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Genotyping of frequent *BRCA1* SNPs in familial breast cancer in Indian population by restriction fragment length polymorphism and sequencing

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ABSTRACT

Breast cancer is one of the most common malignancies affecting women worldwide. In India, breast cancer is the second most common malignant condition among women. Genetic predisposition for familial early onset of breast cancer accounts for approximately 5-10% of all breast cancers. Mutations in two autosomal dominant genes, BRCA1 and BRCA2 have been linked to familial breast cancer. In the present study 15 Indian families with hereditary breast cancer were studied for BRCA1 mutations using PCR RFLP method. The sequencing result showed that there is a single mutation at the EcoR1 restriction site. So RFLP can be used as to find out mutation or SNP in the gene responsible for the cancer development. Gene testing of BRCA1/2 is available as a routine clinical test for diagnosing hereditary breast/ovarian cancer (HBOC) in the US and other Western countries, while only a few reports have been published concerning the prevalence of BRCA1/2 mutations among Indian people. Thus the present study results indicate that BRCA1 mutation play a vital role in breast cancer but a considerable proportion of the early breast cancer and familial breast cancer may be due to genes other than BRCA1 mutation.

Key words: *BRCA*, SNPs, RFLP and Breast Cancer

INTRODUCTION

Germ line mutations in the *BRCA1* (17q chromosome) and *BRCA2* (13q chromosome) genes are considered to be the most important and well-known genetic risk factors for breast and ovarian cancer [1, 2]. It has been known that about 5.0–10.0% of all breast cancer and 10.0–15.0% of ovarian cancer patients carry mutations on one of the *BRCA* genes [3, 4]. In the general population, the prevalence of the BRCA1/2 gene mutation carriers is approximately 0.2% (1/500) [5, 6], but, it can differ considerably in different countries and ethnic groups due to founder effects [7]. The mutations in these genes predispose to a high lifetime risk of breast and ovarian cancer [8, 9]. In women with an inherited *BRCA1* gene mutation [10], the risk of developing breast cancer and ovarian cancer over their lifetime is 65.0–80.0% and 37.0–62.0%, respectively [11,12]. Thereby, the detection of *BRCA1* and *BRCA2* gene mutation carriers is an important step in assessing the risk in each individual [13, 14]. In addition, the incidence and the type of BRCA1 mutations differ extensively and may have different geographic and ethnic distribution [15]

In an attempt to assess the distribution of *BRCA1* gene mutations in the Indian population, the aim of the current study is to screen for *BRCA1* gene mutations using PCR RFLP method in the Indian population [16]

MATERIALS AND METHODS

RBC lysis Buffer were purchased from Vasa Scientific Pvt Ltd, Proteinase-K were purchased from sigmaaldrich scientific pvt ltd. Bioline PCR purification kit were purchased from Bioline, UK. Primers and restriction endonucleases enzymes were purchased from Merck pvt ltd, India. *BRCA1* specific real time primers, PCR reaction mixtures and SYBR Green PCR Master Mix were purchased from Applied Biosystems pvt ltd, Bangalore, India.

Sample collection

Samples were obtained from families at high risk for breast cancer, with three or more individuals with early-onset breast cancer. DNA was extracted either directly from peripheral blood lymphocytes. Blood was collected in EDTA collection tubes and stored at 4° C till use.

DNA isolation and quantification

The DNA extraction was carried out by the Phenol-Chloroform- Isoamyl Alcohol (PCI) method. RBC cells were lyzed and followed by the mixture was centrifuged at 10,000 rpm for 15 min. The precipitated white pellet was washed with PBS and proteins were removed from the sample using Proteinase-K (10mg/mL), DNA extraction buffer, β-mercapto ethanol, 1M DTT (Dithiothreitol). Followed by phenol: chloroform: isoamylalcohol (25:24:1) mixture was added and centrifuged at 10,000 rpm for 15 minutes. From the aqueous layer equal amount of chloroform: Isoamyl alcohol (24:1) was added and mixed gently. After centrifugation at 10,000rpm for 10 min, the aqueous layer aspirated and DNA was precipitated using 70% ethanol. Ethanol was washed out by centrifuging at 10,000 rpm for 10 min and the pellet was dried at RT and dissolved in sterile Milli-Q water and stored at -20°C for further use. The quantity of the isolated DNA was checked in UV-VIS spectophotomer (Viva spec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

PCR Amplification:

The PCR assay was performed using isolated DNA sample in a total reaction volume of 20 µl. The reactions were performed in a Thermo cycler (G-Strom, UK). Thirty amplification cycles were performed using BACA1 FP: 5'CAC CTC CAA GGT GTA TGA AG3' and BACA1 RP: 3'CTC TAG GAT TCT CTG AGC ATG G5'. After amplification, PCR products included 100bp ladder was loaded in 1.0% Agarose gel electrophoresis.

SNP Detection by Restriction fragment length Polymorphism

After amplification of BRCA1 region, the sequence composition of a PCR product can be investigated by restriction enzymes. Digestion of PCR products with restriction endonucleases generates a number of fragments, which can be resolved by 2% Agarose gel electrophoresis, yielding a particular banding pattern. To determine the SNP, RFLP analysis was performed separately by selected Restriction Endonucleases enzyme *Eco*RI (Merck, India).

Direct Sequencing of PCR Products for SNP detection

The samples showing aberrant heteroduplex pattern were reamplified from genomic DNA; amplicons were purified using Bioline PCR purification kit (Bioline, UK) and subjected to cycle sequencing using ABI Protocol using direct incorporation of radiolabel was followed as per manufacturer's instructions. Cycling conditions were 30 cycles of 95°C for 30 s, 56°C for 30 s and 70°C for 1 min. The sequencing primers were the same as those used to amplify the template.

Real time PCR analysis for SNP detection

BRCA1 specific real time primers designed by using PRIMEREXPRESS software (Applied Biosystems). All real-time PCR reactions were performed using the ABI Step One (Applied Biosystems) and the amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems) using the following FP- 5'CCT TAC TTC CAG CCC ATC TGT3' and RP-3'AGC TGA GAG GCA TCC AGA AA5'. The thermal cycling conditions were composed of 50ºC for 2 min followed by an initial denaturation step at 95ºC for 10 min, 45 cycles at 95ºC for 30s, 60ºC for 30s and 72ºC for 30s. The experiments were carried out in triplicate for each data point.

RESULTS

The total genomic DNA was isolated by modified CTAB method from the infected blood samples. A total of seven blood sample was collected from the Government Hospital, Bangalore. After RBC lysis, the obtained pellet was used for the total genomic DNA isolation. The isolated DNA was electrophorized in 1% Agarose gel (Fig.1). The purity of DNA was indicated by A260/A280 ratio, where the value from 1.8 to 2.0 was considered as high purity. Nevertheless, the extracted DNA is considered of adequate purity if A260/A280 is > 1.5.

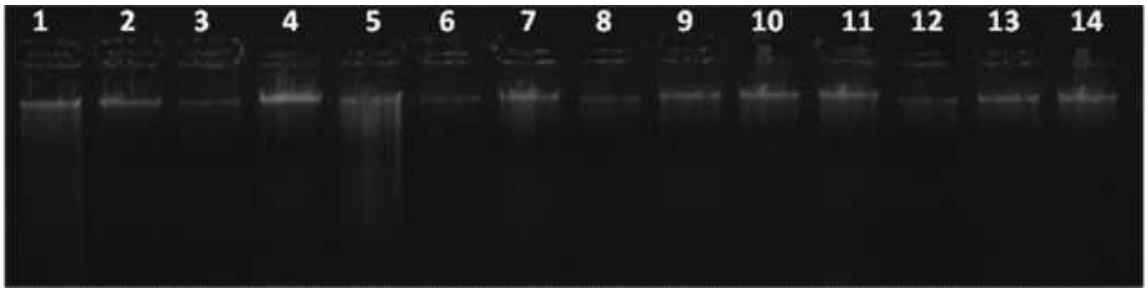


Fig 1. Genomic DNA isolated from patient blood samples (Lane 1-14 - Genomic DNA)

PCR primer designing and PCR amplification of BRCA1 gene

Clinical application of BRCA1/2 gene testing brought a paradigm shift in cancer prevention strategies targeted to at-risk mutation carriers. BRCA1 encodes an 1863 amino acid nuclear protein. Evidence implicates a role for BRCA1 in the control of gene expression, perhaps at the level of transcription. A number of observations have linked BRCA1 to DNA damage response pathways. The regulation and function of BRCA1 is probably very complex. Nevertheless, the finding that the mutations in BRCA1 are associated with hereditary breast cancer is valuable information.

Genetic testing could potentially offer different management options for the BRCA1 mutation carrying high- risk individuals. Analysis of the complete sequence of the *BRCA*1 gene found at NCBI enabled the selection of two new PCR primers. Sequences within the *BRCA*1 gene were aligned, and the most highly conserved regions were selected to design primers for PCR. Specific primers were designed for the *BRCA*1 gene using the sequences available in NCBI GenBank using Primer 3 Software. The predicted primers were validated initially *in silico* and subsequently in wet lab. The primers could yield an amplicon of the expected size of ~ 500bp The PCR product was electrophorized and visualized by 1% agarose gel. (Fig.2) BRCA1 gene (chr 17q21) is a tumor suppressor gene that encodes tumor suppressor protein which acts as a negative regulator for tumor growth.

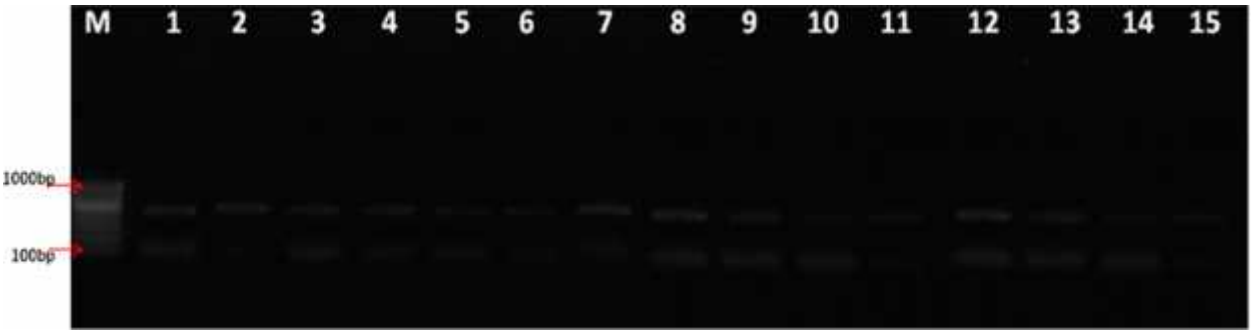


Fig 2. PCR amplification of the BRCA1 partial sequence using specific primer

SNP analysis RFLP method

In this study, 15 Indian families with hereditary breast cancer were studied for BRCA 1 mutations using PCR based RFLP method. Single nucleotide polymorphism (SNP) plays an important role in the study of complex genetic diseases, in pharmacogenetic analysis, in population genetics and evolutionary studies. Gene testing of BRCA1/2 is available as a SNP analysis by direct PCR product sequencing.

The PCR products, after restriction digestion which showed single band, were sequenced by cycle sequencing to identify the nature of mutation. Appropriate control PCR products from normal persons were also sequenced. We were able to identify the mutations in the sample 9. This mutation leads to the inhibition of digestion of the *Eco*R1 restriction enzyme which forms an uncut single band in the RFLP pattern. A woman's risk of developing breast and/or ovarian cancer is greatly increased if she inherits a deleterious (harmful) BRCA1 or BRCA2 mutation. Men with these mutations also have an increased risk of breast cancer. Both men and women who have harmful BRCA1 or BRCA2 mutations may be at increased risk of other cancers routine clinical test for diagnosing hereditary breast/ovarian cancer (HBOC) in the US and other Western countries while only a few reports have been published concerning the prevalence of BRCA1/2 mutations among Indian people as shown in the figure 3, 4 and 5.

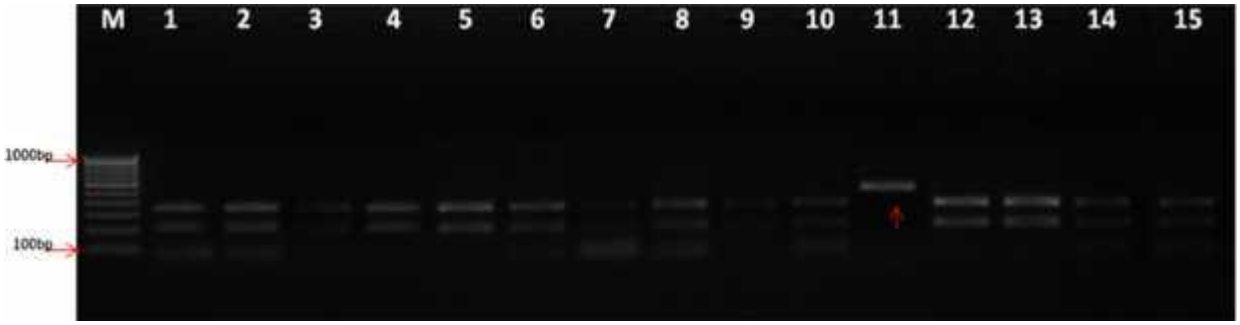
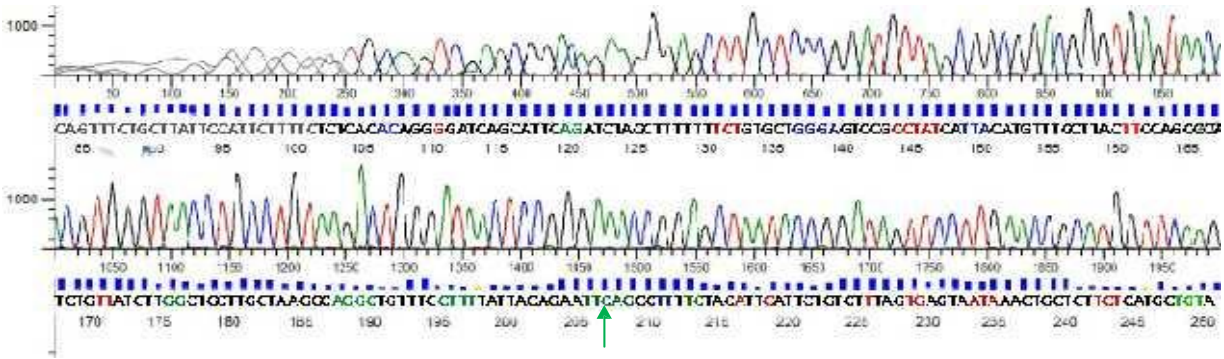


Fig 3. SNP detection by using RFLP analysis of BRCA1 gene using *Eco R*1restriction enzyme

Normal Sample:



5′CAGTTTCTGCTTATTCCATTCTTTTCTCTCACACAGGGGATCAGCATTCAGATCTACCTTTTTTTCTG
TGCTGGGAGTCCGCCTATCATTACATGTTTCCTTACTTCCAGCCCATCTGTTATGTTGGCTCCTTGCTAA
GCCAGGCTGTTTGCTTTTATTACAGAATTCAGCCTTTTCTACATTCATTCTGTCTTTAGTGAGTAATAAA
CTGCTGTTCTCATGCTGTAATGAGCTGGCATGAGTATTTGTGCCACATGGCTCCACATGCAAGTTTGAAA
CAGAACTACCCTGATACTTTTCTGGATGCCTCTCAGCTGCACGCTTCTCAGTGGTGTTCAAATCATTATT
ACTGGGTTGATGATGTTCAGTATTTGTTACATCCGTCTCAGAAAATTCACAAGCAGCTGAAAATATACAA
AAATAACAAGGTACTCAAAAACTGAATTGTCATTAAAAAAATACATA3′

Fig 4. SNP detection by using RFLP analysis of BRCA1 gene in Normal sample

SNP sample- 11:

5′CAGTTTCTGCTTATTCCATTCTTTTCTCTCACACAGGGGATCAGCATTCAGATCTACCTTTTTTTCTG
TGCTGGGAGTCCGCCTATCATTACATGTTTCCTTACTTCCAGCCCATCTGTTATGTTGGCTCCTTGCTAA
GCCAGGCTGTTTGCTTTTATTACAGAATTAAGCCTTTTCTACATTCATTCTGTCTTTAGTGAGTAATAAA
CTGCTGTTCTCATGCTGTAATGAGCTGGCATGAGTATTTGTGCCACATGGCTCCACATGCAAGTTTGAAA
CAGAACTACCCTGATACTTTTCTGGATGCCTCTCAGCTGCACGCTTCTCAGTGGTGTTCAAATCATTATT
ACTGGGTTGATGATGTTCAGTATTTGTTACATCCGTCTCAGAAAATTCACAAGCAGCTGAAAATATACAA
AAATAACAAGGTACTCAAAAACTGAATTGTCATTAAAAAAATACATA3′

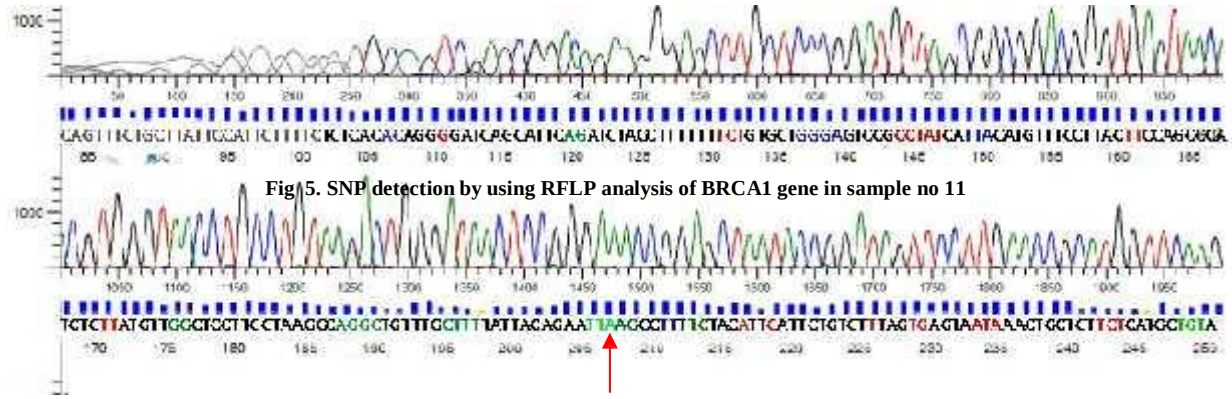


Fig 5. SNP detection by using RFLP analysis of BRCA1 gene in sample no 11

DISCUSSION

In the present study, the study populations with hereditary breast cancer were studied for BRCA1 mutations using PCR RFLP method. Blood sample were collected and total genomic DNA was isolated using modified CTAB method. The isolated DNA was run on 1% Agarose gel to verify the quality and the quantity was estimated by spectrophotometer. BRCA1 specific primers were designed using primer 3 software and synthesized. PCR was done using the specific primer and the gene product was electrophorized in 2% Agarose gel. The amplified product was subjected to restriction digestion using *Eco*R1 and the PCR RFLP, pattern was analyzed. The sample no 11 showed a single band, which is not digested with the restriction enzyme. So it confirmed the mutation in the recognition site of the enzyme. We eluted the PCR product along with two normal sample and send for sequencing. The sequencing result showed that there is a single mutation at the EcoR1 restriction site. So RFLP can be used as to find out mutation or SNP in the gene responsible for the cancer development. Gene testing of BRCA1/2 is available as a routine clinical test for diagnosing hereditary breast/ovarian cancer (HBOC) in the US and other Western countries [17,18], while only a few reports have been published concerning the prevalence of BRCA1/2 mutations among Indian people.

CONCLUTION

To conclude, our study results indicated that BRCA1 mutation has a significant role in breast cancer despite the fact that a considerable proportion of the early breast cancer and familial breast cancer may be due to genes other than BRCA1 mutation. Besides, more analyses on larger cohorts of patients and controls are considered necessary in order to build a high-quality database of genetic *BRCA* gene variants in the Indian population, and to acquire accurate estimates on the association of various polymorphisms with breast cancer risk.

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