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RESEARCH ARTICLE

SCREENING FOR BRCA2 MUTATION IN EXON 11.2 AND 11.3 IN BREAST/OVARIAN CANCER WOMEN OF MADHYA PRADESH, INDIA: IDENTIFIES NOVEL MUTATIONS

Farah Khan[1]*, Ruchira Chaudhary[2],
Ragini Gothalwal[1], Jyoti Jain[3]

1. Department of Biotechnology, Barkatullah University, Bhopal (M.P.) India.
2. Sarojini Naidu Govt. Girls PG College, Bhopal, (M.P.) India
3. Vijayaraje Institute of Science and Management, Gwalior, (M.P.), India

Correspondence
Farah Khan (Research Scholar)
Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

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ABSTRACT

In this study exon 11.2 and 11.3 of BRCA2 gene were analysed on women (N=90) of breast and ovarian cancer of Gwalior region, Madhya Pradesh (India). Direct sequencing was used for identification and confirmation of mutations. Sequences were analyzed for mutation through various computational methods and comparative analysis with reference sequence of BRCA2 gene. Two novel pathogenic heterozygous mutations were detected in exon 11.3 of BRCA2 and none of the cases showed mutation in exon 11.2 of BRCA2 gene. Critical mutations, first one frameshift (c.4878delG) and second one missense (c.4878G>T) were observed in with the frequency of 1.11% in population. The pathogenic effect of mutation in first one is cause of change in amino acid composition of BRCA2 protein due to shift in coding frame and second missense mutation is may be due to structural change in DNA binding domain of BRCA2 protein. Both these mutations were previously not reported in BIC (Breast Cancer Information Core) database.

INTRODUCTION

Cancer is due to failures of the mechanisms that usually control the growth and proliferation of cells. Cancer occurs when the mechanisms that maintain normal growth rates malfunction to cause excess cell division. Breast cancer is the most frequent cancer in women, and after lung cancer the second most frequent cancer in the world. (Parkin et al., 2005).

Cancer is the main source of death in economically developed nations and the second leading cause of death in developing countries (World Health Organization, 2008). Breast cancer is the most widely recognized malignancy among woman and is the second leading reason for disease related death. It represent 23% of all growths among woman though, ovary represents to 30% of all malignancies of the female genital organs (Tavassoli et al., 2003). In India a normal of 80,000 women are determined to have carcinomas of the breast and 40,000 women die of the disease every year consistently (Saxena et al., 2006).

Ovarian cancer is among the most widely recognized cancers in women worldwide with over 200,000 new cases yearly. The incidence rates are most astounding in Europe, North America and Australia/New Zealand (Globocan 2002). Ovarian cancer is the seventh most common malignancy in women (and the 18th most common malignancy overall) around the world. Roughly 239 000 cases were recorded in 2012, representing almost 4 percent of all new cases of cancer in women (2 percent overall).

Cancer of Breast and Ovaries are a standout amongst the most broadly perceived malignancies affecting females around the globe. Differences in two autosomal predominant genes, BRCA1 and BRCA2, have been related with breast and ovary malignancies. Mutations in these two qualities were foreseen to represent to 85–90% of hereditary breast and ovarian cancer cases. Hereditary distinguishing in order to screen might add to danger control a social event of at-risk patients who could benefit by extended screening or prophylactic measures that run with hereditary powerlessness testing for disease. Many risk factors (hormonal, natural) for breast cancer are known yet the majority of the hereditary foundation and molecular mechanisms still stay to be clarified. Despite the fact that mutations in BRCA1 (Hall et al., 1990, Miki et al., 1994) and BRCA2 (Wooster et al., 1994; Wooster et al., 1995) genes are known not a high lifetime risk of breast cancer and ovarian cancer. In the present study the proportion of BRCA2 mutations was evaluated in breast and ovarian cancer of

regions of Madhya Pradesh, India. The aim of the study was to study genetic alteration in BRCA2 gene of breast and ovarian cancer and to find out novel mutations in the population.

METHODS AND MATERIALS

Patient Selection

All the patients included in this study were treated at the Cancer Hospital, Gwalior, Madhya Pradesh, India. Ninety patients with breast or ovarian or breast/ovarian cancer, each from a different family, were recruited for the study. None male breast cancer patient was found to be recruited. Blood samples collected from 10 age-matched, unrelated normal individuals without a family history of breast or ovarian cancers were used as controls. This study was approved by the ethical standards of Institutional Ethical Committee, following ICMR guidelines.

All study participants gave their informed written consent. A questionnaire was prepared and used to collect the information on Clinico-epidermiological characteristics for example age, sex, family history of patient, body mass/menopause status, site of tumour, marital status, blood group etc. from the patients.

Samples Collection

2-5 ml of blood sample was collected from healthy as well as patients by using 5ml disposable syringes in vials containing EDTA as anticoagulant and incidentally put away in to the refrigerator (4ºC) and immediately handled for further examination. Collected blood samples were preserved at 4°C. For storing the samples at room temperature FTA (Flinders Technology Associates) classic card (Whatman Inc, Clifton, NJ) have been used.

Isolation of DNA from Blood samples:

DNA was isolated from all the blood samples using the standard phenol - chloroform protocol (Ithanet.eu 2012) following Sambrook *et al*., (1989).

In Blood sample erythrocyte lysis buffer I (10mM TrisHCl (pH 8.0), 320 mM Sucrose, 5mM $MgCl_2$, 1%Triton X) was added and centrifuged at 3600 rpm for 10min. To the pellet added lysis buffer II (400mM TrisHCl, 60mM EDTA, 150mM NaCl, 1%SDS added after autoclaving) and 5M Sodium perchlorate was added and shaken thoroughly. Equal volume of phenol (phenol, 0.1% 8-Hydroxy Quinoline, 0.5 M TrisHCl (pH 8.0), 0.1 M TrisHCl (pH 8.0).): chloroform: isoamyl alcohol (Phenol: Chloroform: Isoamylalcohol (25:24:1) was added,

mixed gently and centrifuged at 3000 rpm for 10min. The aqueous layer so formed was, mixed thoroughly with equal volume of chloroform and centrifuged at 3000 rpm at 10 min. Again to the aqueous layer ice cold ethanol was added to precipitate DNA. The DNA was pooled out carefully, washed twice with 70% ethanol, air dried and dissolved in TE buffer (10mM TrisHCl (pH 7.5), 1mM EDTA, pH 8.0).

DNA of samples extracted from blood using commercial Fast Technology of Analysis (FTA), an advanced and more efficient technique for DNA preservation, isolation and PCR amplification (Chaudhary *et al.*, 2013).

Sample loaded to FTA Cards and Labeled the Whatman FTA classic cards with thwe appropriate sample identification. Dropped the blood sample about 200-250µl on the circle. Allowed the sample to dry for about for about one hour at room temperature and can be stored at the same. Sample was then ready for downstream processing as punching and PCR analysis.Remove a small disc (1.2 mm or 2.0mm) from dried sample spot and place this into PCR tubes (as per manufacturer's instruction 2-3 discs used for amplification).Washing of the discs in PCR tubes was done in tilted position (as per manufacturer's instruction of washing). Washing reagent was added to PCR tube & incubated for 15-20 minutes at room temperature. Discard all spent FTA reagent by pipetting (as per manufacturer's instruction 100-200 µl of washing reagent to be added to and incubated for 5 min at room temperature). The process was repeated for a total of two washes with FTA purification reagent. The same process was repeated twice with 100µl TE-buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) (as per manufacturer's instruction). The entire spent TE buffer was removed and discarded by pipetting. The disc was allowed to dry at room temp for overnight. The disc is now ready for PCR application.

Polymerization and preparation of DNA:

The extracted DNA was quantified by the UV Spectrophotometer (OD_{260}/OD_{280}, 1.8 to 2.0) method. One ml TE buffer was taken in a cuvette and calibrate the spectrophotometer at 260nm as well as280nm. Added 10 µl of each DNA sample to 900µl TE (Tris-EDTA buffer) and mix well. Used TE buffer as a blank in the other cuvette of the spectrophotometer.OD_{260} and OD_{280} values were noted on spectrophotometer. OD_{260}/OD_{280} ratio were then calculated. The amount of DNA can be quantified using the formula:

DNA concentration (µg/ml) = $\frac{\text{OD260 x 100 (dilution factor) x 50 µg/ml}}{1000}$

The ratio of absorbance at 260 to 280 nm indicates the purity of the sample. This ratio of DNA solutions should range from 1.7 to 1.8. The presence of impurities like proteins or phenol tends to decrease this ratio.

Quantification followed by checking in 0.8% agarose gel by dissolving agarose in 100 ml 0.5X TAE buffer and boiled to dissolve agarose completely.0.7µl Ethidium bromide was added from stock solution to make a final concentration of 0.5µg/ml. Gel was cooled down to 60°C, poured onto a gel tray and was allowed to set. A standard DNA sample of known concentration was also loaded along with the samples to quantify DNA and electrophoresis was carried out at a constant voltage of 80V and after 30 min. of run halfway the gel (containing DNA) and visualized under ultraviolet light (long wave, 340 nm) of UV trans-illuminator and different size marker (ladder) was used in order to estimate the fragment sizes and photographed.

Amplification of BRCA2 gene was carried with, 2 sets of primers (forward and reverse). BRCA2 forward primer (5'-ATCTTGATTATAAAGAAGCACGCCC GCCGCGCCCCGCGCCCGGCCCGCCG CCCCCGCCCG) for exon 11.2, BRCA2 revearse primer (5'-TGAATGTTGTACTGGGTGAC) for exon 11.2, BRCA2 forward primer (5'-AAAAGAAGAGGTCTTGGCTG) for exon 11.3 and BRCA2 revearse primer (5'-GACTAGGTTTGACAGAACACGCCCG CCGCGCCCCGCGCCCGGCCCGCCGC CCCCGCCCG) for exon 11.3 (Table 4), (Purnomosari, 2005).

1U Taq polymerase. The amplification conditions were for BRCA2 exon specific primers (94°C for 5 minutes, 35 cycles (94°C for 1 minutes, 58°C for 1 minutes, 72°C for 30 seconds, 72°C for 10 minutes and 4°C forever for BRCA1and BRCA2 (Lakhotiaet al., 2002 ; Vaidyanathanet al., 2009).

The amplified products the exon specific genes were electrophoresed at 120V in 0.8% agarose gel. The PCR products were then visualized under UV light in transilluminator. On obtaining a single band devoid of any primer-dimer bands the PCR products were then sequenced.

Sequencing of DNA region

Medox easy spin column PCR cleanup Minipreps kit was used for purification of

DNA fragments. Capillary sequencing was done with purified PCR products were sequenced by Samved Biotech Pvt. Ltd, Ahmedabad using the selected primers (forward or reverse) of *BRCA2* (Sigma). DNA sequences of primers were received in the form of ABI and FASTA files. Direct sequencing of the amplified product was carried out in a 96 well plate Sequencing machine (ABI 3730XL), using 100ng of PCR product, 4pmol of primer (forward or reverse separately), 1.8µl of Big Dye Terminator reaction kit (Applied Biosystems, Foster City, CA) and 1.1µl of doubled distilled water to make up the final volume to 5µl. The sequencing conditions are as following: 30 cycles at 96°C for 10 seconds, 55°C for 5 seconds, and 60°C for 4 minutes and 4°C forever.

SNP analysis

Dataset: Sequences of *BRCA2* exon 11.2 and 11.3 regions were sequenced and one references sequence of gene (NM_000059) was obtained from NCBI RefSeq database (http://www. *BRCA2*ncbi.nlm.nih.gov/refseq/).

Sequence Alignment: Multiple sequence alignment was carried out among the sample sequences and reference sequence by the software Clustal-Omega (Sievers *et al*., 2011) (http://www.ebi.ac.uk/Tools/msa/clustalo/) and further alignment was visualized with help of BioEdit V7.2.5 (Hall*et al.,*1999) software to observe the presence of deletions and synonymous mutations. The sequence containing these mutations were detected for further analysis of presence of deleterious mutations (SNP). The mutation position in sample sequence were correlated with position in reference *BRCA2* sequence. The detected position was observed in HCI Database of Prior Probabilities of Pathogenicity for Single Nucleotide Substitutions Information and mutations are notified by BIC numbering system of HCI *BRCA1/2* Prior Probabilities Database.

Confirmation of SNP: The observed single nucleotide mutations were confirmed from the HCI (Huntsman Cancer Institute) *BRCA1/2* Prior Probabilities Database (http://priors.hci.utah.edu/PRIORS/index.php)(Goldgar *et al.,* 2008), *De-novo* Donor site information, protein level information, variant codon and variant amino acid, Align GVGD grade, probability of pathogenicity were analyzed from HGVS (Human Genome Variation Society) substitution nomenclature database. Novelty of mutation of confirmed from LOVD (Leiden Open Variation Database) database (Fokkema *et al.,* 2011). Protein coding frames and point mutations in sample sequences were confirmed from BLAST (BLASTN and BLASTX)

(Altschul *et al*., 1990) analyses.

Analysis of effect of mutations on structural features of BRCA Protein:

Point mutation effect on Structural features of protein sequences were analyzed by HOPE server (Venselaar *et al*., 2010) which compile the related information on various web servers and databases. Further the proteins were also analyzed by PolyPhen-2 server (Adzhubei *et al*., 2013).

Cluster Analysis: To derive the relatedness of the subjects and diversity of exon11.3 and exon11.2 of *BRCA1/2* gene in Madhya Pradesh regions Indian population, cluster analysis was performed using MEGA6 (Kumar *et al*., 2013). Distance matrix method UPGMA (Unweighted Pair Group Method with Arithmetician Mean) method (Sokal and Michener, 1958), mathematical model: Kimura 2 parameter (Kimura*et al.*, 1980) and test of phylogeny: Bootstrap (Hedges*et al.*, 1992) with 500 replicates were used to generate dendogram.

RESULTS AND DISCUSSION

Multiple Sequence Alignment (MSA) of BRCA2 exon 11.3 region of sequences

Total 16 sequences of *BRCA2* gene exon11.3 were aligned with Reference Sequence of *BRCA2* gene (NM_000059) along the region from nucleotide 2138 - 7069 (Exon11 region). Multiple Sequence Alignment of *BRCA2* exon11.3 is presented in Fig. 1, which shows one deletion in breast sample at position 2750 of BioEdit scale and BIC scale 4878. One stop codon is inserted at position 2748 of BioEdit, where nucleotide 'G' is replaced by 'T' in ovarian sample. Only two mutations are shown in this region. Terminal region is not considered for mutation due to poor quality of sequencing.

Mutations in *BRCA2* exon11.3

Two mutations were identified as unique to the Indian population and are distinguish throughout the *BRCA2* gene exon 11.3, thus the mutation frequency was 2.23% (2/91).In our study the percentage of *BRCA2* mutations was low among Indian women, compared to the other Asian Countries (Leide *et al.*, 2002, Balraj *et al*., 2002, De Leon Massuda *et al.*, 2002) however the lower contribution of *BRCA2* mutation is supported by earlier studies from India.(Saxena *et al.* 2002; Rajkumar *et al.* 2003; Valarmathi*et al.* 2004; Saxena *et al.* 2006).

Frameshift Mutation in *BRCA2* exon 11.3

1) Mutation: FKe11.3c4878G

Mutation c.4878delG (Box-1) shows nucleotide 4878 'G' is deleted and coding frame shift by one nucleotide, that changed

the codon patterns and hence the amino acid composition for *BRCA2* protein, onward of amino acid 1550E (Glutamate) in sequence. Codon 1550'GAG' changes to 'GAC', that codes for amino acid Glutamine (Q). Due to frameshift the amino acid 1550 Glutamate (E) is replaced by Aspratate (D) showing no effect at this point and even at codon position 1551, where amino acid Glutamine (Q) is replaced by Lysine (K). But onwards towards C-terminal of protein, the composition changes due to frameshift that impart the changes in protein 3D structure hence protein function. Fig. 2 (A) is electropherogram of mutant breast sample, showing deletion of 'G' in-between the nucleotide positions 108 and 109, whereas in normal 'G' is present at position 108.

Missense Mutation in BRCA2 exon 11.3

2) Mutation: FKe11.3c4876G>T

Mutationc4876G>T (Box-2) isPathogenic missense type of mutation caused due to replacement of 4876 'G' nucleotide by 'T' at first position of codon 1550'GAG'. Codon 1550 'GAG' converted into 'TAG', which is termination codon that stops protein synthesis and partial BRCA2 protein, is produced. From A-GVGD score at protein level it indicates high probability value of pathogenic type (0.99). Fig. 3 (A) is an elegrophoregram of ovary sample showing replacement of nucleotide 'G' to 'T' at position 111 of electrophoregram. Whereas in normal sample GAG is present at 106 - 108.

Diversity of exon-11.3 of *BRCA2* nucleotide sequences

Cluster analysis is multivariate statistical method that group the taxa based on similarity and un-grouping based on dissimilarity. Sequences provide number of characters and options to form number of groups in other word – optimum clustering. Gene sequences are primarily preferred to derive relationship among the taxa or to judge the extent of variations. Sequences of all samples clustered by MEGA6 (Kumar *et al.,* 2013) software, using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method (Sokal and Michener, 1958), evolutionary model – Kimura-2 parameter (Kimura, 1980). To minimize error, test of phylogeny – Bootstrap method (Hedges, 1992) with 1000 replicates was used. Dendogram was divided into two clade and externally linked toreference sequence ‘NM_000059’ and two sample sequences. Clustering pattern in dendogram clearly indicates that closely related taxa are clustered together and with increased dissimilarity taxa separates from each other. Similar type of study was carried out by Lou *et al.*, (2014) to depict the relationship among the *BRCA* sequences from various organisms closely related to human being. It can be observed in Fig.-4

that clades are separated for breast and ovarian cancer samples *BRCA2* sequences with mix-up of few sequences of other types. Maximum taxa possess lesser than 0.1 unit distances or variations indicating conserved nature of sequences of Exon 11.2 *BRCA2*. Two taxa sequences with critical mutations are, 'B22-55-F-*BRCA2'* and 'O22-32-F-*BRCA2'* are clustered together in one clade. All mutations may not affect protein but few mutations become lethal due to its critical position in sequence. Cluster analysis method was used to study the diversity of exon sequences of gene ABO blood group system (Chaudhary *et al.,* 2014) and exon 11.3 of *BRCA1* gene (Chaudhary *et al.,* 2016) in population of Madhya Pradesh, India.

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CONCLUSION

The breast cancer exon 11.3 of *BRCA2* gene from breast and ovarian patients were investigated by evaluating influence of functional SNPs through computational methods. Of total 2 considered SNPs of *BRCA2* 11.3 region, all mutations were predicted to be pathogenic.

Pathogenic 2 SNPs were comprised of 1 INDELs and 1 non-synonymous SNPs (Missense). INDELs were observed to be incorporating in-frame stop codon in coding region resulting into incomplete protein synthesis. Missense mutation was detected as deleterious from the score, 50% - 80% on HOPE server, >0.8 probability score on PlyPHen-2 server and C0-C25 A-GVGD grade score. Entries of these 2 mutations predicted here were not found in BIC (Breast Cancer Information Core) database, though computationally confirmed entries are present in HIC database. Identification of these novel mutation and a wider *BRCA* mutational spectrum suggest that any given population should develop a mutation database for its programme of *BRCA* genetic testing and counselling This study emphasize that there must be regular check-up for chances of breast and ovarian cancer at around the age ≥40. Possible way is through DNA sequencing of exonic regions of *BRCA2* gene, more prone for mutations and its analysis for mutations will provide help in early diagnosis of cancer before onset and necessary medical treatment can be taken.

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Table-1: INDELs and Missense Variants of Unclassified and Polymorphisms Identified In Breast/Ovarian Cancer BRCA2 Gene of Patients from Indian Population (Studied Patients Cases=90, Control=10)**.**

Mutation	**Exon**	**Nucleotide Change**	**Amino Acid Change**	**Mutation type**	**Allel's Frequency**		**Homo/ Heterozygous Cases**		**BIC entries**	**HOPE Disorder %**	**HCI BCGP Probability**	**Probable Influence of Mutation**
					Cases (%) n=90	**Control (%)** ***n*=10**	**Homo** (%)	**Hetero** (%)				
BRCA2												
c.4878delG	11.3	4878delG	p.1550E>D	FS	1 (1.11%)	0	0	1 (1.11%)	No	NA	NA	Pathogenic
c.4878G>T	11.3	4876G>T	p.1550E>*	MS	1 (1.11%)	0	0	1 (1.11%)	No	NA	NA	Pathogenic Stop codon

Note: FS-Frame Shift, MS-Missense, NP-Non pathogenic; HCI BCGP Probability: Huntsman Cancer Institute Breast Cancer Genes Prior Probabilities; BIC: Breast Cancer Information Core

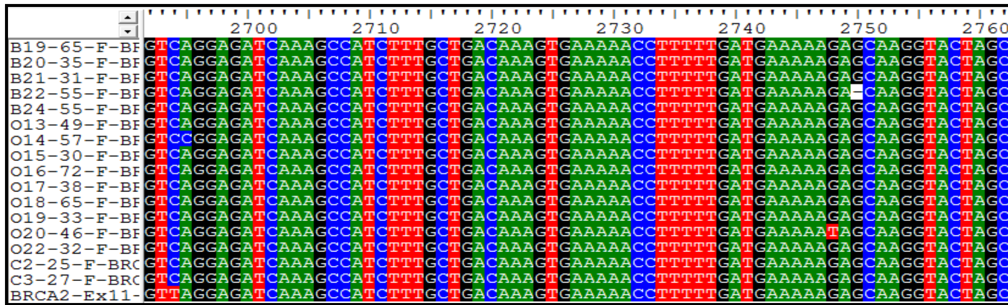
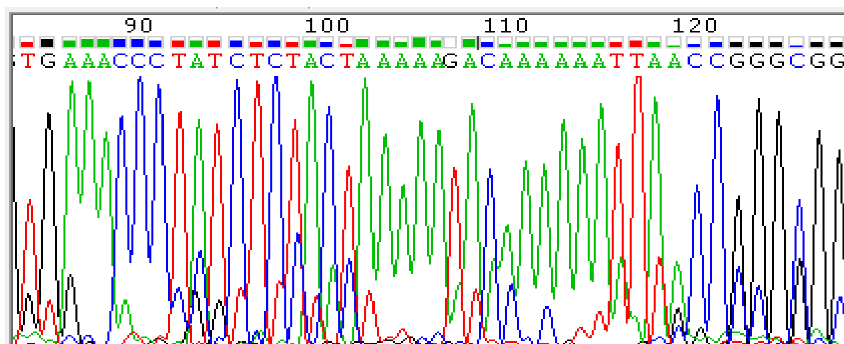


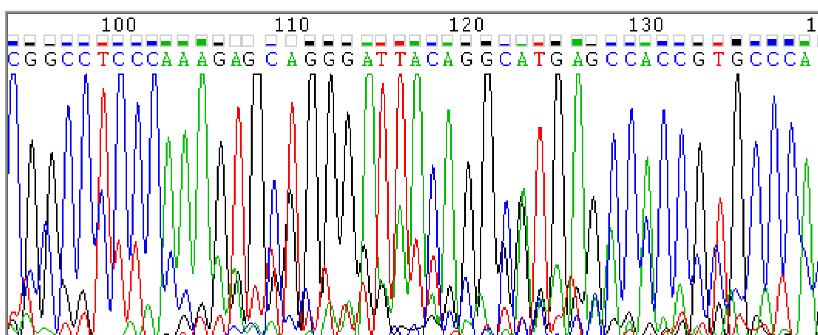
Fig. 1: Multiple Sequence Alignment of BRCA2 from position 2690 to 2765 in three parts (A).

Box-1: Mutation: FKe11.3c4878G (C.4878delG)

4870GAA	**AAA**	**[GA(4878delG)]C**	**AA**	**G**	**GT4884**
1548E K	**p.1550E>D**		**p.1551Q>K**		**p.1552G>V**



A) Mutant (B22-55-F-*BRCA2*-11.3)



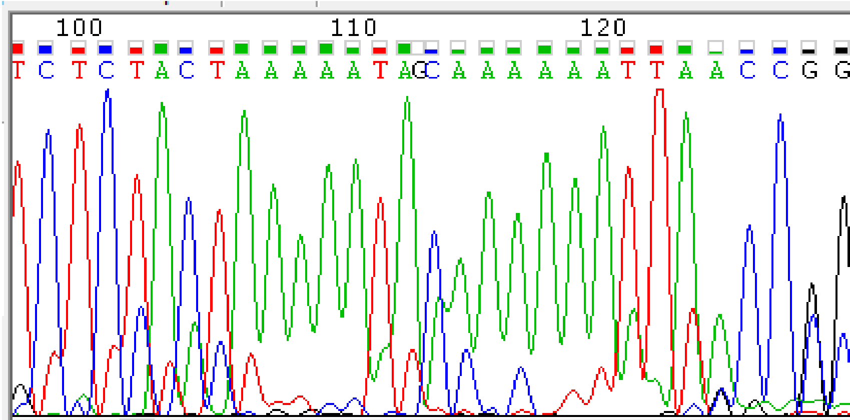
B) Normal (C3-27-F-*BRCA2*-11.3)

Figure 2 (A, B): Electropherogram of mutant and normal subject showing mutation type 'c.1582C>A' at position 367 (of mutant) in Exon 11.3.

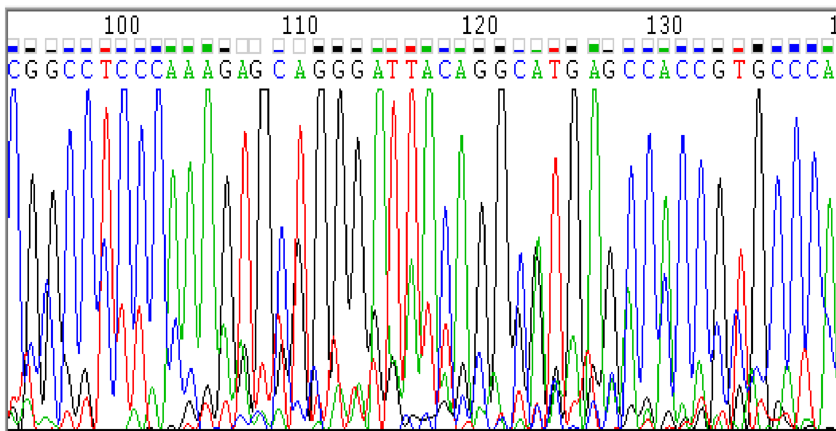
Box-2: Mutation: FKe11.3c4876G>T (c.4878G>T)

4870GAA AAA [(4876G>T)AG)]CAA GGT4884

1548E K p.1550E>* Q G



A) Mutant (O22-46-*BRCA2*-11.3)



B) Normal (C3-27-F-*BRCA2*-11.3)

Figure 3 (A, B): Electropherogram of mutant and normal subject showing mutation type 'c.1582C>A' at position 367 (of mutant) in Exon 11.3.

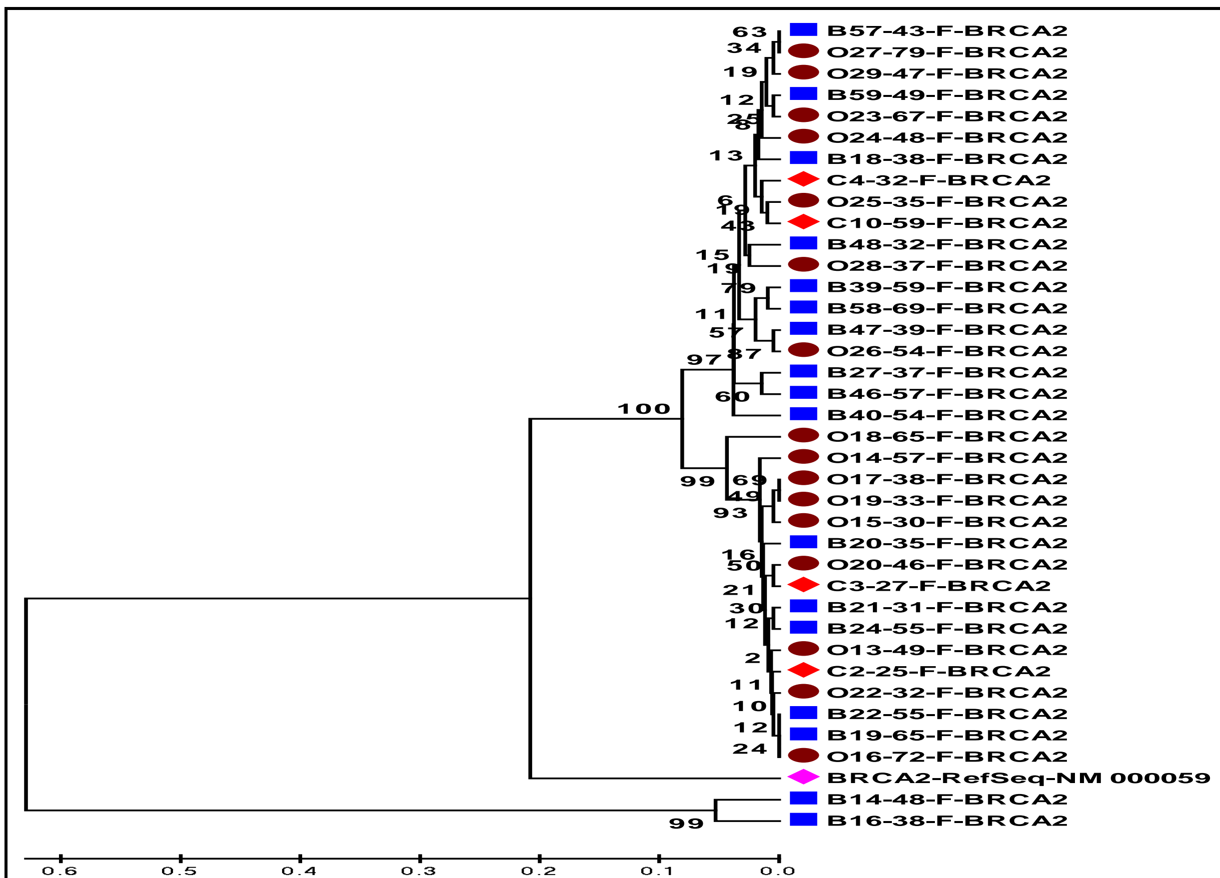


Figure 4, Dendogram: Diversity of exon 11.3 region of BRCA2 *gene among the selected population (Blue Square: Breast cancer, Brown circle: Ovarian cancer, Red diamond: Control, Pink Diamond: RefSeq NM_007294).*