Research paper

Variant Analysis of *ATM, PTEN* and *BRCA2* Genes in Breast Cancer from Karachi, Pakistan

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Abstract:

Breast carcinoma is the cancer of epithelial cells. Most frequent types of breast cancer are ductal and lobular carcinoma. Pakistan has a significant incidence of breast cancer. Single nucleotide variants (SNVs) in *ATM, PTEN* and *BRCA2* genes are reported to be involved in breast cancer. Many SNVs have been identified in different populations along with novel intron variants in *ATM* exon 13, *PTEN* exon 2 and *BRCA2* exon 10. We selected thirteen breast cancer patients from Karachi, Pakistan and their blood and breast tumor samples were collected. Primers were designed to the specific hotspots of breast cancer related genes i.e. *ATM* exon 13, *PTEN* exon 2 and *BRCA2* exon 10. Sanger sequencing was done to sequence the DNA samples. Bioinformatics tools were utilized to analyze the data. In case of *ATM* and *PTEN*, non-coding regions exhibit more variants. Three novel SNVs were observed in case of *ATM* intron 13; among them two were found to be associated with branch point defects that can interfere during mRNA splicing, five in case of *PTEN* and five in case of *BRCA2* which were highly pathogenic in nature.

Keywords: Genetic variants, Breast cancer, *ATM*, *PTEN*, *BRCA2*

Introduction:

Breast cancer is the leading cause of death among the women worldwide. The strongest risk for breast cancer is that for being female and increase in age especially after 40 years of age. But this risk is further increased with the family history of breast cancer[1]. Greater life exposure to estrogen is another cause including many endogenous and exogenous sources[2,3]. Around 80% of the women with breast cancer do not have any family history, while 5-10% is hereditary breast cancer. Some families have inherited breast tumor that inherit in an autosomal dominant manner due to germline mutation BRCA1 and BRCA2, but there are defects in some other genes too that put the women at the risk of breast cancer[4,5]. As breast cancer is increasing public health problem and advances in the introduction of different techniques have been made to predict the elevated risk among the women[6]. The methods of screening have decreased the deaths about one third over past 20 years. Major gaps exist in our knowledge to evaluate the risk of breast cancer in order to diagnose and predict accurately which would be done by using DNA sequencing techniques[7]. Sanger sequencing is considered as gold standard approach in the field of cancer research due to its robustness, reliability and accuracy and help in diagnosis and therapy to enhance personalized management of health care[8]. Sanger DNA sequencing is used to find out single

nucleotide polymorphisms (SNPs), and indels etc. in DNA sequences The data obtained from these studies provide link between genetic mutations and associated disorders. These mutations lead to severe pathological conditions that serves as genetic markers (biomarkers) to predict the risk of breast cancer that leads to therapeutic interventions[9,10].

Here, we report several novel SNVs identified in *ATM, PTEN* and *BRCA2* genes in breast cancer patients in Karachi, Pakistan.

Material and Methods

Sample Collection: The samples were collected from Dr. Ziauddin Hospital cancer building from the patients having breast cancer. Informed consents were obtained from all the patients. This case-control study was approved by Independent Ethics Committee (IEC), International Centre for Chemical and Biological Sciences (ICCBS) and Institutional Review Board (IRB) of Dr. Ziauddin Hospital. Ethical Committee Approval Number: Dr. Ziauddin Hospital, North Nazimabad, Karachi, Pakistan. ZH/ EC-010. Research questionnaire was filled to collect patient's data related to the study. Patient's blood was considered as control while tumor tissue was considered as case to identify genetic variants. Inclusion criteria also includes the point that patients did not contain any family history. Tumor grading, staging, location and menopausal status of the patients were identified by surgeon and pathologist.

All cases were histopathologically reviewed to confirm the diagnosis, most of the cases were classified as ductal and lobular breast carcinomas according to the information provided by surgeon.

DNA Isolation: DNA was isolated from fresh frozen tumor tissue and blood by using CTAB (Cetyl trimethylammonium bromide) method.20

Agarose Gel Electrophoresis: Agarose gel electrophoresis was performed on isolated DNA in 1% ethidium bromide stained gel to check DNA integrity by using Electrophoresis assembly EPS 300X (CBS Scientific) and Gel doc (Enduro GDS touch Labnet).

DNA Quantification: DNA concentration was quantified by using spectrophotometer and by taking the ratio of absorbance with 260 nm and 280 nm to assess the purity of DNA. A ratio of 1.8 is generally considered as pure. Qubit dsDNA HS Assay kit was also used to assess DNA quantity (Qubit™ dsDNA HS Assay Kit, Cat no. Q32851).

Primer Designing: Primers were designed specific to targeted regions that were BRCA2 exon 10 (Forward primer 5' GCC AAG TAC TCA GAA TAA CCC TTT AAA T 3' and reverse primer 5' GAA AAA AAC ACA GAA GGA ATC GTC ATC TAT 3'), PTEN exon 2 (Forward primer 5' GAG ACA GAT TTC TTT CAG CAC TTA A 3' and reverse primer 5' GAT AAA AAT ACT CCA ATC ACT ACT TTC 3'), ATM exon 13 (Forward primer 5'CCA CCT TTA ACT CAG TTA ACT GAA C 3' and reverse primer 5' TCA CAT TAC ATA AAT AAA AGA GAA AGG GT 3') by using Oligocalc. The primers were obtained from Integrated DNA technology (IDT), Biotechnology Company.

Polymerase Chain Reaction (PCR): PCR of these targeted regions of breast cancer patient's blood and tissue samples was done to amplify the regions of interest. PCR program that was optimized for the primers is as follows, initial denaturation: 95ºC for 5

minutes, denaturation: 95ºC for 30 seconds, annealing: 55.5ºC (BRCA2), 52ºC (PTEN), 53.5ºC (ATM) for 30 seconds, extension: 72ºC for 1 minute, final extension: 72ºC for 5 min, hold at 4 ºC for infinity and 30 cycles. 5 µl of these PCR products were loaded on 2% Agarose gel for electrophoresis along with 100 bp ladder (Bioron Inc. Canada) to confirm the amplification of targeted region without any non-specific amplification.

PCR Clean up: PCR clean-up was done to remove dimers by using EZ-10 Spin Column PCR Products Purification Kit Protocol, Cat no. BS363.

Sequencing PCR and Ethanol Precipitation: The samples were sequenced on Beckman Coulter CEQ-8000. DNA was prepared for sequencing reaction by following the protocol from Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter) and (Beckman Coulter GenomeLab kit Protocol, Cat. No. 285501).

Data Analysis: After sequencing, the results were analyzed on UCSC Genome Browser by selecting BLAT (BLAST like alignment tool) which was used to find the sequences of >95% similarity by aligning the DNA sequences with reference human genome GRCh37/hg19. The mutations were observed and found in the patients with breast cancer that were not present in the reference human genome. The variants were compared with the peaks on electropherogram for confirmation. The area of less noise and good resolution peaks were selected to analyze the results. Data was statistically analyzed by using fisher exact test (Graph Pad Prism 7) and p-value <0.05 was considered statistically significant. Variant effect predictor (VEP) tool was used to determine the effect and consequences of variants on genes, by providing information like chromosome number, start coordinate, end coordinate, reference, variant, +/- strand. CRAVAT 4.3 (Cancer-Related Analysis of Variants Toolkit) was utilized for the analysis of variant effect scoring tool (VEST 3.0) that predicts pathogenic nature of a variant. VEST 3.0 tool scores ranges from 0 to 1 indicate its deleterious nature. CHASM 3.0 (Cancer-specific high-throughput annotation of somatic mutations) predicts functional significance of somatic missense mutations that are observed in the genomes of cancer cells. GeneCard and PubMed annotation tool was used to identify gene locus and functional information about given variants. SnvGet predicts substitution features of amino acids. Human Splicing Finder (HSF 3.0) was used to check location of intron variants, either splice sites or branch points

Results

Clinical Characteristics:

The inclusion criteria designed for the selection of breast cancer patients was ages above 30 years without any family history. 13 patients were selected for our study. Clinical characteristics are summarized in (Table 1).

For sanger sequencing PCR of ATM exon 13, PTEN exon 2 and BRCA2 exon 10 was done and the amplified products were purified and 5 µl of product loaded on 2% agarose gel.

ATM gene exon 13 SNVs:

Present study demonstrated that all thirteen patients were found to have ATM gene exon 13 mutations at three nucleotide positions; two of them were repeated in different individuals. Ten patients had a novel deletion at Chr11: 108256138 A del (Figure 1). Six patients had another novel deletion at Chr11: 108256154 A del (Figure 1); while one

Table 1: List of characteristics of the study participants showing their percentage in respective category.

Characteristics	Number of patients
Age groups	
30-39	05
40-49	01
50-59	03
60-69	04
Tumor location	
Left	04
Right	08
Bilateral	01
Tumor stage	
I	00
II	04
III	07
IV	02
Tumor grade	
1 (Well differentiated/low grade)	00
2 (Moderately differentiated)	02
3 (Poorly differentiated/ high grade)	11
4 (Undifferentiated)	00
Histology	
Ductal carcinoma	09
Lobular carcinoma	04
Other kind	00
Family history	
Yes	00
No	13
Menopausal status	
Premenopausal	07
Postmenopausal	06
Ethnic group	
Sindhi	04
Urdu speaking	07
Punjabi	01
Balochi	01

patient showed novel insertion at Chr11: 108256150 T ins (Figure 1). These mutations could not be found in dbSNP and COSMIC databases. These variants were found in both blood and tumor cells. This could be due to the circulating cell free tumor DNA that metastasize to blood stream in many breast cancer patients with 3rd stage and grade of tumor. The Chr11: 108256154 A del mutation was present in only tumor samples of two patients while rest of the four patients had it in both blood and tissue samples. The Chr11: 108256150 T ins mutation was only observed in the tumor sample. Intronic mutations in ATM can affect splice sites that may leads to altered gene expression and alternative splicing. As all thirteen patients showed ATM variants and out of them eight patients had tumor in their right breast, four had tumor in their left breast and one was bilateral (Table 1). Nine patients showed ductal carcinoma and four showed lobular carcinoma. Seven were pre-menopausal while six were post-menopausal. Bilateral breast cancer patient showed Chr11: 108256138 A del mutation in both of its breasts and blood sample as well. The same mutation was also observed in pregnancy based breast cancer that was metastatic in nature. Fisher exact test was applied on ATM variants, none of them were found to be statistically significant (Figure 1).

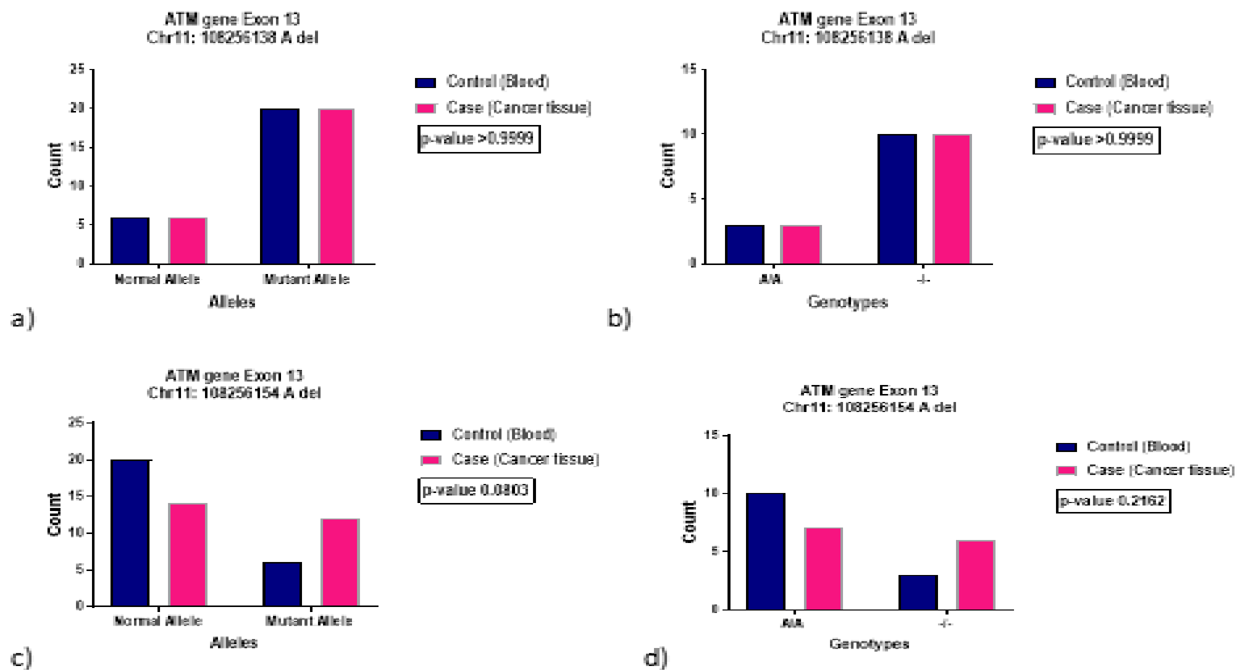


Figure 1: a) Graph showing allele distribution of ATM gene exon 13, Chr11: 108256138 A del in control (blood) and case (cancer tissue). b) Graph showing genotype distribution of ATM gene exon 13, Chr11: 108256138 A del in control (blood) and case (cancer tissue). c) Graph showing allele distribution of ATM gene exon 13, Chr11: 108256154 A del in control (blood) and case (cancer tissue). d) Graph showing genotype distribution of ATM gene exon 13, Chr11: 108256154 A del in control (blood) and case (cancer tissue) of breast cancer patients. (A/A= genotype for homozygous wild type, -/- = homozygous deletion mutant type).

PTEN gene exon 2 SNVs:

Seven patients out of thirteen were found to have mutations in PTEN exon 2 gene. Three patients (patient 1,2 and 5) had T/A intron variant (heterozygous mutation) at chr10: 87866979 (Figure 2). One patient had insertion of C that causes frameshift mutation Chr10: 87867059. One patient had insertion of A that also causes frameshift mutation at Chr10: 87867025. These mutations were found to be novel in our study. While one patient had A/T intron variant (heterozygous mutation) at Chr10: 87867088 and three patients had insertion of C that is a frameshift mutation at Chr10: 87866622. These genetic variants been reported with rs ID's rs41284070 and rs397701299 respectively. The mutation at Chr10:87867088 with rs ID rs41284070 is an intron variant, according to 1000genomes database. The mutation at Chr10: 87866622 with rs ID rs397701299 is also an intron variant according to VEP. Three patients had tumorous growth in their right breast with one patient carried pregnancy associated breast cancer, while

three had in left, the remaining one showed bilateral breast cancer. One patient with pregnancy associated breast cancer, which metastasized to bones had Chr10: 87867088 A/T intron variant (heterozygous mutation) with rs41284070 mutation. The patient with bilateral breast cancer had Chr10: 87866622 C ins (rs397701299) in both breasts and blood sample as well, along with Chr10: 87867025 A/T intron variant (heterozygous mutation) which was only observed in blood sample not in tumor tissue. HSF 3.0 was carried out on PTEN gene mutations, but none of the variants were present at branch points. Fisher exact test was applied on PTEN variants, none of them were found to be statistically significant (Figure 2).

BRCA2 gene exon 10 genetic variants:

In present study, seven out of thirteen patients were observed to have BRCA2 gene exon 10 variants. Among them three patients had germline mutations while the others had somatic mutations. Two patients showed a germline mutation in intronic region at chr13: 32332252 A ins. which was a novel variant (Figure 3). Three patients showed somatic missense mutation which was Chr13: 32332592 A/C. This BRCA2 variant was already reported with rs144848 which was a single nucleotide polymorphism or missense mutation in exon 10. It is a variant of uncertain significance whose significance to the health and function is unknown but found as a result of genetic testing and involved in breast cancer. The evidence of this variant was provided by ExAc and 1000genomes database. One patient showed germline mutation which was a deletion of A in exon region at Chr13: 32332709 (Figure 3). This genetic variant was reported with rs80359269. It is a pathogenic variant with a mutation type frameshift, that causes the change of amino acid from p. Ile411tyr in ClinVar. One patient showed another somatic mutation in exon region at Chr13: 32332625delA. This variant was reported with rs80359265. This BRCA2 variant is also pathogenic in nature because it causes frameshifting and change of amino acid from p. Ile383Ser. Four patients showed a somatic mutation in exon region at Chr13: 32332343. This BRCA2 variant was reported with rs766173. This missense variant is of uncertain significance but found in patients with benign breast tumor. The evidence was provided by ExAc, 1000genomes database and HapMap projects. Four patients showed tumor in their left breast while other three showed tumors in right breast. In patients with bilateral breast tumor and pregnancy based breast tumor, no BRCA2 genetic variant was observed. HSF 3.0 was carried out on BRCA2 gene mutations, but none of the variants were present at branch points. Fisher exact test was applied on BRCA2 variants, only Chr13: 32332343 A/C was found to be statistically significant (Figure 3).

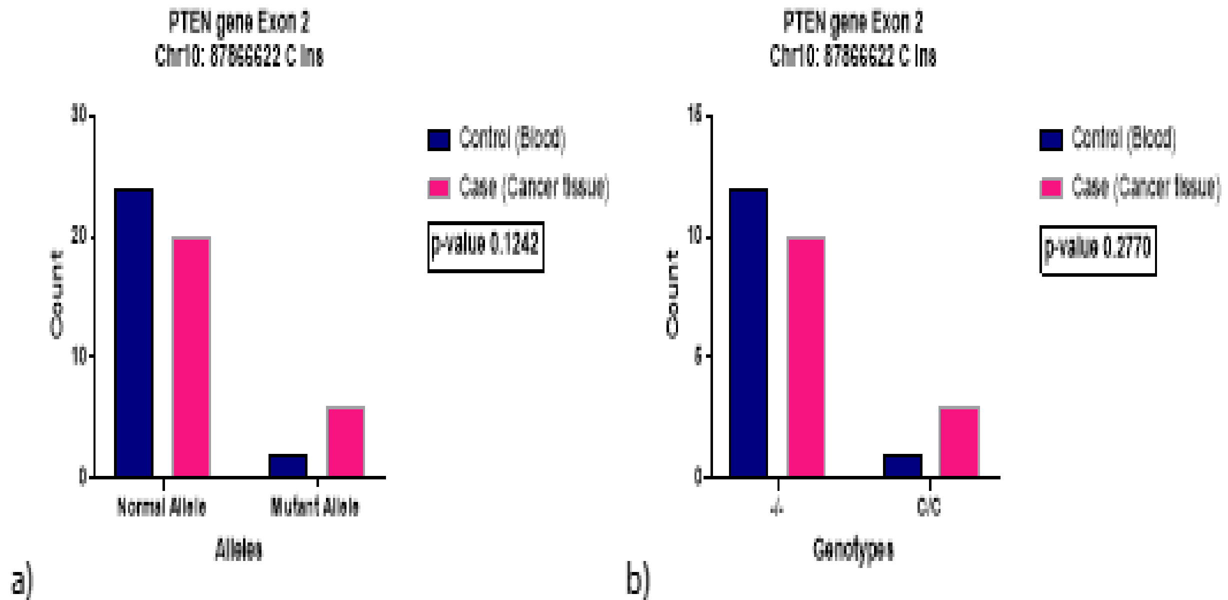
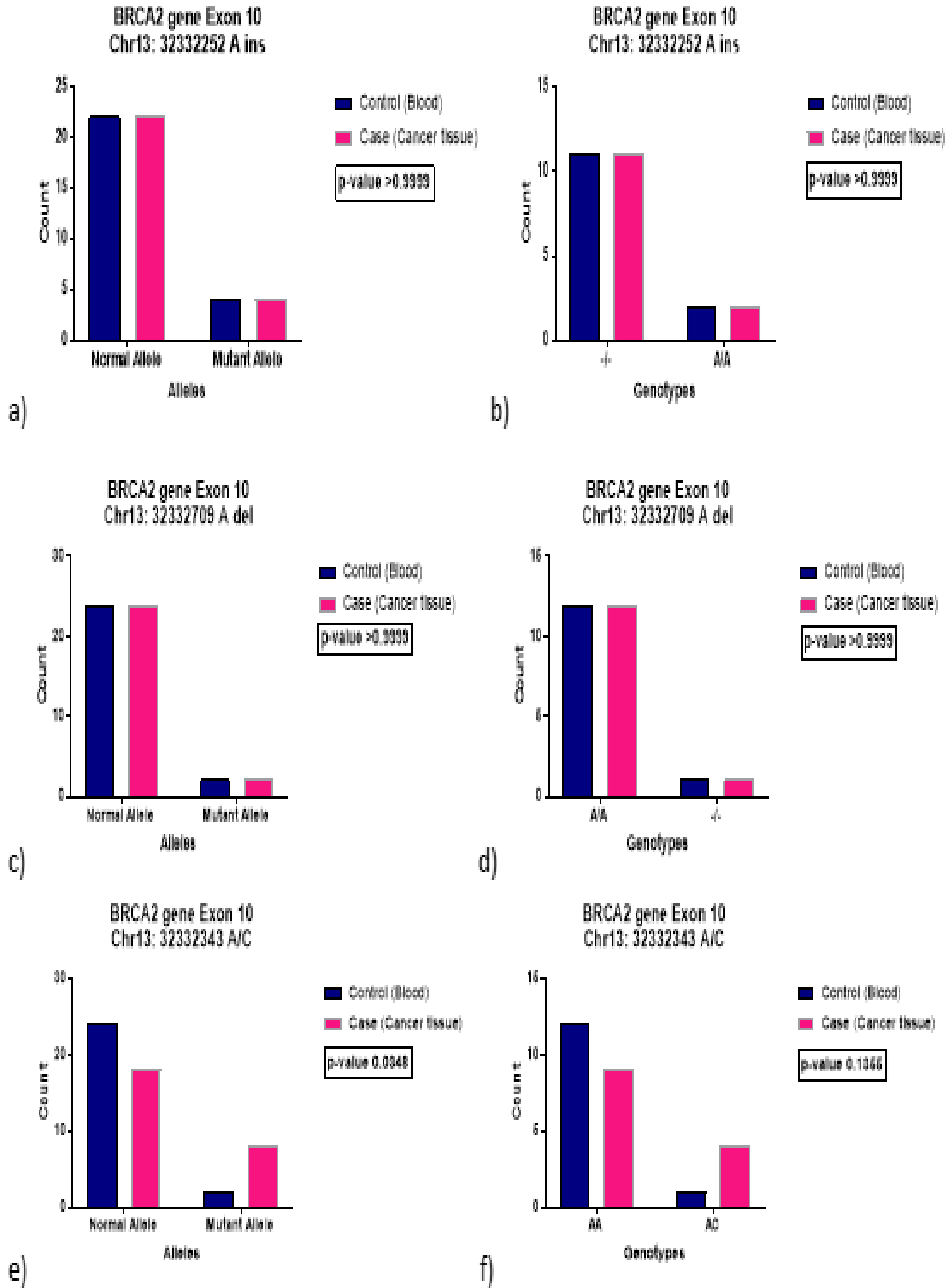


Figure 2: a) Graph showing allele distribution of PTEN gene exon 2, Chr10: 87866622 C ins in control (blood) and case (cancer tissue). b) Graph showing genotype distribution of PTEN gene exon 2, Chr10: 87866622 C ins in control (blood) and case (cancer tissue) of breast cancer patients. (C/C= homozygous insertion mutant genotype, -/- = homozygous reference no insertion).


BRCA2 gene Exon 10
Chr13: 32332252 A ins
Count
Normal Allele
Mutant Allele
Alleles
Control (Blood)
Case (Cancer tissue)
p-value >0.9999
a)
BRCA2 gene Exon 10
Chr13: 32332252 A ins
Count
-/-
A/A
Genotypes
Control (Blood)
Case (Cancer tissue)
p-value >0.9999
b)
BRCA2 gene Exon 10
Chr13: 32332709 A del
Count
Normal Allele
Mutant Allele
Alleles
Control (Blood)
Case (Cancer tissue)
p-value >0.9999
c)
BRCA2 gene Exon 10
Chr13: 32332709 A del
Count
A/A
-/-
Genotypes
Control (Blood)
Case (Cancer tissue)
p-value >0.9999
d)
BRCA2 gene Exon 10
Chr13: 32332343 A/C
Count
Normal Allele
Mutant Allele
Alleles
Control (Blood)
Case (Cancer tissue)
p-value 0.0348
e)
BRCA2 gene Exon 10
Chr13: 32332343 A/C
Count
AA
AC
Genotypes
Control (Blood)
Case (Cancer tissue)
p-value 0.1366
f)

Previous page. **Figure 3**: a) Graph showing allele distribution of BRCA2 gene exon 10, Chr13: 32332252 A ins. in control (blood) and case (cancer tissue). b) Graph showing genotype distribution of BRCA2 gene exon 10, Chr13: 32332252 A ins. in control (blood) and case (cancer tissue). c) Graph showing allele distribution of BRCA2 gene exon 10, Chr13: 32332709 A del in control (blood) and case (cancer tissue). d) Graph showing genotype distribution of BRCA2 gene exon 10, Chr13: 32332709 A del in control (blood) and case (cancer tissue). e) Graph showing allele distribution of BRCA2 gene exon 10, Chr13: 32332343 A/C heterozygous mutation in control (blood) and case (cancer tissue). f) Graph showing genotype distribution of BRCA2 gene exon 10, Chr13: 32332343 A/C heterozygous mutation in control (blood) and case (cancer tissue) of breast cancer patients. (b) A/A= homozygous insertion mutant genotype, -/- = homozygous wildtype no insertion, (d)A/A= genotype for homozygous wild type, -/- = homozygous deletion mutant type, (f) A/A wild type, A/C mutant heterozygous).

CRAVAT analysis:

Cancer-related analysis of variants toolkit contains different tools like VEST, CHASM, SnvGet, GeneCard and PubMed annotation. Input file was provided with the information of all 13 variants, 4 variants (30.8%) out of 13 were found to be coding while 9 variants (69.2%) were found to be non-coding. Cancer genome landscapes showed that ATM, PTEN and BRCA2 genes all are tumor suppressor genes. Sequence ontologies described attributes & features of biological sequences and gave information about 4 reported variants of BRCA2 in coding region, 2 variants were found to be missense and 2 were frameshift mutations. The region of interest where chr13: 32332343, 32332625, 32332709, 32332592 variants are present representing the interaction of this region with PALB2 protein and NPM1 protein. The presence of altered forms of amino acids are deleterious because they might interrupt the binding of these proteins with BRCA2 protein. PALB2 protein binds to BRCA2 and recruits it on DNA breaks and help in DNA homologous recombination repair (HRR) mechanism, while Nucleophosmin which is BRCA2 associated protein product of NMP1 coregulate amplification of centromere. Dysfunction of BRCA2 in a centrosomic region causes abnormalities in progression of cell division.

VEST scores for these BRCA2 variants chr13: 32332343, 32332625, 32332709, 32332592 were found to be deleterious 0.33914000, 0.027, 0.01838000, 0.41271000 respectively. As the score increase between 0 and 1 deleteriousness of variant also increases. Missense variants Chr13: 32332343 and chr13: 32332592 were found to be more deleterious. CRAVAT also gave information that these four variants can be targeted by PARP inhibitors that are toxic for cancer cells and form complex with DNA and halt DNA replication. 1000 genomes Allele frequencies (AF) of Chr13: 32332343 and chr13: 32332592 are 0.07368210 and 0.24940100 respectively. In ExAC AF it is 0.05178370 and 0.27793000 with COSMIC ID COSM3999067 and COSM3753646 respectively. According to GeneCard all four variants were found to be inherited mutations. Both missense variants were found to be prevalent among the individuals of South Asian population with ExAC AF 0.11589400 and 0.35579100 respectively. With reference to ClinVar disease identifier

all four coding variants were found to be related with breast and ovarian cancer. One PTEN intron variant chr10: 87867088 (dbSNP rs41284070) found with 0.00678914 (1000 genomes AF).

Human Splicing Finder (HSF 3.0) analysis:

Human Splicing Finder (HSF 3.0) helps to understand splicing defects and branch point defects by proving reference sequence. The novel intron variants that were found in our study were checked through this tool in order to analyze the exact position of intron variants either they are present at splicing site or branch point. Two novel intron variants of ATM gene, 11: 108256138 A del and 11: 108256154 del A were found to be associated with branch point. The branch point motif ATGTAGA present in region which is upstream at -83 heptamer position from exon 13 with 18.59 consensus value. The other branch point motif CTTACAA present upstream at -69 heptamer position of exon 13 with 51.09 consensus value. The other intron variants were not present in either splice site or branch point. Consensus values (CV) are from 0-100 and the threshold value is defined to be 67. Those branch point motifs that are above the value of 67 are considered to be more potential and lies within 50 nucleotides of upstream region from ATM exon 13 where the lariat structure formation occurs during pre-mRNA editing and removal of introns.

Discussion

Many nucleic acid based techniques are designed to determine the order of nucleotide bases like several platforms of next generation sequencing (NGS) has been developed for high throughput sequencing[11]. However, dideoxy chain termination method or sanger sequencing remains the gold standard for determining the order of nucleotide bases and cannot be fully replaced by NGS[3,11]. The choice of method used for sequencing of desired population and screening of particular exons in the targeted genes of interest can easily done by sanger sequencing. ATM germline mutations were common having moderate risk of breast cancer development[12-15]. Intronic mutations in ATM can affect splice sites that leads to altered gene expression. ATM gene interacts with BRCA1 and BRCA2 genes and give out about twofold increase in the risk of breast cancer[16-19]. ATM gene with its moderate penetrance is considered as non-BRCA gene that could be beneficial for detecting clinical syndromes related to breast cancer and its variants can be utilized as bio-markers for the detection of breast cancer through DNA sequencing technique[20-22]. According to a study penetrance of ATM gene in breast cancer appeared to be 6.02% by the age group of 50 while 32.83% by the age of eighty[14]. Mutations in tumor suppressor gene like PTEN leads to many intracellular abnormalities that causes decrease in cell adhesion, abnormal apoptotic responses and disturbance in the regulation of cell cycle[7]. There is 85% of lifetime risk for breast cancer because of PTEN mutations and the mean age of its diagnosis id between 38-46 years[6]. In young breast cancer patients PTEN mutations are rare[4]. In ductal epithelial cells PTEN altered gene expression was observed and genetic alterations in PTEN gene leads to ductal breast carcinoma[13]. According to the Irish women population with post-menopausal status are highly susceptible to breast cancer risk. Primarily breast cancer is a disease of older people with post-menopausal status[1]. Women with germline mutations either in BRCA1 or BRCA2 have 45-80% risk for breast cancer development[8]. Deficient function of BRCA genes were also

reported because of somatic mutations across exons and introns. Breast cancer somatic genetic signatures are the lead towards its clinical diagnosis[15], because somatic mutations in BRCA genes are very rare and very important biomarkers[11]. After a clinical genetic testing the most common variants to be detected in BRCA genes were missense mutations that are highly pathogenic in nature and increased the risk of breast cancer development[2]. However, 5% of the patients had variants of uncertain significance (VUS) in BRCA2 gene. VUS are also missense mutations where the change in nucleotide codes for different amino acids.

Conclusion

Sanger sequencing was used to identify genetic variants in the targeted regions of particular genes that are hotspots in the development of breast cancer. These genetic variants confer risk in the development of breast cancer. The association of these genetic variants with age showed that 30-39 age group patients were at higher risk of breast cancer development. In case of ATM and PTEN genes most of the patients showed pre-menopausal status while in case of BRCA2 gene almost all patients were at post-menopausal stage. Many reported exon mutations were identified in the population along with novel intron variants in ATM exon 13, PTEN exon 2 and BRCA2 exon 10. It is necessary to study such kind of diseased populations that can provide sequencing data for the estimation and early diagnosis of breast cancer with the help of these biomarkers.

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