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Brief Communication

Detection of clinically important BRCA gene mutations in ovarian cancer patients using next generation sequencing analysis

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Abstract: Ovarian cancer, a complex and aggressive malignancy, remains a significant challenge in clinical oncology due to its heterogeneous nature and limited therapeutic options. In this study, across Pakistani ovarian cancer patients, we conducted a comprehensive analysis of mutations within the BRCA1 and BRCA2 genes to elucidate their potential implications in ovarian cancer susceptibility and progression. Employing Next-Generation Sequencing (NGS), we conducted a comprehensive mutational analysis of BRCA1/2 genes. Kaplan Meier analysis was used to analyze the effect of pathogenic mutations on the survival outcomes of ovarian cancer patients. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Immunohistochemistry (IHC) analyses were conducted to analyze the downstream effect of the pathogenic mutations. Targeted bisulfite sequencing (bisulfite-seq) analysis facilitated the investigation of epigenetic contributions 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 three 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 reverse transcription-quantitative polymerase chain reaction (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 expression dysregulation as well. By uncovering clinically significant pathogenic mutations in BRCA1/2 genes and establishing their link with up-regulated gene expression, this study significantly advances our understanding of ovarian cancer's underlying causes in the Pakistani population.

Keywords: Ovarian cancer, BRCA1, BRCA2, mutation

Introduction

Ovarian cancer remains a significant global health concern, ranking as one of the most lethal gynecological malignancies [1-3]. On a global scale, roughly 295,000 new cases of ovarian cancer are reported every year [4-6]. Ovarian cancer impact is particularly pronounced in high-income countries, where advanced medical facilities enable better detection and treatment [7-9]. In Pakistan, ovarian cancer poses a significant health challenge, although comprehensive data on its prevalence is limited due to inadequate reporting systems and healthcare disparities [10, 11]. Its insidious onset and lack of specific early symptoms often result in a late-stage diagnosis, contributing to the limited success of treatment interventions [12-14]. Genetic factors play a pivotal role in the development of ovarian cancer, with mutations in the breast cancer susceptibility genes, BRCA1 and BRCA2, being among the most well-documented contributors to hereditary ovarian cancer predisposition [15, 16].

The BRCA1 and BRCA2 genes encode essential proteins involved in maintaining genomic stabil-

ity through the regulation of DNA repair mechanisms, homologous recombination, and cell cycle control [17, 18]. Pathogenic mutations in these genes have been identified as key risk factors not only for hereditary breast cancer but also for ovarian cancer, with carriers of these mutations facing a significantly elevated lifetime risk of developing these cancers [19, 20]. Geographical and ethnic variability in the prevalence and spectrum of BRCA1 and BRCA2 mutations have been extensively documented, underscoring the necessity of population-specific studies to better comprehend their impact on ovarian cancer susceptibility [21, 22].

In the Pakistani population, limited research has been conducted to investigate the prevalence and spectrum of germline pathogenic mutations in BRCA1 and BRCA2 genes among ovarian cancer patients [23, 24]. Understanding the genetic landscape of these genes in the context of ovarian cancer in this population is critical for personalized risk assessment, genetic counseling, and the development of targeted therapeutic strategies.

Advances in Next Generation Sequencing (NGS) technologies have revolutionized the study of genetic predisposition to cancer by enabling rapid and accurate profiling of multiple genes simultaneously [25-28]. Targeted NGS approaches offer a cost-effective and efficient means to identify pathogenic mutations in specific genes, allowing for comprehensive analyses of large cohorts [29, 30]. This study aims to utilize a targeted NGS approach to comprehensively assess the prevalence and spectrum of germline pathogenic mutations in the BRCA1 and BRCA2 genes among Pakistani ovarian cancer patients.

By elucidating the genetic alterations underlying ovarian cancer in the Pakistani population, this research seeks to provide valuable insights into the molecular basis of hereditary ovarian cancer predisposition. These findings have the potential to inform clinical practice by enhancing risk stratification, genetic counseling, and early detection strategies. Additionally, a deeper understanding of the genetic factors contributing to ovarian cancer in this population may facilitate the development of novel therapeutic interventions tailored to specific genetic profiles, ultimately improving patient outcomes.

Methods

Enrolment of cancer patients and sample collection

In this research, a group of 25 patients diagnosed with ovarian cancer and who had undergone surgery at District Headquarter (DHQ), D.I.G Khan, Pakistan, were included. Prior to the commencement of the study, meticulous ethical approval was obtained from the concerned department. This was done in strict accordance with the highest standards of research ethics and to ensure the protection of patient rights. A vital foundation of this investigation rested upon the informed and voluntary involvement of the patients who were enrolled. Each participant received comprehensive information about the study's objectives, procedures, potential risks, and advantages. Following this, explicit consent was actively requested and acquired from each participant. This process was undertaken to guarantee the preservation of their rights, personal privacy, and the confidentiality of their information throughout the entire duration of the study.

Tissue samples (cancer and paired control) 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). 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**.

Nucleic acid isolation

DNA extraction was carried out from ovarian cancer tissues (n = 25) and normal control tissues (n = 25) using the organic method [31]. This methodology involves a series of steps aimed at gently breaking open cells and isolating DNA from the rest of the cellular components [31].

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

Sr. no	Characteristics	Sample count (n)
1	Sex	
	Male	0
	Female	25
2	Age	
	>60	2
	< 60	23
3	Treatment	
	Pre-treatment	25
	Post-treatment	0

RNA extraction was accomplished utilizing the TRIzol method [32]. This technique involves a well-defined process where cells are broken open, releasing their RNA content. The extracted DNA and RNA were then purified for further downstream analyses.

We employed the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to assess the concentration and purity of the extracted DNA and RNA, ensuring that the A260/A280 ratio fell within the range of 1.8 to 2.0.

Next generation sequencing analysis

The DNA samples were diluted appropriately according to the recommended input guidelines provided by the AmpliSeq for Illumina BRCA Panel. This panel was used for the subsequent library preparation process. Specific coding and splice-site regions within the BRCA1 and BRCA2 genes (corresponding to NM 007294 and NM 000059, respectively) were amplified. This amplification step utilized primers that were uniquely indexed, following the workflow of the AmpliSeq for Illumina. The resulting DNA fragments that were amplified were then subjected to paired-end sequencing by synthesis, with the goal of achieving a minimum coverage depth of 500X. The sequencing process involved target capture, followed by bridge amplification. This amplification process facilitated signal imaging and extension during the automated 300-cycle sequencing on the V2 flow cell containing clusters. The MiSeq sequencer (Illumina, San Diego, CA, USA) was employed for this sequencing procedure. The raw sequence reads that were obtained underwent thorough analysis to assess both the quality of the bases and the coverage of the amplicons. This analysis ensured the dependability and precision of the subsequent interpretation of the genetic data.

Data analysis and mutation classification

The clean sequencing reads were aligned to the human reference genome hg19/GRCh37 employing the local run manager of the MiSeq sequencer. After the alignment process, reads exhibiting misalignment from the reference were classified as potential mutations, employing the integrated bioinformatics tools provided by Illumina. The identification of genetic mutations was executed using Illumina's Basespace sequence hub mutation caller, which was underpinned by Pisces 5.2.9.23. Subsequent after mutation identification, the annotation of these discerned mutations was conducted utilizing the Basespace mutation interpreter, constructed on the foundation of Annotation Engine 3.1.1.0. Conforming to the guidelines outlined by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology - ACMG/AMP [33], the interpretation of mutations was undertaken. Additionally, the ClinVar database [34] was utilized to assess the clinical significance of the identified mutations.

Mutational frequencies analysis

The Genome Aggregation Database (GnomAD) serves as a comprehensive resource for studying mutational frequencies across the human genome [35]. With its vast collection of exome and genome sequencing data from diverse populations, GnomAD enables researchers to explore the prevalence of genetic variants with unparalleled granularity. This database offers insights into both common and rare variants, aiding in the identification of potentially pathogenic mutations. Researchers and clinicians can leverage GnomAD to assess the frequency of mutations, discerning between benign polymorphisms and disease-associated variants. In this study, the GnomeAD database was used to analyze the frequencies of observed mutations in the Asian population.

Kaplan-Meier analysis

Kaplan-Meier analysis stands as a vital statistical tool for investigating survival disparities between two distinct groups of cancer patients: those with mutated and unmutated profiles

[36]. This analysis method dynamically portrays the survival experience over a specified timeframe, allowing for a comprehensive assessment of differences in survival rates. By plotting cumulative survival probabilities over time, the Kaplan-Meier curve provides an intuitive visualization of how these patient groups fare in terms of survival outcomes. This statistical technique was employed in this study to assess disparities in overall survival (OS) results among ovarian cancer patients with and without genetic mutations, utilizing a significance threshold of $P < 0.05$ for Kaplan-Meier analysis.

Reverse transcription-quantitative polymerase chain reaction (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.) 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 (Hs01-556193_m1), BRCA2 (Hs00609073_m1), and 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^(-ΔΔCq) [37].

Library preparation for targeted bisulfite sequencing (bisulfite-seq) analysis

Concisely, a total of 1 µg of DNA was fragmented into approximately 200-300 bp fragments using a Covarias sonication system from Covarias in Woburn, MA, USA. After purification, the DNA fragments underwent a process of repairing and phosphorylating the blunt ends. This repair was carried out using a combination of T4 DNA polymerase, Klenow Fragment, and T4 polynucleotide kinase. The repaired fragments were subsequently subjected to 3' adenylation using Klenow Fragment (3'-5' exo-), followed by ligation with adapters. These adapters contained 5'-methylcytosine in place of 5'-cytosine, along with index sequences. T4 DNA Ligase was employed for this ligation step. The resulting libraries were quantified using a Qubit fluorometer with the Quant-iT dsDNA HS Assay Kit from Invitrogen in Carlsbad, CA, USA. These libraries were then sent to the Beijing Genomic Institute (BGI), China, for targeted bisulfite sequencing. Post-sequencing, the methylation data underwent normalization to obtain beta values.

Receiver operating curve (ROC) generation

The receiver operating curve (ROC) is a graphical representation used to evaluate the performance of diagnostic tests or classification models [38]. It illustrates the trade-off between sensitivity (the ability to correctly identify positive cases) and specificity (the ability to correctly identify negative cases) as the decision threshold varies. A higher area under the ROC curve indicates better discrimination ability. Based on the RT-qPCR expression and bisulfite-seq-based methylation data, ROC curves of BRCA1/2 expression and methylation levels were generated with the help of SRPLOT web source (https://bioinformatics.com.cn/srplot).

Immunohistochemical analysis of BRCA1/2 expression

For the assessment of BRCA1/2 protein expression, a comprehensive procedure was applied to formalin-fixed paraffin-embedded sections of ovarian cancer. This involved utilizing Anti-BRCA1 antibody (ab238983) for BRCA1 and Anti-BRCA2 antibody (ab216972) for BRCA2. The sections were initially deparaffinized, followed by gradual rehydration through varying alcohol concentrations and a 30-minute treatment with 0.3% H_2O_2 to counteract endogenous peroxidase activity. Epitope retrieval was then carried out in a water bath at 95°C for 20 minutes using 0.1 M citrate buffer. To minimize 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), each for 30 minutes. Following overnight incubation, a biotinylated link antibody was introduced, succeeded by streptavidin (diluted at 1:500). Diaminobenzene was utilized as the chromogen. It is noteworthy that diverse buffers and antigen retrieval methods were explored before finalizing this optimized

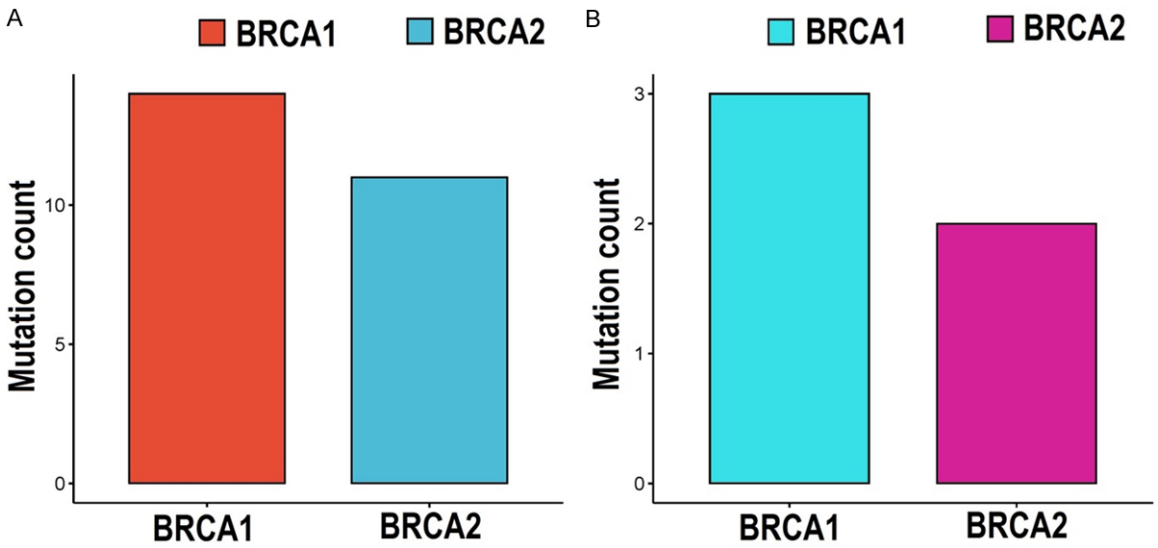

Figure 1. Comprehensive mutation analysis of BRCA1/2 in ovarian cancer patients using next generation sequencing. A. Total mutations detected in BRCA1/2 genes among ovarian cancer patients. B. Count of pathogenic mutations in BRCA1/2 genes in the ovarian cancer patient cohort.

protocol, including microwave treatments at 90°C for 10 minutes and 95°C for 20 minutes.

cBioPortal analysis

The cBioPortal database stands as a pivotal platform for conducting mutational analyses in cancer research [39]. It provides researchers with a user-friendly interface to explore and interpret complex genetic alterations in various cancers. With its extensive collection of multidimensional cancer genomics data, cBioPortal empowers users to investigate the frequency and distribution of mutations across genes and patient cohorts. In the present study, we used this database to analyze clinically significant mutations in BRCA1/2 genes across TCGA ovarian cancer samples.

Enrichment analysis

Metascape database serves as an invaluable tool in the field of biological research. It facilitates the systematic analysis and interpretation of complex biological data by integrating diverse functional annotation resources [40]. Researchers can explore gene lists, pathways, and protein-protein interaction networks to uncover meaningful insights into molecular mechanisms. MetaScape offers interactive visualization tools, enabling the discovery of enriched gene sets, functional annotations, and biological processes. In this study, we used this valuable resource for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the mutated genes. A $P < 05$ was used as the cutoff criterion for the functional enrichment analysis.

Drug prediction analysis

The DrugBank database stands as a pivotal resource in the realm of pharmacology and drug research [41]. It provides a comprehensive and structured repository of information on drugs, their targets, mechanisms of action, interactions, and more. With a vast collection of data, DrugBank aids researchers, clinicians, and pharmacists in making informed decisions regarding drug therapy. In this study, we used the DrugBank database to explore BRCA1/2 expression regulatory drugs.

Results

Sequencing and mutations identification

Sequencing of 25 ovarian cancer cases yielded 25 mutations, including 14 mutations in BRCA1 and 11 mutations in BRCA2 (**Figure 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%. Beyond the in-silico analysis,

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.5445G>A	p.Trp1815Ter	Pathogenic	9
2		NM_007294.4:c.5387C>G	p.Ser1796Ter	Pathogenic	9
3		NM_007294.4:c.5363G>T	p.Gly1788Val	Pathogenic	7
4		NM_007294.4:c.133A>C	p.Lys45Gln	Benign	9
5		NM_007294.4:c.839C>G	p.Ala280Gly	Benign	11
6		NM_007294.4:c.736T>G	p.Leu246Val	Benign	8
7		NM_007294.4:c.427G>A	p.Glu143Lys	Benign	5
8		NM_007294.4:c.397C>T	p.Arg133Cys	Benign	9
9		NM_007294.4:c.370A>G	p.Ile124Val	Benign	11
10		NM_007294.4:c.199G>T	p.Asp67Tyr	Benign	12
11		NM_007294.4:c.133A>C	p.Lys45Gln	Benign	9
12		NM_007294.4:c.891G>T	p.Met297Ile	Benign	11
13		NM_007294.4:c.827C>G	p.Thr276Arg	Benign	10
14		NM_007294.4:c.823G>A	p.Gly275Ser	Benign	7
15	BRCA2	NM_000059.4:c.1A>G	p.Met1Val	Pathogenic	9
16		NM_000059.4:c.97G>T	p.Glu33Ter	Pathogenic	8
17		NM_000059.4:c.502C>A	p.Pro168Thr	Benign	11
18		NM_000059.4:c.865A>C	p.Asn289His	Benign	3
19		NM_000059.4:c.978C>A	p.Ser326Arg	Benign	11
20		NM_000059.4:c.1040A>G	p.Gln347Arg	Benign	13
21		NM_000059.4:c.1141G>A	p.Asp381Asn	Benign	12
22		NM_000059.4:c.1166C>A	p.Pro389Gln	Benign	11
23		NM_000059.4:c.1385A>G	p.Glu462Gly	Benign	9
24		NM_000059.4:c.1538A>G	p.Lys513Arg	Benign	10
25		NM_000059.4:c.1744A>C	p.Thr582Pro	Benign	11

recourse was taken to the ClinVar database to determine the clinical relevance of the resultant mutations. By thoroughly examining mutation calling files from the complete set of cases, a total of 3 pathogenic mutations (21%) alongside 11 benign mutations (79%) were identified within the BRCA1 gene (**Figure 1A** and **Table 2**). Correspondingly, the analysis of the BRCA2 gene unveiled 2 pathogenic mutations (18%) in conjunction with 9 benign mutations (82%) among ovarian cancer patients from Pakistan (**Figure 1B** and **Table 2**).

Clinically significant mutations in BRCA1/2 genes

Pathogenic mutations hold pivotal clinical significance due to their direct involvement in the onset of diseases. These genetic anomalies disrupt the usual cellular processes, resulting in aberrant protein production or function [42]. Within the context of this investigation, a total of three pathogenic mutations (p.Trp1815Ter, p.Ser1796Ter, p.Gly1788Val) with clinical significance were identified within the BRCA1 gene (**Table 2**). Similarly, two pathogenic mutations (p.Met1Val and p.Glu33Ter) were noted within the BRCA2 gene (**Table 2**). These findings underscore the importance of recognizing such mutations for precise diagnosis, prognosis, and personalized treatment of ovarian cancer patients.

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

Pathogenic mutations occurring at low frequencies often possess heightened significance as markers specific to certain populations. Analyzing the frequencies of pathogenic mutations in the BRCA1 gene (p.Trp1815Ter, p.Ser1796Ter, p.Gly1788Val) and the BRCA2 gene (p.Met1Val and p.Glu33Ter) (outlined in **Table 2**) across the GnomeAD database provides valuable insights. Notably, these muta-

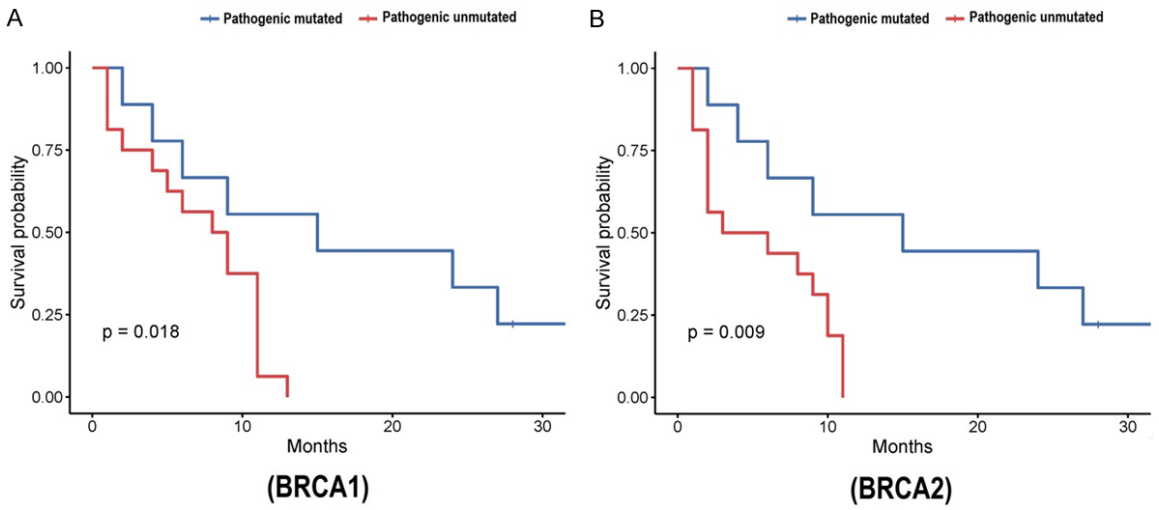

Figure 2. Survival comparison of BRCA1/2 genes in ovarian cancer samples with pathogenic mutations versus those without. The significance level was set at P < 0.05.

tions have not been previously documented among Asian ovarian cancer patients, with their frequencies registering as 0 within this database. This observation implies that these particular mutations exhibit a distinctiveness specific to the Pakistani population.

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 OS between two groups of ovarian cancer patients: one with BRCA1/2 pathogenic mutations and another one non-pathogenic mutation group (**Figure 2**). The group with BRCA1/2 pathogenic mutations experienced worse OS outcomes compared to the pathogenic unmutated group (**Figure 2**). This finding suggests that the presence of pathogenic mutations is associated with a poorer prognosis in ovarian cancer patients, emphasizing the clinical relevance of BRCA1/2 pathogenic mutations in influencing disease progression and survival outcomes.

Expression analysis of BRCA1/2 genes across ovarian cancer samples via RT-qPCR

The assessment of BRCA1/2 gene expression was conducted via RT-qPCR in two distinct subsets of ovarian cancer samples. One subset encompassed samples with established BRCA1/2 pathogenic mutations, while the other subset included samples lacking such mutations, constituting the non-pathogenic mutation group. Upon scrutinizing the outcomes of the RT-qPCR analysis, a noticeable trend emerged: the expression levels of BRCA1/2 genes were significantly elevated within the ovarian cancer samples with BRCA1/2 pathogenic mutations, in stark comparison to the non-pathogenic mutation group (**Figure 3A**). This intriguing observation implies a potential link between BRCA1/2 mutations and the higher expression of these genes in the context of ovarian cancer.

Additionally, the ROC curve analysis rendered notable outcomes. For BRCA1 expression, a marked area under the curve (AUC) of 0.833 (p-value < 0.05) was observed (**Figure 3B**), along with a similar discernible pattern for BRCA2 expression with an AUC of 0.780 (p-value < 0.05). These outcomes underscore significant sensitivity and specificity associated with the potential of these genes as discerning markers.

Promoter methylation analysis of BRCA1/2 genes across ovarian cancer samples via bisulfite sequencing

We conducted a targeted bisulfite-seq analysis to delve into the promoter methylation status of the BRCA1/2 genes within two distinct categories of ovarian cancer samples. The initial group

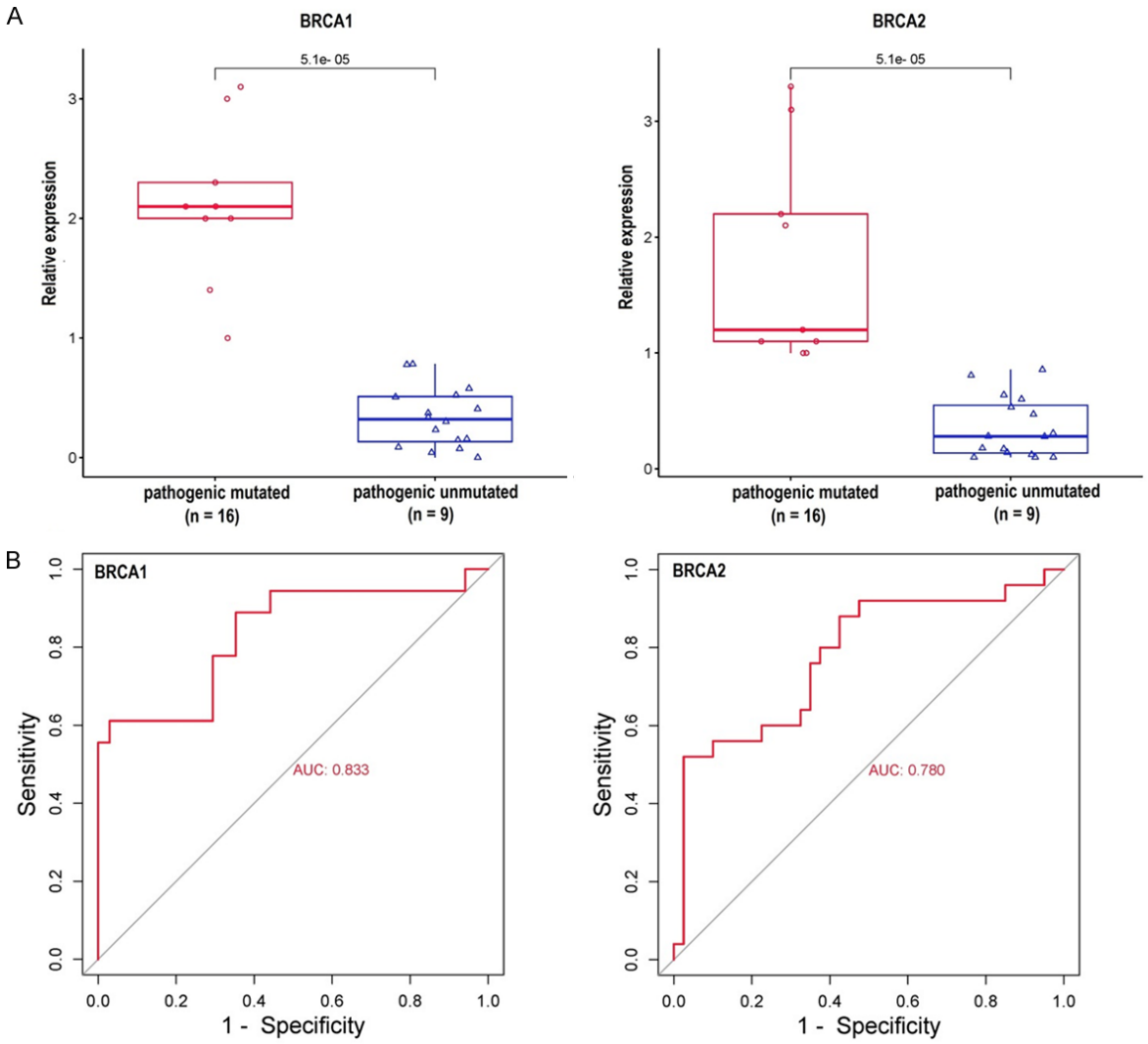

Figure 3. Comparative analysis of BRCA1/2 gene expression via reverse transcription-quantitative polymerase chain reaction and receiver operating curve (ROC) evaluation in ovarian cancer samples with pathogenic mutations versus non-pathogenic mutations. (A) Relative Expression Profiling of BRCA1/2 Genes using RT-qPCR, and (B) ROC Curve Analysis based on RT-qPCR Expression Data for BRCA1/2 Genes. A significance threshold of $P < 0.05$ was applied.

encompassed samples bearing BRCA1/2 pathogenic mutations, while the second group was composed of ovarian cancer samples devoid of such pathogenic mutations, establishing the non-pathogenic mutation group as a comparator. The outcomes of our investigation unveiled a substantial divergence in the patterns of promoter methylation. Specifically, within the ovarian cancer sample group harboring pathogenic mutations, conspicuous hypomethylation was noted within the promoters of both the BRCA1 and BRCA2 genes, presenting a marked contrast in comparison to the non-pathogenic mutation group (**Figure 4A**).

Moreover, our analysis of the ROC curves yielded compelling results. For the BRCA1 promoter methylation level, an appreciable AUC of 0.806 (p-value < 0.05) emerged, while a similar distinct trend was evident for BRCA2, showcasing an AUC of 0.801 (p-value < 0.05, **Figure 4B**).

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 ovarian cancer tissue sample harboring a pathogenic mutation in the BRCA1/2 gene, respectively and another ovarian cancer tissue sample without any pathogenic mutation. The objective was to investigate

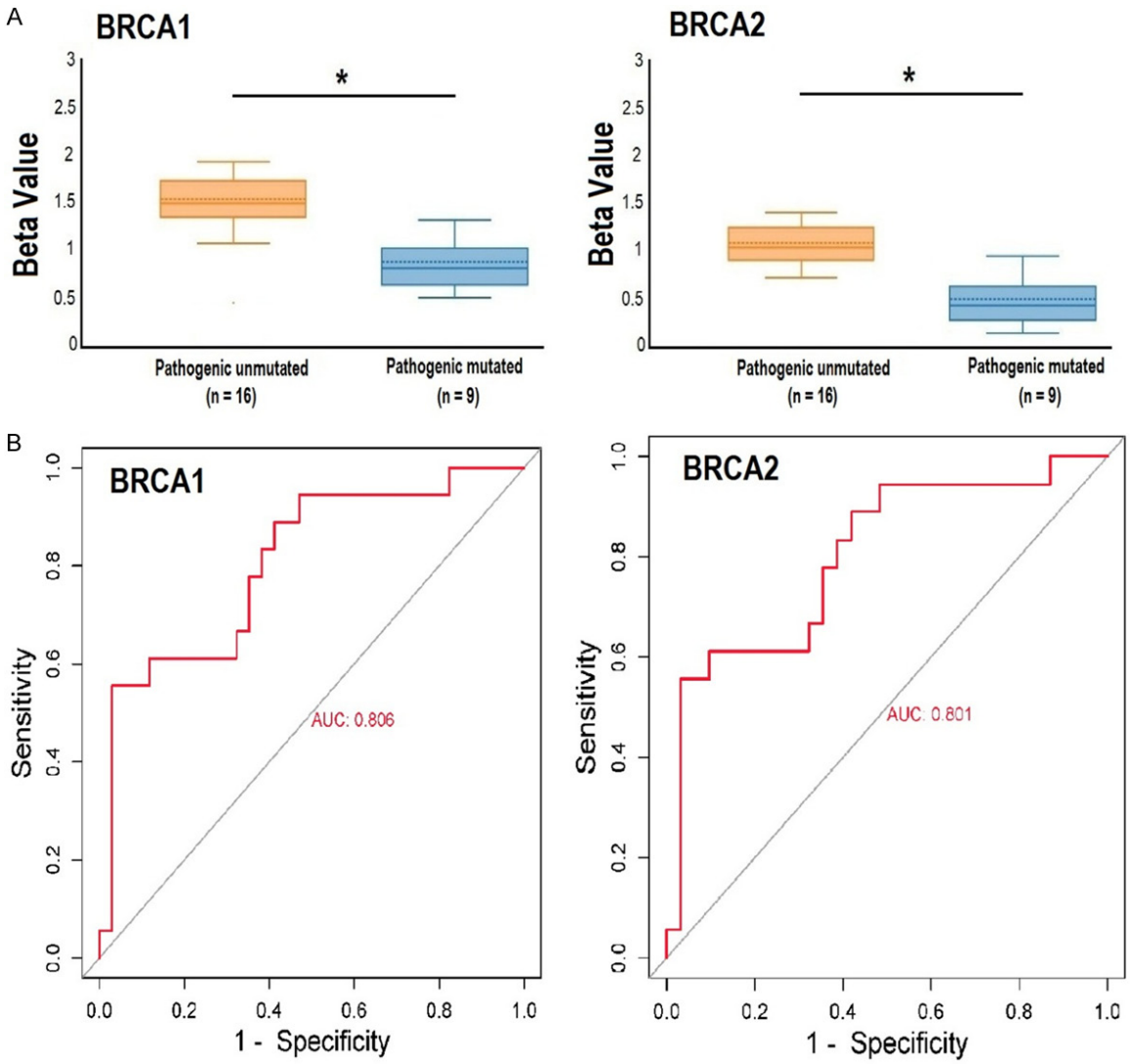

Figure 4. Investigation of methylation levels in BRCA1/2 genes using targeted bisulfite sequencing and receiver operating curve (ROC) analysis in ovarian cancer samples with pathogenic mutations versus non-pathogenic mutations. (A) Assessment of Promoter Methylation in BRCA1/2 Genes through Targeted Bisulfite Sequencing, and (B) ROC Curve Analysis based on Promoter Methylation Levels of BRCA1/2 Genes. Significance was determined at $P < 0.05$.

potential differences in protein expression between these two types of samples.

Upon examining the staining results, a notable pattern emerged (**Figure 5**). 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 (**Figure 5**). 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.

Analysis of clinically important pathogenic mutations in BRCA1/2 across The Cancer Genome Atlas (TCGA) ovarian cancer samples

Subsequently, this study undertook a comprehensive mutational investigation of the BRCA1/2 genes within ovarian cancer samples sourced from the TCGA dataset, utilizing the cBioPortal platform. The primary aim was to discern potential genetic variations and their prevalence across diverse populations. The outcomes of this analysis unveiled a distinct pattern: the pathogenic mutations identified within the BRCA1 gene (p.Trp1815Ter,

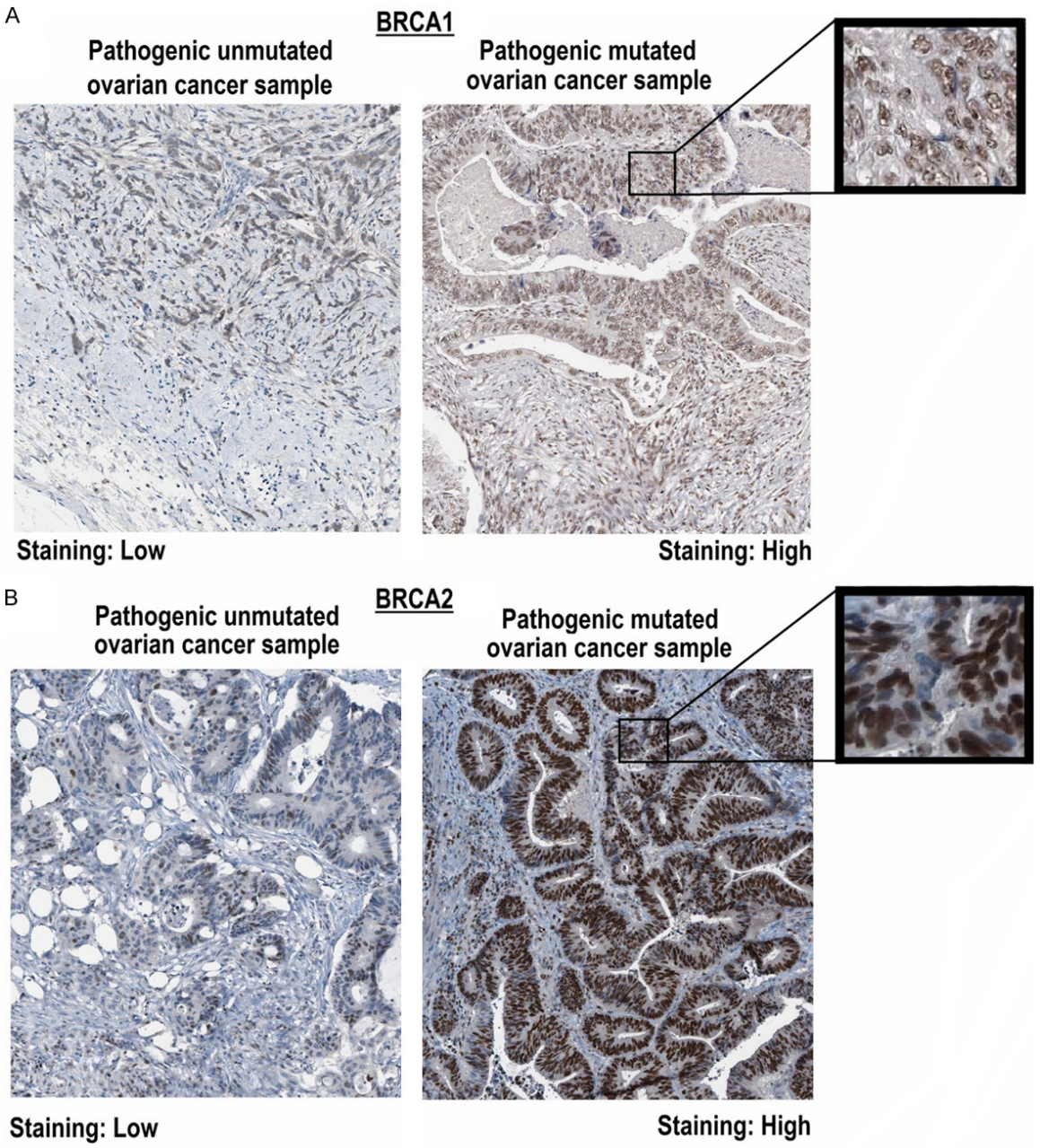

Figure 5. Analysis of proteomic expression of BRCA1 and BRCA2 proteins via immunohistochemistry (IHC) in ovarian cancer samples with pathogenic mutations compared to those without.

p.Ser1796Ter, p.Gly1788Val) and the BRCA2 gene (p.Met1Val and p.Glu33Ter) among ovarian cancer patients of Pakistani origin were conspicuously absent within the TCGA ovarian cancer samples (Supplementary Figure 1). This observation serves to underscore the exceptional character of these identified pathogenic mutations within the Pakistani population. Furthermore, the absence of these specific pathogenic mutations within the TCGA dataset accentuates their exclusivity to the Pakistani cohort. These mutations seem to epitomize distinctive genetic markers linked with the susceptibility to ovarian cancer within individuals of Pakistani descent.

Enrichment analysis of BRCA1/2 genes

Next, we performed GO and KEGG enrichment analyses. Among GO, BRCA1/2 genes were

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	

enriched in "lateral element, synaptonemal structure, synaptonemal complex, condensed nuclear chromosome, and nuclear chromosome" etc., Cellular Components (CC) terms (Supplementary Figure 2A), "tubulin binding, H4 histone acetyltransferase activity, gamma tubulin binding, and acetyltransferase activity" etc., Molecular Function (MF) terms (Supplementary Figure 2B), "DNA damage response, signal transduction by p53 class mediator resulting in transcription of P21 class mediator, DNA damage response, signal transduction resulting in transcription, and histon H3-acetylation" etc., Biological Process (BP) terms (Supplementary Figure 2C), and "homologous recombination, fanconi anemia pathway, breast cancer, and platinum drug resistance in Cancer" etc., KEGG terms (Supplementary Figure 2D).

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). Through our meticulous analysis, we uncovered an array of potential drug candidates, each exhibiting promising attributes to curtail the expression of BRCA1/2. Of particular significance within this pool of candidates are Arecoline, Estradiol, Bortezomib, Doxorubicin, Cyclosporine, Tretinoin, and Tamibarotene (**Table 3**). These compounds demonstrate the potential to effectively regulate the expression levels of the target genes, thus holding substantial potential for innovative therapeutic interventions.

Discussion

Ovarian cancer remains a significant global health challenge [43, 44], 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 the 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 in ovarian cancer patients from the Pakistani population has yielded significant insights through this study. The identification of three clinically significant pathogenic mutations within BRCA1 (p.Trp1815Ter, p.Ser1796Ter, p.Gly1788Val) and two within BRCA2 (p.Met1Val and p.Glu33Ter) 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 [45-48]. 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 [49]. The study's findings that pathogenic mutations within BRCA1 and BRCA2 are associated with poor 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 [50-52]. The incorporation of this survival analysis adds a crucial layer of clinical relevance to the genetic findings, highlighting the potential utility of these mutations as prognostic markers.

The downstream effects of the identified pathogenic mutations have been comprehensively explored through expression assays, including RT-qPCR and 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 [53, 54].

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 [55]. 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 [56-58].

Enrichment analysis, a vital component of the study, provides a broader context for understanding the functional implications of BRCA1 and BRCA2 mutations [59, 60]. By uncovering significant 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 platinum drug resistance 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 capable of modulating BRCA1 and BRCA2 expression regulation through the exploration of DrugBank. 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.

Conclusion

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.

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Disclosure of conflict of interest

None.

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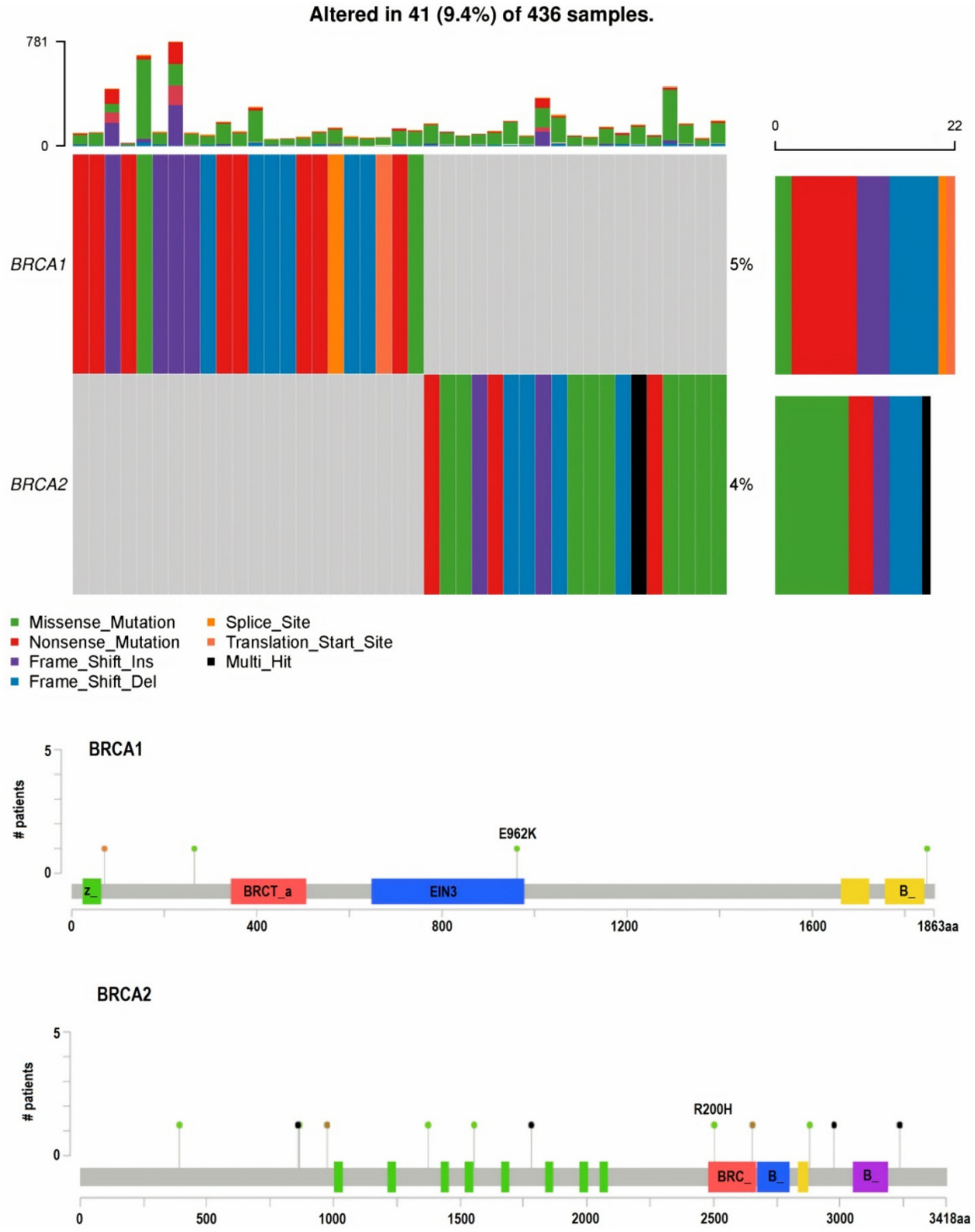
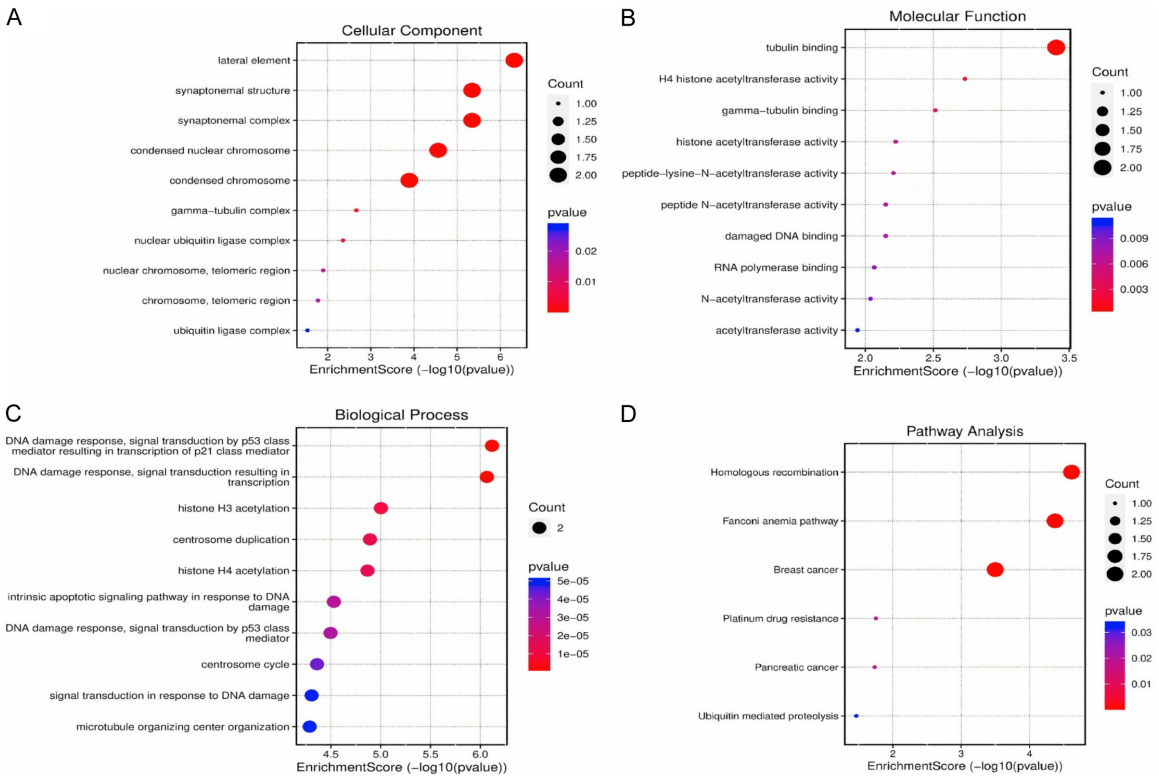


Supplementary Figure 1. Examination of mutations among BRCA1/2 genes in the cancer genome atlas (TCGA) ovarian cancer samples using the cBioPortal platform.



Supplementary Figure 2. Functional enrichment analysis of BRCA1/2 genes using metascape. (A) Cellular Component (CC) Terms Associated with BRCA1/2 Genes, (B) Molecular Function (MF) Terms Related to BRCA1/2 Genes, (C) Biological Process (BP) Terms Linked to BRCA1/2 Genes, and (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways Enriched in BRCA1/2 Genes. Significance was established at $P < 0.05$.