Materials and Methods

MATERIALS AND METHODS

3.1 General Chemicals

Sodium Bisulfite, Hydroquinone, Acrylamide, N, N'-methylene bis-acrylamide, ammonium persulphate (APS), TEMED, TritonX-100, Tween-20, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, glycerol, protein molecular markers, phenyl methyl sulfonyl fluoride (PMSF), trizma base, TRIzol, HEPES, DEPC, SDS, EDTA, MOPS, poly(dI-dC), β-mercaptoethanol, sodium hydroxide, methylene blue, bromophenol blue, isoamyl alcohol, boric acid, IPTG, glucose, glycine, $CaCl_2$, sodium dodecyl sulfate, coomassie brilliant blue R-250, urea, leupeptin, pepstatin, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), foetal bovine serum (FBS), propidium iodide (PI), dNTPs (dATP, dGTP, dCTP, dTTP), ethidium bromide, agarose, sodium pyruvate, sodium bicarbonate and bovine serum albumin (BSA) were purchased from Sigma Chemical Co (St. Louis, MA, USA). Enhanced Chemiluminescence reagent (ECL) was purchased from Santa Cruz, USA. Ethanol, phenol, hematoxylin, eosin, glacial acetic acid and all other reagents used in this study were of analytical grade and obtained from Qualigens and Merck.

3.2. Blotting membranes and Films

Polyvinylpyrolidone difluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). X-OMAT AR, XK-5 films were procured from Kodak (New Delhi, India). X-ray developer and X-ray fixing salt (fixer) from Kodak were used for developing auto radiographs.

3.3. Plastic ware and glassware

Microfuge tubes and pipette tips were purchased from GenAxy (Union city, CA, USA); petri dishes and other plastic ware were obtained from Tarsons (Kolkata, India). Cell culture plastic wares were obtained from Corning-Costar Inc. (Corning, NY, USA) and Falcon-Becton Dickinson (San Jose, CA, USA). 0.45 μM membrane filters for filtering media and other reagents were obtained either from Millipore (MA, USA). All laboratory

glassware was purchased from Schott Duran, Germany.

3.4. Antibodies, Oligonucleotides and Enzymes

Polyclonal antibodies for BRCA1, p16, GSTPi, HIC1 and CDH1 were purchased from Santa Cruz Biotechnology and there details are given in the protocol below **(Table M3)**. Poteinase K, RNaseA and trypsin were purchased from Sigma Chemical Co (St. Louis, MA, USA). Taq Polymerase was purchased from Bangalore Genei. DNA Wizard clean up was purchased system from Promega.

3.5. Biological Specimens

3.5.1 Tissue biopsies

Tissue samples included; (1) Breast tumor tissues and (2) normal breast tissue biopsies from breast cancer patients. A total of 87 breast cancer patients whose diagnosis was confirmed by histopathology were included in the study. The breast tissue biopsies represented different grades of carcinoma of the breast and all were diagnosed with infiltrating ductal carcinoma. Normal breast tissue biopsies (n=87) were obtained adjacent normal tissue to the tumor of the same patient but not infiltrated by tumor cells as confirmed by histopathologists.

To determine the methylation status of a battery of five genes-BRCA1, E-cadherin, p16, GSTPi and HIC-1 and expression of their proteins by Western blotting and Immunohistochemistry, 87 tissue biopsies from clinically and cytopathologically confirmed breast cancer cases along with normal adjacent tissues were collected from surgical OT of Max Superspeciality Hospital, New Delhi with written consent as per the guidelines of the Institutional Ethical Committee. These tissues were used to extract DNA, protein and to make paraffin blocks for histopathology and to carry out immuno-histochemical localization of various proteins indicated above. Non-malignant tissue obtained from the site adjacent to the breast cancer served as control. These biopsies were

collected in sterilized sample collection vials containing chilled Phosphate Buffer Saline (PBS) (pH 7.2) and were immediately used or stored at -70°C until further use.

Inclusion Criteria:

1. Women with primary breast cancer and having age limit between 20 to 70 years were selected for this study.
2. Clinically and cytopathologically confirmed breast cancer cases were recruited.

Exclusion Criteria:

1. Patients who have undergone segmental resection or mastectomy and those who have received radiotherapy or chemotherapy or any other drug before surgery were not included in the study.
2. Women having any other chronic disease other than breast cancer were excluded.
3. Pregnant women were not selected for the study.

3.6. METHODS

3.6.1 DNA isolation from breast tissue specimens

The fresh tissue sample was properly minced in petri-dish containing 200µl of 1x TE. To the minced tissue 400 µl of 1x TE was further added. After mixing well, 200 µl of tissue lysis buffer (3% SDS in 2x TE) was added followed by addition of 6 µl proteinase K. after overnight incubation at 50 °C equal amount (600 µl) of TE equilibrated phenol was added and subjected to overhead shaker for 15 minutes at room temperature. It was then centrifuged at 10,000 rpm at 4°C for 10 minutes and supernatant carefully aspirated with the help of micropipette. To the supernatant equal volume of phenol and chlorofom-isoamyl alcohol in the ratio 25:24:1 was added (Das *et al., 1992*). After overhead shaking for 15 minutes at room temperature it was centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was again collected carefully and to this an equal amount of chloroform isoamyl alcohol was added and shaked overhead for 15 minutes at room temperature. After centrifugation at 10,000 rpm at 4°C for 10 minutes the supernatant

was aspirated and to this around 1/10th volume of chilled Sodium Acetate (~50 µl) and equal volume of isopropanol was added. After keeping at -70°C for 2 hrs or at -20°C for overnight, it was centrifuged for 15 minutes at 10,000 rpm at 4°C. The pellet was washed with 70% ethanol by centrifuging at 8,000 rpm at 4°C for 5 minutes. The pellet was air dried at room temperature overnight and then dissolved in 200 µl of 1x TE.

3.6.2 Quantitation of genomic DNA using agarose gel electrophoresis

Agarose gel was prepared by dissolving 1 gm of agarose in 100 ml of 1x TAE buffer and boiling for 2 minutes in a microwave oven. To this 3 µl of Ethidium Bromide dye was added and the gel casted. In each well, a mixture 1 µl of DNA dissolved in 1x TE and 9 µl of 6x bromophenol blue gel running dye was loaded. After running the gel at 50V for 30 minutes the gel was examined on a transilluminator and the DNA quantified.

3.6.3 Sodium Bisuphite modification of DNA

To 10 µl of DNA 40 µl of water was added in a 1.5 ml eppendorff and heat snapped at 95°C for 2 minutes. 5.5 µl of 2M NaOH was added and incubated at 37°C for 10 minutes to create single stranded DNA. 520 µl of freshly prepared 3M Sodium Bisulphite was further added and after thorough mixing incubated at 50°C for 16 hrs. The tubes were kept at room temperature and 1 µl of DNA Wizard clean up from Promega was added to each tube. The mixture was transferred to miniprep column provided with the kit using 1 ml pipette. The solution was seived using 2 ml piston of syringe in miniprep column. The filter was removed from the miniprep column and fitted in decapped eppendorff tubes. The tubes were microfuged at 12,000 rpm for 2 minutes. The filter was washed with 80% isopropanol using piston of same mini prep column. The filter was again fitted to clean labelled capless 1.5 ml eppendorf tubes and microfuged at 12,000 rpm for 2 minutes. To the filter still attatched to the eppendorf tubes, 50 µl of water heated to 70°C was added and the tubes centrifuged at 12,000 rpm for 2 minutes. To the filtrate in each tube 5.5 µl of 3M NaOH was added and the tubes incubated at room temperature for 5 minutes. Then 1 µl of glycogen as carrier was added followed by 33 µl of 10 M Ammonium Acetate and

300 µl of chilled absolute ehtanol. After incubating overnight at -20°C the DNA was precipitated by centrifuging at 14,000 rpm for 20 minutes then washed with 70% ethanol. The pellet so obtained was air dried and resuspended in 20 µl distilled water and stored at -20°C until further use.

3.6.4 Methylation specific polymerase chain reaction (MSP)

Methylation specific PCR was carried out on the bisulfite modified DNA samples. The PCR mixture contained lx PCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM MgCl2/10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) or unmodified DNA (50-100 ng) in a final volume of 50 µl. 1.25 units of Taq polymerase (Genei) was used for the final volume. Amplification was carried out in a Biorad thermal cycler for 39 cycles (30 sec at 95°C, 30 sec at the annealing temperature listed in Table 2, and 30 sec at 72°C), followed by a final 7-min extension at 72°C. Controls without DNA were performed for each set of PCRs. Each PCR product along with loading dye (10 µl + l µl) was directly loaded onto 2% Agarose gels, stained with ethidium bromide, and directly visualized under UV illumination.

Table M1: Reaction protocol for MSP

Reaction	Time	Temp (°C)
Initial denaturation	5’	95
Denaturation	30”	95
Annealing	30”	X*
Extension	30”	72
Final extension	7’	72
Hold reaction	Forever	4

***Annealing temparature**

Table M2: PCR primer sequence used in the study of gene promoter methylation in breast cancer

Gene	Methylation / Unmethylation	Sequence (5'–3')		Size (bp)	*Tm*, °C
BRCA1	Unmethylated	F	TTG GTT TTT GTG GTA ATG GAA AAG TGT	86	55
		R	CAA AAA ATC TCA ACA AAC TCA CAC CA		
	Methylated	F	TCG TGG TAA CGC AAA AGC GC	75	55
		R	AAA TCT CAA CGA ACT CAC GCC G		
E-cadherin	Unmethylated	F	TAA TTT TAG GTT AGA GGG TTA TTG T	97	53
		R	CAC AAC CAA TCA ACA ACA CA		
	Methylated	F	TTA GGT TAG AGG GTT ATC GCG T	116	57
		R	TAA CTA AAA ATT CAC CTA CCG AC		
P16	Unmethylated	F	TTA TTA GAG GGT GGG GTG GAT TGT	151	60
		R	CAA CCC CAA ACC ACA ACC ATA A		
	Methylated	F	TTA TTA GAG GGT GGG GCG GAT CGC	150	65
		R	GAC CCC GAA CCG CGA CCG TAA		
GSTPi	Unmethylated	F	GAT GTT TGG GGT GTA GTG GTT GTT	91	59
		R	CCA CCC CAA TAC TAA ATC ACA ACA		
	Methylated	F	TTC GGG GTG TAG CGC TCG TC	99	59
		R	GCC CCA ATA CTA AAT CAC GAC G		
HIC-1	Unmethylated	F	TTG GGT TTG GTT TTT GTG TTT TG	95	57
		R	CAC CCT AAC ACC ACC CTA AC		
	Methylated	F	TCG GTT TTC GCG TTT TGT TCG T	118	57
		R	AAC CGA AAA CTA TCA ACC CTC G		

3.6.5 Preparation of Protein Extract

Protein extracts from all breast biopsies (cancer and normal adjacent control) and cell lines were prepared by the method of (Dignam, 1990) with minor modification described earlier (Mishra et al., 2006). Briefly, frozen tissues were minced (in case of cell lines pellet was directly used) and resuspended in ice-cold buffer A [20 mM HEPES (pH 7.6), 20% (v/v) Glycerol, 10mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 1mM DTT, 1mM PMSF, 2µg/ml Leupeptin and 10µg/ml Aprotinin]. The lysates were microfuged at 4,000 rpm for 10 min at 4°C after incubating them for 15 min on ice. The supernatant was transferred in a new tube and designated as cytoplasmic extracts. The pellet containing isolated nuclei was resuspended in the 2 times pellet amount of extraction buffer B

[20mM HEPES (pH 7.6), 25% (v/v) Glycerol, 500mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 1mM DTT, 1mM PMSF, 2µg/ml Leupeptin and 10µg/ml Aprotinin]. The extraction mixture was microfuged after 1 hr at 14,000 rpm at 4°C for 25 min. The resulting supernatant was designated as nuclear extract. The protein concentration of the extracts was determined by spectrophotometric method and the extract was stored at –70 °C deep freezer or in liquid nitrogen until use. The protein was estimated by diluting in solubilising buffer and measuring its absorbance at 230nm and 260nm against a solubilising buffer as a blank. The amount of total protein in µg/ml of protein extract was calculated using the following formula:

$[(183 \times A_{230}) - (75.8 \times A_{260})] \times$ Dilution factor =Protein (µg/ml)

Or was measured by using Nanodrop spectrophotometer ND-100

Table M3: List of Antibodies used for the study

S. No.	Antibodies	Epitope corresponding to the amino acids
1	BRCA1	rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of BRCA1 of human origin
2	P16	mouse monoclonal antibody raised against amino acids 1-167 representing full length p16 of mouse origin
3	HIC1	mouse monoclonal antibody raised against recombinant HIC-1 of human origin
4	GSTPi	mouse monoclonal antibody raised against purified truncated recombinant GSTP1 of human origin
5	CDH	mouse monoclonal antibody raised against amino acids 600-707 mapping within an extracellular domain of E-cadherin of human origin

All these antibodies were obtained from Santa Cruz Biotechnology, Inc., California, USA.

3.6.6 WESTERN BLOTTING AND ECL DETECTION

3.6.6.1 *SDS-Poly-acrylamide gel electrophoresis*

For SDS-Polyacrylamide gel electrophoresis 10% SDS-Polyacrylamide resolving gel was prepared by adding following components **Table M4.**

Table M4: Preparation of 10% SDS-Poly-acrylamide gel

Resolving gel 10%		**Stacking gel 5%**	
30% Acrylamide (29:1)	13.3 ml	30% Acrylamide (29:1)	1.7 ml
1.5mM Tris-HCl (pH.8.8)	10.0 ml	1.5mM Tris-HCl (pH.6.8)	1.25 ml
10% SDS	0.04 ml	10% SDS	0.1 ml
10% APS	0.04 ml	10% APS	0.1 ml
TEMED	0.016 ml	TEMED	0.01 ml
Distilled Water	15.9 ml	Distilled Water	6.8 ml
Total Volume	40 ml	Total Volume	10 ml

After assembling the glass plates using 1mm spacers and comb, the resolving gel solution was poured and allowed to polymerize. Water saturated butanol (1ml) was layered over the resolving gel solution which allows formation of uniform level of resolving gel and also prevents its contact with air. Stacking gel solution was then poured on the top of resolving gel and was also allowed to polymerize for another 30 min. When the gel was ready 50µg protein samples mixed with an equal volume of 2x Lammelis buffer (100mM Tris-HCl, pH 8.0, 20mM EDTA pH 8.0, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue), were loaded on the gel along with protein molecular weight marker after denaturation by boiling at 100°C for 5min. Electrophoresis was done in at 4°C in 1x Tris-glycine buffer (25mM Tris-HCl, pH 8.0, 250mM glycine, pH 8.3, 0.1% SDS) at a constant current of 20mA till the protein sample reaches resolving gel. The current was then increased to 40mA for electrophoresis in resolving gel.

3.6.6.2 *Immunoblotting-Semi-dry transfer and ECL detection*

For semi-dry transfer of proteins on to a PVDF membrane (Immobilon-P membrane, Millipore Corporation, USA) the following transfer sandwich **(Fig. M1)** was made in a semi-dry transfer apparatus (Biometra, Germany). Briefly, the PVDF membrane was cut in appropriate size (that is size equal to gel) and was kept in methanol for 3min, given a brief rinse in distilled water and finally transferred to Buffer II (0.025M Tris-HCl, pH 8.0, 20% Methanol) for 15min. Appropriately sized 3mm Whatman papers were also cut and soaked in three different transfer buffers such as Buffer I (0.3M Tris-HCl, pH 8.0, 20% Methanol), Buffer II and Buffer III. Similarly gel was also transferred to Buffer III (0.025M Tris-HCl, pH 8.9, 20% Methanol, 0.04M D-L-Norleucine) for 15min and the semi-dry electro-blotting was done by applying a constant current of 1mA/cm^2 in a cold room (4°C) for 1 hr. While making the transfer sandwich care was taken that no air bubble are trapped in.

After the protein transfer was complete, the membrane was stained with a reversible staining solution of Ponceau's S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid, [3-hydroxy-4-(2-sulpho-4-[4-sulphophenylazo]-phenyl-azo)-2,7-napthalenedisulfonic acid] to check the transfer and equality in the amount of protein loaded in all the lanes. When the pre-stained protein molecular weight markers were used they were also transferred on to the membrane and obtained the molecular weight were marked by pencil.

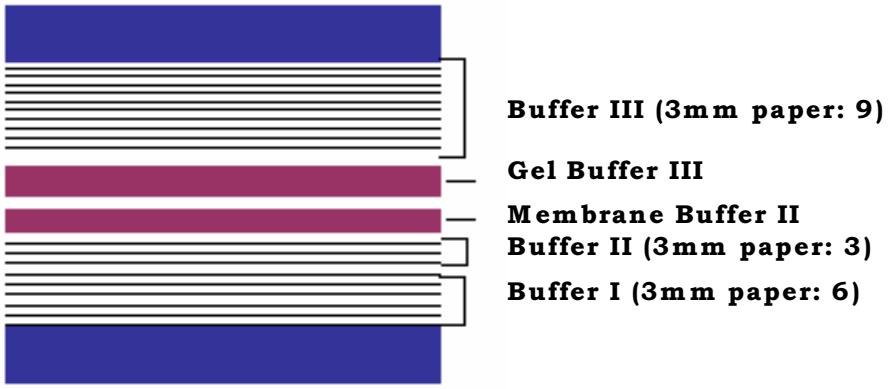

Figure M1: Assembly for semi-dry transfer of proteins from gel to PVDF membrane

The membrane was destained by washing the stain in sterile double distilled water for 1 min and processed for detection a specific protein.

3.6.6.3 ***Blocking and antibody interaction***

For detection of a specific protein on the membrane and blocking of all the non-specific sites it was incubated in a solution of 10% fat-free milk in PBS-T (PBS containing 0.05% Tween 20) at 4°C for 30 min at a low speed on a rocker platform. The membrane was then washed three times for 5min each with fresh changes of PBS-T, incubated overnight at 4°C in primary antibody specific for the respective protein (1:5000 dilution of 100µg/ml solution) (in 5% fat-free milk in PBS-T). The membrane was again washed four times for 5 min each with PBS-T and kept for incubation in the horseradish peroxidase labeled antirabbit-IgG (1:5000) (in 5% fat-free milk in PBS-T) for 2hrs at 4 °C. After final washing of the membrane four times each for 5min with PBS-T, it was processed for ECL detection using the Santa Cruz Luminol detection system (Santa Cruz Biotechnology, Inc., California, USA).

3.6.6.4 ***Enhanced chemiluminescence (ECL) detection***

For ECL detection of protein, the processed membrane was briefly dried on a piece of 3MM Whatman paper. In the meantime, equal volume of ECL Solution I was mixed with an equal volume of ECL Solution II (Santa Cruz Biotechnology, Inc., California, USA). The final volume of the mixture was made which was sufficient to cover the membrane completely. The solution was poured on the entire surface of the membrane and immediately processed for autoradiography after covering the membrane in a cassette with saran wrap. The specific protein bands of AP-1 and NF-kB were identified and a comparison was made between tumor and normal adjacent controls. The blots were stripped and re-probed for β-actin levels to confirm equal loading and normalization. The expression levels of different AP-1 members were evaluated densitometrically by Alpha Digidoc version 4.1.0 (Alpha Innotech Corporation, IL) on a scale of 0-255 and the averaged pixel values were re-grouped for analysis on an arbitrary scale as strong = >

50%; medium = 10-50%; weak=1-10% and nil/not detectable < 1% as described earlier (Prusty & Das, 2005).

3.6.7 IMMUNO-HISTOCHEMISTRY (IHC)

To determine the micro-heterogeneity and distribution of proteins of BRCA1, p16, GSTP1, HIC1 and CDH1 in breast carcinogenesis, fresh tissue biopsies of different grades were treated with 10% formalin and embedded in paraffin blocks. The tissue was cut into thin sections (~ 5 μm thick) using a microtome and was placed on poly L-lysine coated slides and were heat fixed. After deparaffinization using fresh xylene and rehydration using different grades of alcohol, the tissue sections were boiled in 1X citrate buffer to retrieve the antigen. The endogenous peroxidase enzymatic activity was quenched in 2% hydrogen peroxide for 30 minutes. The slides were washed with 1X PBS and then blocking serum was added and incubated at 37^0C in a humidified chamber in order to block the non-specific interactions. The slides were then rewashed thrice with 1X PBS (pH=7.4) and sections were incubated with respective primary polyclonal antibodies with the respective standardised dilutions for overnight at 4^0C. Next day slides were washed with 1X PBS (pH=7.4) and incubated with the biotinylated secondary antibody for 30 minutes followed by avidin-biotin labelled tertiary antibody (Santa Cruz Biotechnologies, USA) for another 30 minutes after washing with 1X PBS. The sections were then stained with chromogen; Diamino benzidine, (DAB) (Sigma-Aldrich, USA) for 2-5 minutes in dark, rinsed in tap water and counterstained with Mayer's haematoxylin, properly dehydrated in methanol, cleared in xylene and finally mounted in DPX. The slides were visualized under bright field microscope (Olympus, 1X81, Japan). Images were captured at different magnifications using image pro software. The quantitation of the images was done using (Image J software version 1.38, NIH (http://rsb. info. nih.gov/ij/), USA).

3.6.8 STATISTICAL ANALYSIS

The χ^2-test was used to determine associations between the methylation of individual genes and various phenotypic or molecular features of breast cancer. Fisher's exact test was used when individual cell numbers were less than 5. All P values were derived from two-tailed statistical tests and significance was assumed at <0.05. All analyses were performed using the SPSS 10.0 (Chicago, IL) statistical software package.