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Original Article

Next-generation sequencing uncovers crucial mutated genes and potential therapeutic targets in ovarian cancer patients

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Abstract: Objectives: Ovarian cancer is a highly lethal gynecological malignancy, often diagnosed late, resulting in high mortality. While BRCA1 and BRCA2 mutations are known risk factors, the broader genetic landscape needs comprehensive profiling to identify additional diagnostic markers or therapeutic targets. The current study aims to explore the genetic landscape of various cancer-susceptible genes in ovarian cancer patients. Methods: The genetic landscape of ovarian cancer was investigated by analyzing 27 genes via next-generation sequencing (NGS) in 50 ovarian cancer patients. Results: Mutations were detected in four genes: Breast Cancer 1 (BRCA1) (62%), Cyclin-Dependent Kinase 4 (CDK4) (58%), MutS Homolog 2 (MSH2) (48%), and Phosphatase and Tensin Homolog (PTEN) (22%). Pathogenic mutations were identified in BRCA1 (p.Tyr1853Ter and p.Gln1848Ter), CDK4 (p.Arg24His), and PTEN (p.Tyr29Ter), occurring in 11 patients. Interestingly, these pathogenic mutations were absent in The Cancer Genome Atlas (TCGA) dataset and the gnomAD for the Asian population, suggesting their unique presence in the Pakistani cohort. Functional assays revealed that these mutations significantly reduced the mRNA and protein expression levels of BRCA1, CDK4, and PTEN, as demonstrated by Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) and Immunohistochemistry (IHC) analyses. Receiver Operating Characteristic (ROC) curve analysis confirmed the potential of these genes as biomarkers, with downregulated expression accurately distinguishing between normal and cancerous tissues. Structural validation of mutated proteins using Ramachandran plots and Protein Structure Analysis (ProSA-web) analysis confirmed the stability of the mutations. Drug prediction and molecular docking identified Resveratrol as a potential therapeutic agent, indicating strong binding affinities with BRCA1, CDK4, and PTEN proteins. Conclusion: These findings provide novel insights into the genetic underpinnings of ovarian cancer in the Pakistani population and suggest potential targets for therapeutic intervention.

Keywords: Ovarian cancer, NGS, pathogenic mutations, biomarker, expression

Introduction

Ovarian cancer remains a formidable challenge in the realm of women's health, posing a significant global burden due to its high mortality rates and elusive nature [1, 2]. Traditionally, the diagnosis and prognosis of ovarian cancer patients often depend on the detection of BRCA1/2 mutations [3, 4]. The low survival rate of ovarian cancer patients indicates that, beyond BRCA1/2 mutations, additional gene mutations may play a role in ovarian cancer development. Moreover, recent studies in genomic research have substantiated evidence of the participation of numerous other genes in the onset and progression of ovarian cancer [5, 6]. Consequently, there is a growing imperative to explore the broader spectrum of genetic alterations to better comprehend the etiology of ovarian cancer and pave the way for more effective diagnostic and therapeutic interventions [7-10].

The quest to unravel the complex genetic underpinnings of ovarian cancer has led to an expanding understanding of the role played by non-BRCA genes in disease susceptibility [11]. Beyond the well-established BRCA1/2 mutations, a myriad of other genes (CDH1, PALB2, PTEN, STK11, TP53, ATM, CHEK2, BARD1,

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Multi-gene panel—next generation sequencing	
ATM	*BARD1*
BRCA1	*BRCA2*
BRIP1	*CDK12*
CHEK2	*NBN*
PALB2	*TP53*
EPCAM	*RAD51C*
RAD51D	*MSH2*
APC	*CDH1*
CDKN2A	*MKH1*
MSH6	*NF1*
PMS2	*PTEN*
CDK4	*MUTYH*
POLD1	*POLE*
SMAD4	

Figure 1. This illustrates the compilation of the 27 ovarian cancer susceptibility genes analyzed in this study through Next-Generation Sequencing (NGS).

BRLP1, RAD51C, and RAD51D) have emerged as potential contributors to ovarian cancer development [12, 13]. This paradigm shift has prompted a comprehensive exploration of genetic mutations in the additional 27 genes (**Figure 1**) implicated in ovarian cancer susceptibility, necessitating advanced molecular techniques for thorough genomic profiling.

The selected panel of genes encompasses diverse functions, ranging from DNA repair mechanisms to cell cycle regulation, reflecting the multifaceted nature of ovarian cancer susceptibility. By scrutinizing these genes with high-throughput NGS, we aim to identify genetic mutations, uncover potential gene interactions, and elucidate the intricate network of genetic factors contributing to ovarian cancer development in Pakistani patients.

Furthermore, understanding the broader genetic landscape of ovarian cancer holds profound implications for personalized medicine. Unraveling the specific genetic alterations in individual patients can guide tailored therapeutic strategies, optimize surveillance protocols, and enhance overall patient outcomes. As we delve into the unexplored genetic intricacies of ovarian cancer, our study not only seeks to expand the current knowledge base but also strives to pave the way for more targeted and effective interventions in the diagnosis, prevention, and treatment of this devastating disease.

Methodology

Sample collection

We collected a total of 50 ovarian cancer tissue samples and 10 adjunct control tissue samples, sourced from patients who underwent surgery at the Nishtar Hospital, Multan, Pakistan. The ethical aspects of our study were meticulously overseen, with approval granted by the Ethics Committee of Nishtar Medical College in adherence to the Helsinki guidelines [14]. Before collecting the samples, each patient provided informed consent, ensuring transparency and respect for individual autonomy.

Inclusion and exclusion criteria

The inclusion criteria for ovarian cancer patients in the current study included individuals with a confirmed diagnosis of ovarian cancer, as histologically verified by biopsy reports, who were willing to participate in the research. Exclusion criteria included patients with a history of other malignancies, those currently undergoing treatment for ovarian cancer, and those unable or unwilling to provide informed consent. Patients with incomplete medical records or insufficient tissue samples for genetic analysis were also excluded from the study. Clinical information of the included prostate cancer patients is detailed in **Table 1**.

Nucleic acid isolation

DNA extraction was conducted using the organic method [15], while RNA isolation followed the TRIzol method [16]. The 260/280 ratios were meticulously examined to determine the purity of both the DNA and RNA samples, ensuring the quality and integrity of the genetic material obtained using these extraction methods.

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	50
2	Age	
	>60	0
	<60	50
3	Treatment	
	Pre-treatment	50
	Post-treatment	0

Next-generation sequencing (NGS)

NGS was conducted using the Ion Torrent S5 system (Thermo Fisher Scientific, Waltham, MA, United States), with automated library preparation facilitated by Ion Chef (Thermo Fisher Scientific, Waltham, MA, United States). The Ion Chef system encompasses the following stages: fragmentation, adapter ligation onto PCR products, and clonal amplification. Following library preparation, DNA libraries were quantified using the Real-Time Step One PCR System (Thermo Fisher Scientific, Waltham, MA, United States). Prepared samples were then loaded onto an Ion 530™ chip using Ion Chef (Thermo Fisher Scientific, Waltham, MA, United States). Sequencing was conducted on the Ion S5™ Plus instrument (Thermo Fisher Scientific, Waltham, MA, United States).

For NGS data analysis on the Torrent Suite 5.14.0 platform, specific plugins such as "SampleId" and "Coverageanalysis" were employed. The uniformity of base coverage exceeded 98% in all batches, and the base coverage was consistently over ×20 across all target regions. It is worth mentioning that this NGS method is limited to detecting variations within the ±10 nucleotide coding sequence and may not capture variations beyond this range.

Variant classification

The genetic variations identified in patients were classified into five classes: benign (C1), likely benign (C2), variant of uncertain significance (VUS, C3), likely pathogenic (C4), and pathogenic (C5). This classification adhered to the guidelines outlined by the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) (https://enigmaconsortium.org/). Our focus was directed specifically towards mutations with clinical relevance.

Variants were denoted by the nomenclature recommendations of the Human Genome Variation Society (https://www.hgvs.org). The clinical significance of the genetic variants identified in the current study was evaluated using resources such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Varsome (https://varsome.com), and Franklin Genoox (https://franklin.genoox.com).

gnomAD database

gnomAD, the Genome Aggregation Database (https://gnomad.broadinstitute.org/), is a comprehensive genomic resource providing numerous genetic variants from over 140,000 exomes and 15,000 genomes [17]. It offers valuable insights into human genetic diversity, enabling researchers to evaluate variant frequencies, annotate functional impacts, and enhance our understanding of rare and common genetic variations across diverse populations. In the present study, this database was utilized to analyze the frequencies of the observed pathogenic mutations in Asian ovarian cancer patients.

cBioPortal database

cBioPortal (https://www.cbioportal.org/) is a web-based platform offering interactive exploration of complex cancer genomics data [17]. It facilitates the visualization and analysis of multidimensional cancer genomics datasets, assisting researchers in comprehending genetic alterations, pathways, and clinical correlations. cBioPortal significantly contributes to the advancement of cancer research by providing an intuitive interface for data interpretation. The cBioPortal database was employed in the present research to examine the occurrence of the observed pathogenic mutations in The Cancer Genome Atlas (TCGA) of ovarian cancer patients.

Transcription quantitative polymerase chain reaction (RT-qPCR)-based expression analysis

cDNA was synthesized using the cDNA Synthesis Mix Kit from Innovagene (Hunan, China). Real-time PCR was carried out employing the

SYBR Green qPCR Mix, which was also provided by Innovagene. The expression profiles of PTTG genes were calculated and analyzed using the $2^{-\Delta\Delta Cq}$ method. To ensure normalization, glyceraldehyde-3-phosphate dehydrogenase (GADPH) was employed as an internal control. The following primers were utilized for the amplification of GAPDH-mutated genes (BRCA1, CDK4, and PTEN).

GAPDH-F 5'-ACCCACTCCTCCACCTTTGAC-3'; GAPDH-R 5'-CTGTTGCTGTAGCCAAATTCG-3'. BRCA1-F: 5'-CTGAAGACTGCTCAGGGCTATC-3'; BRCA1-R: 5'-AGGGTAGCTGTTAGAAGGCTGG-3'. CDK4-F: 5'-AGGGTAGCTGTTAGAAGGCTGG-3'; CDK4-R: 5'-GGTCGGCGCAGTTGGGCTCC-3'. PTEN-F: 5'-TGAGTTCCCTCAGCCGTTACCT-3'; PTEN-R: 5'-GAGGTTTCCTCTGGTCCTGGTA-3'.

Receiver operating characteristic (ROC) analysis

ROC curves for the expression levels of the mutated genes were constructed using the SRPLOT web resource available at https://bioinformatics.com.cn/srplot.

Immunohistochemical analysis

A total of six tissue specimens, including two adjacent normal tissues, two mutated ovarian cancer tissues with pathogenic mutations, and two other ovarian cancer tissues lacking pathogenic mutations, underwent formalin fixation, paraffin embedding, and subsequent sectioning into 3 μm thick slices. After deparaffinization and neutralization of endogenous catalase, the sections underwent boiling in sodium citrate buffer for antigen exposure. Subsequently, a 1-hour incubation in 5% normal goat serum blocked non-specific binding. The sections were then treated with anti-BRCA1, anti-CDK4, and anti-PTEN antibodies (1:50 dilution; CAT#: TA322776, and CAT#: TA324611) at 4°C overnight, and binding was visualized using the avidin-biotin-peroxidase method. Hematoxylin counterstaining followed, and two experienced researchers independently assessed the results.

Gene enrichment and drug prediction analyses

DAVID (https://david.ncifcrf.gov/) is a bioinformatics resource that empowers researchers to explore the functional significance of gene lists [18]. It integrates various biological data and offers tools for gene functional annotation, pathway analysis, and functional annotation clustering, assisting in the interpretation of high-throughput genomics experiments for a deeper understanding of biological processes. In the present study, this resource was utilized to perform the gene enrichment analysis of the mutated genes.

DrugBank (https://go.drugbank.com/) is a comprehensive pharmacological database that consolidates information on drug actions, interactions, and targets [19]. It is a valuable resource for researchers, clinicians, and pharmacologists, providing detailed data on drug properties, mechanisms of action, and associated pathways. DrugBank assists in drug discovery, development, and personalized medicine initiatives. This database was utilized in the current study to identify mutated gene expression regulatory drugs.

Molecular docking analysis

Ligand and receptor preparation and docking analysis: To assess the binding affinities between the chosen drug, Resveratrol, and the mutated BRCA1, CDK4, and PTEN proteins, we employed a molecular docking approach facilitated by the CB-DOCK web server (http://clab.labshare.cn/cb-dock/php/) [20]. The SDF structure of Doxorubicin was obtained from the PubChem database, and for the mutated BRCA1, CDK4, and PTEN proteins, we generated PDB structures using the SwissModel tool. Furthermore, both PROCHECK web server (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK) [32] and ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) [33] were utilized to verify the stability of the mutated BRCA1, CDK4, and PTEN-based generated models for further docking. Molecular docking involves several key steps, including ligand (Resveratrol) pre-processing, removal of excess ligands from the target proteins (macromolecules), elimination of crystal water molecules, and addition of hydrogen atoms. The molecular docking was carried out using the CB-DOCK platform to calculate the binding energies of the molecules across various conformations. The conformation with the highest hydrogen bond energy was selected as the active component of the protein interaction. For visualization purposes, PyMol software (version 2.5.2) was employed.

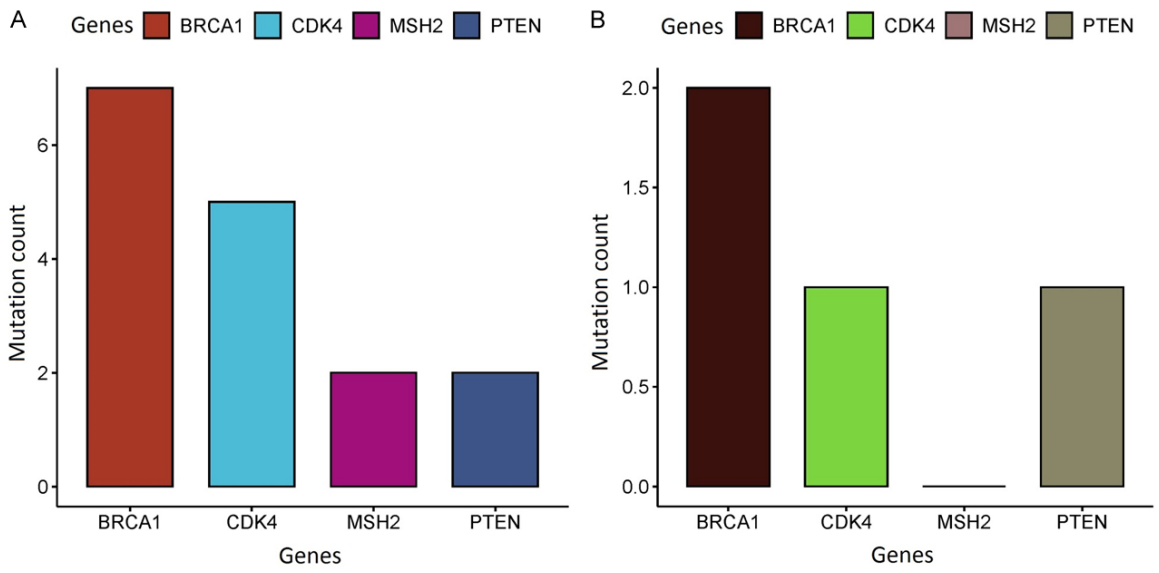

Figure 2. Total count of overall detected mutations and pathogenic mutations in BRCA1, CDK4, and PTEN genes across ovarian cancer patients via Next-Generation Sequencing (NGS). (A) An overall count of detected mutations in BRCA1, CDK4, MSH2, and PTEN genes across ovarian cancer patients, and (B) A count of detected pathogenic mutations in BRCA1, CDK4, MSH2, and PTEN genes across ovarian cancer patients.

Sanger sequencing analysis

Five ovarian cancer samples with pathogenic mutations were sequenced for the entire coding region of BRCA1 using Sanger sequencing. The sequencing was conducted with the ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit and analyzed on an ABI 3100 Genetic Analyzer from Applied Biosystems, following the protocol described by Coppa et al. [21]. The reference sequence used for BRCA1 was NM_007294.3.

Results

In the present study, a total of 50 ovarian cancer cases were enrolled from January 2021 to December 2022. Out of the 27 analyzed genes via NGS, various genetic mutations were detected in four genes: BRCA1, CDK4, MSH2, and PTEN. The BRCA1 gene was mutated in 31 patients (62%), the CDK4 gene was found to be mutated in 29 patients (58%), the MSH2 gene was mutated in 24 patients (48%), and the PTEN gene was mutated in 11 patients (22%) (**Figure 2A** and **Table 2**).

In the BRCA1 gene, a total of 7 mutations were detected, of which 6 (85%) were benign/likely benign mutations and one (15%) was a pathogenic mutation. In the CDK4 gene, a total of 5 mutations were detected, of which 4 (80%) were benign mutations and one (20%) was a pathogenic mutation. In both cases of the MSH2 and PTEN genes, a total of 2 mutations were detected. In the MSH2 gene, both mutations were benign (100%), while in the PTEN gene, one mutation (50%) was benign and one was pathogenic (50%) (**Figure 2B** and **Table 2**).

Eleven ovarian cancer patients showed three clinically important pathogenic mutations in BRCA1, MSH2, and PTEN genes

Pathogenic mutations possess clinical significance due to their association with the development or progression of diseases [22, 23]. These mutations often disrupt normal cellular functions, resulting in abnormal processes that contribute to a pathological condition [24]. In the present study, a total of eleven ovarian cancer patients had pathogenic mutations in BRCA1 (p.Tyr1853Ter and p.Gln1848Ter), CDK4 (p.Arg24His), and PTEN (p.Tyr29Ter) genes. Meanwhile, no pathogenic mutation was detected in the MSH2 gene. The distribution of pathogenic mutations in ovarian cancer patients has been reported in **Figure 2**.

Table 2. Count and type of mutations observed in BRCA1, CDK4, MSH2, and PTEN genes across ovarian patients

Sr. no	Gene	NM:c.DNA	Protein	Nature (ClinVar)	Nature (In silico analysis)	No. patients
1	BRCA1	NM_007294.4:c.5559C>G	p.Tyr1853Ter	Pathogenic	DC	11
2		NM_007294.4:c.5542C>T	p.Gln1848Ter	Pathogenic	DC	11
3		NM_007294.4:c.5587T>G	p.Tyr1863Asp	Likely benign	Non-DC	31
4		NM_007294.4:c.5530C>T	p.Leu1844Phe	Likely benign	Non-DC	28
5		NM_007294.4:c.5347A>T	p.Met1783Leu	Benign	Non-DC	22
6		NM_007294.4:c.5198A>G	p.Asp1733Gly	Benign	Non-DC	24
7		NM_007294.4:c.5158A>G	p.Thr1720Ala	Benign	Non-DC	27
8	CDK4	NM_000075.4:c.71G>A	p.Arg24His	Pathogenic	DC	11
9		NM_000075.4:c.-60C>G	-----	Benign	Non-DC	29
10		NM_000075.4:c.219-31G>A	-----	Benign	Non-DC	14
11		NM_000075.4:c.522+8G>A	-----	Benign	Non-DC	21
12		NM_000075.4:c.447A>G	p.Thr149=	Benign	Non-DC	29
13	MSH2	NM_000251.3:c.593A>G	p.Glu198Gly	Benign	Non-DC	20
14		NM_000251.3:c.949G>A	p.Val317Ile	Benign	Non-DC	16
15	PTEN	NM_000314.8:c.87T>A	p.Tyr29Ter	Pathogenic	DC	11
16		NM_000314.8:c.1197A>G	p.Gln399=	Benign	Non-DC	8

DC = Disease causing.

Sanger sequencing analysis of BRCA1 mutations

Following the initial identification of two pathogenic mutations, NM_007294.4:c.5559C>G (p.Tyr1853Ter) and NM_007294.4:c.5542C>T (p.Gln1848Ter), in the BRCA1 gene among ovarian cancer patients using NGS, we proceeded with validating these mutations using Sanger sequencing. This secondary validation step was critical to ensure the accuracy of our findings. The Sanger sequencing results unequivocally confirmed the presence of both pathogenic mutations across five ovarian cancer patients (**Figure 3**), reinforcing their potential role in the genetic predisposition to ovarian cancer in the studied cohort.

Analysis of the pathogenic mutations in the TCGA dataset of ovarian cancer patients

Next, to analyze the prevalence of the pathogenic mutations in BRCA1 (p.Tyr1853Ter and p.Gln1848Ter), CDK4 (p.Arg24His), and PTEN (p.Tyr29Ter) genes across TCGA dataset, we conducted a thorough analysis using cBioPortal database. The analysis results revealed various other mutations in TCGA ovarian cancer patients (**Figures 4A**, **4B** and **5**). Most importantly, none of the pathogenic mutations deciphered during the present study was found in the TCGA ovarian cancer patients.

Analysis of mutational frequencies across Asian ovarian cancer patients

The mutational frequencies of the pathogenic mutations in BRCA1 (p.Tyr1853Ter and p.Gln1848Ter), CDK4 (p.Arg24His), and PTEN (p.Tyr29Ter) genes were analyzed using the gnomAD database. The frequencies of the observed pathogenic mutations were 0 in the gnomAD database for the Asian population. This scenario declares that the observed pathogenic mutations in BRCA1, CDK4, and PTEN genes are being reported for the first time in Asian ovarian cancer patients, particularly in the Pakistani population.

Analyzing the functional consequences of the pathogenic mutations in BRCA1, CDK4, and PTEN genes

Effect of the observed pathogenic mutations on the mRNA expression level of BRCA1, CDK4, and PTEN: To assess the impact of the observed mutations on the mRNA expression of BRCA1, CDK4, and PTEN genes, we performed RT-qPCR analysis in two ovarian cancer sample groups and one control sample group. The normal con-

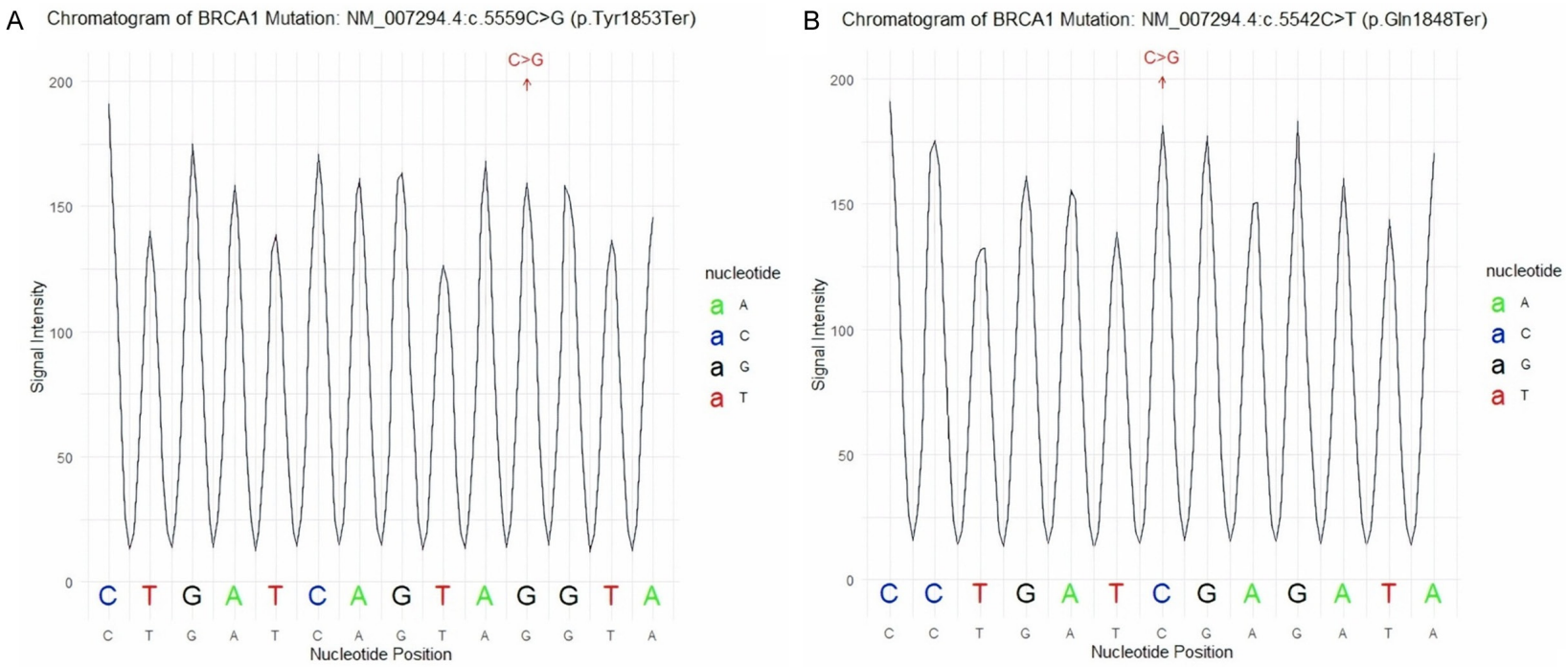

Figure 3. Validation of pathogenic mutations in BRCA1 gene through Sanger sequencing. A. Chromatogram of the BRCA1 mutation NM_007294.4.5559C>G (p.Tyr1853Ter), showing a substitution from cytosine (C) to guanine (G) at nucleotide position 5559, indicated by the red arrow. B. Chromatogram of the BRCA1 mutation NM_007294.4.5542C>T (p.Gln1848Ter), showing a substitution from cytosine (C) to thymine (T) at nucleotide position 5542, also indicated by a red arrow.

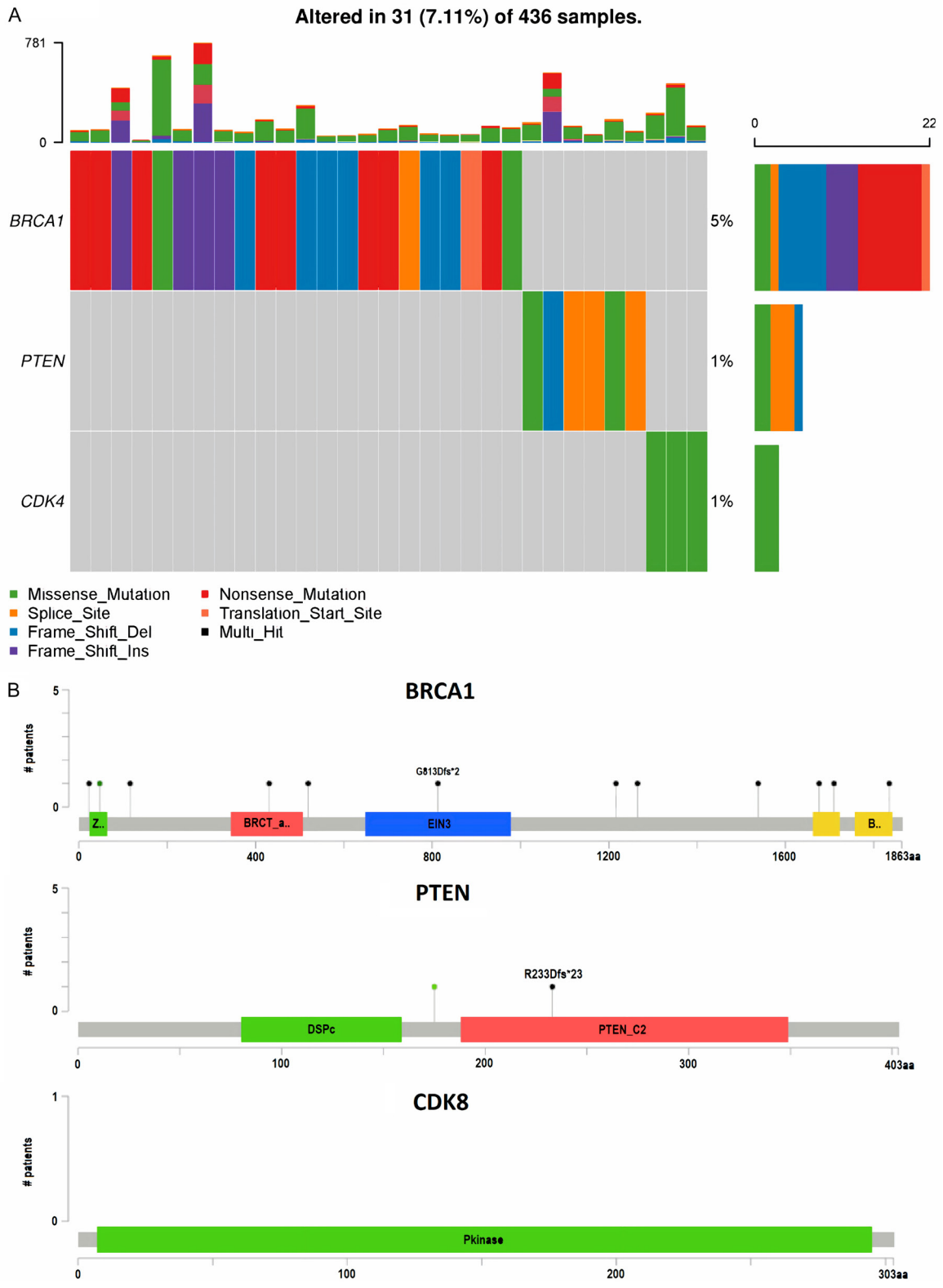

Figure 4. Oncoplot and lollipop plot-based visualization of the observed BRCA1, CDK4, and PTEN mutations across The Cancer Genome Atlas (TCGA) ovarian cancer patients. (A) Three rows showed percentage of ovarian cancer samples which are positive for BRCA1, CDK4, and PTEN mutations, and (B) lollipop plots highlighted amino acid change due to mutation at the protein level.

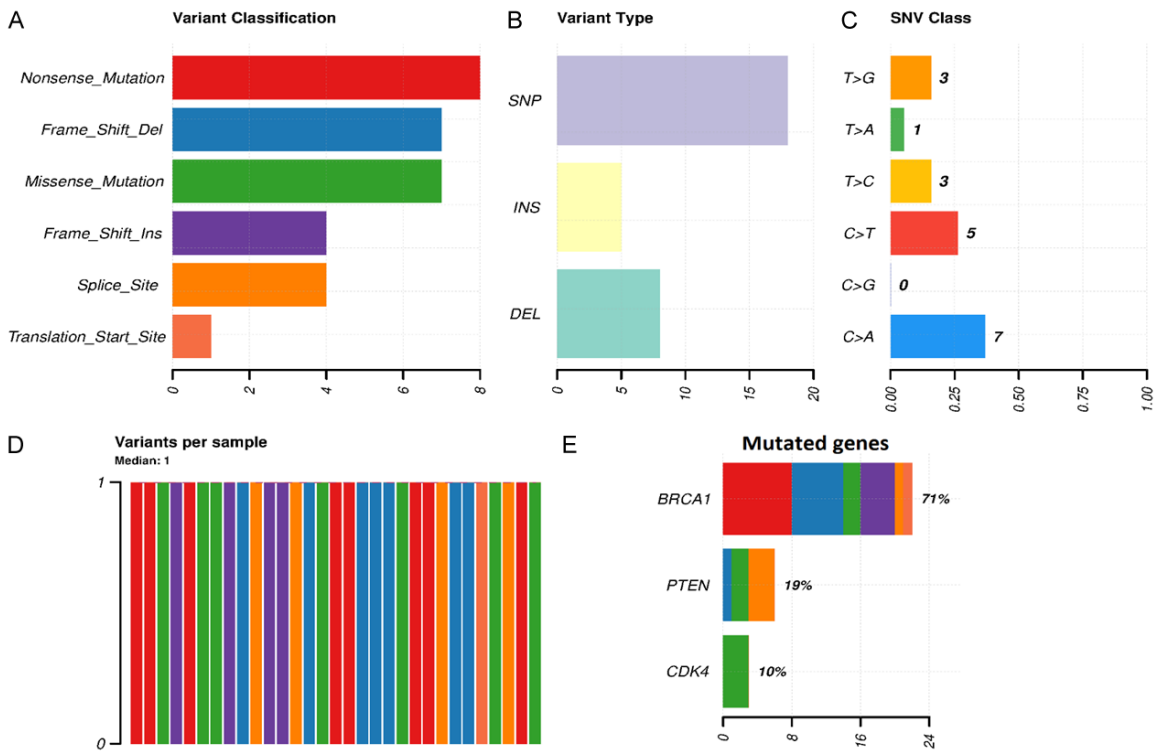

Figure 5. Detailed summery of the genetic mutations observed in BRCA1, CDK4, and PTEN across The Cancer Genome Atlas (TCGA) ovarian cancer patients. A. This panel shows the categorization of the types of genetic variants found. B. This panel highlights the distribution of variant types. C. This panel shows classifies single nucleotide variants (SNVs) based on their nucleotide changes, such as T>G, T>A, T>C, C>T, C>G, and C>A. D. This panel illustrates the number of variants found in each sample. E. Stacked bar graph in this panel displays the proportion of mutations found in specific genes.

trol sample group consisted of ten adjacent control samples. The first ovarian cancer sample group comprised samples with pathogenic mutations in BRCA1, CDK4, and PTEN genes, while the second group lacked such mutations. The results revealed no significant difference in the mRNA expression of BRCA1, CDK4, and PTEN genes between the normal group and the ovarian cancer sample group lacking pathogenic mutations (**Figure 6A**). On the contrary, a significant reduction in the mRNA expression of BRCA1, CDK4, and PTEN genes was revealed in the ovarian cancer sample group having pathogenic mutations as compared to the other two groups, including the normal sample group and ovarian cancer sample group lacking pathogenic mutations in the group of these genes (**Figure 6A**). Moreover, ROC curve analysis demonstrated that downregulated BRCA1, CDK4, and PTEN genes can accurately differentiate between normal and ovarian cancer patients (**Figure 6B**).

Effect of the observed pathogenic mutations on the proteomic expression level of BRCA1, CDK4, and PTEN: To assess the impact of the observed pathogenic mutations on the protein expression of BRCA1, CDK4, and PTEN, IHC analysis was conducted on six tissue samples. This included two adjacent normal control samples, two ovarian cancer samples with pathogenic mutations in BRCA1, CDK4, and PTEN, and two ovarian cancer samples lacking such mutations. The results indicated high staining for BRCA1, CDK4, and PTEN proteins in normal tissue and ovarian cancer samples without pathogenic mutations (**Figure 7**). On the contrary, the two ovarian cancer samples with pathogenic mutations exhibited low staining for BRCA1, CDK4, and PTEN proteins (**Figure 7**). Collectively, these results indicated a reduction in the expression of BRCA1, CDK4, and PTEN at the protein level in the ovarian cancer tissue samples having pathogenic mutations compared to the other two counterparts.

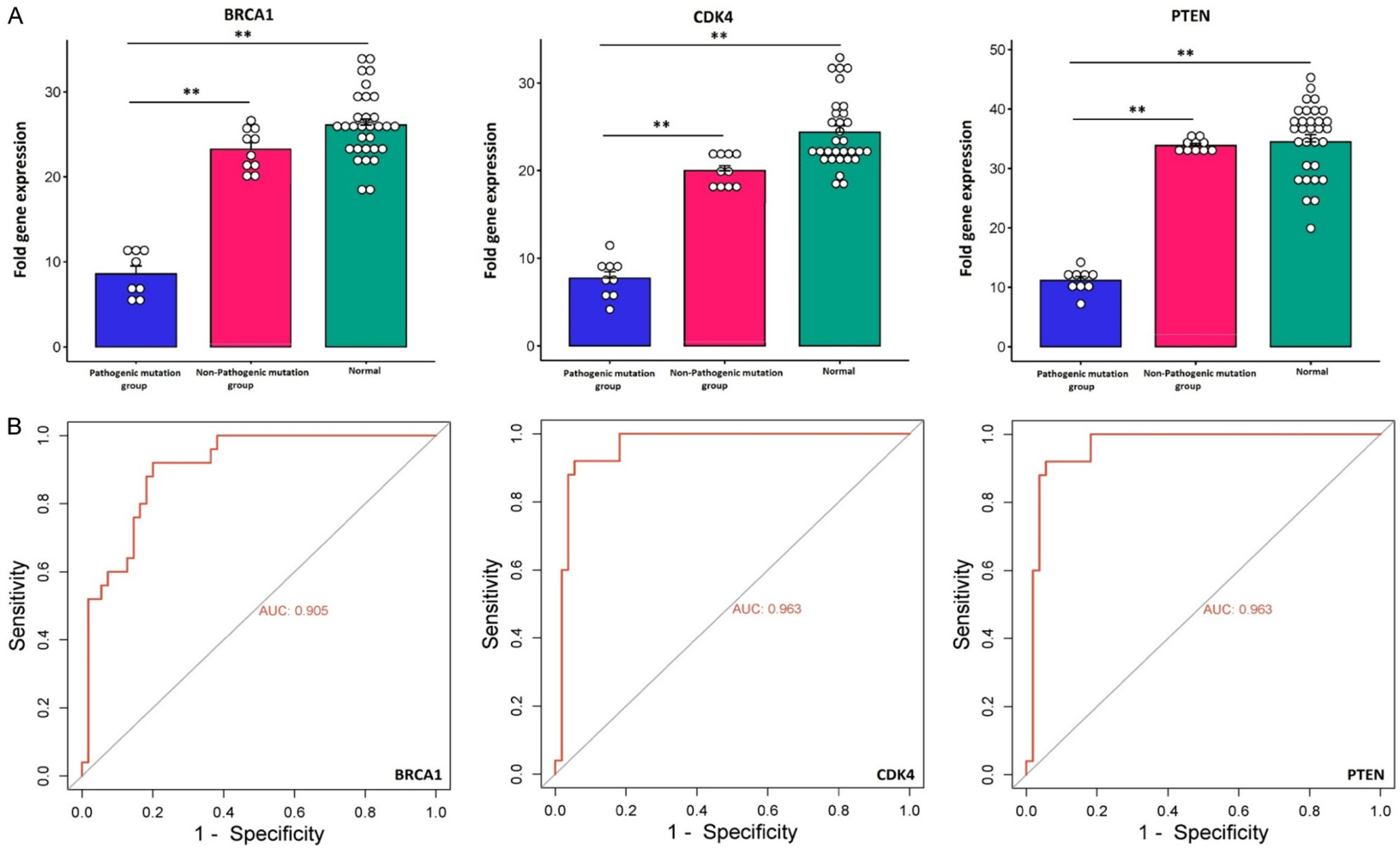

Figure 6. Relative expression and receiver operating characteristic (ROC) curve analysis of BRCA1, CDK4, and PTEN genes between normal, pathogenic mutated, and non-pathogenic mutated ovarian cancer sample groups. (A) Relative expression analysis of BRCA1, CDK4, and PTEN genes via Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR), and (B) RT-qPCR expression-based ROC curves of BRCA1, CDK4, and PTEN genes. ***P*-value <0.01.

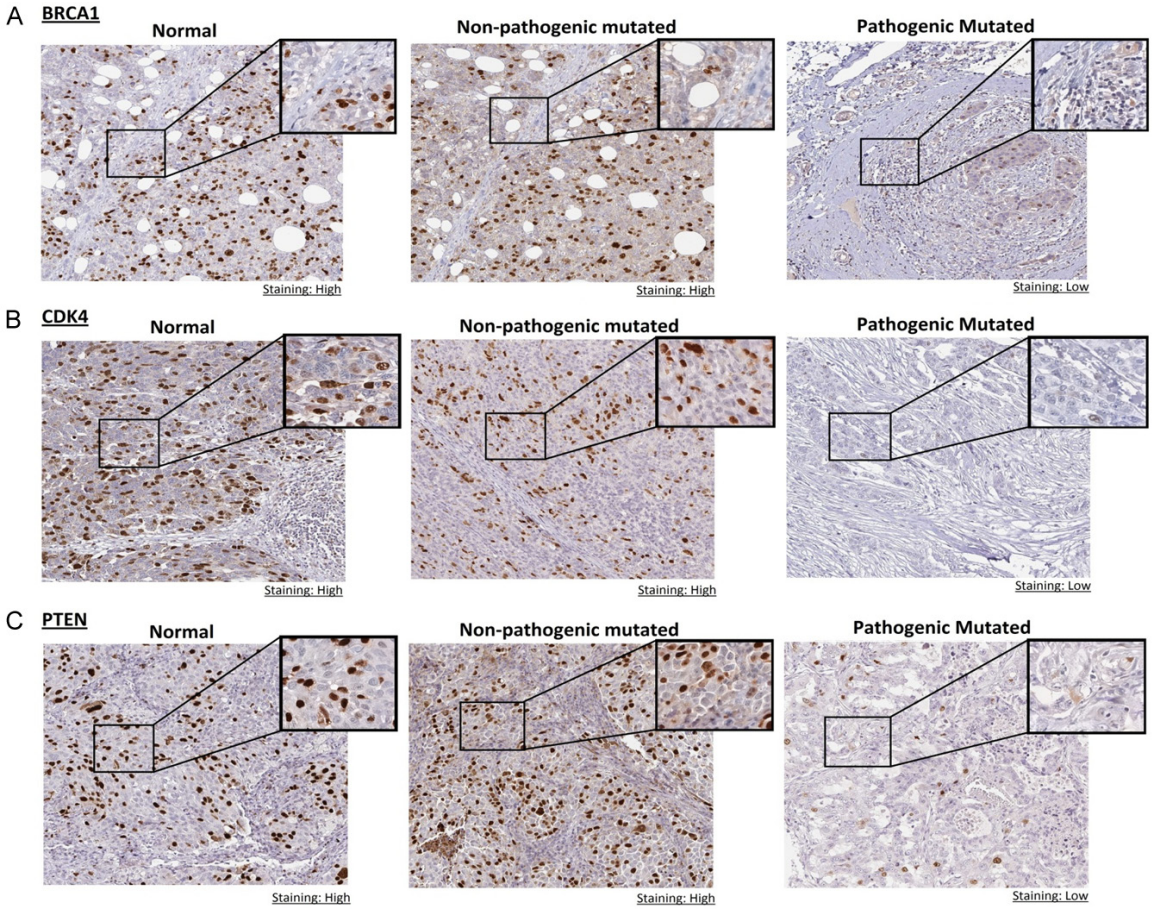

Figure 7. Immunohistochemistry (IHC)-based proteomic expression analysis of BRCA1, CDK4, and PTEN proteins between normal, pathogenic mutated, and non-pathogenic mutated ovarian cancer samples. A. BRCA1 expression. B. CDK4 expression. C. PTEN expression. Expression differences were measured based on the staining intensities.

Gene enrichment analysis of BRCA1, CDK4, and PTEN

Subsequently, comprehensive GO and KEGG enrichment analyses were conducted to gain insights into the functional implications of BRCA1, CDK4, and PTEN genes. In the realm of Gene Ontology (GO), our analysis revealed significant enrichments of BRCA1, CDK4, and PTEN genes in various Cellular Component (CC) terms, such as "BRCA1-BARD1 complex, Cyclin D2-CDK4 complex, Lateral element, and BRCA1-A complex" (**Figure 8A**). Within Molecular Function (MF) terms, enrichments were observed in categories such as "H3 histone acetyltransferase activity, RNA polymerase II CTD heptapeptide repeat kinase activity, Cyclin-dependent protein serine/threonine kinase activity, and cyclin binding" (**Figure 8B**). Furthermore, Biological Process (BP) terms exhibited enrichments related to "Histone H3 acetylation, Histone H4 acetylation, centrosome duplication, and cellular response to alcohol" etc. (**Figure 8C**). In the context of KEGG pathways, BRCA1, CDK4, and PTEN genes were notably enriched in pathways encompassing "Homologous recombination, Fanconi anemia pathway, pancreatic cancer and melanoma" etc. (**Figure 8D**).

Ramachandran plots-based validation

The Ramachandran plot assesses the energy associated with stable conformational angles (psi, ψ, and phi, Φ) of each amino acid. Structure validation for mutated BRCA1, CDK4, and PTEN indicated over 90 percent of residues falling within favorable and allowed regions (**Figure 9A**). ProSA-web analysis evaluated the quality and potential flaws in the 3D models, assigning Z-scores of -3.5, -5.3, and -7.3 for the peptide models of mutated BRCA1, CDK4, and PTEN, respectively (**Figure 9B**).

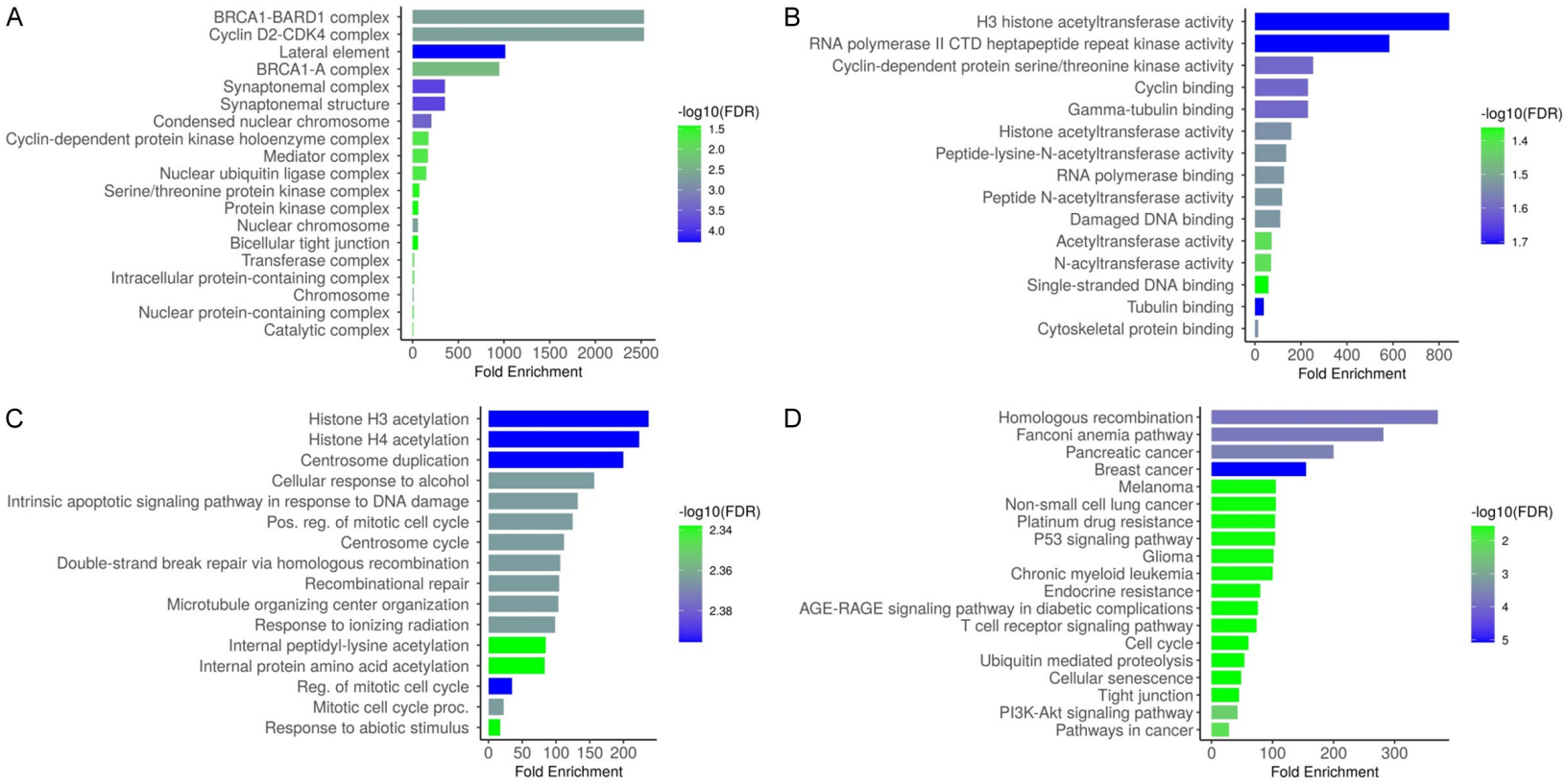

Figure 8. Gene enrichment analysis of BRCA1, CDK4, and PTEN genes via DAVID. (A) BRCA1, CDK4, and PTEN genes-related cellular components (CC) terms, (B) BRCA1, CDK4, and PTEN genes-related molecular function (MF) terms, (C) BRCA1, CDK4, and PTEN genes-related biological process (BP) terms, and (D) BRCA1, CDK4, and PTEN genes-related Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. A $P<0.05$ was used as the cut-off criterion.

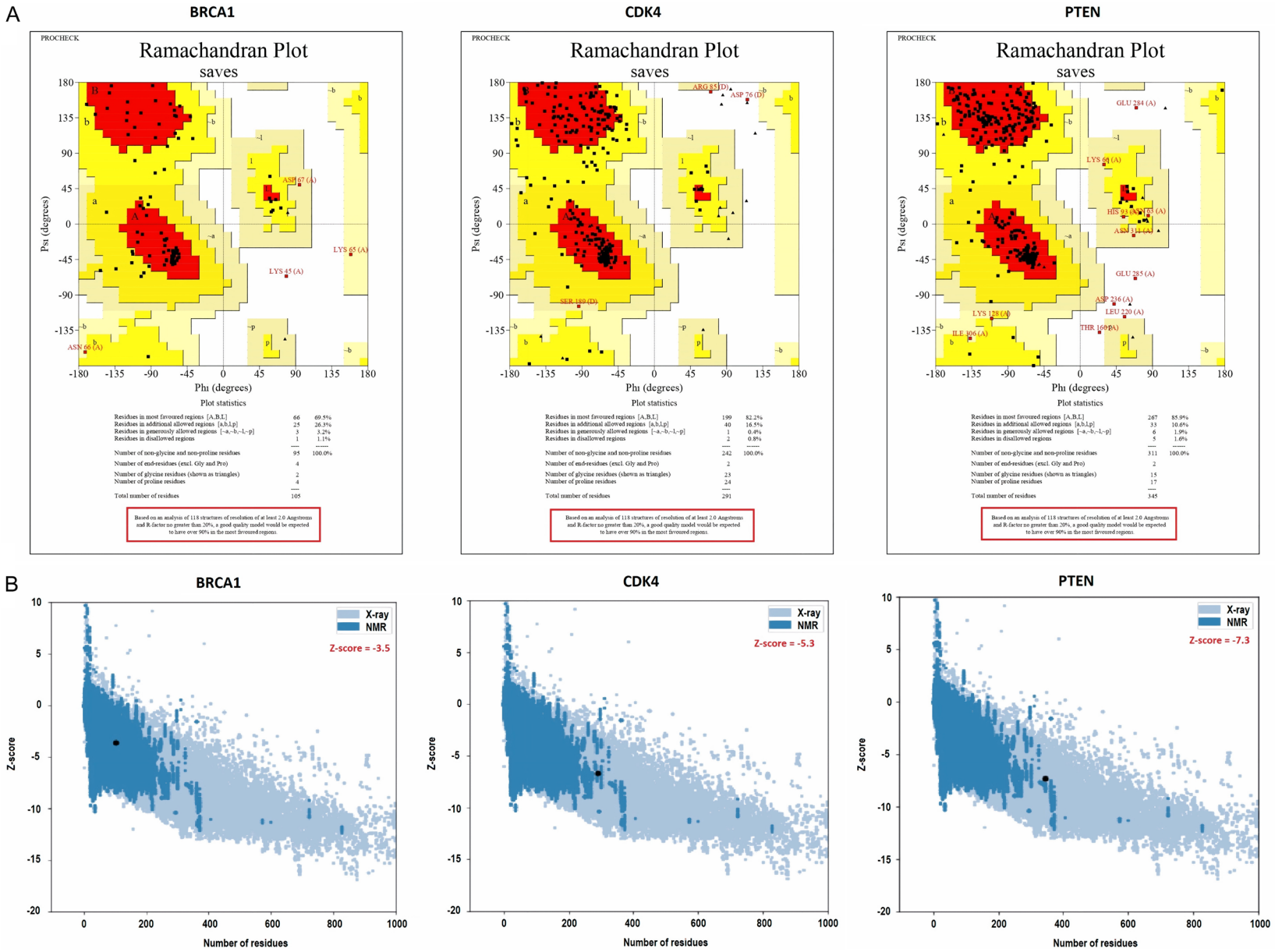

Figure 9. This figure presents the structural validation of mutated BRCA1, CDK4, and PTEN protein. (A) The Ramachandran plots for the final models indicate that over 90% of the amino acids reside within favorable regions, and (B) The ProSA-web plot of the models yield a Z-score.

Drug prediction and molecular docking analyses

The DrugBank database was employed to explore potential drugs with the capacity to enhance the expression of BRCA1, CDK4, and PTEN proteins in the context of ovarian cancer treatment. Within this repository, Resveratrol emerged as a promising drug with the potential to reduce the expression of BRCA1, CDK4, and PTEN proteins. To validate the impact of Resveratrol on expression enhancement, molecular docking analysis was conducted. Docking results unveiled varying binding affinities of Resveratrol with BRCA1, CDK4, and PTEN, spanning from -6.1 to -7.3 kcal/mol (**Figure 10**). These binding affinities, falling within the range of -6.1 to -7.3 kcal/mol, signify a relatively robust interaction between Resveratrol and the BRCA1, CDK4, and PTEN proteins (**Figure 10**).

Discussion

In the current study, by performing NGS analysis, we conducted the mutational analysis of 27 susceptibility genes known to play crucial roles in the etiology and progression of ovarian cancer. Among all the analyzed 27 genes, only four genes (BRCA1, CDK2, MSH2, and PTEN) were found mutated in ovarian cancer patients. Most importantly, clinically significant mutations were observed in BRCA1, CDK4, and PTEN genes.

In the case of mutated BRCA1, the compromised DNA repair mechanisms led to genomic instability, heightening the susceptibility to additional mutations and facilitating tumor development, particularly in breast and ovarian tissues [25-27]. Mutated CDK4 disrupted cell cycle regulation, fostering uncontrolled cell proliferation and cooperation with oncogenes, thereby promoting tumorigenesis in various cancers [28-30]. Mutated PTEN, on the other hand, unleashed the PI3K/AKT pathway, fostering cell survival, growth, and angiogenesis while impeding apoptosis [31-33]. This multifaceted disruption of cellular processes in the presence of BRCA1, CDK4, and PTEN pathogenic mutations emphasizes their pivotal roles in the development of ovarian cancer.

Next, the functional consequences of pathogenic mutations in BRCA1, CDK4, and PTEN were assessed at both the mRNA and protein expression levels. The downregulation of BRCA1, CDK4, and PTEN genes at the mRNA level in ovarian cancer samples with pathogenic mutations suggests a potential role of these mutations in suppressing the expression of tumor-suppressive genes. The ROC curve analysis further validates the potential of these genes as diagnostic markers for ovarian cancer. At the protein level, the IHC analysis revealed reduced staining for BRCA1, CDK4, and PTEN in ovarian cancer samples with pathogenic mutations, indicating a correlation between genetic mutations and protein expression. Moreover, previous studies reported that pathogenic mutations predominantly manifest as loss-of-function mutations, culminating in the downregulation of gene expression among cancer patients [34-38]. This pattern emphasizes a fundamental mechanism by which these mutations exert their deleterious effects on cellular functions. The diminished expression of these proteins in the presence of pathogenic mutations highlights their role as tumor suppressors and suggests a potential avenue for targeted therapies.

Furthermore, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses provided insights into the functional implications of BRCA1, CDK4, and PTEN genes. Enrichments in terms related to DNA damage response, signal transduction, and histone acetylation signify the multifaceted roles of these genes in cellular processes. The involvement of these genes in pathways such as homologous recombination and melanoma further supports their significant contribution to ovarian cancer development. The role of these pathways in cancer development is already well studied [39-43].

Moreover, the exploration of potential drugs using the DrugBank database identified Resveratrol as a promising candidate with the capacity to enhance the expression of BRCA1, CDK4, and PTEN proteins. Molecular docking analysis reinforced the potential interactions between Resveratrol and the target proteins, suggesting its therapeutic relevance in ovarian cancer treatment [44]. Previous preclinical studies have illuminated its diverse mechanisms of action, positioning Resveratrol as a promising candidate in the realm of cancer therapeutics [45, 46]. Its multifaceted proper-

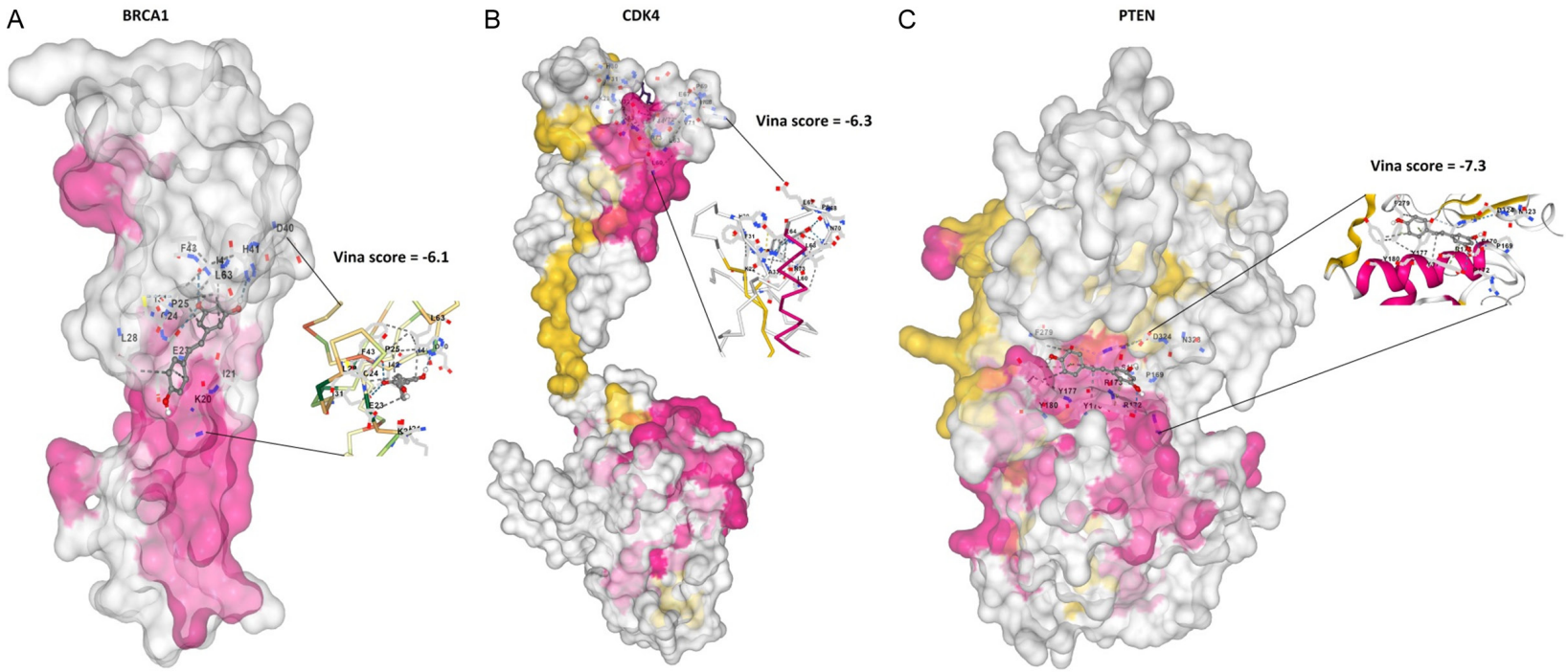

Figure 10. This figure displays the molecular docking results of Resveratrol with mutated BRCA1, CDK4, and PTEN proteins. A. BRCA1-Resveratrol docking. B. CDK4-Resveratrol docking. C. PTEN-Resveratrol docking. The structures of BRCA1, CDK4, and PTEN proteins are depicted in yellow, pink and gray, while the Resveratrol drug is represented by gray and pink molecules, illustrating their docking interactions with the target proteins.

ties include anti-inflammatory, antioxidant, and antiangiogenic effects, collectively contributing to its ability to impede cancer development and progression [47, 48]. Resveratrol has demonstrated the capacity to induce apoptosis, or programmed cell death, in cancer cells while leaving healthy cells largely unaffected. Importantly, the ability of Resveratrol to selectively target cancer cells, induce cell cycle arrest, and mitigate metastatic potential has been observed across various cancer types, including breast, prostate, and colon cancers [47, 49, 50]. Identifying Resveratrol candidate drugs against mutated BRCA1, CDK4, and PTEN opens avenues for ovarian cancer treatment.

Conclusion

In conclusion, the present study provides comprehensive understanding of genetic mutations in BRCA1, CDK4, MSH2, and PTEN genes among ovarian cancer patients. The identification of pathogenic mutations in BRCA1, CDK4, and PTEN, their clinical significance, and the downstream effects on mRNA and protein expression levels contribute to the ongoing efforts in unraveling the complexities of ovarian cancer biology. The population-specific nature of these mutations highlights the importance of diverse genetic studies for personalized cancer care. Further validation studies, functional assays, and clinical trials are warranted to translate these findings into actionable insights for improved diagnostic and therapeutic strategies in ovarian cancer.

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Disclosure of conflict of interest

None.

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References

[1] Győrffy B. Discovery and ranking of the most robust prognostic biomarkers in serous ovarian cancer. Geroscience 2023; 45: 1889-1898.

[2] Li L, Zhang F, Zhang J, Shi X, Wu H, Chao X, Ma S, Lang J, Wu M, Zhang D and Liang Z. Identifying serum small extracellular vesicle microRNA as a noninvasive diagnostic and prognostic biomarker for ovarian cancer. ACS Nano 2023; 17: 19197-19210.

[3] Weiderpass E and Tyczynski JE. Epidemiology of patients with ovarian cancer with and without a BRCA1/2 mutation. Mol Diagn Ther 2015; 19: 351-364.

[4] Usman M, Okla MK, Asif HM, AbdElgayed G, Muccee F, Ghazanfar S, Ahmad M, Iqbal MJ, Sahar AM, Khaliq G, Shoaib R, Zaheer H and Hameed Y. A pan-cancer analysis of GINS complex subunit 4 to identify its potential role as a biomarker in multiple human cancers. Am J Cancer Res 2022; 12: 986-1008.

[5] Pietragalla A, Arcieri M, Marchetti C, Scambia G and Fagotti A. Ovarian cancer predisposition beyond BRCA1 and BRCA2 genes. Int J Gynecol Cancer 2020; 30: 1803-1810.

[6] Minion LE, Dolinsky JS, Chase DM, Dunlop CL, Chao EC and Monk BJ. Hereditary predisposition to ovarian cancer, looking beyond BRCA1/BRCA2. Gynecol Oncol 2015; 137: 86-92.

[7] Hodgson DR, Dougherty BA, Lai Z, Fielding A, Grinsted L, Spencer S, O'connor MJ, Ho TW, Robertson JD, Lanchbury JS, Timms KM, Gutin A, Orr M, Jones H, Gilks B, Womack C, Gourley C, Ledermann J and Barrett JC. Candidate biomarkers of PARP inhibitor sensitivity in ovarian cancer beyond the BRCA genes. Br J Cancer 2018; 119: 1401-1409.

[8] O'Sullivan CC, Moon DH, Kohn EC and Lee JM. Beyond breast and ovarian cancers: PARP inhibitors for BRCA mutation-associated and BRCA-like solid tumors. Front Oncol 2014; 4: 42.

[9] Ahmad M, Khan M, Asif R, Sial N, Abid U, Shamim T, Hameed Z, Iqbal MJ, Sarfraz U, Saeed H, Asghar Z, Akram M, Ullah Q, Younas QUA, Rauf L, Hadi A, Maryam S, Hameed Y, Khan MR, Tariq E and Saeed S. Expression characteristics and significant diagnostic and prognostic values of ANLN in human cancers. Int J Gen Med 2022; 15: 1957-1972.

[10] Usman M, Hameed Y and Ahmad M. Does human papillomavirus cause human colorectal cancer? Applying Bradford Hill criteria postulates. Ecancermedicalscience 2020; 14: 1107.

[11] Eoh KJ, Kim JE, Park HS, Lee ST, Park JS, Han JW, Lee JY, Kim S, Kim SW, Kim JH, Kim YT and Nam EJ. Detection of germline mutations in

patients with epithelial ovarian cancer using multi-gene panels: beyond BRCA1/2. Cancer Res Treat 2018; 50: 917-925.
[12] Shah PD, Patil S, Dickler MN, Offit K, Hudis CA and Robson ME. Twenty-one-gene recurrence score assay in BRCA-associated versus sporadic breast cancers: differences based on germline mutation status. Cancer 2016; 122: 1178-1184.
[13] Fanale D, Incorvaia L, Filorizzo C, Bono M, Fiorino A, Calò V, Brando C, Corsini LR, Barraco N, Badalamenti G, Russo A and Bazan V. Detection of germline mutations in a cohort of 139 patients with bilateral breast cancer by multi-gene panel testing: impact of pathogenic variants in other genes beyond BRCA1/2. Cancers (Basel) 2020; 12: 2415.
[14] General Assembly of the World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. J Am Coll Dent 2014; 81: 14-18.
[15] Gupta N. DNA extraction and polymerase chain reaction. J Cytol 2019; 36: 116-117.
[16] Rio DC, Ares M Jr, Hannon GJ and Nilsen TW. Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb Protoc 2010; 2010: pdb.prot5439.
[17] Gudmundsson S, Singer-Berk M, Watts NA, Phu W, Goodrich JK, Solomonson M; Genome Aggregation Database Consortium, Rehm HL, MacArthur DG and O'Donnell-Luria A. Variant interpretation using population databases: lessons from gnomAD. Hum Mutat 2022; 43: 1012-1030.
[18] Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, Lane HC, Imamichi T and Chang W. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). Nucleic Acids Res 2022; 50: W216-W221.
[19] Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, Gautam B and Hassanali M. DrugBank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res 2008; 36: D901-906.
[20] Liu Y, Grimm M, Dai WT, Hou MC, Xiao ZX and Cao Y. CB-Dock: a web server for cavity detection-guided protein-ligand blind docking. Acta Pharmacol Sin 2020; 41: 138-144.
[21] Coppa A, Nicolussi A, D'Inzeo S, Capalbo C, Belardinilli F, Colicchia V, Petroni M, Zani M, Ferraro S, Rinaldi C, Buffone A, Bartolazzi A, Screpanti I, Ottini L and Giannini G. Optimizing the identification of risk-relevant mutations by multigene panel testing in selected hereditary breast/ovarian cancer families. Cancer Med 2018; 7: 46-55.
[22] Feng Y, Spezia M, Huang S, Yuan C, Zeng Z, Zhang L, Ji X, Liu W, Huang B, Luo W, Liu B, Lei Y, Du S, Vuppalapati A, Luu HH, Haydon RC, He TC and Ren G. Breast cancer development and progression: risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes Dis 2018; 5: 77-106.
[23] Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. Endocr Rev 2007; 28: 742-762.
[24] Xu X, Chang CW, Li M, Liu C and Liu Y. Molecular mechanisms of the RECQ4 pathogenic mutations. Front Mol Biosci 2021; 8: 791194.
[25] Ali RMM, McIntosh SA and Savage KI. Homologous recombination deficiency in breast cancer: implications for risk, cancer development, and therapy. Genes Chromosomes Cancer 2021; 60: 358-372.
[26] Venkitaraman AR. Linking the cellular functions of BRCA genes to cancer pathogenesis and treatment. Annu Rev Pathol 2009; 4: 461-487.
[27] Cantor SB. Revisiting the BRCA-pathway through the lens of replication gap suppression: "Gaps determine therapy response in BRCA mutant cancer". DNA Repair (Amst) 2021; 107: 103209.
[28] McDonald ER 3rd and El-Deiry WS. Cell cycle control as a basis for cancer drug development. Int J Oncol 2000; 16: 871-957.
[29] Shostak A. Circadian clock, cell division, and cancer: from molecules to organism. Int J Mol Sci 2017; 18: 873.
[30] Sherr CJ. Cancer cell cycles. Science 1996; 274: 1672-1677.
[31] Luongo F, Colonna F, Calapà F, Vitale S, Fiori ME and De Maria R. PTEN tumor-suppressor: the dam of stemness in cancer. Cancers (Basel) 2019; 11: 1076.
[32] Zhao HF, Wang J, Shao W, Wu CP, Chen ZP, To ST and Li WP. Recent advances in the use of PI3K inhibitors for glioblastoma multiforme: current preclinical and clinical development. Mol Cancer 2017; 16: 100.
[33] Chalhoub N and Baker SJ. PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol 2009; 4: 127-150.
[34] Flanagan SE, Patch AM and Ellard S. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. Genet Test Mol Biomarkers 2010; 14: 533-537.
[35] Jiao SY, Yang YH and Chen SR. Molecular genetics of infertility: loss-of-function mutations in humans and corresponding knockout/mutated mice. Hum Reprod Update 2021; 27: 154-189.
[36] Derks MFL, Gjuvsland AB, Bosse M, Lopes MS, van Son M, Harlizius B, Tan BF, Hamland H,

Grindflek E, Groenen MAM and Megens HJ. Loss of function mutations in essential genes cause embryonic lethality in pigs. PLoS Genet 2019; 15: e1008055.

[37] Khan M and Hameed Y. Discovery of novel six genes-based cervical cancer-associated biomarkers that are capable to break the heterogeneity barrier and applicable at the global level. J Cancer Res Ther 2023.

[38] Xu W, Li H, Hameed Y, Abdel-Maksoud MA, Almutairi SM, Mubarak A, Aufy M, Alturaiki W, Alshalani AJ, Mahmoud AM and Li C. Elucidating the clinical and immunological value of m6A regulator-mediated methylation modification patterns in adrenocortical carcinoma. Oncol Res 2023; 31: 819-831.

[39] Kim KB, Soroceanu L, de Semir D, Millis SZ, Ross J, Vosoughi E, Dar AA, Nosrati M, Desprez PY, Ice R, Chen M, Chetal K, Bhattacharjee A, Moretto J, Leong SP, Singer MI, Parrett BM, Minor DR, McAllister S, Miller JR 3rd, Salomonis N and Kashani-Sabet M. Prevalence of homologous recombination pathway gene mutations in melanoma: rationale for a new targeted therapeutic approach. J Invest Dermatol 2021; 141: 2028-2036, e2022.

[40] You Z, Lv M, He X, Pan Y, Ge J, Hu X, Zheng Y, Huang M, Zhou C and You C. Homologous recombination repair gene mutations as a predictive biomarker for immunotherapy in patients with advanced melanoma. Front Immunol 2022; 13: 871756.

[41] Chan WY, Brown LJ, Reid L and Joshua AM. PARP inhibitors in melanoma-an expanding therapeutic option? Cancers (Basel) 2021; 13: 4520.

[42] Hu H, Umair M, Khan SA, Sani AI, Iqbal S, Khalid F, Sultan R, Abdel-Maksoud MA, Mubarak A, Dawoud TM, Malik A, Saleh IA, Al Amri AA, Algarzae NK, Kodous AS and Hameed Y. CDCA8, a mitosis-related gene, as a prospective pan-cancer biomarker: implications for survival prognosis and oncogenic immunology. Am J Transl Res 2024; 16: 432-445.

[43] Luo M, Rehman A, Haque S, Izhar S, Perveen F, Haris M, Abdel-Maksoud MA, Saleh IA, Zomot N, Malik A, Alamri A, Kodous AS, Aufy M, Zaky MY, Zaeem M, Hameed Y and Li J. Thorough examination of the potential biological implications of the cuproptosis-related gene LIPT2 in the prognosis and immunotherapy in pan-cancer. Am J Transl Res 2024; 16: 940-954.

[44] Xu XL, Deng SL, Lian ZX and Yu K. Resveratrol targets a variety of oncogenic and oncosuppressive signaling for ovarian cancer prevention and treatment. Antioxidants (Basel) 2021; 10: 1718.

[45] Jiang Z, Chen K, Cheng L, Yan B, Qian W, Cao J, Li J, Wu E, Ma Q and Yang W. Resveratrol and cancer treatment: updates. Ann N Y Acad Sci 2017; 1403: 59-69.

[46] Ren B, Kwah MX, Liu C, Ma Z, Shanmugam MK, Ding L, Xiang X, Ho PC, Wang L, Ong PS and Goh BC. Resveratrol for cancer therapy: challenges and future perspectives. Cancer Lett 2021; 515: 63-72.

[47] Ko JH, Sethi G, Um JY, Shanmugam MK, Arfuso F, Kumar AP, Bishayee A and Ahn KS. The role of resveratrol in cancer therapy. Int J Mol Sci 2017; 18: 2589.

[48] Fulda S. Resveratrol and derivatives for the prevention and treatment of cancer. Drug Discov Today 2010; 15: 757-765.

[49] San Hipólito-Luengo Á, Alcaide A, Ramos-González M, Cercas E, Vallejo S, Romero A, Talero E, Sánchez-Ferrer CF, Motilva V and Peiró C. Dual effects of resveratrol on cell death and proliferation of colon cancer cells. Nutr Cancer 2017; 69: 1019-1027.

[50] Carter LG, D'Orazio JA and Pearson KJ. Resveratrol and cancer: focus on in vivo evidence. Endocr Relat Cancer 2014; 21: R209-R225.