

BREAST CANCER PATIENTS;

Identification of mutation(s) in BRCA1 gene

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ABSTRACT.. Objective: Genetic factors contribute to the high rates with breast cancer patients. Our objective was to screen the mutations in the BRCA 1 gene in exon 20. **Design:** A diagnostic study. **Place and Duration of Study:** The study was carried out in molecular biology lab, Department of Zoology, GC University Lahore and Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore over a period of one year from July 2011 to Aug 2012. **Patients and Methods:** To screen for mutation in the BRCA1 gene, blood samples were collected from 22 different patients suffering from breast cancer from the Anmol Hospital and Sir Ganga Ram Hospital Lahore. The collected samples were processed to screen any mutation in exon 20 which is indicative of the fact that exon 20 is not a hotspot for mutations. **Results:** In our study of 22 females, we have found no mutation in the gene. It is becoming increasingly clear that breast cancer is a multifaceted and heterogeneous disease and histopathological characteristics of breast cancer are controlled by subsets of genetic alterations, providing convincing hints of genotypic–phenotypic correlations between morphological patterns and molecular changes. BRCA has emerged as the master regulator of the genome through its ability to regulate and coordinate various steps of DNA damage response. Women who carry a mutation of the gene have greatly increased chance of developing breast cancer. The population of Pakistan has been substantially screened for somatic and germline mutations in BRCA. **Conclusions:** Breast cancer is the most common cancer of women in Pakistan. One every 8th women is found to carry the disease. A female may develop the disease through inherited mutations in the BRCA1 gene. The absence of mutation maybe attributed to small sample size of the study or may be due to the fact that the size of the gene is so large that a single axon may not be enough to screen for mutations.

Key words: Breast Cancer; Gene BRCA1; Pakistan; Risk Factors.

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INTRODUCTION

Breast cancer (BC) is reported to be the most common type of cancer and involves approximately one third of malignancies in the females worldwide and is responsible for approximately one quarter of cancer related deaths in females¹. Young females (less than age 35) are reported to have more aggressive form of disease than the older ones (less than age 75) thereby causing a higher mortality rate in the former case². Approximately ninety-five percent of breast carcinomas arise from breast epithelial elements. Invasive (infiltrating) breast cancers spread outside the membrane that lines a duct or lobule, invading the surrounding tissues. The cancer cells can then travel to other parts of the body, such as the lymph nodes¹.

Different risk factors associated with the increased incidence of breast cancer include gender (females have a higher rate of incidence than men), hormonal

history (cumulative exposure to estrogen and progesterone), hormone replacement therapy, cousin marriages, tobacco, excess alcohol consumption, obesity, excessive sunlight exposure, diet, physical activity, environmental toxins, certain types of infections and environmental exposure to different chemicals and radiations³. Hereditary breast cancer cases account for about 5-10% while 90-95% of other breast cancer cases are sporadic⁴. Genetic mutations in Breast Cancer Anti-estrogen resistance-1 genes (BRCA1), Breast Cancer Anti-estrogen resistance-2 gene (BRCA2), Ataxia Telangiectasia mutant gene (ATM), Phosphate and Tensin homology (PTEN) and Tumor suppressor Protein (P53) and several genes are also involved in the onset of BC^{5,7}. Approximately 80% of the cases of hereditary breast cancer are due to mutations in either BRCA1 (40–45%) or BRCA2 (35–40%). Mutations in the various genes are associated with the some of the remaining 20% of cases: e.g.,

Cowden syndrome (the PTEN tumor suppressor), Li-Fraumeni syndrome (p53), and the androgen receptor⁸.

BRCA1 gene is located on the q arm of chromosome 17 and at band 21. BRCA1 gene is a very huge gene that spread over almost 100 kb of total genomic DNA. The gene consists of 24 exon among which 1 and 4 are non-coding and are not analyzed, and codes for a protein of 1863 amino acids which produces a nuclear protein of about 220 kd⁹. The gene is mainly involved in the maintenance of genomic stability as well as functions as tumor suppressor gene. However any transformation in the gene results in serious malfunctioning; thus leading to breast cancer development⁴. Normally BRCA1 gene repairs a broken PTEN gene by sewing it back together. When BRCA 1 is mutated, it stops repairing PTEN gene, hence contributing towards development of cancer and metastasis.

No repair of PTEN gene by BRCA1 results in cell growth, proliferation, cell death inhibition, cell migration, new blood vessels sprout and metastasis. Mutations in the BRCA1/2 gene are found in most families with a positive history of breast cancer or with many cases of early onset breast cancer, especially if they also include one or more patients with ovarian cancer³. However, BRCA1 somatic mutations have seldom been reported in sporadic breast cancer¹⁰. There are more than 600 different mutations that have been identified in BRCA1 gene and 450 mutations in BRCA2¹¹. Mutation of BRCA1 is thought to account for approximately 45% of families with significantly high breast cancer incidence and at least 80% of families with increased incidence of both early-onset breast cancer and ovarian cancer¹². It has been recognized for several years that BRCA1 related breast cancers are more likely to be estrogen receptor (ER) negative¹³. Women diagnosed with high-grade, estrogen receptor negative breast cancer before the age of 30 years have a 40-45% chance of harboring a BRCA1 mutation,

compared with a 4-5% chance if these parameters are not met¹³.

There are many genetic tests for BRCA1 and BRCA 2 mutations but techniques based on sequencing and denaturing high performance liquid chromatography, denaturing gradient gel electrophoresis offer the greatest accuracy; but these techniques are expensive. The breast cancer screening recommendations are based on data from families with cancer predisposing BRCA1 mutations, which shows that elevated breast cancer risk begins in the late 20s or early 30s. Screening should be started with monthly breast self-examination in early adulthood, annual clinical examination at age 25-35 years and annual mammography at age 25-35 years¹⁴.

Mammography, ultrasound and magnetic resonance imaging (MRI) are usually recommended for the diagnosis of disease. Surgery may also be done to remove the 'at risk' tissues¹⁵.

Chemoprevention is also used to reduce the risk of developing breast cancer or to reduce the risk of re-getting the cancer. For example, the drug tamoxifen has been proved effective in the treatment of diagnosed breast cancer as well as to lower the risk of breast cancer in BRCA1 mutation carriers. Tamoxifen appears to be effective in reducing both local recurrence and contralateral breast cancer in women with BRCA1 mutations⁸.

At present, in Pakistan breast cancer is the most common type of cancer occurring in women, followed by the cancer of oral cavity and the ovary¹⁶. In Asia, Pakistan has one of the highest rates of breast cancer, and majority of the cases occur below age 40^{16,17}. Only one study from Pakistan has described relationship between consanguinity and the risk of breast cancer¹⁸. The objective of this study was to find association/linkage of BRCA1 gene in breast cancer patients. The mutations in Breast Cancer Susceptibility

(BRCA1) gene in exon 20 in Pakistani breast cancer females were studied.

MATERIALS AND METHODS

To screen for mutation in the BRCA1 gene, blood samples were collected from different patients suffering from breast cancer. The sampling was done from the Anmol Hospital and Sir Ganga Ram Hospital Lahore. About 3ml of blood sample from each patient was collected in blood vacutainer EDTA tubes. The collected samples were stored at -20°C in molecular biology lab, Department of Zoology, GC University

Type of cancer	Age at diagnosis	Duration of cancer
CA Breast	35 years	3 months
CA Breast	47 years	1 year
CA Breast	31 years	5-6 months
CA Breast	37 years	2 year
CA Breast	40 years	2 months
CA Breast	33 years	3-4 months
CA Breast	35 years	6 months
CA Breast	38 years	5-6 months
CA Breast	45 years	1 year
CA Breast	40 years	1 year
CA Breast	38 years	4 months
CA Breast	50 years	3 months
CA Breast	42 years	3 months
CA Breast	35 years	6-7 months
CA Breast	49 years	3 months
CA Breast	36 years	4 months
CA Breast	44 years	4 months
CA Breast	39 years	8 months
CA Breast	33 years	2 months
CA Breast	35 years	6 months
CA Breast	31 years	3 months
CA Breast	28 years	5 months

Table-I.I. History of blood samples.

Lahore and Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore. History of patients is as follows:

DNA EXTRACTION

Blood obtained from the patients was subjected to DNA extraction to get genomic DNA. The modified phenol chloroform method was used to extract Genomic DNA¹⁹.

GEL ELECTROPHORESIS

Horizontal gel electrophoresis method was used for the confirmation of extracted DNA (John and Stowell, 2006).

AMPLIFICATION OF GENE

The amplification of gene was done by using the method as described by Chan *et al.*, 2001¹².

PRIMER DESIGNING

To amplify the exons 20, two sets of primers were designed from already reported sequence of BRCA1 gene²¹. The sequences of designed primers are given below:

BRCA1 gene	Exon	Primer sequence (5'-3')	Exon size
	E20F	5' ATATGACGTG TCTGCTCCAC3'	401bp
	E20R	5' GGGAATCCAA ATTACACAGC3'	401bp

Table-II. Primer sequence.

POLYMERASE CHAIN REACTION

The gene was amplified by Polymerase Chain Reaction (PCR) with a final volume of 25 μl with thermocycling conditions as described by Dufresen *et al.*, 2006

Cycling condition
Initial denaturation at 97 for 4 min
Followed by 40 cycles of denaturation at 94 for 1 min
Annealing at 57 for 1 min, extension at 72 for 1.5 min
Final extension at 72 for 8 min
Table-III. PCR cycling condition

AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCT

Amplification of specific gene fragments was confirmed by agarose gel electrophoresis. For this 3µl of 6X DNA loading dye was added to 5µl PCR product. Hundred milliliters (100ml) of 1% agarose gel was prepared in 1X TAE buffer (prepared from 50X TAE). Amplified products were loaded and the gel was run for 40 minutes at 90 volts. UV transilluminator was used to visualize the amplified product.

GENE CLEAN AND DNA QUANTIFICATION

To purify the amplified PCR fragment from the gel desired bands of PCR amplified products were cut from agarose gel by using a sterilized razor and saved in eppendorf tubes. Each fragment was weighed using digital electronic balance. Equal volume of Binding buffer (GB buffer) was added to the eppendorf having slice of amplified DNA and was incubated at 55°C for 10min or until gel melted completely. Dissolved gel was transferred to the gene clean column and was centrifuged at 10000rpm for 1 minute. To remove the contamination 500µl of wash buffer were added to each column and centrifuged it for 1 minute at 10000rpm. Filtrate was discarded. This step was repeated again to ensure the removal of all contaminants. Empty column was dried by centrifuging at 10000rpm for 1minute. The column was transferred to new labelled micro-centrifuge tube and 50µl of elution buffer was added to the center of column membrane and allowed to stand for 2 minutes. Amplified DNA was collected in eppendorf tubes by centrifugation of columns at 10000rpm for 2

minutes. Purified PCR product was stored at -20°C.

After gene clean 5µl of product was loaded on 1% agarose gel. Six microliters of DNA Ladder Mix were loaded for comparing the intensity of DNA bands.

SEQUENCING

About 30ng of each gene clean product was sent to CAMB (Center of Advanced Molecular Biology) for sequencing along with E20F and E20R primers. The sequencing results showed their homology with exon 20. Sequences were be further analyzed for mutations.

SEQUENCING ANALYSIS

Peaks of the sequences received from CAMB were analyzed carefully for their quality and then blasted in Nucleotide Blast of NCBI.

RESULTS

To look for mutations in BRCA1 gene blood samples were collected from Inmol Hospital Lahore and Sir Ganga Ram Hospital Lahore. About 3ml of blood was taken from the patients and transferred to EDTA vials. The samples were brought to Molecular Biology Lab of Zoology Department and Institute of Molecular and Biotechnology (IMBB) of University of Lahore and stored at -20°C.

ISOLATION OF GENOMIC DNA

The genomic DNA was isolated from blood samples of patients suffering from breast cancer using a modified phenol chloroform protocol. Sodium dodecyl sulphate (SDS) was used to lyse the WBCs and denature the proteins. Pretease K was used to lyse nuclear membrane. After isolation the genomic DNA was stored at -20°C. About 5µl of isolated genomic DNA along with 3µl 6X loading dye is loaded in wells on 1%agarose gel for electrophoresis. After 40 minutes the gel was visualized on UV trans-illuminator. Bright bands of DNA were observed at high molecular weight.

PCR RESULT OF BRCA1 GENE

After primer designing exon 20 of BRCA1 gene was amplified using progene thermocycler. Different optimized conditions were used for the amplification of the exon. Optimized products were obtained by PCR at 50°C by using 0.64mM dNTPs and 2mM MgCl₂ in the presence of 1X Taq buffer with (NH₄)₂SO₄. The product was again loaded on 1% agarose gel for electrophoresis. Sharp bands of DNA were visualized at 401 bp. Following figures are showing the results of PCR amplification:

RESULTS OF SEQUENCING

The sequence obtained is homologous to normal human genome sequence. No mutation is observed in our case study of breast cancer patients.

DISCUSSION

Breast cancer is the most common malignancy of females affecting every one in eight women during their lifetime and about one million females are diagnosed with breast cancer every year^{5,24}. The disease is more common and severe in young females than the older ones². Most of the breast cancer cases reported today are sporadic and have no genetic lineage in their families. However, about 5% of cases have been considered hereditary and run in families through their genetic linkage. In Pakistan, breast carcinomas are the most common carcinomas among women with incidence rates similar to those reported in the west⁷. In Karachi, Pakistan the age standardized rates (ASR) of BC are reported to be 51.7/100,000 per year, the highest ASR reported for any Asian population. In other Asian populations, these rates are reported to be <40 per100,000 per year^{6,16}. Confluence of information suggested that BRCA1 acted as a scaffold in the assembly of a multi-protein complex and had functionalities in gene transcription, DNA damage repair and transcription-coupled DNA damage repair.

In different areas of Punjab, families have different

rates of mutations, e.g; Punjabi families have 1128 cases of mutations (4184del4; axon20), 1071 have mutations (2041insA; axon 20). In Pashtun families (2080insA; axon20) mutations in 90 cases are found. At international level British families have one case of (ivs20+60 ins12bp) in axon 20. Seven families have axon 20 deletions. Four cases have 5382inSC, which also occurs in axon 20. The major (non genetic) risk factors for breast and ovarian cancer are reproductive and dietary factors. Pakistani women have a low frequency of these traditional risk factors. The female population, on average, has high levels of fertility, early age at first pregnancy, multiple births, and prolonged breast-feeding. Although the age at first marriage has been gradually rising, women in consanguineous unions marry at earlier ages and are less likely to use modern contraceptive methods than are women from nonconsanguineous marriages²³. Generally, Pakistani women do not use exogenous hormones (oral contraception or estrogen-replacement therapy). They generally do not smoke tobacco products, although some women practice chewing pan and pan tobacco. Furthermore, the population of Pakistan is predominantly Muslim (97%), and alcohol consumption is uncommon⁷. Our study did not report any mutation in exon 20 of BRCA. It needs to be investigated in detail among breast cancer subtypes. Our sampling was random and we did not include data of the chemotherapeutic drugs. Moreover we did not evaluate BRCA mutations in breast cancer subtypes. Moreover, mutations in other regulators of DNA damage signaling and downstream effectors of cell signaling were not screened. The expression of BRCA in our study was not determined as there is a possibility of a transcriptional down regulation or miRNA mediated targeting of BRCA. All these aspects need detailed investigation.

CONCLUSIONS

Breast cancer is the most common cancer of women in Pakistan. One every 8th women is found to carry the disease. A female may develop the disease through

inherited mutations in the BRCA1 gene. In our case study of 22 females, we have found no mutation in the gene. The absence of mutation maybe attributed to small sample size of the study or may be due to the fact that the size of the gene is so large that a single axon may not be enough to screen for mutations.

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**Talent wins games,
but teamwork and intelligence
wins championships.**

Michael Jordan