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IDENTIFICATION OF MUTATIONAL PATTERNS IN DIFFERENT ONCOGENES AND TUMOR SUPPRESSOR GENES PLAY ROLE IN HUMAN BREAST CANCER. A STUDY FROM PUNJAB, PAKISTAN

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Abstract
Background: Cancer is an unequal growth of cells that have capability to invade and spread in parts of an organism. It starts from the breast tissues, frequently from inner covering of milk channels.
Objective: The objective of study was to identify novel somatic alternations in oncogenes and tumor suppressor genes of breast cancer**.**
Methods: Whole-exome sequencing was performed in DNA extracted from tumor samples of people from Jinnah hospital Lahore, Pakistan.
Results: There were nineteen people of age group 27 to 73 and tissue specimens were collected from six patients. Age group, ER and PR status both show non-significant difference. The frequency of 20 mutated tumor suppressor genes includes BRCA1 (66.67%), BRCA2 (83.33%), CARS (50%), CHEK2 (33.33%), DDX5 (50%), FH (66.67%), MEN1 (33.33%), NF1 (66.67%), NF2 (33.33%), NUP98 (66.67%), PALB2 (66.67%), PTEN (50%), SUFU (33.33%), TP53 (50%) and VHL (33.33%, and 22 mutated oncogenes were ABL1 (33.33%), AKT2 (83.33%), ATF1 (83.33%), BCL2 (50%), BCL3 (50%), BCL6 (50%), BCR (50%), BRAF (50%), NUP214 (83.33%), PIK3CA (83.33%), PIM1 (50%), and USP6 (50%). More number of Synonymous SNV mutations was observed in both oncogenes and tumor suppressor genes. Amino acid variations and deletion was detected in various exonic regions of genes.

Conclusion: All people show invasive ductal breast carcinoma. Synonymous SNV mutations show high frequency. BRCA2 as tumor suppressor and AKT2, ATF1, NUP214 and PIK3CA as oncogene show high mutated frequency. More data is needed to clearly state the role of these altered genes in breast cancer patients.

Keywords: Breast cancer, Oncogenes, Tumor suppressor genes, Somatic mutations, Exons, Whole exome sequencing

Introduction

Breast cancer is very frequent disease in females on global scale (1-3). In spite of progresses in existence, breast cancer remains the 2nd important reason of death in women. It is frequent in Pakistan then other Western countries (4). There is an increase in frequency and cancer-related death in India (5-8). It is recognized that BC genome harbors complex mutational backgrounds, often categorized by point mutation, small insertions and deletions and large structural variations through the genome (1, 3, 9-12). Major genetic variations found in human BC falls into two groups: gain-of-function variations in proto-oncogenes, which stimulates cell evolution, division, and existence and loss-of function variations in TSGs that usually helps to avoid uncontrolled cell progression and promotes DNA repair and cell cycle checkpoints stimulation (13, 14). Such DNA mutations in body cells in tumor genomes may results to activate TSGs and activate OGs. Complete identification of these variations is essential to clearly know the biological process of cancer-causing genes and cancer development.

Breast cancer is very complicated malignancy; many patients are non-inherited, while many are hereditary. Variations in BRCA1 and 2 comprises of 25% while 5 TO 10% comprises of all breast cancer patients (15-18). GWAS have recognized common variations in about 70 locations related with BC, which explains 14 percent of inherited threat to disorder (19). According to BC, its subtypes are categorized into ER, PR, HER-2, Luminal A, and Luminal type B, HER2 overexpression, basal-like, and normal breast-like subtypes, which account for 23.7%, 52.8%, 11.2%, 12.3%, and 7.8% of cases, respectively (20-22).

Currently, large scale genome-wide sequencing developments in breast tumors have recognized many important BC associated genes including PIK3CA, AKT1as oncogenes and TP53, PTEN, MAP3K1, CDH1, CDKN1B, RB1, GATA3 as tumor suppressor genes (1, 11). This was clearly observed in people of Europe. BRCA1/2 accounts for below 20% of inherited threat of BC with other less penetrant genes i.e., TP53, ATM, and PTEN accounts for below 5% (23). Lifetime evaluations of BC threat in BRCA1/2 carriers about 36-90% and OC 24-59% and 8-35% in BRCA1/2, respectively (24-28). Alterations in BRCR1 and BRCA2 accounting many families with genetic vulnerability to BC and OC (29-32). Identification of BRCA1 or 2 alterations allows the employment of prevention approaches, includes MRI screening or threats during biopsy, which expands existence of patients (33, 34). Mutations in genes other than BRCA tumor suppressors account for less than 1% of all inherited BCs (35-38). Genetic testing for other high threat BC vulnerability genes, like TP-53 (Li-Fraumeni disorder), PTEN (Cowden's disease), and CDH-1 (inherited gastric carcinoma), is usual in suitable individuals (39). Moreover, germline variations in DNA repair genes like BRIP-1, RAD-51C, and RAD-51D are related with enhanced threat of OC (40-46). At present, work evaluates the incidence of variations in moderate-penetrance BC disposition genes have been directed in selected BC people includes African Americans (47), people with (TNBC) (48), and many patients observed in high-threat heritable hospitals (49-52).

Next-generation sequencing (NGS) can play an important role in diagnosis, prognosis, and treatment. A number of NGS platforms are available and have different properties, such as throughput (Gb), maximum read length (bp), reads, running time, and error profile. NGS platforms use two types of sequencing mechanisms: ligation or synthesis(53) . The exome comprises approximately 1% of the total human genome, so WES is considered an outstandingly powerful tool for medical genetic research (54).

This study was aimed to identify novel somatic alternations in oncogenes and tumor suppressor genes of breast cancer; whole-exome sequencing was performed in breast tumors.

Materials and Methods

Patient selection

Breast tissue specimens were obtained from the Jinnah hospital Lahore, Pakistan. Declaration of Helsinki was followed during the study. Signed consent form was obtained. The certificate was obtained from Institutional Review Board University of Okara. The study size was arrived at Jinnah Hospital Lahore or Allama Iqbal Medical College Lahore. Questionnaire Performa was designed to ask questions about patient's background including age, sex, tumor size, TNM stage, cancer type, subtype, ER/PR and HER-2 status is shown in table 1. Those who do not included in the study were not included in the inclusion criteria of the study. All authors have access to the data that could classify specific members during or after the collection.

The participants included in the study were observed with HBC, having family history with two cases of breast cancer in 1^{st} and 2^{nd} degree relatives, having bilateral BC, BC detected earlier at 40 years of age (55). The tumors were categorized to (ER) or Estrogen receptor, (PR) or progesterone receptor, and human epidermal growth factor receptors 2 (HER-2) status (56). The information lost during the study has been removed from this study.

Immunohistochemistry and DNA extraction

Immunohistochemistry test was completed according to the standard protocols (57, 58). Breast cancer was categorized into 4 groups depends on IHC status ER (positive or negative), PR positive or negative), and Her2/neu (positive or negative) expressions. If ER/PR+, Her2+ then it is Luminal B type, if ER and PR+, Her-2- then Luminal A, if ER and PR-, Her-2+ and ER and PR-, Her-2-then it is called triple negative or basal-like tumor (59).

DNA extraction

DNA was extracted by Phenol chloroform (PC) method. The standard protocol followed by (60, 61) was used to extract DNA. Agarose gel electrophoresis and DNA quantification was done by the procedure followed by Ghatak S, and their colleagues. Further the image was made by UV Trans-Illuminator bio Doc Analyzer (62, 63). The DNA was quantified by Thermo scientific Multi Skan Go Apparatus. 260/280 ratio shows the quality while concentration was shown in ng/ul (64).

Whole exome sequencing (WES)

Whole exome sequencing was performed by the method followed by (65).

Validation of Mutations

For the validation of changes, (PCR) was performed. The precise PCR primers were designed by Primer3 database (http:// bioinfo.ut.ee/primer3-0.4.0/). Results were sequenced by ABI PRISM BigDye Kit on ABI 3130 DNA sequencer. Further outcomes were examined by Chromas version 2.23 (65).

SNV detection

The WGS sequences were united to human genome (hg19) by BWA-MEM. Duplication reads were marked and filtered with Mark Duplicates and Duplicate Read Filter, respectively. Haplotype caller was used for germline variations. SNVs were obtained by using MuTect2. The final SNVs were functionally annotated by using ANNOVAR. To identify the nature of mutations, SIFT, and PMUT servers were employed.

Statistical analysis

Variant calling was performed using Mutect (GATK) tool. The input files were the fasta files of the mention genome and BAM files with marked duplicates. The output of this tool was a variant call format (VCF) file which contains diverse recognized variants. Annotation of the variants was achieved by ANNOVAR tool. The inputs were predicted variants in VCF file. ANNOVAR is a fast and effective technique to annotate functional consequences of genetic variations from high-throughput sequencing data. Further analysis was performed using MS excel 2010 (65).

Nucleotide sequences

The Nucleotide sequences were submitted to online database NCBI (ncbi.nlm.nih.gov).

Results

This study was directed from Jinnah hospital Lahore to collet breast tissue specimens during biopsy. Six specimens were obtained from patients and information of nineteen patients were obtained. Histopathological reports of all patients were obtained. WES was done on all specimens. The total of 20 tumor suppressor genes and 22 oncogenes were targeted to find the novel mutations. The tumor suppressor genes includes ATM, BRCA1, BRCA2, CARS, CDK6, CHEK2, DDX5, FH, IL2, MAP2K4, MEN1, NF1, NF2, NUP98, PALB2, PTEN, RUNX1, SUFU, TP53, and VHL while oncogenes includes ABL1, AKT1, AKT2, ATF1, BCL11A, BCL2, BCL3, BCL6, BCR, BRAF, CBLB, NUP214, PAX8, PIK3CA, PIM1, PPARG, PTPN11, RAF1, SMO, TFG, USP6, and MYB were targeted.

Patient characteristics and sequencing

The frequency of females was more than males. The deamographic information and disease related data i.e., number of tumors, location of tumors, TNM-stages, estrogen receptors, progesterone receptors, and (HER-2) status of people was gathered. Number of tumors vary i.e., 1, 2, 3, 11 and sometimes 15 in people. The metastatic sites include place of tumors either obtained from left or right side of breast. The TNM stages also vary i.e., grade 2, 3, 4 and 5. BC subtypes show triple negative BC and some with HER-2 overexpressed. All people show Invasive ductal carcinoma. The status of ER/PR either positive or negative and HER2 also vary. Table 1 shows the details.

Table 1: Shows the characteristics of breast cancer patients

Characteristics	**Number**	**Percentage**
Patients with tumor samples	6	31.58
Patients with clinical information	19	100.00
Age (median, range)	(45,46)	
TNM stage %		
0-I	0	0.00
II	6	31.58
III	11	57.89
IV	2	10.53
Tumor location		
Left side	12	63.16
Right side	7	36.84
ER status %		
Positive	4	21.05
Negative	15	78.95
PR status %		
Positive	4	21.05
Negative	15	78.95

HER2 status %		
Positive	4	21.05
Negative	15	78.95
Cancer type		
Invasive ductal carcinoma	19	100.00
Subtype		
Triple Negative	12	63.16
HER2-overexpressed	7	36.84

Extraction and Quantification of DNA

DNA was extracted from 4 tissue specimens with IDs 5859, 5836, 5729 and 5852 were run into gel as shown in figure no. 1. Gel electrophoresis was performed using 1% agarose gel and the composition included 1 gram agarose which was dissolved in 100 ml of 1X TAE buffer (Tris Acetic acid EDTA). Ladder of 1KB was loaded in first well along with DNA samples in next wells. DNA is of highly intact and of more than 20kb size. The DNA quantity was measured by Thermo scientific Multi Skan Go Instrument.

Figure 1: Gel electrophoresis of extracted DNA

Whole exome sequencing and Exonic variant analysis

This was performed to analyze the exonic variations in patients.

Tumor suppressor genes and Oncogenes

In this study, 20 tumor suppressor genes and 22 oncogenes were targeted to find the novel mutations. TSGs play important role in the suppression of tumor development while OGs are the cancer causing genes and play significant role in gene mutation. The TSGs includes ATM, BRCA1, BRCA2, CARS, CDK6, CHEK2, DDX5, FH, IL2, MAP2K4, MEN1, NF1, NF2, NUP98, PALB2, PTEN, RUNX1, SUFU, TP53, and VHL were targeted. The more frequently mutated TSGs includes BRCA1 (66.67%), BRCA2 (83.33%), CARS (50%), CHEK2 (33.33%), DDX5 (50%), FH (66.67%), MEN1 (33.33%), NF1 (66.67%), NF2 (33.33%), NUP98 (66.67%), PALB2 (66.67%), PTEN (50%), SUFU (33.33%), TP53 (50%) and VHL (33.33%, were considered for further analysis. The OGs includes ABL1, AKT1, AKT2, ATF1, BCL11A, BCL2, BCL3, BCL6, BCR,

BRAF, CBLB, NUP214, PAX8, PIK3CA, PIM1, PPARG, PTPN11, RAF1, SMO, TFG, USP6, and MYB were targeted. The more frequently mutated OGs includes ABL1 (33.33%), AKT2 (83.33%), ATF1 (83.33%), BCL2 (50%), BCL3 (50%), BCL6 (50%), BCR (50%), BRAF (50%), NUP214 (83.33%), PIK3CA (83.33%), PIM1 (50%), and USP6 (50%) were considered for further analysis. The frequency of mutated genes is sown in table 2. The more frequently mutated genes were targeted for further study.

Table 2: Shows the frequency of mutated genes

Tumor suppressor genes					**Oncogenes**				
Sr no.	**Gene**	**Chromosome no.**	**No. of people**	**Frequency %**	**Sr no.**	**Gene**	**Chromosome no**	**No. of people**	**Frequency %**
1	ATM	11	1	16.67	1	ABL1	9	2	33.33
2	BRCA1	17	4	66.67	2	AKT1	14	1	16.67
3	BRCA2	13	5	83.33	3	AKT2	19	5	83.33
4	CARS	11	3	50.00	4	ATF1	12	5	83.33
5	CDK6	7	1	16.67	5	BCL11A	2	1	16.67
6	CHEK2	22	2	33.33	6	BCL2	18	3	50.00
7	DDX5	17	3	50.00	7	BCL3	19	3	50.00
8	FH	1	4	66.67	8	BCL6	3	3	50.00
9	IL2	4	1	16.67	9	BCR	22	3	50.00
10	MAP2K4	17	1	16.67	10	BRAF	7	3	50.00
11	MEN1	11	2	33.33	11	CBLB	3	1	16.67
12	NF1	17	4	66.67	12	NUP214	9	5	83.33
13	NF2	22	2	33.33	13	PAX8	2	1	16.67
14	NUP98	11	4	66.67	14	PIK3CA	3	5	83.33
15	PALB2	16	4	66.67	15	PIM1	6	3	50.00
16	PTEN	10	3	50.00	16	PPARG	3	1	16.67
17	RUNX1	21	1	16.67	17	PTPN11	12	1	16.67
18	SUFU	10	2	33.33	18	RAF1	3	1	16.67
19	TP53	17	3	50.00	19	SMO	7	1	16.67
20	VHL	3	2	33.33	20	TFG	3	1	16.67
					21	USP6	17	3	50.00
					22	MYB	6	1	16.67

Validation of mutations in Oncogenes and Tumor suppressor genes

The oncogenes that were frequently mutated include ABL1, AKT2, ATF1, BCL2, BCL3, BCL6, BCR, BRAF, NUP214, PIK3CA, PIM1, and USP6 were considered for further analysis. The table 1 in supplementary material shows the complete mutational detail including the nucleotide change, amino acid change their exonic sites and the type of mutation in genes. The tumor suppressor genes that were frequently mutated includes BRCA1, BRCA2, CARS, CHEK2, DDX5, FH, MEN1, NF1, NF2, NUP98, PALB2, PTEN, SUFU, TP53 and VHL is shown in table 2 in supplementary material. This shows the complete mutational analysis their accession number their chromosome no and their exonic mutations with the detail of oncogenes.

Type of gene mutation

During the study, different type of mutations was observed in patients under study. These mutations include stopgain mutations, Frameshift deletion, Nonframeshift substitution, Nonframeshift deletion, Synonymous SNV, and Nonsynonyymous SNV. Three stopgain mutations, six frameshift deletion, eight nonframeshift substitution, zero nonframeshift deletion, hundred synonymous SNV and forty two nonsynonyymous SNV were observed in oncogenes. Eleven stopgain mutations, ten frameshift deletion, ninteen nonframeshift substitution, four nonframeshift deletion, one hundred fifteen synonymous SNV and eighty nonsynonyymous SNV were observed in tumor suppressor genes. Table 5 shows the details in it.

Table 5: Shows the type of mutations in genes

Mutation type	**Breast cancer patients (n=6)**	
	No. of mutations	
	Oncogenes (22)	**Tumor suppressor genes (22)**
Stopgain	3	11
Frameshift deletion	6	10
Nonframeshift substitution	8	19
Nonframeshift deletion	0	4
Synonymous SNV	100	115
Nonsynonyymous SNV	42	80

The figure 2 shows the frequency of mutation types in oncogenes and tumor suppressor genes.

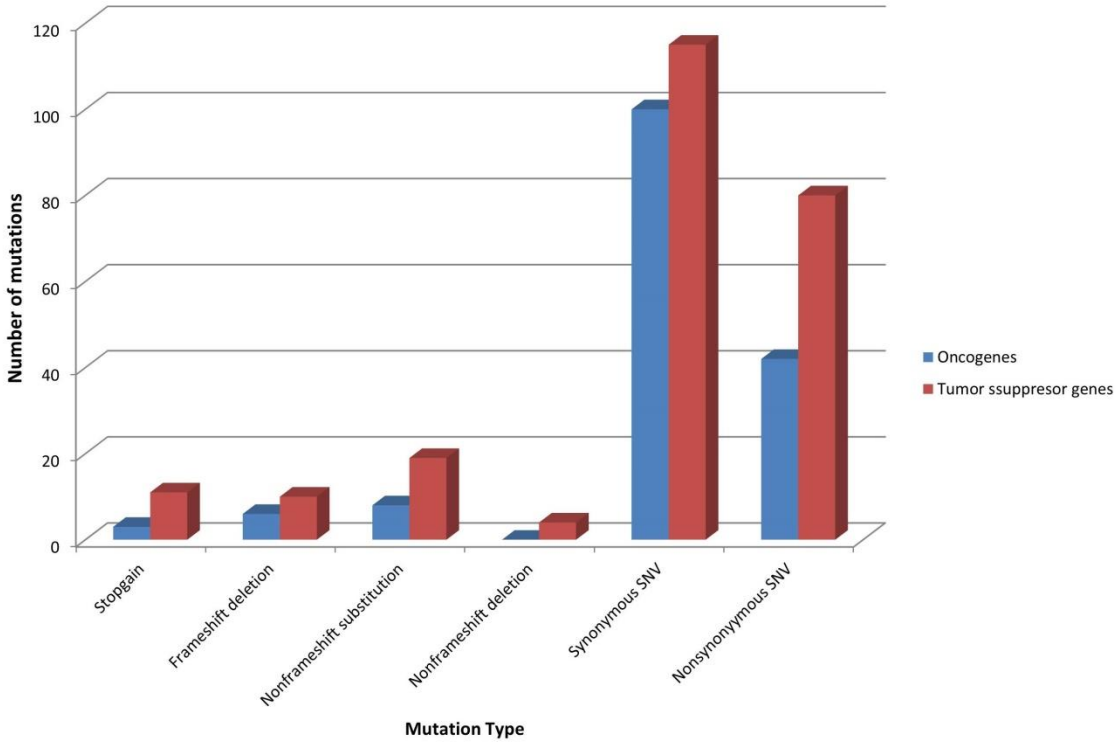

Figure 2: Shows the type of mutation in genes

Nucleotide sequences

The sequence of nucleotides of these oncogenes and tumor suppressor genes were submitted online (https://www.ncbi.nlm.nih.gov/).

Discussion

This study was directed in Jinnah Hospital, Pakistan to evaluate the histological features and single nucleotide polymorphisms in targeted genes including 20 tumor suppressor genes and 22 oncogenes. People of India and Pakistan from Asia are the growing racial groups in United States with high frequency of breast cancer than Caucasian people (66, 67). They worked by collecting data from SEER records for patients examined from 1988 to 2006 and found high ratio of BC in young females than 40y of age associated with Caucasian people. They found females belong to Asia shows high frequency of breast cancer then other racial group in United states resulted in an unequal investigation by age (68). I worked in Pakistan and found the age dispersal of people with breast cancer and compared below 50y and above 50y of age. I observed non-significant differences.

Earlier work on the biology of BC defined again into sets of various types. Every group show details associated with various usual past, therapeutic results and analyses (69-72). Already explained work on breast cancer in people of India did not work on the incidence of histological features of breast cancer invasive ductal, lobular and inflammatory breast cancer. Every type has varied function and analysis (73-75). Inflammatory breast cancer is very dangerous subtype with less danger frequency for 5y of its presence in their multivariate study and found mostly among Indian people from Asia than Caucasian. Lobular invasive cancer may observe with bilateral malignancies. The invasive ductal cancer is one-sided and risk of reoccurrence lessens as time moves from primary examination (68). I observed 2 patients having IV stage. People of stage III were eleven, and 6 with stage II. Frequency of people affected with stage III was high in this study. All people were affected with invasive ductal cancer subtype.

Their SEER investigation shows that IDC is commonly analyzed in people of India and Pakistan who belong to Asian women's than Caucasian women's while investigation of lobular cancer is constantly low (68). I found the highest frequency of IDC in Pakistani people belonging to Asian country.

Highest frequency of breast cancer in women of Asian country than Caucasian people (30.6% vs. 21.8%, $p < 0.0095$) was ER, PR negative (68). I found the equal distribution of people with ER/PR positive and negative with non-significant difference. HER-2 profile was also studied. Total of fifteen people with HER-2 negative and four with HER-2 positive was observed.

Some researchers observed the frequency of deleted BRCA1 and BRCA2 alterations in inherited BC and ovarian cancer was 9.4% in Southern Chinese high-risk related (n Z 1427) by the grouping of PCR-dependent Sanger sequencing, NGS, and MLPA (76). I worked to identify the amino acid variations and deletions in selected oncogenes and tumor suppressor genes in BC patients from Pakistan. This was achieved by WES method. This is shown in supplementary material.

They distinguish variations by next generation sequencing method that was before lost by high resolution melting. They conducted biggest work on genetic disposition broadcast of BC explained in people of China. In general, 126 pathogenic differences were observed in BRCA1 and BRCA2 gene; moreover, alterations in TP53 and PTEN were observed in 5 and 2 families, respectively. Broad study on the mutational analysis showed that seventeen types of mutations were identified, which contributes 48.8% of all observed variants. This suggested about one-half of Southern Chinese mutations. They exposed nine population-specific SNPs from study, included eight in BRCA2 and one in BRCA1, found and useful in the arrangement of VUS (76).

WES was performed to classify the variations or SNPs in genes. I observed, amino acid changes and deletions were detected in different exonic regions with various types of alteration including stopgain, synonymous SNV mutation, nonsynonymous SNV mutation, frameshift deletion, nonframeshift deletion, nonframeshift substitution The most common used technique to show mutations in gene is IHC classifies only variations that encourage proteins growth, and different alterations. The TTGE/sequencing observed 15% of TP53 variations outside exons 5 to 8; associates the significance of observing the whole gene not only these exons explained in previous work (77).

Conclusions

In conclusion People of Pakistan from Asia shows high frequency of invasive ductal breast cancer. Patients with ER and PR (+,-) shows equality. Triple negative breast cancer and HER-2 status was overexpressed. Whole exome sequencing identifies many variations include synonymous SNV, non-synonymous SNV, stopgain, non-frameshift deletion, frameshift deletions and non-frameshift substitutions in various exonic sites of genes. High frequency of Synonymous SNV alterations were found in both oncogenes and tumor suppressor genes. The amino acid changes and deletions were also identified in patients. For detailed exome research on breast carcinoma variations, we require more research and broad investigations.

Supplementary materials
Supplementary material will be available any time.

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Author contributions
All authors contributed equally in this manuscript.

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Institutional Review Board Statement
This study was performed in accordance to the Declaration of Helsinki. Approval was obtained by the Ethical review board of University of Okara, Punjab, Pakistan.

Consent to participate
Informed consent was signed from patients during data collection.

Consent to publish
Informed consent was signed from patients to publish data in journals.

Conflict of interest:
Authors declare no conflict of interest.

List of abbreviations
OGs: Oncogenes
TSGs: Tumor suppressor genes
BC: Breast cancer
OC: Ovarian cancer
SNPs: Single nucleotide polymorphisms
NGS: Next generation sequencing
WES: Whole exome sequencing
TNBC: Triple negative breast cancer

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