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ORIGINAL RESEARCH

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Next generation sequencing analysis of *BRCA1/2* genes reveals clinically significant pathogenic mutations in ovarian cancer patients

Yang Gao[1], Shengqi Su[1], Rida Naz[2,*], Xufei Yao[1,*]

[1]Lishui Hospital of traditional Chinese medicine, 317312 Lishui, Zhejiang, China
[2]Regional Blood Centre, KPK Health Program, 29111 Dera Ismail Khan, Pakistan

***Correspondence**
dr.ridaanaz@gmail.com (Rida Naz);
13600606060@139.com (Xufei Yao)

Abstract

Ovarian cancer is a formidable global health concern, necessitating extensive research to unravel its underlying genetic and epigenetic complexities. This study focuses on dissecting the role of Breast Cancer Gene 1 (*BRCA1)* and Breast Cancer Gene 2 (*BRCA2)* genes within the context of ovarian cancer in the Pakistani population. Employing Next-Generation Sequencing (NGS), we conducted a comprehensive mutational analysis of *BRCA1/2* somatic mutations. Kaplan Meier analysis was used to analyze the effect of pathogenic mutations on the survival outcomes of the ovarian cancer patients. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Immunohistochemistry (IHC) analyses were conducted to analyze the down-stream effect of the pathogenic mutations. Targeted bisulfite sequencing (bisulfite-seq) analysis facilitated the investigation of epigenetic contribution to gene expression regulation. Enrichment analysis was conducted to uncover significant Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with *BRCA1/2*. Exploring DrugBank, we identified potential drugs capable of modulating *BRCA1/2* expression regulation. NGS analysis identified four clinically significant pathogenic mutations within the *BRCA1* gene and two within the *BRCA2* gene, shedding light on their potential involvement in ovarian cancer susceptibility and progression. Kaplan Meier analysis unveiled poor overall survival (OS) associated with the identified pathogenic mutations, accentuating their prognostic value. Expression analysis using RT-qPCR and IHC demonstrated a significant up-regulation of *BRCA1* and *BRCA2* genes in ovarian cancer samples harboring pathogenic mutations. Bisulfite-seq revealed a significant hypomethylation within promoter regions of mutated *BRCA1* and *BRCA2* genes in ovarian cancer samples, compared to non-mutated cases with pathogenic mutations, indicating the role of epigenetics in in expression dysregulation as well. We unveil clinically important pathogenic mutations and demonstrate their association with altered gene expression. These findings collectively contribute to a deeper comprehension of ovarian cancer etiology, potentially paving the way for personalized therapeutic interventions.

Keywords

Ovarian cancer; *BRCA1*; *BRCA2*; Mutation

1. Introduction

Ovarian cancer remains a significant global health concern due to its high morbidity and mortality rates [1]. It ranks as the eighth most common cancer in women and is responsible for the highest mortality among all gynecological malignancies [2, 3]. The underlying genetic predisposition to ovarian cancer has gained substantial attention in recent years, with the identification of mutations in the *BRCA1* and *BRCA2* genes being among the most critical factors contributing to ovarian cancer susceptibility [4].

The *BRCA1* and *BRCA2* genes are crucial players in maintaining genomic stability through their involvement in DNA repair and homologous recombination mechanisms [5]. Mutations in these genes can result in the impairment of these repair mechanisms, leading to the accumulation of genetic aberrations and ultimately contributing to tumorigenesis [6, 7]. Somatic pathogenic mutations in *BRCA1* and *BRCA2* genes have been well-established as strong risk factors for not only ovarian cancer but also breast cancer [8, 9]. These mutations are associated with a significantly increased lifetime risk of developing ovarian cancer, ranging from 16% to 60%, depending on the specific mutation and other factors [10].

The prevalence of somatic *BRCA1* and *BRCA2* pathogenic

mutations exhibits substantial variability across different populations [11, 12]. While these mutations are well-studied in populations of European descent [13–15], there is a paucity of comprehensive data regarding their prevalence and spectrum in other ethnic groups. The Pakistani population, characterized by its unique genetic makeup and diverse ancestral backgrounds, presents an intriguing opportunity to study the prevalence and impact of *BRCA1* and *BRCA2* mutations in ovarian cancer predisposition.

Only a few studies have indicated that *BRCA1* and *BRCA2* mutations in Pakistani individuals are associated with an increased risk of both breast and ovarian cancers [16, 17]. However, the extent and spectrum of these mutations within the Pakistani ovarian cancer population remain largely unexplored. Investigating the prevalence of these mutations in Pakistani ovarian cancer patients holds immense potential for improving genetic counseling, risk assessment, and tailored therapeutic strategies in this population.

Next-generation sequencing (NGS) technologies have revolutionized the field of genetics by enabling the rapid and cost-effective screening of large genomic regions for mutations [18–22]. Targeted NGS approaches, such as those aimed at capturing the exons and splice site regions of specific genes, have become instrumental in identifying genetic mutations underlying various diseases, including cancer [23, 24]. The application of targeted NGS to the *BRCA1* and *BRCA2* genes provides an opportunity to comprehensively analyze a wide range of potential pathogenic somatic mutations in these genes within a single assay.

The primary objective of this original research article is to investigate the prevalence and spectrum of somatic pathogenic mutations in the *BRCA1* and *BRCA2* genes among Pakistani ovarian cancer patients using a targeted NGS approach. This study seeks to contribute crucial insights into the genetic landscape of ovarian cancer predisposition in the Pakistani population and shed light on the role of *BRCA1* and *BRCA2* mutations in the etiology of this disease.

2. Methods

2.1 Enrolment of cancer patients and sample collection

In this study, a total of 30 ovarian cancer patients were enrolled who underwent surgery in Mufti Mehmood Memorial Teaching Hospital, D.I.G Khan, Pakistan.

Tumor tissue samples were carefully collected from each participant during their respective surgeries, ensuring the preservation of the biological material for downstream analysis. To maintain the integrity of the samples and prevent degradation of nucleic acids, each collected tissue specimen was immediately immersed in RNA later solution (ThermoFisher, AM7021, Waltham, MA, USA). This specialized preservation solution serves to stabilize RNA and DNA molecules, effectively halting any enzymatic degradation processes that could compromise the genetic material's quality. Following proper preservation, the samples were promptly stored in a controlled environment at a temperature of −80 degrees Celsius until nucleic acid extraction. Detailed information about the collected samples is given in Table 1.

TABLE 1. An overview of ovarian cancer patient's characteristics in the present study.

Sr.no	Characteristics	Sample count (n)
	Sex	
1	Male	0
	Female	30
	Age	
2	>60	3
	<60	27
	Treatment	
3	Pre-treatment	30
	Post-treatment	0

2.2 Nucleic acid isolation

To extract DNA from the preserved tissue samples, an organic extraction method [25] was employed. This method involves the use of specialized organic solvents and reagents to separate the DNA from other cellular components. The preserved tissue samples were processed using a series of steps that included cell lysis, protein removal, and DNA precipitation. The organic extraction method was chosen for its efficiency in isolating DNA of high quality and purity, which is essential for accurate genetic analysis [25].

Conversely, for RNA isolation, the TrIzol method [26] was utilized. This method involves the use of TrIzol reagent, which aids in the separation of RNA from DNA and proteins. The tissue samples preserved in RNA later solution were homogenized to release cellular components, and the resulting lysate was mixed with TrIzol reagent. The addition of chloroform facilitated the separation of the RNA-containing aqueous phase from the rest of the lysate. Subsequent precipitation steps allowed for the isolation and purification of RNA molecules from the extracted aqueous phase [26].

2.3 Next Generation Sequencing analysis for somatic mutation detection in *BRCA1/2* genes

The DNA samples were appropriately diluted following the recommended input guidelines provided by the AmpliSeq for Illumina BRCA Panel, which was utilized for the subsequent library preparation process. Amplification of the coding and splice-site regions within the *BRCA1* and *BRCA2* genes (corresponding to NM 007294 and NM 000059, respectively) was conducted using uniquely indexed primers in accordance with the AmpliSeq for Illumina workflow. The resulting amplified DNA fragments were then subjected to paired-end sequencing by synthesis, aiming for a minimum coverage depth of 500×. The process encompassed target capture, followed by bridge amplification, which facilitated signal imaging and extension during the automated 300-cycle sequencing on the clusters-bearing V2 flow cell. This sequencing procedure

was performed using the MiSeq sequencer (Illumina, San Diego, CA, USA). The acquired raw sequence reads underwent comprehensive analysis to evaluate base quality and amplicon coverage, ensuring the reliability and accuracy of the subsequent genetic data interpretation.

2.4 Data analysis and mutation classification

The cleaned reads were aligned to the human reference genome hg19/GRCh37 by the MiSeq sequencer's local run manager. Following alignment, mismatched reads were designated as mutations utilizing the integrated bioinformatics tools provided by Illumina. The identification of genetic mutations was accomplished through the use of Illumina's Basespace sequence hub mutation caller, which was based on Pisces 5.2.9.23. Subsequently, the annotation of these identified mutations was carried out using the Basespace mutation interpreter, which was built upon Annotation Engine 3.1.1.0. As per the guidelines of American College of Medical Genetics and Genomics and the Association for Molecular Pathology—ACMG/AMP [27], mutation interpretation was done. Moreover, ClinVar database [28] was employed to check the clinical significance of the mutations.

2.5 Mutational frequencies analysis

The Genome Aggregation Database (GnomAD) is a vital resource for genomics research, offering a comprehensive collection of genetic mutations from diverse populations [29]. This database encompasses exome and genome sequencing data, collated from thousands of individuals. Researchers globally rely on GnomAD to investigate the frequency and distribution of genetic variations, aiding in the identification of disease-causing mutations and the interpretation of genetic findings. In this study, GnomeAD database was used to analyze the frequencies of observed mutations in Asian population.

2.6 Kaplan-Meier analysis

The exploration conducted using Kaplan-Meier analysis within groups of patients with mutations and those without mutations provides crucial understandings regarding survival differences [30]. We conducted KM survival analysis to determine the effect of pathogenic mutations in ovarian cancer patients by comparing the survival duration of patients with pathogenic mutations to those without using the Kaplan-Meier (KM) method. To determine if the observed differences are statistically significant, the log-rank test was applied.

2.7 Real time quantitative PCR (RT-qPCR)

The TaqMan® gene expression assay (Applied Biosystems, Foster City, CA, USA) in combination with the 7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was employed to amplify template cDNA. The PCR mixture, totaling 20 μL, comprised 2X Premix ExTaq (Probe qPCR; Takara Bio, Inc., Otsu, Japan), 1 μL of cDNA, and 1 μL each of the primers and probes targeting *BRCA1* (Hs01556193_m1), *BRCA2* (Hs00609073_m1), and Actin, Beta (*ACTB)* (Hs99999903_m1) (cat. no. 4331182). Amplification was carried out in a 96-well optical plate, initiating at 95 °C for 30 seconds, followed by 45 cycles at 95 °C for 5 seconds and 60 °C for 34 seconds. This experiment was triplicated, and relative gene expression was determined using the comparative Cq method. The final mRNA expression level was calculated through the formula: mRNA expression level = $2^{-\Delta\Delta Cq}$ [31].

2.8 Receiver operating curve (ROC) generation

Based on the RT-qPCR expression and bisulfite-seq based methylation data, receiver operating curve (ROC) curves of *BRCA1/2* expression and methylation levels were generated with the help of SRPLOT web source (https://bioinformatics.com.cn/srplot).

2.9 Library preparation for targeted bisulfite sequencing analysis

In brief, total DNA (1 μg) was fragmented into approximately 200–300 bp fragments using a Covaris sonication system (Covaris, Woburn, MA, USA). Following purification, the DNA fragments underwent repair and phosphorylation of blunt ends using a mixture of T4 DNA polymerase, Klenow Fragment, and T4 polynucleotide kinase. The repaired fragments were then 3′ adenylated using Klenow Fragment (3′-5′ exo-) and ligated with adapters containing 5′-methylcytosine instead of 5′-cytosine and index sequences using T4 DNA Ligase. The constructed libraries were quantified using a Qubit fluorometer with the Quant-iT dsDNA HS Assay Kit (Q33120, Invitrogen, Carlsbad, CA, USA) and sent to Beijing Genomic Institute (BGI), China for targeted bisulfite sequencing. Following sequencing, the methylation data was normalized into beta values.

2.10 Immunohistochemical analysis of BRCA1/2 expression

To examine BRCA1/2 protein expression, formalin-fixed paraffin-embedded ovarian cancer sections underwent a comprehensive procedure using, including Anti-BRCA1 antibody (ab238983) for BRCA1 protein and Anti-BRCA2 antibody (ab216972) for BRCA2 protein. The ovarian cancer sections were deparaffinized, followed by a stepwise rehydration process involving varying alcohol concentrations and 0.3% H_2O_2 for 30 minutes to counteract endogenous peroxidase. Subsequently, epitope retrieval was conducted in a water bath at 95 °C for 20 minutes using 0.1 M citrate buffer. To prevent nonspecific binding, rabbit anti-mouse serum was applied prior to monoclonal antibodies (AB-1, AB-8F7), and swine anti-rabbit serum was used before polyclonal antibodies (AB-D20, AB-C-terminus) for 30 minutes. Post overnight incubation, a biotinylated antibody (link antibody) was introduced, followed by streptavidin (diluted at 1:500). Chromogen employed was diaminobenzene. Notably, diverse buffers and antigen retrieval methods were experimented with before arriving at this optimsized protocol, including microwave treatments at 90 °C for 10 minutes and 95 °C for

20 minutes.

2.11 cBioPortal analysis

The cBioPortal database stands as a pivotal platform in cancer genomics research [32]. This online resource empowers researchers with a user-friendly interface to explore and analyze complex genomic data across The Cancer Genome Atlas (TCGA) samples of different cancer types. By offering tools to visualize alterations in genes, pathways, and clinical outcomes across a plethora of cancer types, cBioPortal aids in unraveling the intricate genetic landscape of cancer. This enables scientists and clinicians to identify potential driver mutations, therapeutic targets, and prognostic markers. In the present study, we used this database to analyze clinically significant mutations across TCGA ovarian cancer samples.

2.12 Enrichment analysis

MetaScape database serves as a vital resource in the realm of functional enrichment analysis [33]. This platform offers researchers a comprehensive suite of tools to explore and interpret biological datasets, enabling the identification of enriched pathways, gene ontologies, and protein interactions. In this study, we used this valuable resource for Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses of the mutated genes. A $p < 0.05$ was used as the cutoff criterion for the functional enrichment analysis.

2.13 Drug prediction analysis

The DrugBank database stands as a comprehensive and accessible resource in the realm of pharmaceuticals [34]. It serves as a reservoir of meticulously curated information about drugs, their mechanisms, interactions, and associated targets. This resource proves invaluable for researchers, healthcare professionals, and the pharmaceutical industry, facilitating the exploration of drug properties, indications, side effects and pathways. In this study, we used DrugBank database to explore *BRCA1/2* expression regulatory drugs.

3. Results

3.1 Sequencing analysis and somatic mutation detection in *BRCA1/2* genes

Sequencing of 30 ovarian cases yielded 35 somatic mutations, including 20 mutations in *BRCA1* and 15 mutations in *BRCA2* (Fig. 1A), all holding a mutation quality score of 100. The sequencing reads exhibited coverage of 96.6%, while the average Quality score (Q30) reached 95%. Apart from conducting *in-silico* analysis, the ClinVar database was consulted to ascertain the clinical significance of the resulting mutations. Through examination of mutation calling files from all cases, a total of 4 pathogenic mutations (20%) and 16 benign mutations (80%) within the *BRCA1* gene were identified (Fig. 1A and Table 2). Correspondingly, the *BRCA2* gene revealed 2 pathogenic mutations (13%) and 13 benign mutations (87%) among a subset of ovarian cancer patients from Pakistani population (Fig. 1B and Table 2).

3.2 Clinically significant mutations in *BRCA1/2* genes

Pathogenic mutations are deemed clinically significant due to their direct association with the development of diseases. These genetic mutations disrupt normal cellular functions, leading to abnormal protein production or function [35]. In this study, a total of four clinically significant pathogenic mutations (p.Glu1836Ter, p.Trp1815Ter, p.Ser1797Ter and p.Ser1797Ter) were detected in *BRCA1* gene (Table 2), while two clinically significant pathogenic mutations (p.Tyr57Ter and p.Val211Leu) were observed in *BRCA2* gene (Table 2). These findings underscore the importance of recognizing such mutations for precise diagnosis, prognosis, and personalized treatment of ovarian cancer patients.

3.3 Frequencies of the clinically significant mutations in *BRCA1/2* genes across Asian ovarian cancer patients by GnomAD database

Low-frequency pathogenic mutations are often more important as populations-specific molecular markers. Frequencies analysis of the pathogenic mutations in *BRCA1* pathogenic mutations (p.Glu1836Ter, p.Trp1815Ter, p.Ser1797Ter and p.Ser1797Ter) and *BRCA2* (p.Tyr57Ter and p.Val211Leu) (Table 2) genes across GnomeAD database reveals that these mutations have not been previously reported among Asian ovarian cancer patients, as their frequencies were 0 in this database. This suggests that these specific mutations are unique to a subset of ovarian cancer patients from Pakistani population.

3.4 Survival analysis of ovarian cancer patients having pathogenic mutations in *BRCA1/2* genes

In the current research, the Kaplan-Meier survival analysis indicates a significant difference in overall survival (OS) between two groups of ovarian cancer patients: one with *BRCA1/2* pathogenic mutations and another without *BRCA1/2* mutation group (Fig. 2). The ovarian cancer group with *BRCA1/2* mutations experienced significant worse OS outcomes compared to the group without *BRCA1/2* mutation (Fig. 2). No doubt, beside the presence of pathogenic mutation in *BRCA1/2* genes, OS of the ovarian cancer patients can be influenced by a multitude of factors, such as surgical quality (extend of cytoreduction), type of chemotherapy given, number of lines of chemotherapy, utilization of novel agents (such as immunotherapy, *etc.*). Therefore, we encourage further research that incorporates these additional clinical variables to better elucidate the complex interplay between *BRCA1/2* pathogenic mutations and these factors in determining patient OS.

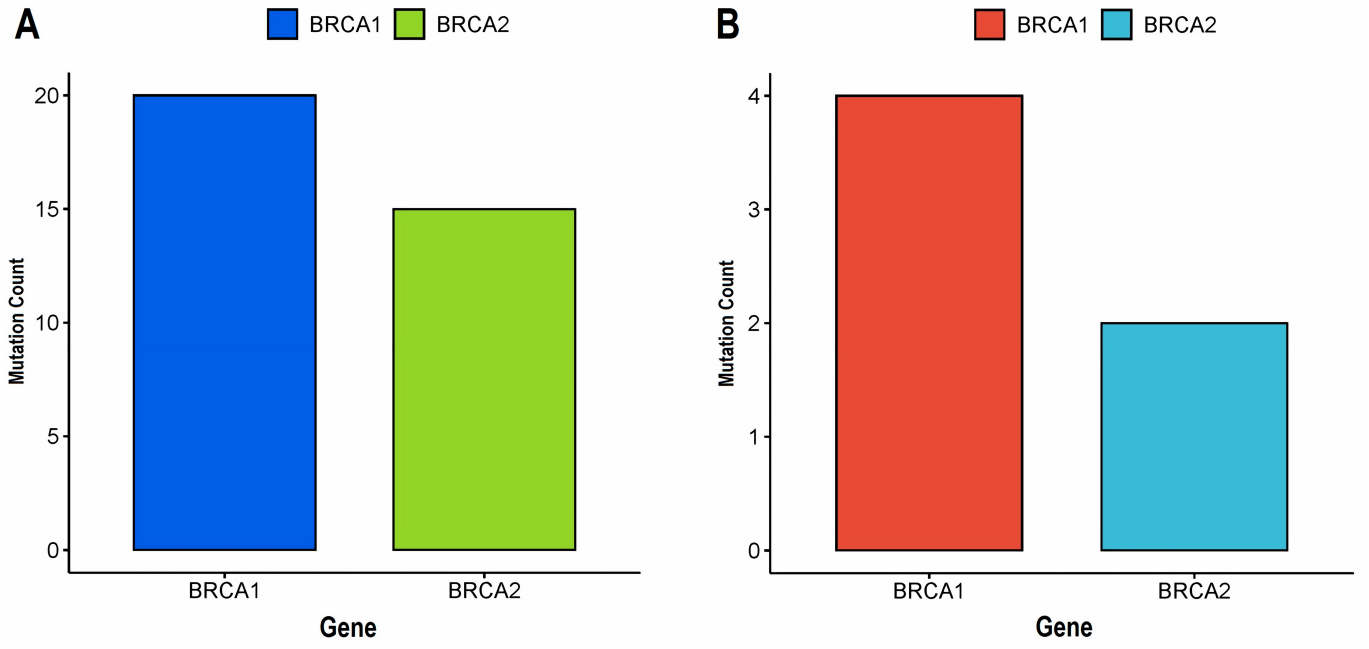

FIGURE 1. Total count of overall detected mutations and pathogenic mutations in *BRCA1/2* across ovarian cancer patients *via* WES. (A) An overall count of detected mutations in *BRCA1/2* genes across ovarian cancer patients, and (B) A count of detected pathogenic mutations in *BRCA1/2* genes across ovarian cancer patients. BRCA: Breast cancer.

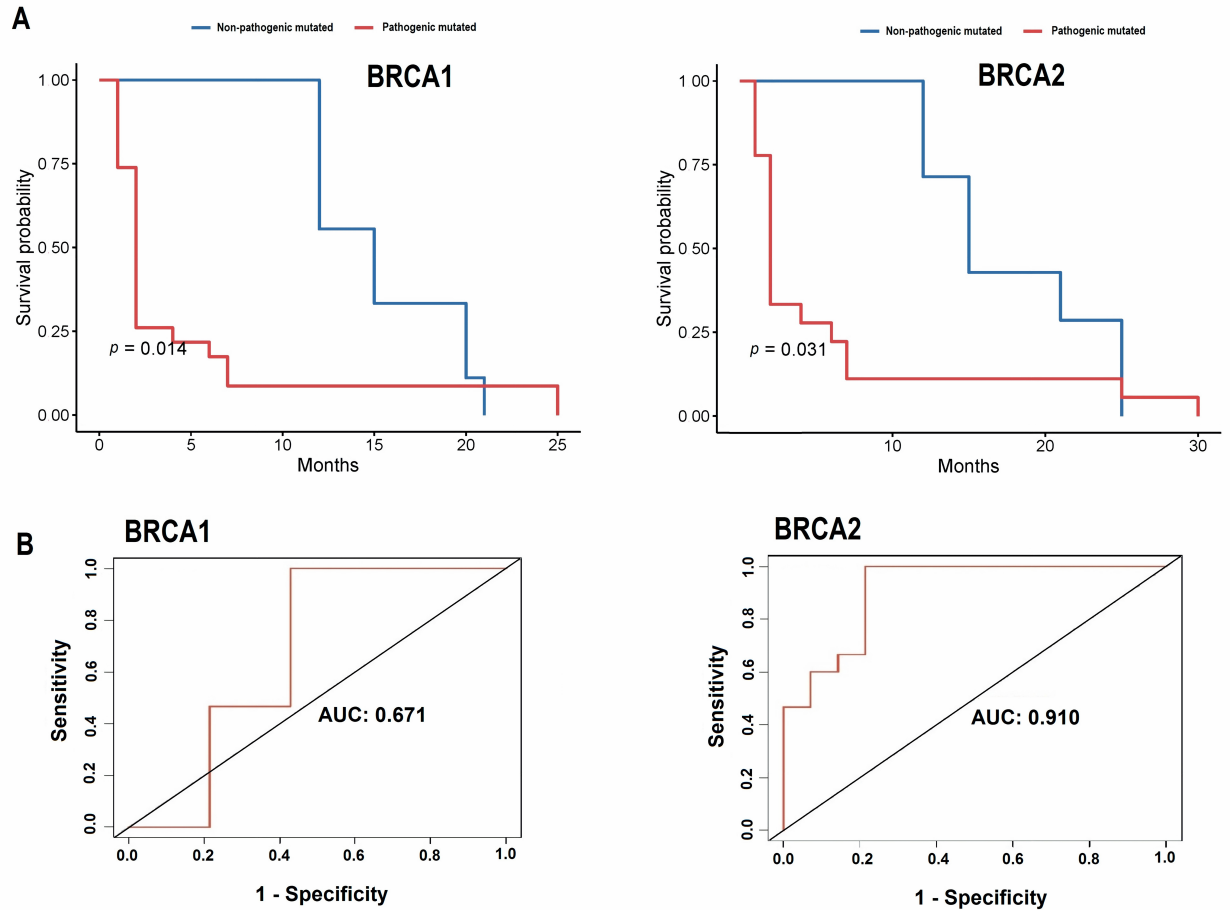

FIGURE 2. Survival analysis of *BRCA1/2* genes between pathogenic mutated and non-pathogenic mutated ovarian cancer sample groups. (A) Survival analysis curves, and (B) ROC curves. A $p < 0.05$ was used as the cut-off criterion. BRCA: Breast cancer; AUC: Area under the curve; ROC: Receiver operating curve.

TABLE 2. Count and type of mutations observed in *BRCA1/2* genes across ovarian cancer patients.

Sr. no	Gene	NM:c.DNA	Protein	Nature	No. patients
1	*BRCA1*	NM_007294.4:c.5506G>T	p.Glu1836Ter	Pathogenic	8
2		NM_007294.4:c.5444G>A	p.Trp1815Ter	Pathogenic	8
3		NM_007294.4:c.5390C>A	p.Ser1797Ter	Pathogenic	8
4		NM_007294.4:c.5390C>G	p.Ser1797Ter	Pathogenic	8
5		NM_007294.4:c.5576C>G	p.Pro1859Arg	Benign	14
6		NM_007294.4:c.5456A>G	p.Asn1819Ser	Benign	16
7		NM_007294.4:c.5402G>A	p.Gly1801Asp	Benign	11
8		NM_007294.4:c.4840C>T	p.Pro1614Ser	Benign	2
9		NM_007294.4:c.4837A>T	p.Ser1613Cys	Benign	14
10		NM_007294.4:c.4682C>T	p.Thr1561Ile	Benign	8
11		NM_007294.4:c.4636G>T	p.Asp1546Tyr	Benign	10
12		NM_007294.4:c.4520G>C	p.Arg1507Thr	Benign	12
13		NM_007294.4:c.4402A>C	p.Asn1468His	Benign	5
14		NM_007294.4:c.4327C>G	p.Arg1443Gly	Benign	8
15		NM_007294.4:c.4255G>C	p.Glu1419Gln	Benign	2
16		NM_007294.4:c.4039A>G	p.Arg1347Gly	Benign	11
17		NM_007294.4:c.3797G>C	p.Ser1266Thr	Benign	9
18		NM_007294.4:c.3748G>A	p.Glu1250Lys	Benign	14
19		NM_007294.4:c.3724A>G	p.Thr1242Ala	Benign	14
20		NM_007294.4:c.3601G>A	p.Gly1201Ser	Benign	11
21	*BRCA2*	NM_000059.4:c.171C>G	p.Tyr57Ter	Pathogenic	10s
22		NM_000059.4:c.631G>C	p.Val211Leu	Pathogenic	11
23		NM_000059.4:c.865A>C	p.Asn289His	Benign	11
24		NM_000059.4:c.943T>A	p.Cys315Ser	Benign	9
25		NM_000059.4:c.1798T>C	p.Tyr600His	Benign	13
26		NM_000059.4:c.1810A>G	p.Lys604Glu	Benign	3
27		NM_000059.4:c.2138A>T	p.Gln713Leu	Benign	15
28		NM_000059.4:c.2803G>A	p.Asp935Asn	Benign	15
29		NM_000059.4:c.3396A>C	p.Lys1132Asn	Benign	12
30		NM_000059.4:c.3869G>A	p.Cys1290Tyr	Benign	11
31		NM_000059.4:c.5312G>A	p.Gly1771Asp	Benign	13
32		NM_000059.4:c.5744C>A	p.Thr1915Lys	Benign	7
33		NM_000059.4:c.6290C>T	p.Thr2097Met	Benign	14
34		NM_000059.4:c.6455C>A	p.Ser2152Tyr	Benign	9
35		NM_000059.4:c.6935A>T	p.Asp2312Val	Benign	5

BRCA: Breast cancer.

3.5 Expression and promoter methylation analyses of *BRCA1/2* genes across ovarian cancer samples *via* RT-qPCR and bisulfite sequencing

The expression and promoter methylation analyses of *BRCA1/2* genes were conducted in two distinct groups of ovarian cancer samples. One group consisted of samples with confirmed *BRCA1/2* pathogenic mutations, while the other group comprised samples without pathogenic mutations (non-pathogenic mutation group). Upon analyzing the RT-qPCR and bisulfite-seq results, it was observed that the expression levels of *BRCA1/2* genes were significantly up-regulated (Fig. 3A) and their promoter methylation level was lower (Fig. 3B) in the ovarian cancer sample group with BRCA1/2 pathogenic mutations when compared to non-pathogenic mutation group. This finding suggests a potential association between *BRCA1/2* mutations, the higher expression and lower promoter methylation level of these genes in the context of ovarian cancer.

Furthermore, the ROC curves displayed noteworthy levels of sensitivity and specificity for *BRCA1* (AUC: 0.671, p-value < 0.05) and *BRCA2* (AUC: 0.910, p-value < 0.05), based on their expression profiles (Fig. 3C). Similarly, based on their promoter methylation levels (Fig. 3D), *BRCA1* (AUC: 0.615, p-value < 0.05) and *BRCA2* (AUC: 0.744, p-value < 0.05), the ROC curves also exhibited significant sensitivity and specificity.

3.6 Immunohistochemical analysis of BRCA1/2 protein expression

We conducted an IHC analysis to assess the proteomic expression of BRCA1/2 proteins in ovarian cancer tissue samples. Specifically, we examined one tissue sample harboring a pathogenic mutation in *BRCA1/2* gene, respectively and another tissue sample without any pathogenic mutation. The objective was to investigate potential differences in protein expression between these two types of samples.

Upon examining the staining results, a notable pattern emerged (Fig. 4). The ovarian cancer tissue samples containing pathogenic mutations exhibited significantly higher expression levels of the BRCA1/2 proteins compared to their counterpart lacking the pathogenic mutations (Fig. 4). This observation suggests that the presence of a pathogenic mutation in the *BRCA1/2* genes may be associated with an elevation in the expression of these proteins across ovarian cancer.

3.7 Analysis of clinically important pathogenic mutations in BRCA1/2 across TCGA ovarian cancer samples

Next, this study involved a comprehensive mutational analysis of the *BRCA1/2* genes in ovarian cancer samples from the TCGA dataset, employing the cBioPortal platform. The objective was to discern any potential genetic variations and their prevalence across different populations. The analysis outcomes showed that the pathogenic mutations identified within the *BRCA1* (p.Glu1836Ter, p.Trp1815Ter, p.Ser1797Ter and p.Ser1797Ter) and *BRCA2* (p.Tyr57Ter and p.Val211Leu) genes among ovarian cancer patients of Pakistani origin were notably absent within the TCGA ovarian cancer samples. Detail of observed *BRCA1/2* mutations, including their types, numbers, and position in the encoded proteins across TCGA ovarian cancer patients can be seen in Fig. 5.

Overall this finding highlights the unique nature of these detected pathogenic mutations within a subset of ovarian cancer patients from Pakistani population. These mutations appear to represent distinctive genetic markers associated with ovarian cancer susceptibility in individuals of Pakistani descent.

3.8 Enrichment analysis of *BRCA1/2* genes

Next, we performed GO and KEGG enrichment analyses. Among GO, *BRCA1/2* genes were enriched in "Peroxisomal Matrix, Microbody Lumen, chromosome, telomeric region, condensed chromosonme, and Intracellular Non-Membrane-Bounded Organelle" *etc.*, Cellular components (CC) terms (Fig. 6A), "Tubulin binding, RNA Polymerase Binding, Histone H4 Acetyltransferase Activity, and Acetyltransferase Activity" *etc.*, Molecular function (MF) terms (Fig. 6B), "Response to Ionizing Radiations, Regulation of Cellular Response to Stress, and Double Strand Break Repair" *etc.*, BP terms (Fig. 6C), and "Homologous Recombination, Fanconi Anemia Pathway, Breast Cancer, and MicroRNA in Cancer" *etc.*, KEGG terms (Fig. 6D).

3.9 Drug prediction analysis of *BRCA1/2* genes

In this thorough investigation, we utilized the DrugBank database to conduct a methodical exploration of therapeutic avenues aimed at mitigating the expression of down-regulated, mutated genes (*BRCA1 and BRCA2*). Our rigorous analysis yielded a spectrum of potential drug candidates with promising capabilities to decrease *BRCA1/2* expression. Noteworthy among these candidates are Arecoline, Estradiol, Bortezomib, Doxorubicin, Cyclosporine, Tretinoin and Tamibarotene (Table 3). These compounds exhibit the potential to effectively modulate the expression levels of the target genes, thus holding promise for novel therapeutic interventions.

4. Discussion

Ovarian cancer remains a significant global health challenge [36, 37], demanding comprehensive research efforts to decode its intricate genetic and epigenetic underpinnings. The present study takes a focused approach, investigating the crucial roles of *BRCA1* and *BRCA2* genes in DNA repair and tumor suppression, within the specific context of ovarian cancer in the Pakistani population. The integration of NGS technique, survival analysis, expression assays, epigenetic investigations, and pathway enrichment analyses has facilitated a deeper understanding of the involvement of *BRCA1* and *BRCA2* mutations in ovarian cancer susceptibility and progression.

The utilization of NGS to analyze the mutational landscape of *BRCA1* and *BRCA2* a subset of ovarian cancer patients from Pakistani population has yielded significant insights through

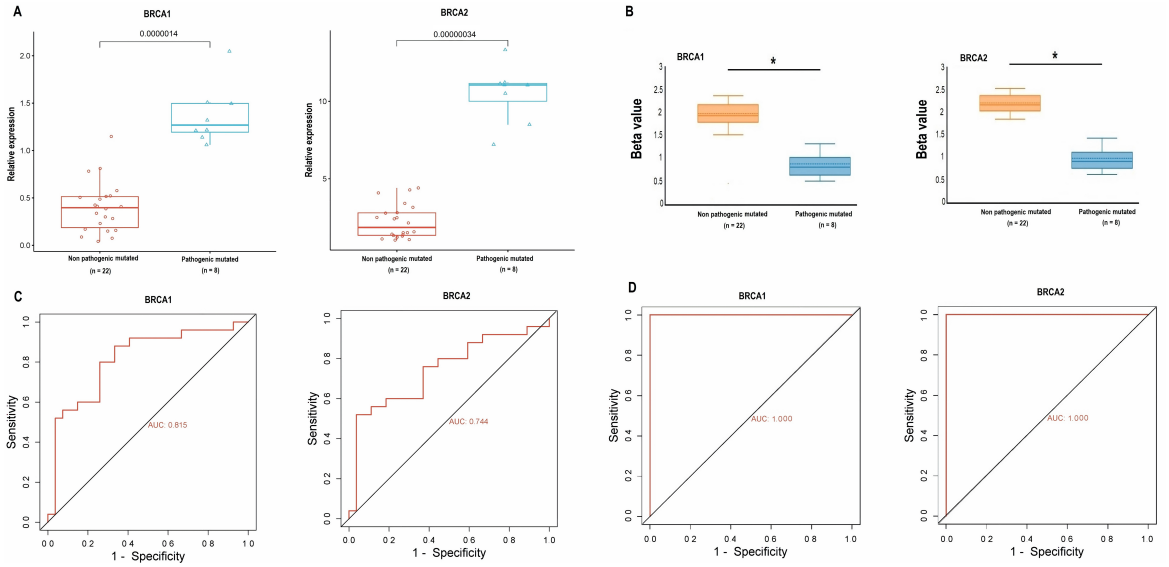

FIGURE 3. Relative expression, promoter methylation, and ROC curve analysis of *BRCA1/2* genes between pathogenic mutated and non-pathogenic mutated ovarian cancer sample groups. (A) Relative expression analysis of *BRCA1/2* genes *via* RT-qPCR, (B) Promoter methylation analysis of *BRCA1/2* genes *via* bisulfite-seq, (C) RT-qPCR expression-based ROC curves of *BRCA1/2* genes, and (D) Promoter methylation level-based ROC curves of *BRCA1/2* genes. A $p < 0.05$ was used as the cut-off criterion. A $p^* < 0.05$ indicates the cut-off criterion for significant results. BRCA: Breast cancer; AUC: Area under the curve.

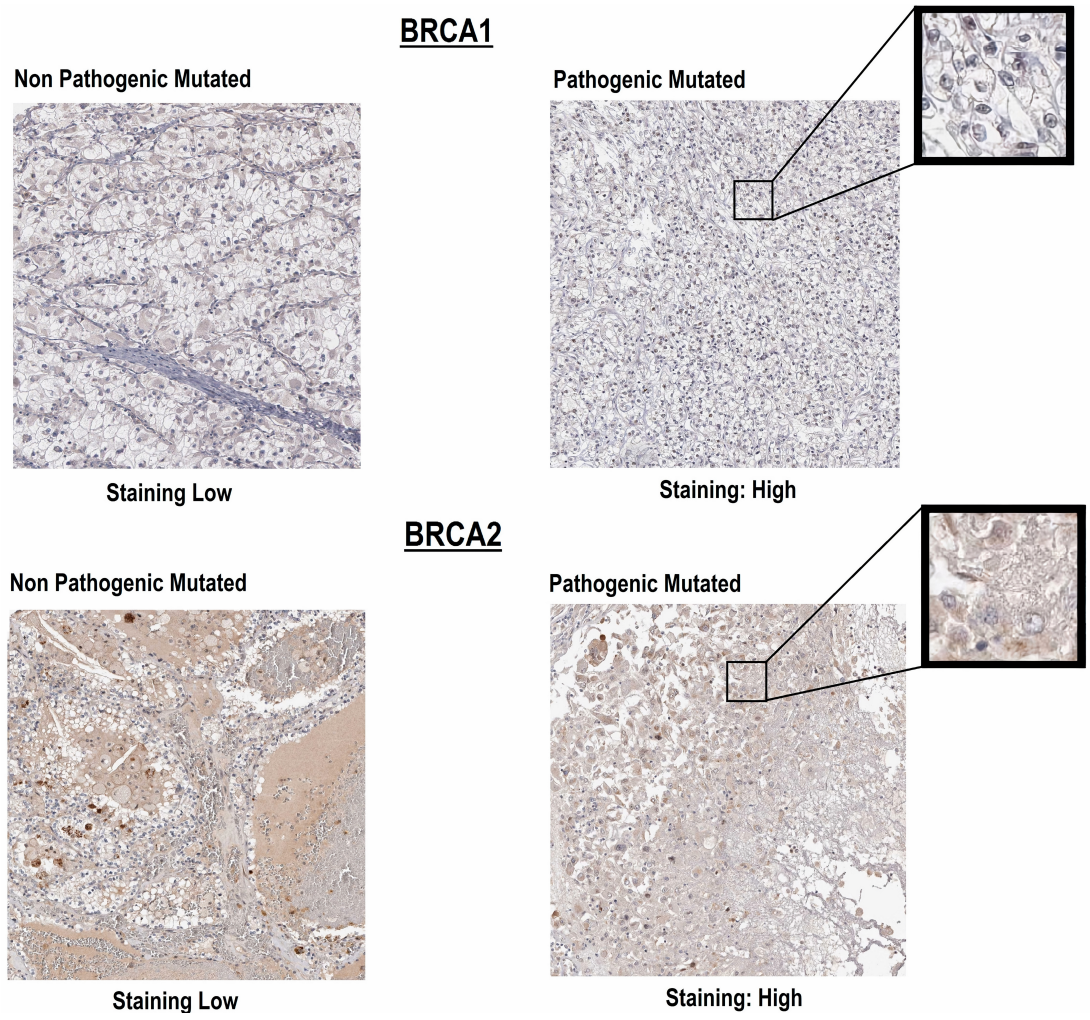

FIGURE 4. IHC-based proteomic expression analysis of BRCA1/2 proteins between pathogenic mutated and non-pathogenic mutated ovarian cancer samples. BRCA: Breast cancer.

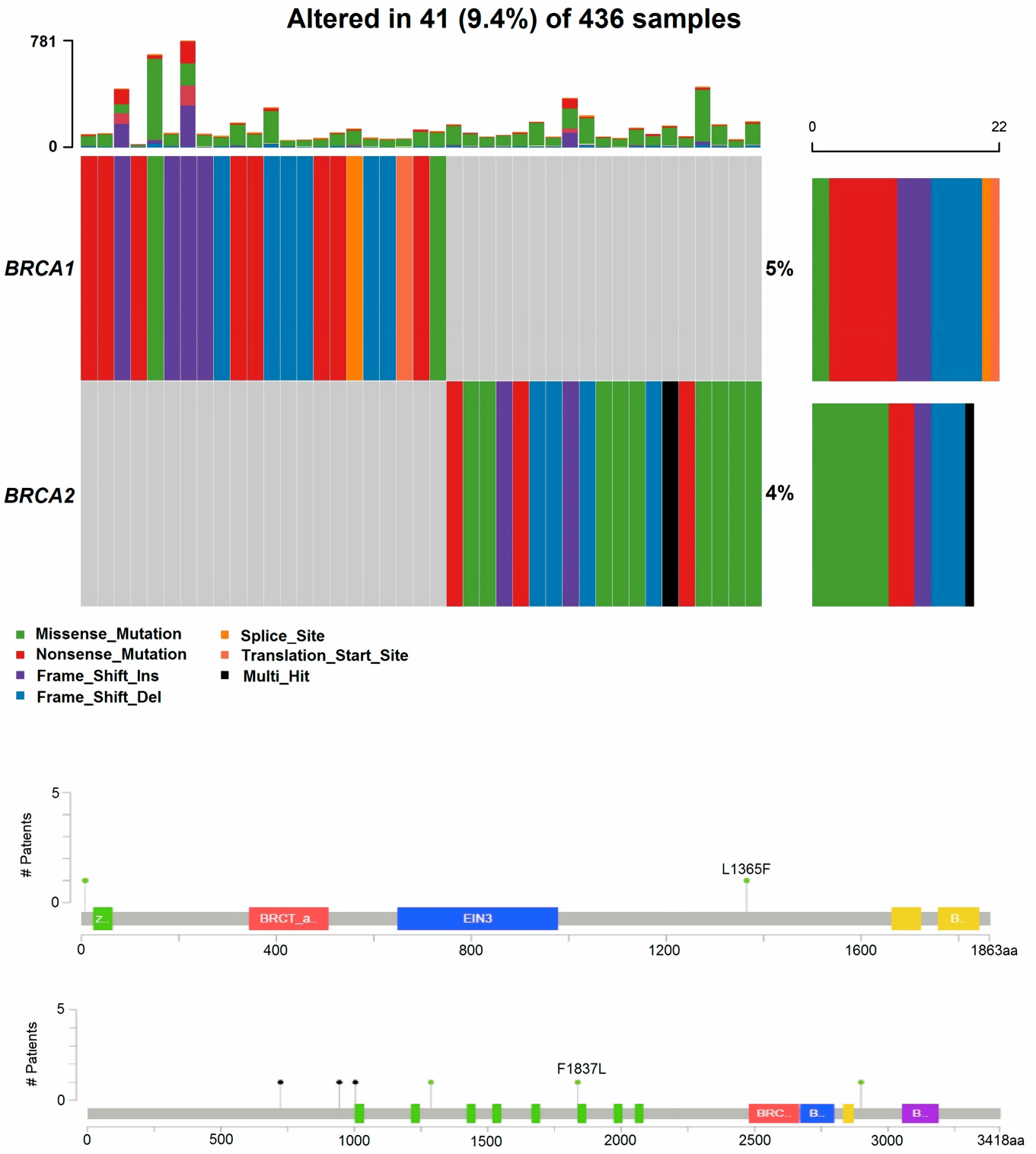

FIGURE 5. Oncoplot and lollipop plot-based visualization of the observed *BRCA1/2* mutations across TCGA ovarian cancer patients. Two rows showed percentage of ovarian cancer samples which are positive for BRCA1/2 mutations, and lollipop plots highlighted amino acid change due to mutation at the protein level. BRCA: Breast cancer.

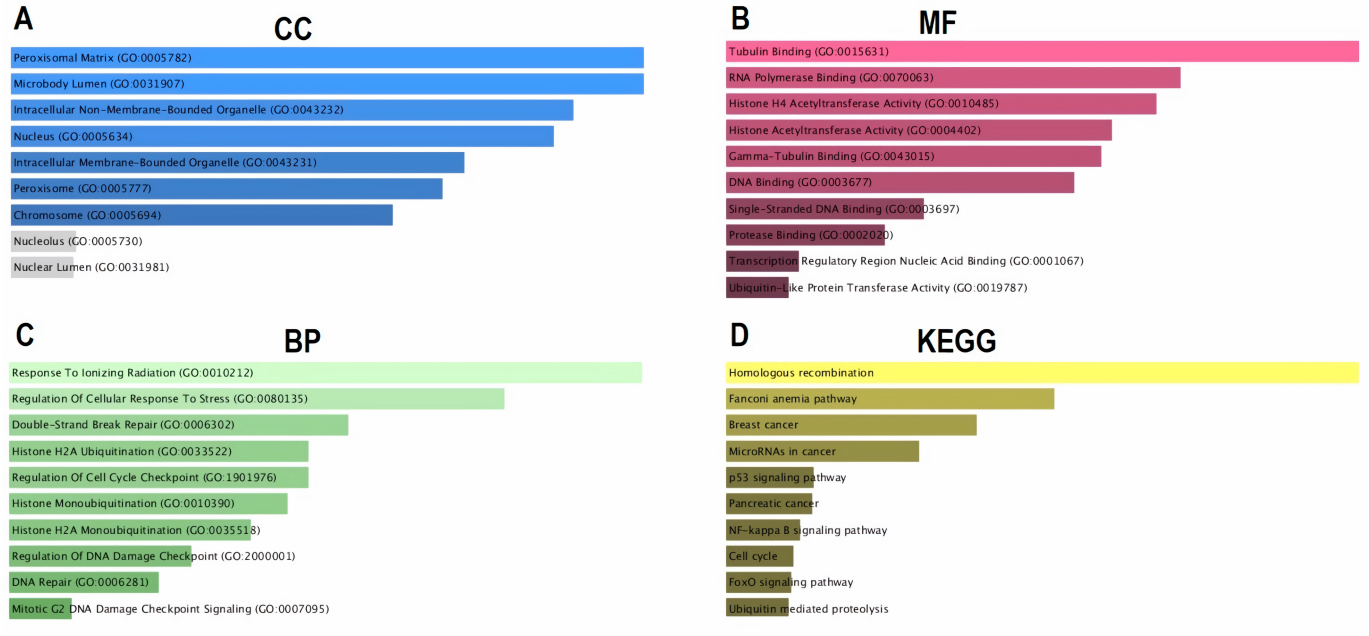

FIGURE 6. GO and KEGG analyses of *BRCA1/2* genes *via* Metascape. (A) *BRCA1/2* genes-related CC terms, (B) *BRCA1/2* genes-related MF terms, (C) *BRCA1/2* genes-related BP terms, and (D) *BRCA1/2* genes-related KEGG terms. A $p < 0.05$ was used as the cut-off criterion. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; CC: Cellular components; MF: Molecular function; BP: Biological process; BRCA: Breast cancer.

TABLE 3. DrugBank-based *BRCA1/2* associated drugs.

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	*BRCA1*	Arecoline	Decrease expression of *BRCA1* mRNA	A20694	Approved
		Estradiol		A21155	
		Bortezomib		A21448	
		Cyclosporine		A20661	
2	*BRCA2*	Bortezomib	Decrease expression of *BRCA2* mRNA	A21448	Approved
		Doxorubicin		A21498	
		Estradiol		A21155	
		Cyclosporine		A20661	
		Tretinoin		A24376	
		Tamibarotene		A24376	

BRCA: Breast cancer.

this study. The identification of four clinically significant pathogenic mutations within *BRCA1* (p.Glu1836Ter, p.Trp1815Ter, p.Ser1797Ter and p.Ser1797Ter) and two within *BRCA2* (p.Tyr57Ter and p.Val211Leu) underscores their potential as key players in ovarian cancer development. This observation is in line with existing knowledge that highlights the role of pathogenic mutations within *BRCA1/2* genes in the development and progression of different human cancers [38–41]. The discovery of these mutations not only expands the spectrum of known pathogenic variants but also emphasizes the relevance of *BRCA1* and *BRCA2* in the context of ovarian cancer in the Pakistani population.

Survival analysis using the Kaplan-Meier method is a powerful tool to assess the impact of genetic mutations on clinical outcomes [42]. The study's findings that pathogenic mutations within *BRCA1/2* genes are associated with poor overall survival (OS) emphasize their clinical significance. These results align with previous research indicating that pathogenic mutations in *BRCA1/2* genes can influence disease progression and patient outcomes [43, 44]. In case of ovarian cancer, some studies also suggest that ovarian cancer patients with *BRCA1/2* mutations may have a better prognosis compared to those without these mutations [45–47]. However, this improved prognosis could be partly attributed to the improved surgical respectability, improved chemo responsiveness, and intrinsic growth rate, which can extend OS in certain cases. To the best of our understanding, this research is the first to report association between pathogenic mutations within *BRCA1/2* genes and poor OS rate in a subset of Pakistani ovarian cancer patients.

The downstream effects of the identified pathogenic mutations have been comprehensively explored through expression assays, including RT-qPCR and immunohistochemistry (IHC). The substantial up-regulation of *BRCA1 and BRCA2* genes in ovarian cancer samples harboring these mutations indicates their potential role in driving aberrant gene expression patterns. This observation further supports the hypothesis that mutations in these genes contribute to ovarian cancer progression through dysregulated gene expression. Similar to our results, previous studies also linked pathogenic mutations within *BRCA1/2* genes with their expression dysregulation in cancer patients [48, 49].

The study's exploration of epigenetic contributions to gene expression regulation through targeted bisulfite-seq is also noteworthy. The significant hypomethylation observed within promoter regions of mutated *BRCA1* and *BRCA2* genes suggests that epigenetic modifications play a crucial role in gene expression dysregulation. Epigenetic alterations, such as DNA methylation, have been increasingly recognized as key factors influencing gene expression patterns in cancer [50]. The findings in this study reinforce the intricate interplay between genetic and epigenetic mechanisms in ovarian cancer pathogenesis. Prior research has also established a connection between promoter hypomethylation of *BRCA1/2* genes and their aberrant expression in diverse cancer types [51, 52].

Enrichment analysis, a vital component of the study, provides a broader context for understanding the functional implications of *BRCA1* and *BRCA2* mutations. By uncovering significant Gene Ontology (GO) terms and KEGG pathways associated with these genes, the study highlights potential molecular mechanisms that underlie their roles in ovarian cancer. In this study, we decipher some important signaling pathways related to *BRCA1/2* genes, including "Homologous Recombination, Fanconi Anemia Pathway, Breast Cancer, and MicroRNA in Cancer", *etc*. The role of these pathways in the development and progression of different cancer types is already well known.

One of the study's notable contributions is the identification of potential drugs (Arecoline, Estradiol, Bortezomib, Doxorubicin, Cyclosporine, Tretinoin, and Tamibarotene) capable of modulating *BRCA1* and *BRCA2* expression regulation. Among these identified drugs, Cyclosporin is a commonly tested chemotherapeutic drug in the treatment of different cancers. Cyclosporin, a noncytotoxic immunosuppressant, was initially discovered in the 1970s and primarily employed to manage immunosuppression post organ and bone marrow transplantation [53]. It is reported in the medical literature that Cyclosporin has been recognized as one of the initial-generation multidrug resistance (MDR) modulators, aimed at counteracting MDR and enhancing the effectiveness of chemotherapy [54]. When used in conjunction with chemotherapy, Cyclosporin has been shown to elevate the plasma levels of chemotherapy drugs while reducing the clearance of substances like digoxin and etoposide, which are substrates of P-glycoprotein (P-gp) [55]. On the other hand, Cyclosporin is an inhibitor, but not a substrate for breast cancer resistance protein (BCRP) and BRCA proteins in breast cancer [55, 56]. Due to its primary clinical applications in immunosuppression for preventing organ transplant rejection, exploring Cyclosporin's potential role as a chemotherapeutic drug against *BRCA1/2*-related breast cancer would indeed require further research and investigation. This insight offers a glimpse into the potential for targeted therapies aimed at restoring normal gene expression patterns in the presence of pathogenic mutations. The prospect of personalized therapeutic interventions tailored to the genetic and epigenetic profiles of individual patients holds immense promise for improving treatment outcomes and patient survival.

5. Conclusions

In conclusion, the study's comprehensive approach sheds light on the intricate interplay between genetic and epigenetic factors in the context of ovarian cancer in the Pakistani population. The identification of clinically significant pathogenic mutations within *BRCA1* and *BRCA2*, coupled with their associations with altered gene expression and poor survival outcomes, strengthens our understanding of ovarian cancer etiology. By unraveling these complexities, the study paves the way for potential personalized therapeutic interventions that target the underlying genetic and epigenetic dysregulations. The findings not only contribute to the broader field of ovarian cancer research but also exemplify the importance of diverse population-based studies in uncovering the nuances of cancer biology. As research progresses, these insights will likely fuel the development of novel diagnostic tools and targeted therapies, ultimately improving the prognosis and quality of life for ovarian cancer patients in the Pakistani population.

AVAILABILITY OF DATA AND MATERIALS

The data could be obtained by contacting corresponding author.

AUTHOR CONTRIBUTIONS

RN and YG—designed the research study. SQS and XFY—performed the research. YG—analyzed the data. SQS, XFY, YG, and RN—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Prior to commencing the study, ethical approval (ref # KPHD/3456) was meticulously obtained from the Khyber Pakhtunkhwa, Health Department, Pakistan adhering to the highest standards of research ethics and patient protection. Additionally, a fundamental cornerstone of this study was the informed and voluntary participation of the enrolled patients. Each participant was provided with detailed information about the study's purpose, procedures, potential risks, and benefits. Subsequently, their explicit consent was sought and obtained, ensuring that their rights, privacy, and confidentiality were fully upheld throughout the study's duration.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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