water up to 1000 ml

3.8.2 **Borate Buffered Saline (BBS), PH 8.3**

- Borax - 9.435 g
- Boric Acid - 6.61 g
- Sodium Chloride - 4.35 g

Water up to 1000 ml.
3.8.3 Tris-Glycine Buffer, PH 9.5
Glycine  -  2.88 g
Tris    -  0.6 g
Water upto 1000 ml

3.8.4 Lower Gel Buffer for SDS-PAGE, PH 8.8
Tris    -  90.825 g
SDS     -  20 ml from 10% Stock solution
Volume is adjusted upto 500 ml with water.

3.8.5 Upper Gel Buffer for SDS-PAGE, PH 6.8
Tris    -  30.275 g
SDS     -  20 ml from 10% stock solution
Volume is adjusted upto 500 ml with water.

3.8.6 Sample Buffer for SDS-PAGE (4 X Conc) PH 6.8
Tris    -  1.574 g
SDS     -  20 ml of 10% stock solution
Glycerol -  20 ml
Bromophenol Blue -  1 mg
Volume is adjusted upto 50 ml with water.

3.8.7 Running Buffer for SDS-PAGE, PH 8.8
Tris    -  3.0 g
Glycine -  14.4 g
SDS     -  5 ml from 10% stock solution
Volume is adjusted upto 1000 ml with water.

3.8.8 Carbonate - Bicarbonate Buffer, PH 9.6

Stock Solutions
A : 0.2 M solution of anhydrous Sodium Carbonate (21.2 g in 1000 ml of water).
B : 0.2 M solution of Sodium Bicarbonate (16.8 g in 1000 ml of water).
### 3.8.9 Phosphate – Citrate Buffer, PH 5.0

**Stock solutions**
- **A**: 0.2 M dibasic Sodium Phosphate (35.6 g in 1000 ml of water).
- **B**: 0.1 M Citric Acid (21.01 g in 1000 ml of water)

**Mixture**
<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml)</th>
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<tbody>
<tr>
<td>A</td>
<td>25.7</td>
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<td>B</td>
<td>24.3</td>
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Water upto 100 ml

### 3.8.10 Transblot Buffer, PH 8.3

<table>
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<th>Component</th>
<th>Volume (g)</th>
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</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.03</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>200</td>
</tr>
</tbody>
</table>

Water upto 1000 ml

### 3.8.11 Glycine– Hydrochloric Acid Buffer, 0.1M, PH 2.8

**Materials**
- Glycine 0.2 M (15.01 g in 1000 ml), Hydrochloric Acid 0.2 N

The pH of 500 ml of 0.2 M Glycine was titrated to 2.8 with 0.2 N HCl and the volume is made upto 1000 ml with water.

### 3.9 REAGENTS

#### 3.9.1 PAGE in Tris–Glycine buffer System

**Solution A**
- Tris          : 1.815 g
- 1(N) HCl      : 2.4 ml
- TEMED         : 0.023 ml

Diluted upto 5 ml with water.
Solution B

Acrylamide - 3.0 g
Bis-acrylamide - 0.08 g
Potassium ferricyanide - 0.0015 g

Diluted upto 10 ml with water.

Solution C

Ammonium Persulfate - 0.015 g
Water - 10 ml

Mixture

A : B : C : H₂O
1 : 2 : 4 : 1

3.9.2 SDS-PAGE system

Bis-acrylamide solution (30% Acrylamide solution)

Bis - 0.8 g
Acrylamide - 30.0 g

Water upto 100 ml

Running Gel solution

30% Acrylamide solution - 10 ml
Lower Gel buffer - 7.5 ml
Water - 11.3 ml
Ammonium Persulfate (15 mg/ml) - 1 ml
TEMED - 40 μl

Stacking Gel solution

30% Acrylamide solution - 1 ml
Upper Gel buffer - 2.5 ml
Water - 5.5 ml
Ammonium Persulfate (15 mg/ml) - 1 ml
TEMED - 20 μl