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Research article

IDENTIFICATION, ISOLATION AND AMPLIFICATION OF BRCA1 GENE INVOLVED IN BREAST CANCER

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ABSTRACT: Cancer is a disease that begins in the cells of the body which is characterized by uncontrolled, uncoordinated and undesirable cell division. If a cell accumulates critical mutations in five or six of the proto-oncogenes, tumour suppressor genes and DNA repair genes are likely to result in a fully malignant cell, capable of forming a tumour. In this work we described the isolation and amplification of the *BRCA1* gene. Primers were designed and synthesised later used to amplify the BRCA1 gene. The total new workflow includes all steps from purified DNA to data analysis, and includes PCR for all amplicons covering the gene, PCR cleanup, cycle sequencing, electrophoresis, and data analysis. To simplify workflows and decrease the time-to-result, we focused on the method "one sample, one assay" approach. The success of this workflow was the 24-well plate design, which contained prespotted PCR primers covering the gene and also included multiplex nontemplate controls. The workflow was developed using a Genetic Analyzer and bands were observed.

Key Words: Cancer, BRCA1, Primer design and PCR

INTRODUCTION

Cancer harms the body when the cells divide uncontrollably to form lumps of tissue called tumors. Tumors can grow and interfere with the digestive, nervous, and circulatory systems by releasing hormones that alter body function. Identification of BRCA1 has led to major changes in the breast and ovarian cancer treatment of women. These clinical changes has become the innovative feature in identification of genetic approach to high-risk women. The medical and surgical options offered to high-risk women were not helpful in appropriate curing. Ultimately, understanding the pathways of BRCA1 and BRCA2 in normal breast cells and in breast tumorigenesis will help in the basis of non-invasive intervention for women at risk. *BRCA1* plays an important role in repairing damaged DNA and preventing cells from growing. *BRCA* gene mutations are distributed throughout the coding region and were responsible for cause of Cancer (Casey G 1997). BRCA1 and BRCA2 proteins are involved in controlling of double-strand break repair in response to DNA damage and homologous recombination (Scully, R. et al 2000; Scully, R. et al 2000; Wang, Q et al 2000; Zheng, L et al 2000). Regulations of BRCA1 may leads to functional inactivation of BRCA genes which ultimately leads to Cancer. It is known that the normal protein products of BRCA1 were involved in the cellular processes of transcriptional regulation and maintaining genomic integrity. Mutations in BRCA1 were responsible for Cancer cause and were with hereditary susceptibility to breast and ovarian cancer (Hall, J et al 1990; Miki, Y et al 1994; Wooster, R et al 1994; Wooster, R et al 1995). The breast and ovarian cancer phenotypes related to mutations in BRCA1 and BRCA2 are similar. BRCA genes are tumor suppressors as Tumorigenesis in individuals with germline BRCA mutations requires somatic inactivation of the remaining wild-type allele (Smith, S.A et al 1996; Collins, N et al 1995). BRCA mutations leads to breast cancer in males and females, also ovarian, pancreatic, prostatic, bile duct, gall bladder, and stomach cancers and melanoma. Recent genetic studies indicate that BRCA1 mutation leads to a lifetime risk of breast cancer which is more than 80% (The New York Breast Cancer Study Collaborative Group 2001).

In the case of BRCA2 mutations lifetime breast cancer risk approaches that of BRCA1 carriers (Schubert, E.L et al 1997). Women with BRCA1 mutations have an increased risk of ovarian cancer other than breast cancer and males have an lifetime risk of prostatic cancer (Ford, D et al 1994). The phenotypes of BRCA1 and BRCA2 suggest a commonality of function. Multiple functions of BRCA1 And BRCA2 were revealed by biochemical, genetic and cytological studies and from these studies proteins involved in the interaction with BRCA1 and BRCA2 have been identified (Deng, C.X. et al 2000; Irminger-Finger, I et al 1999; Welcsh, P.L et al 2000). Ongoing *BRCA1* and *BRCA2* research has showed the need for the workflow of simple and robust mutation detection. Automated Sanger sequencing of DNA is widely known as the standard for mutation detection and characterization. It is a highly referenced technique that delivers long read lengths and the ability to sequence anywhere from a few to several hundred samples in a single day. The protocols involved are generally straightforward and the assays are cost effective. Genetic Analyzers are widely used in life science research laboratories for DNA sequence analysis as well as for numerous DNA fragment analysis assays. Here we highlight the workflow for analysis of the *BRCA1* gene in a preclinical research setting, which was developed using 24 human DNA samples derived from whole blood.

MATERIALS AND METHODS

Blood samples from 24 human cancer subjects were collected by clinical molecular geneticists at the Indo-American Cancer institute, Hyderabad and were analyzed in this blinded study. Genomic DNA was isolated from 7 mL of fresh whole blood using a Chemagen magnetic particle processor and the Chemagic kit (Chemagen, Baesweiler, Germany) according to the manufacturer's instructions. Amplification, cycle sequencing, and purification were performed and analyzed the reactions on the Genetic Analyzer.

Primer Designing

Making use of the Human Genome build NCBI36 (*BRCA1*: Acc. nr: cDNA: NM_007294.3, *BRCA2*: Acc. nr: NM_000059.3) PCR primers were designed using primer3 server and optimized for 100% coverage of the coding sequences of the *BRCA1* gene, including sections of approximately 50 bp up and downstream of each exon. All amplicons were produced and sequenced using a universal set of PCR conditions. *BRCA1* comprises 23 exons. In order to scan the exons for mutations, the gene was divided into 34 amplicons using specific primer pairs. To enable a single sequencing approach, all forward primers include the forward primer sequence and all reverse primers include the reverse primer sequence.

Amplification Reactions and Conditions

The amplifications were performed using 100 ng of the extracted DNA with AmpliTaq Gold 360 Master Mix on a Veriti 96-well, 0.2 mL Thermal Cycler. AmpliTaq Gold 360 delivered good overall amplification, even in difficult-to-sequence regions such as exon of the *BRCA1* gene. Using AmpliTaq Gold 360 and producing target amplicons that varied in size from 300 bp to 530 bp ensured that good coverage of the *BRCA1* genes was achieved. To facilitate a single DNA screening approach, primer pairs for both genes are prespotted and dried down in the wells of 96-well PCR plates. Multiplex non template controls for every amplicon are included in the plate to ensure that any amplification observed is specific.

Step-by-Step Protocol:

1. Amplification Reactions and Cycling Conditions for Each Plate

Prepare for each sample:

• Sample amplification mix:

1.05 mL AmpliTaq Gold 360 Master Mix

966 µL H2O (ultrapure)

84 µL human genomic DNA (100 ng/µL)

• Nontemplate control mix:

200 µL AmpliTaq Gold 360 Master Mix

200 µL H2O (ultrapure)

• Use prespotted plate with *BRCA1* and *BRCA2* primers

• Add 25.0 µL of the sample amplification mix

• Add 25.0 µL of the nontemplate control mix

Amplification cycling protocol
Initial denaturation 95°C for 10 minutes
Denaturation 95°C for 30 seconds
Annealing 60°C for 30 seconds
Extension 72°C for 1 minute
Final extension 72°C for 7 minutes
Hold temperature 10°C

2. Purification of PCR Products

Add 75 µL of H2O to every well in the PCR plate (to make a 1:4 dilution).
• Prepare ExoSAP-IT Mix
200 µL ExoSAP-IT Mix
600 µL H2O
Following the plate layout, prepare the forward and reverse sequencing plates. Transfer 1 µL of the diluted PCR product to the sequencing plate. Add 4 µL ExoSAP-IT mix to every well.
• Process the plates on the Veriti thermal cycler following this protocol:
15 minutes at 37°C
15 minutes at 80°C
Hold at 4°C

3. Cycle Sequencing

Prepare forward and reverse sequencing mixes:
• Forward mix:
200 µL BigDye Terminator v1.1
100 µL 5X Sequencing buffer
200 µL M13 forward primer (8 pmol/µL)
Add 5 µL sequencing mix to every well of the forward primer PCR plate. The final volume in each well is 10 µL.
• Reverse mix:
200 µL BigDye Terminator v1.1
100 µL 5X Sequencing buffer
200 µL M13 reverse primer (8 pmol/µL)
Add 5 µL sequencing mix to every well of the M13 reverse primer PCR plate. The final volume in each well is 10 µL.
Perform cycle sequencing on a Veriti thermal cycler.
Initial denaturation 96°C for 1 minutes
Denaturation 96°C for 10 seconds
Annealing 50°C for 5 seconds
Extension 60°C for 2 minutes
Hold temperature 10°C

4. Purification of the Sequencing Reactions

• Prepare BigDye XTerminator mix:
9 mL SAM solution
2 mL bead mix
Add 55 µL of the BigDye XTerminator mix to every well of the two sequencing plates. Vortex for 15 minutes at 1,800 rpm. Centrifuge the plates for 2 minutes at 1,000 RCF.

5. Electrophoresis and Analysis

Purified sequencing reactions were analyzed using Genetic Analyzer. The sequence results for each sample were analyzed independently. At the Rayalu's biotech pvt ltd software was used for data analysis.

RESULTS

From the blood samples human DNA was isolated and analyzed in gel doc viewer (Figure 1).

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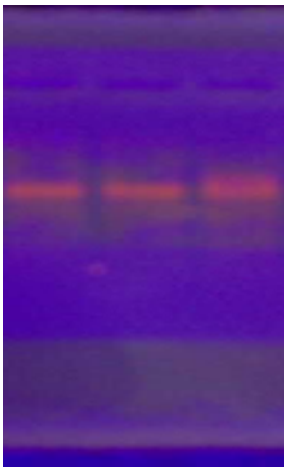

Figure 1: Human DNA isolation

By using the developed optimized sequencing protocol all amplicons that comprise the *BRCA1* gene were amplified with AmpliTaq Gold 360 and sequenced using BigDye Terminator v1.1 chemistry. The protocol has been subsequently verified on 24 human DNA samples. Following sequence analysis by both teams, the mutations observed using the Genetic Analyzer. Software v1.1 provides easy review of mutations, with clear comparison to the reference sequence and the resulting variants, which reduces the time required for data review (Figure 2).

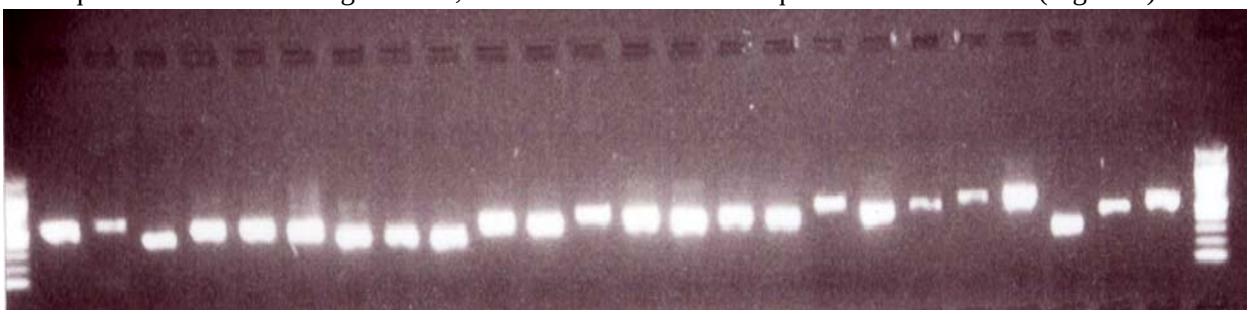

Fig 2: amplified BRCA1 genes

Figures 1 and 2 show that genes isolated and amplified genes of BRCA1.

CONCLUSION

Using the approach described in this publication, mutation scanning of multiple exon genes by direct sequencing becomes very feasible. At the laboratory, skipping the prescreening step accelerated analysis, reduced labor time, and simplified laboratory setup. The AmpliTaq Gold 360 Master Mix gave reproducible and robust results.
Below is a summary of the advantages of this direct sequencing workflow:

• Obtaining sequence data on all exons makes it possible not only to confirm known SNPs, but also to identify other possible variations
• With Sanger sequencing, amplicon length can be designed longer than 500 bp to obtain additional sequence context information, especially in regions around exon-intron boundaries.
• The instrument platform is flexible, allowing complementary studies on the same instrument.
• When amplicons are generated using tailed primers, a single protocol can be implemented in the lab for automated sequencing.
• Samples can be processed as soon as they come into the lab, without the need for batching; this significantly increases the speed of analysis.
• The primer design enables a single protocol to be used for both the PCR and sequencing steps, facilitating automated sequencing.
• This automated workflow significantly reduces hands-on time and increases the speed with which results are obtained.

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