MATERIAL AND METHODS
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1. Study I: Analyzing the association of a promoter region SNP, BRCA1 rs11655505 (c.-2265 C/T) with the predisposition of breast cancer

rs11655505 (c.-2265 C/T) is a variation located in BRCA1 promoter. That has been found in a protective association with the occurrence of breast cancer in a Chinese population whereas, that has not shown any association with the disease in other studies. Our first attempt was to determine if there is any association between the SNP and breast cancer in an Indian population. In this view in mind the following methods were performed to find out the association.

1.1 Selection of Patients

Irrespective of their age and menopausal status 352 female breast cancer patients were selected for the present study. Screening for BRCA1 (c.-2265C/T) rs11655505 variation was performed in selected patients. Later for the ease of statistical analysis they were grouped according to their age, menopausal status and the familial history. Histological verification and diagnosis were done for the selected patients at Indo-American Cancer Institute and Research Center, Hyderabad, India. Mean age of selected breast cancer patients were 44.86 ±10.53 years, ranging from 18 to 68 years. Total 139 (39.48%) patients had
attained menopause while rest 213 (60.51%) were in pre-menopausal status. Breast cancer family history was reported from 73 (20.73%) patients while rest of the patients had no familial history of breast cancer. If the patients had at least one additional first or second degree relative with breast cancer diagnosed before 50 years were regarded as familial breast cancer patients. For control, 380 healthy women with no history of breast cancer up to first or second degree relative also participated in this study voluntarily. The mean age of healthy women was 43.81±10.33 years. Blood samples from healthy women were used as control for the study. Oral consent was taken from patients and healthy volunteers after discussing the importance of this study with them.

1.2. Sample collection

About 5 mL of venous blood was drawn from patients and healthy women. Samples were then transferred to sterile, lavender-top vacutainer containing anticoagulant. Samples were stored at 4°C until processed for DNA extraction. DNA was extracted within 24h of collection of samples from the patients.

1.3. Isolation of DNA

Blood samples were subjected to the genomic DNA extraction by using a standard salting out procedure (Miller et al. 1988), (Appendix I). The concentration of extracted DNA was determined by using a NanoDrop 1000 (Thermo Fisher Scientific, MA, USA). To determine the quality of DNA
agarose gel electrophoresis was performed (Schwark et al. 2011). Extracted DNA was stored at -80°C until used for genotyping (Appendix II).

1.3. Mutation Analysis

For the single nucleotide polymorphism (SNP) analysis TaqMan® SNP Genotyping Assays were performed with a slight modification. TaqMan® SNP Genotyping Assays is a real-time PCR allelic discrimination technique. The assay accounts for non-labeled forward and reverse primers and two fluorescent TaqMan oligonucleotide probes. For the ease of allelic discrimination, allele 1-specific probe labeled with VIC fluorophore, allele 2-specific probe labeled with FAM (6-carboxy- fluorescein fluorophore) were used. Both the reporter dyes, VIC and FAM are covalently attached to the 5’ terminal base of the two respective probes and the nonfluorescent quencher dye is attached near the 3’ ends. The probes differentially bind to the amplicons generated during PCR and selectively report their respective alleles (Livak, 1999). All PCRs were run in duplicate and contained 50 ng (10 µL) DNA, 9 µL TaqMan genotype PCR master mix, and 1 µL allelic discrimination mix. Real-time PCR was performed on an ABI 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Condition for the allelic discrimination PCRs were; 50°C for 2 min, 95°C for 10 min, and then 40 cycles of amplification (92°C denaturation 15 s, 62°C annealing, extension for 60 s). The annealing temperature was empirically determined to promote high binding specificity of the probes without the loss of assay sensitivity. For each cycle, the SDS software
determined the ΔRn, which is the normalized (i.e., compared with a passive reference fluorophore) fluorescent signal from the VIC or FAM labeled probe. For the present study, we used the ΔRn value after the final cycle because it proved more reliable than the cycle at which the threshold was crossed (Ct value) (Appendix II).

1.4 Statistical analysis

Deviation of genotypes in whole case and control groups; menopausal case and control groups; premenopausal case and control groups; non-familial cases and whole control groups and; familial case and whole control groups were compared. To determine the deviation of genotype frequencies χ2 test was done for heterogeneity (two degrees of freedom) and test for trend (one degree of freedom), in order to evaluate the ethnicity-specific effect. Genotype specific risks were estimated as odds ratio (ORs) using unconditional logistic regression. If no statistically significant differences were found (data not shown), the results were combined. The risk associated with each SNP was estimated by allelic, dominant and recessive OR and associated 95% confidence intervals (CI). ORs with 95% confidence intervals (CIs) were calculated to assess the strength of the association between polymorphism and breast cancer risk. Then we explored the association for co-dominant model, dominant model, recessive model and allele versus allele, respectively. All statistical tests were based on two-sided probabilities using IBM SPSS v.19. The priori p value for an association was considered to be p ≤0.05.
2. Study 2: Determining association of *BRCA1* promoter methylation with rs11655505 (c.-2265 C/T) variation and gene expression in sporadic breast cancer

DNA methylation is epigenetic event which may cause the gene silencing without altering the gene and genetics. The aim was to find any significant association (i) between *BRCA1* promoter methylation and gene expression; (ii) gene expression and the variation rs11655505 (c.-2265 C/T) and; (iii) variation rs11655505 (c.-2265 C/T) and promoter methylation in sporadic breast cancer patients. Following methods were carried out to determine the association of *BRCA1* promoter methylation with rs11655505 (c.-2265 C/T) variation and gene expression.

2.1. Collection of samples

Twenty-nine sporadic breast tumors and twenty six breast biopsy materials were collected from the pathology archives and tumor bank at the Indo-American Cancer Research Institute and Mahavir Hospital, Hyderabad. Of the 29 breast tumors, 18 were paraffin embedded specimens and 11 were fresh frozen specimens. Samples containing >50% tumor representative sections from each case were selected after microscopic examination. 26 frozen breast biopsy specimens containing normal breast epithelia were used as controls. Count of breast cancer tissues according to their tumor stages are shown in table below. Blood samples from healthy women with no history of any type of cancer in last
two generation and up to second cousins were also collected. Study was approved by the ethical committees of Indo American Cancer Hospital & Research Institute and study proposal was approved by the Director and Review Committee, R & D Center, Bharathiar University. All the demographic and pathological data were obtained from the patient’s record.

**Number of breast cancer tissue samples according to the stages**

<table>
<thead>
<tr>
<th>Tumor Stages</th>
<th>Number of Tissue Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage-I</td>
<td>5</td>
</tr>
<tr>
<td>Stage-II</td>
<td>11</td>
</tr>
<tr>
<td>Stage-III</td>
<td>9</td>
</tr>
<tr>
<td>Stage-IV</td>
<td>4</td>
</tr>
</tbody>
</table>

**2.2. DNA and RNA Extraction**

Genomic DNA and total RNA was extracted from paraffin-embedded tissue using AllPrep DNA/RNA FFPE Kit (Qiagen, Inc., Valencia, CA). For frozen tissues, MagMAX™ DNA Multi-Sample Kit (Applied Biosystems, Foster City, CA) was used to extract genomic DNA whereas RNA was extracted by using TRIzol protocol (Invitrogen, Carlsbad, CA). All the extractions were performed according to the manufacturer’s instructions. The quantity of extracted DNA and RNA was determined by NanoDrop 1000 (Thermo Fisher Scientific, MA, USA). Agarose gel electrophoresis was performed for the quality determination (Schwark et al. 2011). DNA from the
blood of healthy women was isolated by salting out method (Appendices III, IV and V).

2.3. **BRCA1 rs11655505 (c.–2265C/T) genotyping**

For the single nucleotide polymorphism (SNP) analysis, TaqMan® SNP Genotyping Assays were performed according to the manufacturer’s instructions with a slight modification (Appendix II). All PCRs were run in duplicate and contained 50 ng (10 µL) DNA, 9 µL TaqMan genotype PCR master mix, and 1 µL allelic discrimination assay mix. Real-time PCR was performed on ABI 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Condition for the allelic discrimination PCRs were; 50ºC for 2 min, 95ºC for 10 min, and then 40 cycles of amplification (92ºC denaturation 15 s, 62ºC annealing, extension for 60 s). The annealing temperature was empirically determined (data not shown) to promote high binding specificity of the probes without the loss of assay sensitivity. For each cycle, the SDS software determines ΔRn, which is the normalized (i.e., compared with a passive reference fluorophore) fluorescent signal from the VIC or FAM labeled probe. For the present study, we used ΔRn value after the final cycle because it proved more reliable than the cycle at which the threshold was crossed (CT value) (Alshatwi et al. 2012b).
2.4. Detecting the methylation status of BRCA1 promoter (nucleotides -150 to +32)

Methylation status of the BRCA1 promoter region was determined by methylation specific PCR after sodium bisulphite modification of DNA (Herman et al. 1996). For sodium bisulphite modification of DNA and purification MethylSEQuTM (Applied Biosystems, Foster City, CA, USA) was used according to the manufacture’s recommendations. The modified DNA was amplified through earlier reported sets of primers for BRCA1 promoter (nucleotides -150 to +32) (Baldwin et al. 2000). Primer sequences for methylated promoter were (F) GGTTAATTAGAGTTTCGAGAGACG and (R) TCAACGAACCTCAGCCGCAGCAATCG; unmethylated promoter was (F) GGTTAATTAGAGTTTTGAGAGATG and (R) TCAACAAACTCACACCAATCA. DNA extracted from blood of healthy women and DNA after in vitro methylation was used as controls. The amplification of methylated and unmethylated promoters AmpliTaq 360 DNA polymerase reagents and protocols were used. The primer sets and AmpliTaq 360 kits were obtained from Applied Biosystems, Foster City, CA, USA. Instructions of manufacture were followed with modifications.

The PCR solution (25 µL) contained 5 µL (<1µg) of modified DNA, 2 µL (10pM) of both the primers, 2 µL magnesium chloride (2mM), 2 µL (100µM) dNTP mix, 2 µL GC enhancer, 0.25 µL (1.25U) AmpliTaq 360 DNA polymerase, 2.5 µL 10X AmpliTaq 360 buffer and 7.25 µL PCR grade water.
The qPCR was carried out in ABI 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the following conditions: one cycle of 94°C for 10 minutes followed by 40 cycles of 95°C for 30s, 65°C for 30s and 72°C for 60s, ending with one cycle of 72°C for 7 minutes. For each bisulphite modified DNA sample, same reactions were carried out with the methylated and unmethylated sets of primer. A blank reaction (water) was also run parallelly. Then, 6 µL of the PCR product were mixed with 6 µL of 1X loading buffer (98% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 10 mM EDTA) and electrophoresed on 0.8% agarose gels. (Appendices VI, VII and VIII).

2.5. cDNA conversion and qPCR analysis for gene expression

Extracted RNA from tissues was subjected to cDNA conversion. At this point, all the mRNAs get converted as cDNA. RT² First Strand Kit (Qiagen, Inc., Valencia, CA) was used for cDNA conversion. All the manufacturer’s instructions were followed except the incubation period of the reaction mixture, which was kept as 20min. mRNA levels of BRCA1 and the reference gene GAPDH was assayed using gene-specific SYBR Green-based QuantiTect® Primer assays (QIAGen, Inc., Valencia, CA.). Quantitative real-time RT-PCR was performed in a reaction volume of 25 µL according to the manufacturer’s instructions.

To each well of a PCR plate 12.5 µL of the master mix, 2.5 µL of primer assay (10×) and 10 µL of template cDNA (100 µg) were added. After a brief
centrifugation, the PCR plate was subjected to 35 cycles at the following conditions: PCR activation at 95°C for 5 minutes, denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 10 seconds. All samples and controls were run in triplicates on an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA). The quantitative RT-PCR data was analyzed by a comparative threshold (Ct) method. ΔCt was calculated by subtracting the Ct-value of GAPDH from BRCA1. The mean ΔCt of patients and control samples were 1.80 and 2.22. ΔCt of each patient and control sample was subtracted from that. Value less than the mean ΔCt was regarded as the low expression (LE) whereas, equal or more than mean ΔCt as the normal expression (NE) of BRCA1 gene (Wei et al. 2005) (Appendix IX and X).

2.6. Statistical analyses

χ²-tests were performed for genotypic and allelic frequencies and methylation status. Odds Ratio (ORs) was calculated to find out the association between rs11655505 (c.-2265C/T) and methylation status of BRCA1 promoter with the disease. Fisher Exact tests were used to calculate the differences among three genotypes and methylated and unmethylated status for each clinico-pathological parameter. Fisher Exact tests were performed to calculate the methylation status of BRCA1 promoter among three genotypes, level of gene expression among three different genotypes and the effect of methylation status on gene expression. In addition to all the tests statistical analyses were carried out using Sigma Plot (v11.0). Multiple logistic regressions (MLR) were
performed to evaluate the association of BRCA1 genotypes and gene expression level with methylation status of BRCA1 promoter for patients and control samples separately. MLR performed with the help of IBM SPSS Statistics (v19.0).