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BRCA1 Status in Pakistani Breast Cancer Patients with Moderate Family History

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ABSTRACT

Objective: To determine BRCA1 status in breast carcinoma patients of Pakistani origin.

Study Design: Observational study.

Place and Duration of Study: The Oncology Clinics of the Aga Khan University Hospital, Karachi, between May 2005 and December 2009.

Methodology: Fifty three breast cancer patients based on clinical and laboratory diagnosis were recruited for this study. Moderate family history was defined as having a close relative (mother, daughter, sister) diagnosed with breast cancer under 45 years. Peripheral blood samples were collected from each patient in a 5 ml tube containing EDTA as anticoagulant. Subsequent to DNA extraction, mutational analysis of BRCA1 exons 2, 5, 6, 16, 20 and 22 was carried out using single strand conformation polymorphism (SSCP) assay while protein truncation test (PTT) was used to examine mutations in exon 11. All BRCA1 sequence variants were confirmed by DNA sequencing.

Results: Twenty-three patients were diagnosed with early onset breast cancer, 30 patients had moderate family history. At the time of diagnosis, the median age of enrolled patients was 39 years (range 24-65 years). Out of 53 patients, analyzed by SSCP assay, mobility shift was detected in exon 6, 16 and 20 of three patients, whereas one patient was tested positive for mutation in exon 11 by PTT assays. All patients with BRCA1 mutations were further confirmed by DNA sequencing analysis. In exon 16 c.4837A > G was confirmed, which is a common polymorphism reported in several populations including Asians. Moreover, mutations in exon 6 (c.271T > G), exon 20 (c.5231 delG) and exon 11 (c.1123 T > G) were reported first time in the Pakistani population.

Conclusion: Several BRCA1 mutations were observed in Pakistani breast cancer patients with moderate family history. Therefore, mutation-based genetic counselling for patients with moderate family history can facilitate management, if one first or second degree relative or early onset disease is apparent.

Key words: BRCA1. Mutation. Pakistani women. Breast cancer. Familial.

INTRODUCTION

Cancer remains one of the leading causes of death worldwide. In Pakistan, like other parts of the world, breast cancer is the most common malignancy in women followed by oral cavity and ovarian cancer.^{1,2} In addition, the number of breast cancer cases is on the rise, for instance in 2004, Karachi Cancer Registry has reported 69.1% ASR per 100,000 from 1998 to 2000 in comparison to 53% per 100,000 during 1995 to 1997.¹ Bhurgri *et al.* also reported an association of family history in 10% of the early onset (less than 45 years) breast cancer patients in Pakistan.³

Both BRCA1 and BRCA2 genes are important to familial breast cancer and reportedly confer 80% life time risk of breast cancer and 40% life time risk of ovarian cancer.⁴

Mutations in BRCA1 and BRCA2 genes have been reported worldwide, but the type of mutations differ considerably from country to country. Rashid *et al.* studied 176 Pakistani breast cancer patients and reported thirty germ-line mutations.⁵ Most of the studies in familial breast cancer patients were carried out in large pedigree families with multiple affected relatives over many generations; the parameters of such studies cannot be applied to interpret small pedigree families.⁶⁻⁸ Likewise, breast cancer patients with moderate family history have not been studied in the Pakistani population.

The aim of the present study was to determine BRCA1 mutations in early onset cancer patients (age < 45 years) from Pakistan with and without moderate family history.

METHODOLOGY

Overall, 53 breast cancer patients confirmed on the basis of clinical and laboratory diagnosis were recruited from the Oncology Clinics of the Aga Khan University Hospital, Karachi, between May 2005 and December 2009. The criteria for inclusion were based on the involvement of first degree and/or second degree

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Table I: Distribution of sequence variants in BRCA1 exons.

Patient ID	Age	BRCA1 exon	Nucleotide change	Amino acid change	Reported in BIC	Mutation type ¹	Ethnicity	Family history	Other cancer history
3	28	11	c.1123T > G	stop at 374	Novel	FM	Pashtun	Pos	Neg
19	29	20	c.5231delG	ORF	Novel	FM	Mohajir	Pos	Neg
30	39	16	c.4837A > G	S1613G	Reported	PM	Mohajir	Pos	Neg
48	48	6	c.271T > G	C91W	Novel	MS	Mohajir	Pos	Neg

¹FM= frame shift mutation; PM= polymorphism; MS = missense mutation.

relatives with breast and/or ovarian cancer and age at the time of diagnosis. The participants (breast cancer patients) were divided into three groups; (a) women with early onset (< 38 years) breast cancer without first degree relatives with breast cancer, (b) women with breast cancer under the age of 45 years with at least one first or second degree relative with breast cancer, (c) women with bilateral breast cancer having first degree relative with breast or ovarian cancer. Out of 53 patients, 23 did not report family history but demonstrated early onset breast cancer and their ages ranged between 20 and 37 years. On the other hand, remaining 30 patients exhibited moderate family history. Peripheral blood samples were collected from patients in 5 ml tubes containing EDTA as anti-coagulant. This study was approved by the institution's ethical review committee.

DNA was extracted from the blood samples by Wizard DNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. It was diluted to 100 ng/μl concentration in molecular biology grade deionized water. PCR assay for BRCA1 exons 2, 5, 6, 20 and 22 was carried out according to the published protocols. The amplified products were verified on agarose gel and analyzed by SSCP assay that was performed for the screening of BRCA1 mutations as described by Futreal *et al.*⁹ Briefly, 10 μl of PCR product was mixed with 10 μl of gel loading dye (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and xylene cyanol) and was applied to 0.5 x MDE gel (FMC Bioproducts, USA). After electrophoresis at room temperature in 0.6 x TBE, the gel was silver stained and photographed. Exon 11 of BRCA1 was analyzed by PTT assay according to Hogevoorst protocol.¹⁰ It was amplified as three overlapping fragments with 5' primers modified to carry a T7 promoter site. Amplicons were used as template for *in vitro* transcription and translation for producing a ³⁵S methionine labelled protein. Protein products were separated on a 10% polyacrylamide gel, fixed and autoradiographed. DNA samples revealing sequence variations by SSCP or PTT assay were confirmed by cycle sequencing (CEQ 8000, Beckman, USA).

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 19.0 for windows. Fisher's exact test was used to observe the

Table II: Comparison of risk factors among BRCA1 negative familial, BRCA1 mutation carrier and non-familial (< 45 years old) breast cancer patients.

Variable	Family history group			p-value
	Familial (n=26)	Non-familial (n=23)	BRCA1 (n=4)	
Age at onset ¹	41.0 (34.8, 49.2)	33.0 (30.0, 35.0)	35.5 (28.2, 42.8)	0.018
First degree relation				
Yes	23 (88.5%)	0	3 (75%)	< 0.001
No	3 (11.5%)	23 (100%)	1 (25%)	
Second degree relation				
Yes	7 (26.9%)	0	1 (25%)	0.017
No	19 (73.1%)	23 (23%)	3 (75%)	
Other cancer history				
Yes	19 (73%)	21 (91.3%)	3 (75%)	0.300
No	6 (23%)	2 (8.7%)	1 (25%)	
Age at menarche ²				
< 12	2 (7.7%)	3 (13%)	0	0.309
12	14 (53.8%)	6 (26.1%)	1 (25%)	
> 12	9 (34.6%)	13 (56.6%)	2 (50%)	
Menopause status				
Yes	5 (19.2%)	4 (17.4%)	0	1.000
No	21 (80.8%)	19 (82.6%)	4 (100%)	
Age at first child birth				
< 30 years	16 (61.5%)	13 (56.5%)	2 (50%)	0.159
> 30 years	1 (3.8%)	–	–	
No birth	–	6 (26.1%)	1 (25%)	
Unknown	9 (34.6%)	4 (17.47%)	1 (25%)	

¹Median (inter-quartile range); ² n=50 (3 observations are missing)

association of degree of relationship (first and second), other cancer history, age at menarche, menopausal status and age at first child birth with the family history group. Age at onset was found non-normally distributed, while age at menopause was found to be normally distributed using Kolmogorov-Smirnov and Shapiro Wilk methods. Kruskal Wallis test was used to compare age at onset by family history group. Results were considered significant for p < 0.05.

RESULTS

Fifty three patients recruited on the basis of early onset breast cancer were screened for mutations in BRCA1 exons by PTT and SSCP techniques. Mobility shift was identified in exons 6, 16 and 20 of the BRCA1 gene in three patients. Figure 1 (a-f) shows a representative gel electrophoresis patterns of 3 positive patients for mutations in exons 6, 16, and 20. These exons were

further amplified and sequenced to confirm the location of mutations. As shown in Table I, BRCA1 mutation was identified in exon 11 in a Pathan patient, while mutations in exon 6, 16, 20 were identified in Urdu speaking (Mohajir) families. Figure 1 illustrates the sequence analysis of exons 6, 16, and 20. Except for a polymorphism identified in exon 16 (c.4837 A > G), the

other two mutations in exon 6 (c.271 T > G) and 20 (c.5231 del G) were novel mutations. Exon 11 of BRCA1 gene was screened for missense mutations using PTT assay. As presented in Table I, in one subject a nucleotide exchange (T > G) at position c.1123 was confirmed, which resulted in conversion of amino acid Leucine at position 374 to stop codon.

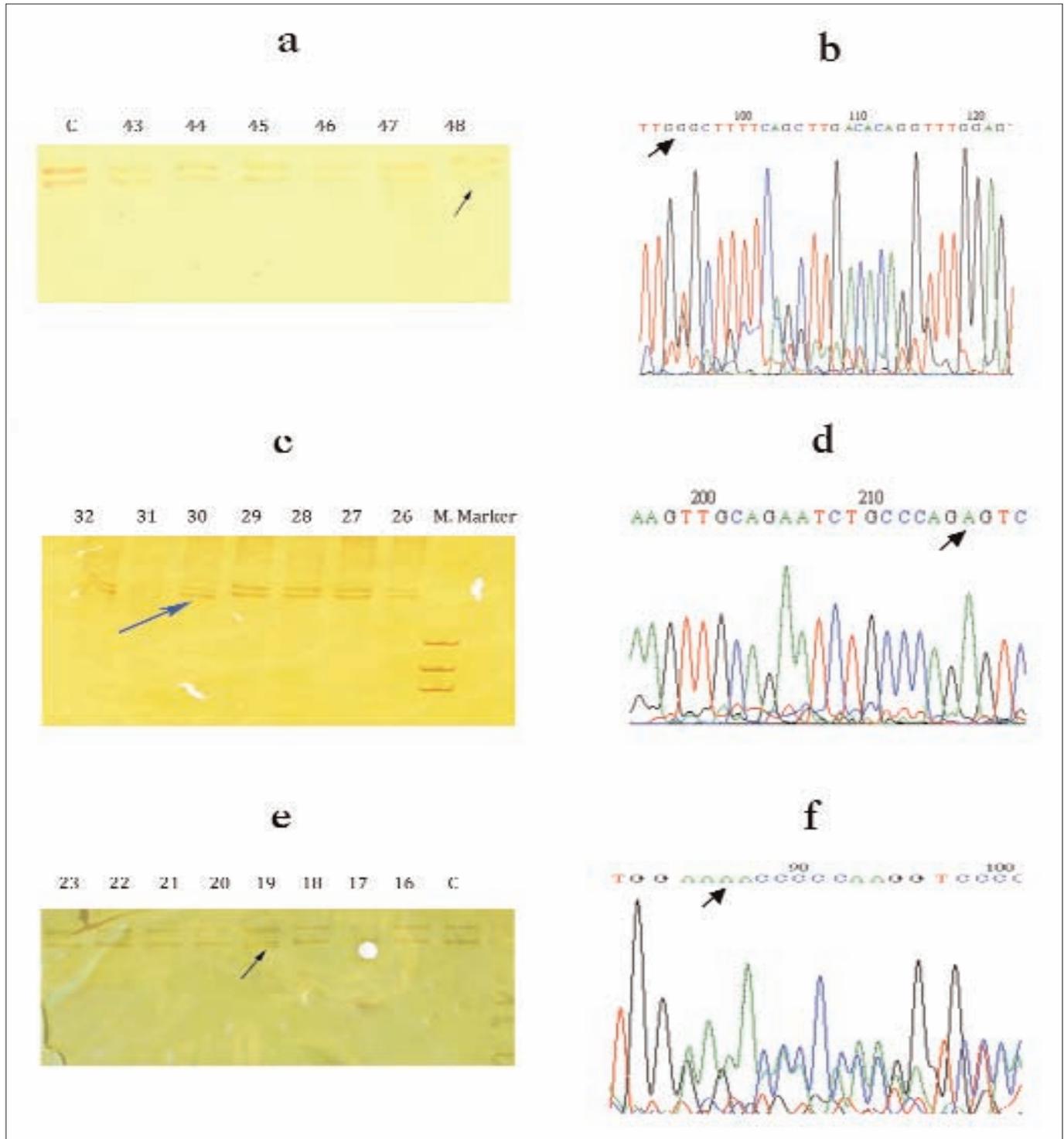


Figure 1 (a-f): Single strand conformation polymorphism (SSCP) assay for germ-line mutation in exons 6, 16, 20 (a, b, c) differential migration pattern of patient sample marked by arrow on SSCP gel conformed by direct sequencing (d) a germ-line mutation in the BRCA1 (exon 6). A missense substitution at c.271 G > T. (e) mutation in the BRCA1 (exon16). A missense substitution at c.4837G > A. (f) a germ-line mutation in the BRCA1 (exon 20). One bp deletion in BRCA1 at c.5231 del G is marked by an arrow.

Table II shows the distribution of breast cancer patients by family history group. The first group consisted of 26 familial breast cancer patients; their median age at time of diagnosis was 41 years. In this group, 43.3% cases acquired the disease after 40 years of age and a large proportion of their second degree relatives (37%) were diagnosed for breast cancer at less than 38 years of age. The median age of the onset of breast cancer in BRCA1 positive (35.5 years) and non-familial breast cancer patients (33 years) was comparatively lower than the familial group (41 years) and it was statistically significant ($p=0.018$). In addition, comparison of risk factors among the patient groups illustrated in Table II shows more first and degree relatives affected by the disease in BRCA1 mutation carriers and non-carriers familial patients (first degree relatives, $p < 0.001$). Although, family members 3/4 BRCA1 positive patients reported malignancies other than breast cancer, nevertheless, the difference was not statistically significant ($p=0.300$). In addition, the difference in risk factors such as age at menarche and menopause or age at first child birth did not appear significant among the groups or when compared with the Western populations. Furthermore, uterine and ovarian cancers were frequently reported in the family members of BRCA1 positive patients.

DISCUSSION

In the last several decades, the issue of familial breast cancer has received immense attention due to advancements in early detection and treatment options as well as discovery of breast cancer associated genes e.g. BRCA1 and BRCA2. The data from earlier BRCA1 studies represented large pedigrees with multiple affected relatives over several generations.

In the current study, pre-menopausal breast cancer patients (less than 45 years) with moderate family history were selected for the analysis of BRCA1 gene. The exons of BRCA1 gene selected for analysis were 2, 5, 6, 11, 16, 20 and 22. Many published reports have shown higher frequency of mutations in these exons.¹¹⁻¹³ The frequency of BRCA1 mutations in our patients was 8%, which is in agreement with the mutation rate (6%) reported by Yassaee *et al.* in the Iranian population.¹¹ However, if patients with moderate family history are included then the mutation rate would increase to 13%. Many studies have shown BRCA1 mutation rate ranging from 6-45%.¹⁴⁻¹⁷ The lower mutation rate in this study highlights the importance for entire BRCA1 gene analysis. Overall, four mutations were detected, three were substitutions and one was deletion. Moreover, except for one substitution at exon 16, all other mutations were first time reported. A novel mutation identified in exon 6 at position 271 results in cytosine to tryptophan substitution at codon 91. This substitution has the potential to disrupt formation of BRCA1/BARD1 heterodimers, which is an important step for retention of

BRCA1 in the nucleus for promoting DNA repair process.¹⁸ In another patient, A > G substitution was identified at position 1613 in exon 16, which was reported in other populations as well, and according to Breast Information Core (BIC) database, it is classified as a polymorphism. Two mutations were detected in exon 11 and 20. In exon 11, a single base substitution T > G at position 1123 led to the creation of a stop codon at amino acid position 375 leading to truncation of BRCA1 protein. In addition, a frame shift mutation at position 5231 was detected in exon 20. A linker region is encoded by exon 20, which is positioned between N-terminal and C-terminal BRCT regions of BRCA1 protein.¹⁹

The average age of patients carrying BRCA1 mutations at the time of diagnosis was 36 years and 50% of the patients were below 30 years, further confirming the fact that BRCA1 mutations are associated with early onset breast cancer. Several other studies have documented a higher prevalence of BRCA1 mutations in patients diagnosed at an early age.²⁰ A meta-analysis of 22 breast cancer studies performed by Antoniou *et al.* concluded that penetrance of mutated BRCA1 gene depends upon index case's age, mutation site and number of affected breast cancer relatives.²¹ In addition, Al-Mulla *et al.* in 2008 published a study on penetrance of BRCA1 and reported that different BRCA1 mutations have different effect on the onset age of breast and ovarian cancer.²²

In the present study, BRCA1 mutations were not detected in patients with early onset breast cancer without family history and in a majority of patients with moderate family history. These observations are consistent with Peto *et al.* who also have demonstrated lower penetrance of breast cancer genes in their population.²⁰ It is becoming evident that moderate families which are characterized by less striking history showed increased risk of disease. These families may be affected by a mixture of low penetrance genes, genetic factors and shared environmental factors.

This data was classified into two groups based on the presence of BRCA1 mutations. The results suggested that majority of BRCA1 mutation positive patients had statistically significant ($p < 0.001$) involvement of first degree relatives in comparison to second degree relatives. These findings are also supported in previous studies, which have shown strong association of family history with disease and showed that the risk of disease vary according to the nature of family history.²³ On the contrary, Kim *et al.* showed that most of their Korean breast cancer patients with BRCA1 mutations did not have a family history, which is in sharp contrast to the well-known perception.²⁴

Reproductive factors including early menarche, pre- and postmenopause and age at first child birth were evaluated in this study, but correlation with breast cancer were inconsistent when compared with previously

reported studies. In addition, recently published studies also failed to find a correlation between early menarche and appearance of breast cancer at an early stage. Our findings also demonstrated early menarche before 12 years of age in 3/23 of non-familial breast cancer patients. Conversely, no case of early menarche was observed in BRCA1 positive group. These findings are consistent with studies that reported no correlation of early menarche with the appearance of breast cancer in early age.^{24,25}

CONCLUSION

Familial breast cancer is a heterogeneous disease in which hormonal, and environmental factors can confound risks associated with BRCA1 mutations. We found increased frequency of BRCA1 mutations in breast cancer patients with moderate family history. Pakistani patients can benefit from selective screening of BRCA1 gene and mutation based counselling for small and moderate families if one first or second degree relative is affected or early onset disease is apparent.

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