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Brief Communication

Deciphering DNA repair gene mutational landscape in uterine corpus endometrial carcinoma patients using next generation sequencing

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Abstract: Uterine Corpus Endometrial Carcinoma (UCEC) is a significant health concern with a complex genetic landscape impacting disease susceptibility and progression. This study aimed to unravel the spectrum of DNA repair gene mutations in Pakistani UCEC patients through Next Generation Sequencing (NGS) and explore their potential functional consequences via downstream analyses. NGS analysis of genomic DNA from 30 UCEC patients was conducted to identify clinically significant pathogenic mutations in DNA repair genes. This analysis revealed mutations in 4 key DNA repair genes: BRCA1, BRCA2, APC, and CDH1. Kaplan-Meier (KM) analysis was employed to assess the prognostic value of these mutations on patient overall survival (OS) in UCEC. To delve into the functional impact of these mutations, we performed RT-qPCR, immunohistochemistry (IHC), and western blot analyses on the mutated UCEC samples compared to their non-mutated counterparts. These results unveiled the up-regulation in the expression of the mutated genes, suggesting a potential association between the identified mutations and enhanced gene activity. Additionally, targeted bisulfite sequencing analysis was utilized to evaluate DNA methylation patterns in the promoters of the mutated genes. Strikingly, hypomethylation in the promoters of BRCA1, BRCA2, APC, and CDH1 was observed in the mutated UCEC samples relative to the non-mutated, indicating the involvement of epigenetic mechanisms in the altered gene expression. In conclusion, this study offers insights into the genetic landscape of DNA repair gene mutations in Pakistani UCEC patients. The presence of pathogenic mutations in BRCA1, BRCA2, APC, and CDH1, coupled with their down-regulation and hypermethylation, suggests a convergence of genetic and epigenetic factors contributing to genomic instability in UCEC cells. These findings enhance our understanding of UCEC susceptibility and provide potential avenues for targeted therapeutic interventions in Pakistani UCEC patients.

Keywords: Uterine corpus endometrial carcinoma, next generation sequencing, DNA repair gene, pathogenic mutations

Introduction

Uterine corpus endometrial carcinoma (UCEC) represents a complex and increasingly prevalent gynecological malignancy that affects the endometrial lining of the uterus [1-3]. In 2022, UCEC prevalence exhibited a steady increase and this disease declared as one of the most commonly diagnosed gynecological malignancies worldwide [4, 5]. Factors such as obesity, hormonal imbalances, and an aging population contributed to this rising trend [6-8]. With a diverse clinical spectrum ranging from indolent to aggressive forms, UCEC poses significant health challenge [9, 10]. While early-stage UCEC is often associated with a favorable prognosis, a subset of cases manifests as high-grade tumors with aggressive behavior, necessitating a deeper understanding of the underlying molecular mechanisms that drive its progression [11, 12].

DNA repair gene mutations are pivotal drivers of cancer development [13, 14]. These genes, responsible for correcting DNA damage, safeguard the genome against mutations [15]. When they acquire mutations themselves, the DNA repair process becomes compromised,

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

Sr. no	Characteristics	Sample count (n)
1	Sex	
	Male	0
	Female	30
2	Age	
	>60	1
	<60	29
3	Treatment	
	Pre-treatment	30
	Post-treatment	0

allowing the accumulation of genetic alterations, which can ultimately lead to the initiation and progression of cancer.

Defects in DNA repair genes, such as BRCA1, BRCA2, and others, are particularly associated with an increased cancer risk [16, 17]. These mutations can result in impaired repair of DNA double-strand breaks, making cells susceptible to genomic instability and the formation of malignant tumors. Given the clinical and therapeutic implications, elucidating the genetic and epigenetic landscape of DNA repair genes in UCEC has become paramount. So far, less research work has been done on exploring mutational spectrum of DNA repair genes in Pakistani UCEC patients. Therefore, investigating DNA repair gene mutations in the Pakistani UCEC patient's context will address population-specific variations influencing disease susceptibility. Furthermore, the investigation into gene expression changes and epigenetic alterations, including hypomethylation in gene promoters, will enhance our understanding of the intricate molecular mechanisms underlying UCEC in the Pakistani context. In the current study, by harnessing cutting-edge Next Generation Sequencing (NGS) technology, we delve deep into the genetic makeup of UCEC, unearthing clinically significant mutations in a selective group of DNA repair genes across Pakistani UCEC patients' cohort. These findings not only serve as prognostic markers but also shed light on the molecular terrain within UCEC cells.

Beyond genetic mutations, we explore the functional repercussions of these alterations, investigating how they may affect the expression of DNA repair genes. Through state-of-the-art techniques like RT-qPCR and immunohistochemistry, we unveil a potential link between these mutations and reduced gene activity, raising intriguing questions about the compromised DNA repair mechanisms in UCEC cells. Moreover, we also explored epigenetic aspect of the mutated genes, examining their DNA methylation status.

Method

Ethical approval and sample collection

This research was conducted in accordance with Helsinki guidelines [18] and approved by the institutional review board. Informed consent was obtained from all patients participating in the study. A cohort of 30 UCEC patients, who were underwent for surgical resection in the DHQ, Teaching Hospital, Dera Ismail Khan, KPK were recruited for this study (**Table 1**). Patients were selected based on clinical and histopathological criteria, ensuring diverse disease stages and grades. After resection, UCEC tissue samples were immediately collected and snap-frozen in liquid nitrogen for subsequent analysis.

Genetic analysis

Genomic DNA extraction: Genomic DNA was extracted from tissue samples using a commercially available kit (Easy Genomic DNA Extraction Kit, Thermo Fischer). DNA concentration and purity were measured by NanoDrop 2000 Spectrophotometer and Qubit 3.0 Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA).

Next generation sequencing (NGS) analysis: In total of the coding region of 27 DNA repair genes, including BRCA1, BRCA2, APC, ATM, BARD1, BMPR1A, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, EPCAM, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PMS2, PTEN, RAD50, RAD51C, RAD51D, SMAD4, STK11, and TP53 were analyzed. Multiplex polymerase chain reaction (PCR) was performed using 50-100 ng of genomic DNA and involved 17 cycles. A premixed primer pool and Ion AmpliSeq Library Kit 2.0 were used for this purpose, as previously detailed [19]. Subsequently, the PCR amplicons underwent treatment with 2 µL of FuPa reagent, leading to partial digestion of primer sequences and phosphorylation of the amplicons. The amplicons underwent liga-

tion to adapters, incorporating diluted barcodes from the Ion Xpress Barcode Adapters kit (Life Technologies). Following this step, adaptor-ligated amplicon libraries were subjected to purification using Agencourt AMPure XP reagents (Beckman Coulter, Tokyo, Japan). Subsequently, library concentrations were assessed using an Ion Library Quantitation Kit (Life Technologies). Each library was then diluted to a concentration of 8 pM, and equal amounts of these libraries were combined for a single sequencing reaction. Emulsion PCR was subsequently conducted employing the Ion OneTouch System and Ion PI Template OT2 200 Kit v2 (Life Technologies), adhering to the manufacturer's protocols. The Ion Sphere Particles, confirmed as template-positive, were subsequently enriched through the employment of Dynabeads MyOne Streptavidin C1 Beads (Life Technologies) via the Ion OneTouch ES system (Life Technologies). After this purification process, the Ion Sphere particles were loaded onto an Ion PI Chip v2. Sequencing process, conducted on an Ion Proton System (Life Technologies), employed the Ion PI Sequencing 200 Kit v2. This sequencing procedure utilized 500 flow runs, resulting in the generation of reads approximately 200 base pairs in length.

Data analysis and mutation classification: The clean sequencing reads were aligned to the human reference genome hg19/GRCh37. After the alignment process, reads exhibiting misalignment from the reference were classified as potential mutations. Subsequent to 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 [20], the interpretation of mutations was undertaken. Additionally, the ClinVar database [21] was utilized to assess the clinical significance of the identified mutations.

Mutational frequencies analysis: The Genome Aggregation Database (gnomAD) is a comprehensive and widely utilized genetic resource. It compiles and shares exome and genome sequencing data from diverse populations, enabling researchers to explore genetic variations and their frequencies [22]. In this study, GnomeAD database was used to analyze the frequencies of observed mutations in Asian population.

Survival analysis (Kaplan-Meier)

Kaplan-Meier survival curves [23] were generated to assess the impact of DNA repair gene mutations on overall survival (OS) in UCEC patients. These curves visually depicted the survival probability over time for two distinct groups: UCEC patients with the identified DNA repair gene pathogenic mutations and those without. To determine the statistical significance of these survival differences, the log-rank test, a widely recognized statistical method for comparing survival distributions, was employed. This test assessed whether the observed differences in OS between the two groups were statistically meaningful, providing valuable insights into the prognostic implications of these mutations in UCEC.

Functional consequences of mutations

RNA extraction: Total RNA was extracted from UCEC tissue samples was extracted using kit method (GeneJET RNA Purification Kit, Thermo Fischer), following instructions of manufacturer. RNA concentration and purity were measured by NanoDrop 2000 Spectrophotometer and Qubit 3.0 Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR): The whole RNA was converted into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kits from Applied Biosystems. Subsequently, RT-qPCR was conducted employing Platinum PCR SuperMix High Fidelity from Life Technologies. Each reaction was replicated three times for accuracy. To determine the mRNA expression levels, the following formula was employed: mRNA expression level =2^(-ΔΔCq) [24]. A student t-test was employed to find expression differences between two groups.

Receiver operating curve generation

Based on the RT-qPCR expression and bisulfite-seq based methylation data, ROC curves of DNA repair gene expression and methylation levels were generated with the help of SRPLOT

web source (https://bioinformatics.com.cn/sr-plot).

Immunohistochemistry (IHC)

Tissue sections were deparaffinized, and antigen retrieval was performed by heat treatment in EDTA (ethylenediaminetetraacetic acid) solution pH 8.0. Protein expression of the mutated genes in UCEC tissues samples were evaluated on 4-μm-thick, formalin-fixed, paraffin-embedded (FFPE) sections with anti-BRCA1 (EPR19433, abcam), anti-BRCA2 (EPR23442-43, abcam), anti-APC (EP701Y, abcam), and anti-CDH1 (EP700Y, abcam) monoclonal antibodies using the Ventana BenchMark XT staining system (Roche, Tokyo, Japan). In this analysis, non-pathogenic mutated tissue samples served as comparison counterpart. A pathologist determined the tumors to be positive when nuclear staining in tumor tissue was present or negative when the nuclear stain was absent. Protein expression was observed based on staining intensity.

Western blotting

Protein extraction from two samples, encompassing one UCEC sample with pathogenic mutations and another UCEC sample devoid of pathogenic mutations, was carried out using a kit method (ab270054). The protein concentration was quantified using the BCA Protein Assay reagent (Beyotime, Shanghai, China). Subsequently, the samples underwent separation on an SDS-polyacrylamide gel and were transferred onto polyvinylidene difluoride (PVDF) membranes. Signal detection was accomplished using ECL western blotting detection reagent (Thermo, USA). The antibodies employed in this process included anti-BRCA1 (ab238983), anti-BRCA2 (ab123491), anti-APC (ab40778), anti-CDH1 (ab219332), and β-actin (ab6302).

Targeted bisulfite sequencing analysis

Library preparation: In brief, total DNA (1 μg) was fragmented into approximately 200-300 bp fragments using a Covarias sonication system (Covarias, 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 (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.

cBioPortal analysis: cBioPortal is a user-friendly, open-access platform designed for cancer genomics research [25]. It offers a suite of powerful tools to explore complex cancer genomic datasets. Researchers can easily visualize, analyze, and interpret genetic alterations in various cancers, enhancing our understanding of the disease. In the present study, we used this database to analyze clinically significant mutations across TCGA UCEC samples.

Enrichment analysis: MetaScape is a versatile bioinformatics tool widely used for KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) analysis [26]. It streamlines the exploration of biological pathways, functions, and molecular interactions within large-scale datasets. In this study, we used this valuable resource for GO and KEGG analyses of the mutated genes. A $P<05$ was used as the cutoff criterion for the functional enrichment analysis.

Drug prediction analysis: DrugBank is a comprehensive and authoritative resource in the field of pharmacology [27]. It serves as an essential repository of information on drugs, drug targets, and drug interactions, encompassing both approved pharmaceuticals and investigational compounds. In this study, we used DrugBank database to explore mutated genes' expression regulatory drugs.

Results

Mutation identification via NGS

The analysis of 30 UCEC cases' sequencing data revealed a total of 31 mutations, distributed as follows: 10 mutations in BRCA1, 7 mutations in BRCA2, 6 mutations in APC, and 8 mutations in the CDH1 gene (**Figure 1A**). Notably, all detected mutations exhibited a

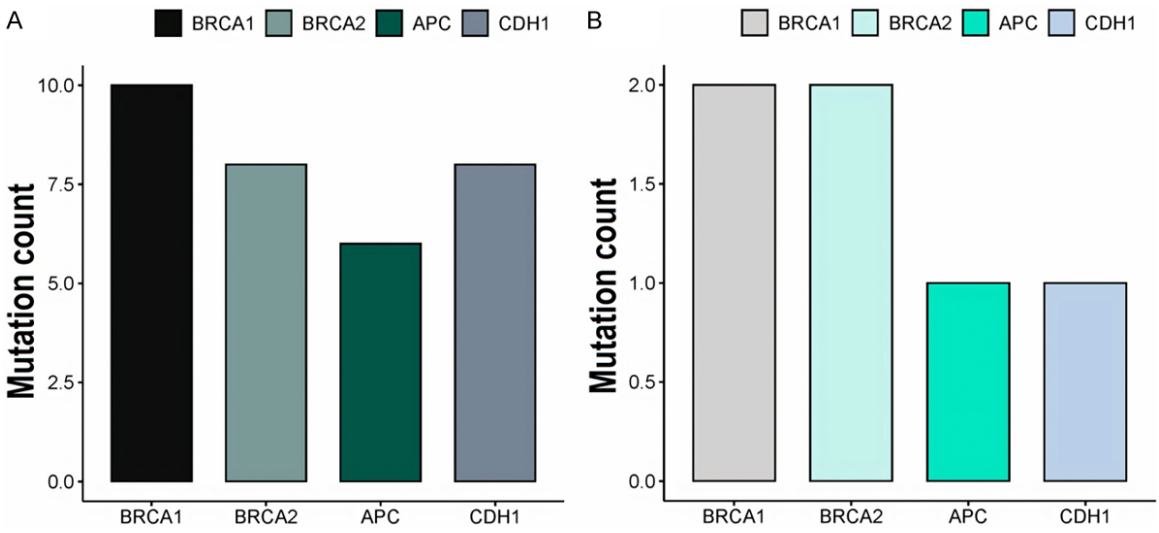

Figure 1. Comprehensive overview of mutations in BRCA1, BRCA2, APC, and CDH1 genes in UCEC samples via NGS. (A) Total count of identified mutations in BRCA1, BRCA2, APC, and CDH1 genes across UCEC samples, and (B) Number of pathogenic mutations detected in BRCA1, BRCA2, APC, and CDH1 genes across UCEC samples.

high mutation quality score of 100. The sequencing reads provided an impressive coverage rate of 97.1%, and the average Quality score (Q30) reached an impressive 97%. In addition to the computational analysis, we utilized the ClinVar database to assess the clinical significance of these observed mutations.

Upon a comprehensive evaluation of mutation calling files for the entire cohort of cases, we identified a sum of 2 pathogenic mutations (constituting 20%) and 8 benign mutations (comprising 80%) within the BRCA1 gene. Similarly, in the BRCA2 gene, we found 2 pathogenic mutations (constituting 25%) and 5 benign mutations (constituting 75%). For the APC gene, our analysis revealed 1 pathogenic mutation (constituting 15%) and 5 benign mutations (constituting 85%). Lastly, within CDH1 gene, we detected 1 pathogenic mutation (constituting 12%) and 7 benign mutations (constituting 88%) in some of the analyzed UCEC samples (**Figure 1B** and **Table 2**).

Clinically valuable mutations

Pathogenic mutations carry critical clinical importance as they directly contribute to disease initiation. These genetic abnormalities disrupt normal cellular processes, leading to abnormal protein production or function [28]. In terms of clinical significance, our investigation identified a total of 2 pathogenic mutations (p.Glu1817Ter and p.Trp1815Ter) in BRCA1, 2 pathogenic mutations (p.Gly173Arg and p.Val211Ile) in BRCA2, 1 pathogenic mutation (p.Gln208Ter) in APC, and 1 pathogenic mutation (p.Asp254Tyr) in CDH1 (**Figure 1B** and **Table 2**).

Screening frequencies of the clinical valuable mutations across Asian UCEC patients via GnomAD database

Low-frequency pathogenic mutations are valuable as population-specific biomarkers due to their ability to identify distinctive genetic variations prevalent in particular populations [29]. To confirm the uniqueness of the pathogenic mutations observed in our studied population, we examined their frequencies in the GnomeAD database. Remarkably, these pathogenic mutations, including BRCA1 (p.Glu1817Ter and p.Trp1815Ter), BRCA2 (p.Gly173Arg and p.Val211Ile), APC (p.Gln208Ter), and CDH1 (p.Asp254Tyr), have not been previously documented in Asian UCEC patients, registering a frequency of 0 in GnomAD database. This suggests that these mutations are specific to the Pakistani population.

Survival outcomes of the UCEC patients harboring pathogenic mutations in BRCA1, BRCA2, APC, and CDH1 genes

In this study, the Kaplan-Meier survival analysis reveals a notable disparity in overall survival (OS) between two cohorts of UCEC patients:

Table 2. Detail of the mutations observed in four DNA repair genes across UCEC patients

Sr. no	Gene	NM:c.DNA	Protein	Nature	No. patients
1	BRCA1	NM_007294.4:c.5449G>T	p.Glu1817Ter	Pathogenic	14
2		NM_007294.4:c.5445G>A	p.Trp1815Ter	Pathogenic	14
3		NM_007294.4:c.5117G>C	p.Gly1706Ala	Benign	17
4		NM_007294.4:c.4985T>C	p.Phe1662Ser	Benign	11
5		NM_007294.4:c.4910C>T	p.Pro1637Leu	Benign	11
6		NM_007294.4:c.4837A>G	p.Ser1613Gly	Benign	19
7		NM_007294.4:c.4816A>G	p.Lys1606Glu	Benign	15
8		NM_007294.4:c.4682C>T	p.Thr1561Ile	Benign	21
9		NM_007294.4:c.4636G>A	p.Asp1546Asn	Benign	24
10		NM_007294.4:c.4535G>T	p.Ser1512Ile	Benign	24
11	BRCA2	NM_000059.4:c.517G>C	p.Gly173Arg	Pathogenic	14
12		NM_000059.4:c.631G>A	p.Val211Ile	Pathogenic	14
13		NM_000059.4:c.5640T>G	p.Asn1880Lys	Benign	15
14		NM_000059.4:c.6943A>T	p.Ile2315Leu	Benign	13
15		NM_000059.4:c.7534C>T	p.Leu2512Phe	Benign	19
16		NM_000059.4:c.7731A>T	p.Lys2577Asn	Benign	23
17		NM_000059.4:c.7902G>A	p.Met2634Ile	Benign	23
18	APC	NM_000038.6:c.622C>T	p.Gln208Ter	Pathogenic	14
19		NM_000038.6:c.295C>T	p.Arg99Trp	Benign	15
20		NM_000038.6:c.715G>C	p.Ala239Pro	Benign	23
21		NM_000038.6:c.995G>A	p.Arg332Gln	Benign	22
22		NM_000038.6:c.1240C>T	p.Arg414Cys	Benign	21
23		NM_000038.6:c.2608C>T	p.Pro870Ser	Benign	21
24	CDH1	NM_004360.5:c.760G>T	p.Asp254Tyr	Pathogenic	14
25		NM_004360.5:c.820G>A	p.Gly274Ser	Benign	23
26		NM_004360.5:c.892G>A	p.Ala298Thr	Benign	15
27		NM_004360.5:c.1018A>G	p.Thr340Ala	Benign	23
28		NM_004360.5:c.1162G>A	p.Glu388Lys	Benign	21
29		NM_004360.5:c.1298A>G	p.Asp433Gly	Benign	16
30		NM_004360.5:c.1409C>T	p.Thr470Ile	Benign	21
31		NM_004360.5:c.2077G>A	p.Gly693Ser	Benign	11

one characterized by the presence of pathogenic mutations in BRCA1, BRCA2, APC, and CDH1 (n=14), and the other comprising patients without pathogenic mutations (n=16) (**Figure 2**). The group harboring pathogenic mutations in BRCA1, BRCA2, APC, and CDH1 exhibits worst OS outcomes when contrasted with the non-mutated group (**Figure 2**). This discovery underscores the clinical significance of BRCA1, BRCA2, APC, and CDH1 pathogenic mutations in influencing disease progression and survival prospects, indicating an association with poorer prognosis in UCEC patients.

Expression analysis of BRCA1, BRCA2, APC, and CDH1 genes

We conducted RT-qPCR assessments of BRCA1, BRCA2, APC, and CDH1 gene expression in two distinct subsets of UCEC samples. One subset comprised samples (n=14) with confirmed pathogenic mutations in BRCA1, BRCA2, APC, and CDH1, while the other subset (n=16) consisted of samples lacking these mutations, forming the non-pathogenic mutation group. Our RT-qPCR analysis revealed a marked increase in the expression levels of these mutated genes in UCEC samples harboring patho-

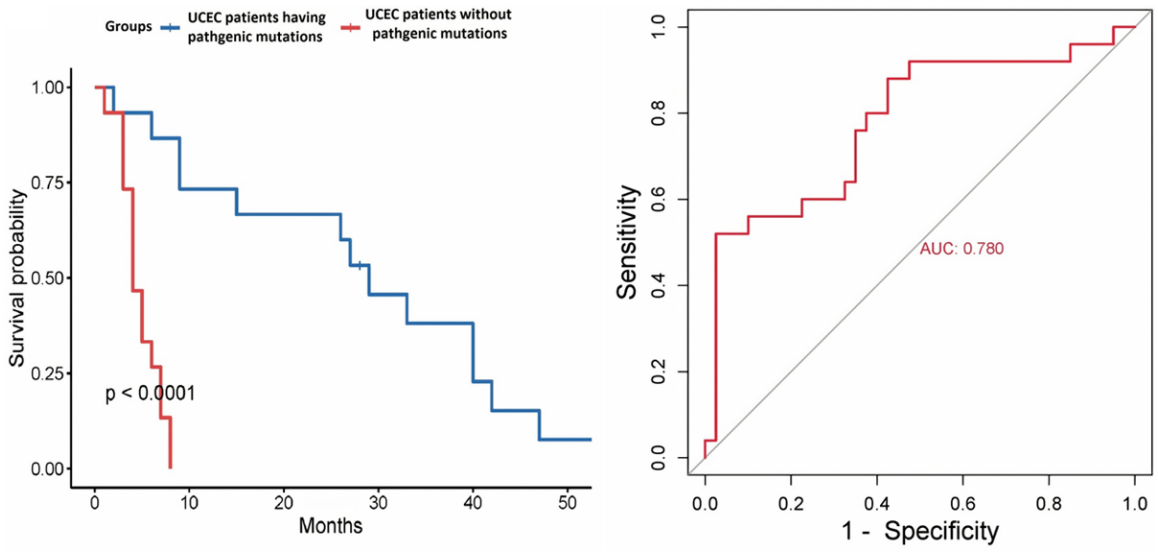

Figure 2. Kaplan-Meier survival analysis of UCEC patients. A significance threshold of P<0.05 was applied to indicate significant outcomes.

genic mutations when compared to UCEC samples devoid of such mutations (**Figure 3A**). Furthermore, the analysis of ROC curves yielded significant results. For BRCA1, BRCA2, APC, and CDH1 expression, a prominent AUC of 1 (*p*-value <0.05) was observed (**Figure 3B**).

Promoter methylation analysis of BRCA1, BRCA2, APC, and CDH1 genes

We carried out a promoter methylation analysis using targeted bisulfite-seq to explore the promoter methylation status of BRCA1, BRCA2, APC, and CDH1 genes within two distinct categories of UCEC samples. The first group comprised samples containing pathogenic mutations in BRCA1, BRCA2, APC, and CDH1, while the second group consisted of UCEC samples lacking such pathogenic mutations, as a counterpart. Our investigation revealed a significant contrast in promoter methylation patterns. In the UCEC sample group with pathogenic mutations, we observed substantial hypomethylation within the promoters of BRCA1, BRCA2, APC, and CDH1 genes as compared to the non-pathogenic mutation group of UCEC samples (**Figure 4**).

Immunohistochemical and western blot analyses of BRCA1, BRCA2, APC, and CDH1 protein expressions

We performed an IHC analysis to evaluate the expression of BRCA1, BRCA2, APC, and CDH1 proteins in UCEC samples. Specifically, we examined two tissue sample containing pathogenic mutations in each of the BRCA1, BRCA2, APC, and CDH1 genes, and one tissue sample without any pathogenic mutations. The objective was to discern potential differences in protein expression between these two types of samples. Upon analyzing the staining results, a noticeable trend became evident. UCEC tissue samples with pathogenic mutations displayed significantly higher levels of BRCA1, BRCA2, APC, and CDH1 proteins compared to their counterparts lacking pathogenic mutations (**Figure 5A**). In addition to the IHC, western blot analysis also revealed that the protein expression of the BRCA1, BRCA2, APC, and CDH1 was significantly higher in UCEC sample with pathogenic mutations as compare to those without pathogenic mutations (**Figure 5B**).

Analysis of clinically valuable pathogenic mutations across TCGA dataset

Following this, our study embarked on an extensive exploration of mutations within the BRCA1, BRCA2, APC, and CDH1 genes in UCEC samples obtained from the TCGA dataset, utilizing the cBioPortal platform. The primary objective was to identify potential genetic variations and their prevalence across diverse populations. The results of this analysis revealed a distinct pattern: the pathogenic mutations detected in BRCA1 (p.Glu1817Ter and p.Trp1815Ter), BR-

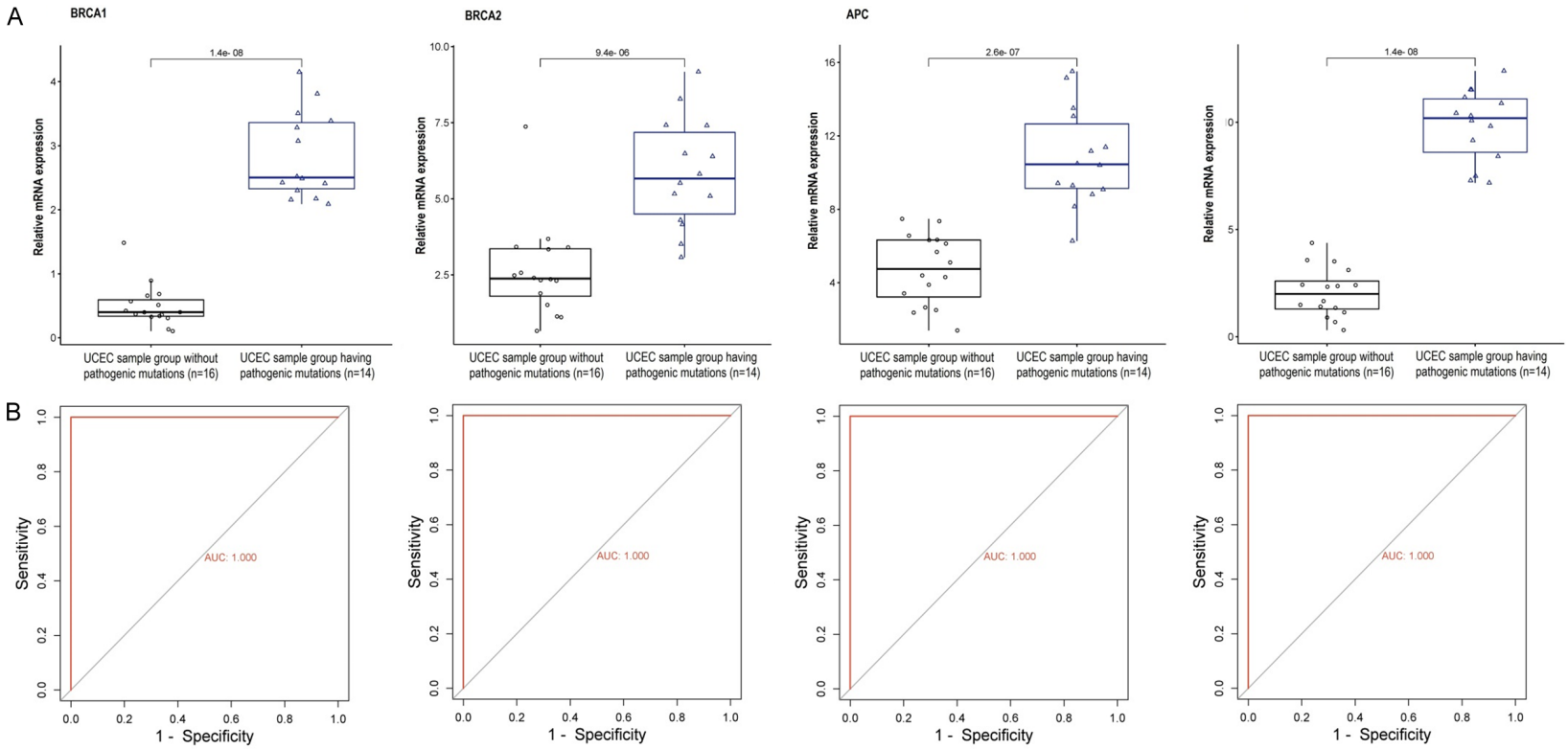

Figure 3. Comparative expression and ROC curve analysis of BRCA1, BRCA2, APC, and CDH1 genes in pathogenic mutated and non-pathogenic mutated UCEC sample groups. (A) Evaluation of relative expression levels of BRCA1, BRCA2, APC, and CDH1 genes through RT-qPCR, and (B) ROC curve analysis based on RT-qPCR expression data for BRCA1, BRCA2, APC, and CDH1 genes. A significance threshold of $P<0.05$ was applied to indicate significant outcomes.

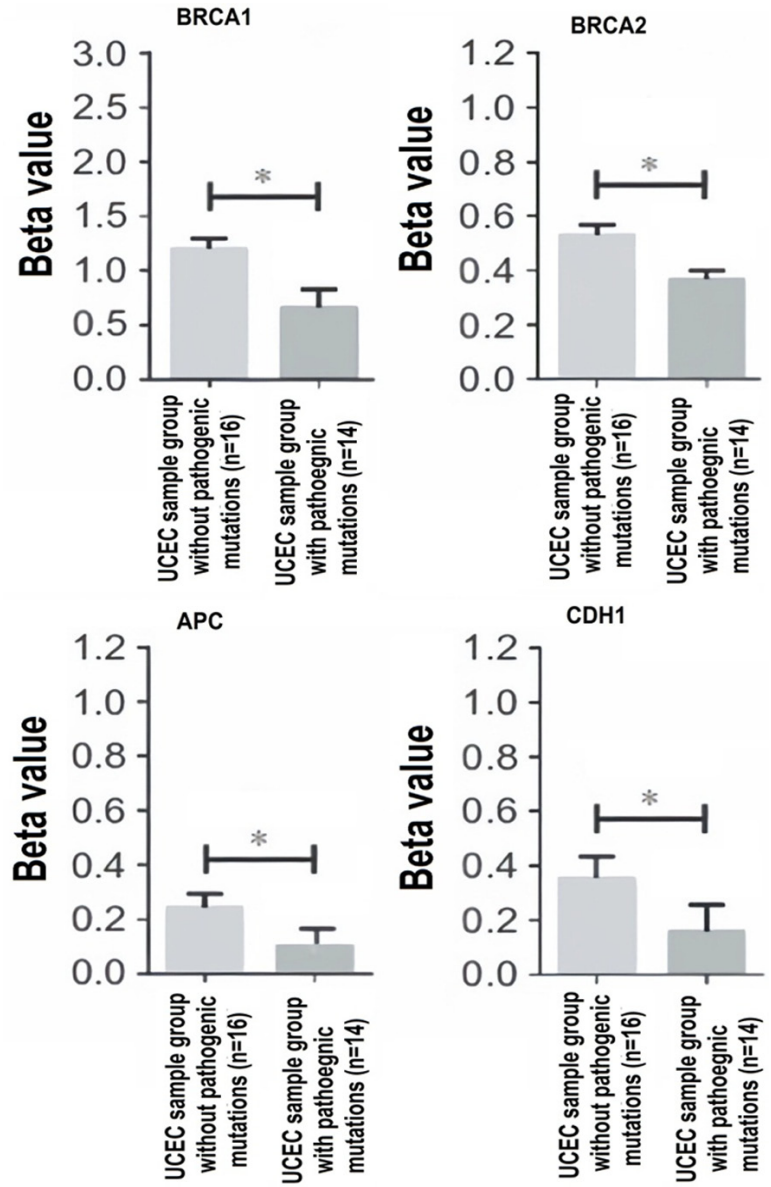

Figure 4. Assessment of methylation levels in BRCA1, BRCA2, APC, and CDH1 genes via targeted bisulfite sequencing in pathogenic mutated and non-pathogenic mutated UCEC sample groups. A significance threshold of $P<0.05$ was applied to indicate significant outcomes.

CA2 (p.Gly173Arg and p.Val211Ile), APC (p.Gln208Ter), and CDH1 (p.Asp254Tyr) in UCEC patients of Pakistani origin were conspicuously absent within the TCGA UCEC samples (**Figure 6**). The absence of these specific pathogenic mutations in the TCGA dataset underscores their unique occurrence within the Pakistani cohort.

Enrichment analysis outcomes

Next, we performed GO and KEGG enrichment analyses. Among GO, BRCA1, BRCA2, APC, and CDH1 genes were enriched in "BRCA1-BARD1 complex, lateral element, BRCA1-A complex, and flotillin complex" etc., CC terms (**Figure 7A**), "gamma-catenin binding, H3 histone acetyltransferase activity, H4 histone acetyltransferase activity, and GTPase activated protein binding" etc., MF terms (**Figure 7B**), "cell cycle DNA replication maintenance of fidelity, response to indole-3-methanol, cellular response to indole-3-methanol, and histon H3-acetylation" etc., BP terms (**Figure 7C**), and "homologous recombination, fanconi anemia pathway, breast cancer, endometrial cancer, basal cell mcarcinoma, and platinum drug resistance in Cancer" etc., KEGG terms (**Figure 7D**).

Drug prediction analysis outcomes

In this comprehensive investigation, we employed the DrugBank database for a systematic exploration of therapeutic strategies aimed at attenuating the expression of underexpressed and mutated genes (BRCA1, BRCA2, APC, and CDH1). Our meticulous analysis unveiled a range of potential drug candidates, each displaying promising attributes for suppressing the expression of BRCA1, BRCA2, APC, and CDH1. Among these candidates, Dasatinib, Doxorubicin, Bortezomib, Cyclosporine, Resveratrol, Lucanthone, Estradiol, Curcumin, Quercetin, Tretinoin (**Table 3**) hold particular significance. These compounds exhibit the potential to effectively modulate the expression levels of the target genes, thus representing promising avenues for innovative therapeutic interventions.

Discussion

Uterine Corpus Endometrial Carcinoma (UCEC) represents a significant public health concern,

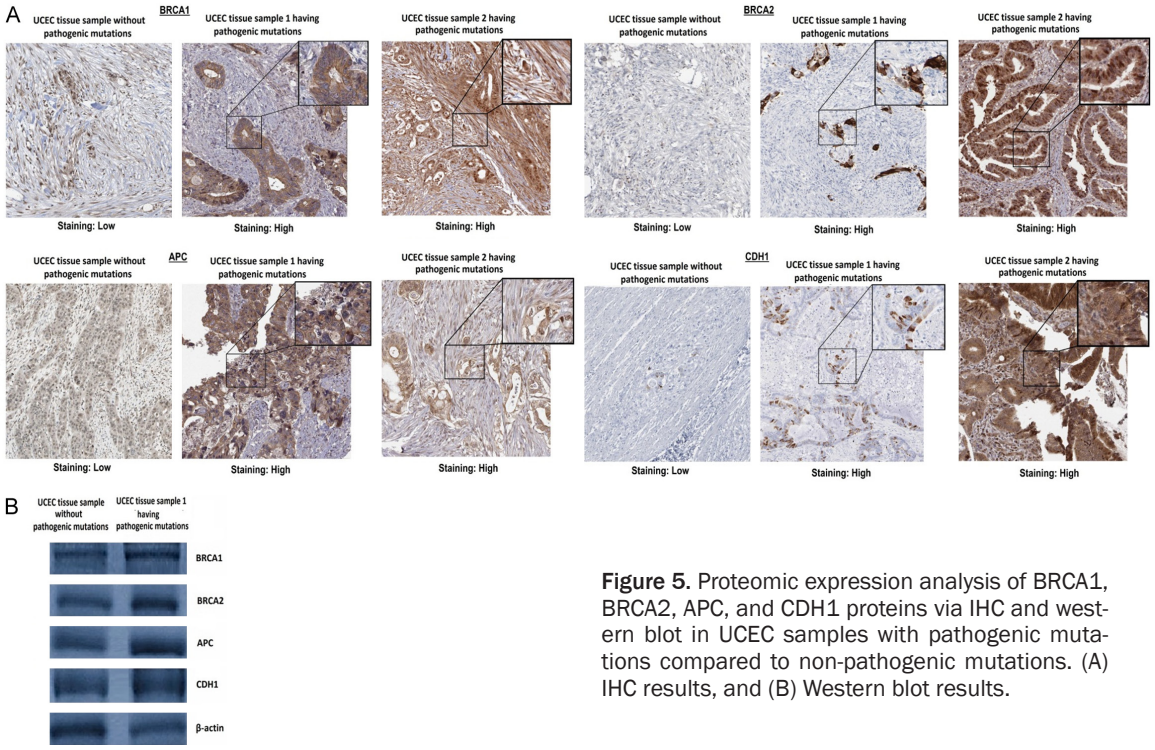

Figure 5. Proteomic expression analysis of BRCA1, BRCA2, APC, and CDH1 proteins via IHC and western blot in UCEC samples with pathogenic mutations compared to non-pathogenic mutations. (A) IHC results, and (B) Western blot results.

particularly among women, due to its increasing incidence and impact on morbidity and mortality [30-32]. UCEC, a subtype of endometrial cancer, is the most common malignancy of the female reproductive tract in developed countries, making it a substantial healthcare burden [5, 33, 34]. Its incidence has been steadily rising, partly due to factors such as obesity, hormone therapy, and an aging population [35-38].

Genetic factors also play a crucial role in UCEC development [39]. Mutations in DNA repair genes can lead to genomic instability, a hallmark of cancer [40, 41]. Among these genes, BRCA1 and BRCA2 have garnered attention due to their association with hereditary breast and ovarian cancers [42, 43]. However, their involvement in UCEC has been less explored, particularly in populations like Pakistan. Therefore, the present study focuses on unraveling the genetic and epigenetic factors contributing to UCEC susceptibility and progression in Pakistani patients.

In the current study, we conducted a comprehensive analysis of 27 major DNA repair gene mutations in Pakistani UCEC patients using NGS. The results revealed Pakistani population-specific pathogenic mutations in four key DNA repair genes: BRCA1 (p.Glu1817Ter and p.Trp1815Ter), BRCA2 (p.Gly173Arg and p.Val211Ile), APC (p.Gln208Ter), and CDH1 (p.Asp254Tyr). Similar to our study, previous studies have identified germline and somatic mutations in BRCA1 and BRCA2 genes in UCEC, emphasizing their role in endometrial cancer susceptibility and treatment response [44, 45]. Additionally, studies have explored the correlation between specific morphological features and microsatellite instability (MSI) status in UCEC, shedding light on the presence of APC pathogenic mutations in this context [46, 47]. Furthermore, investigations have also investigated germline pathogenic mutations in CDH1 gene and their implications in hereditary diffuse gastric cancer, which also has implications for UCEC given CDH1 mutations' broader significance [48, 49].

Furthermore, we assessed the possible functional consequences of these pathogenic mutations in BRCA1, BRCA2, APC, and CDH1 through RT-qPCR and immunohistochemistry (IHC) analyses in UCEC samples. The up-regulation of mutated BRCA1, BRCA2, APC, and CDH1 genes in mutated UCEC samples as compared to the non-mutated samples sug-

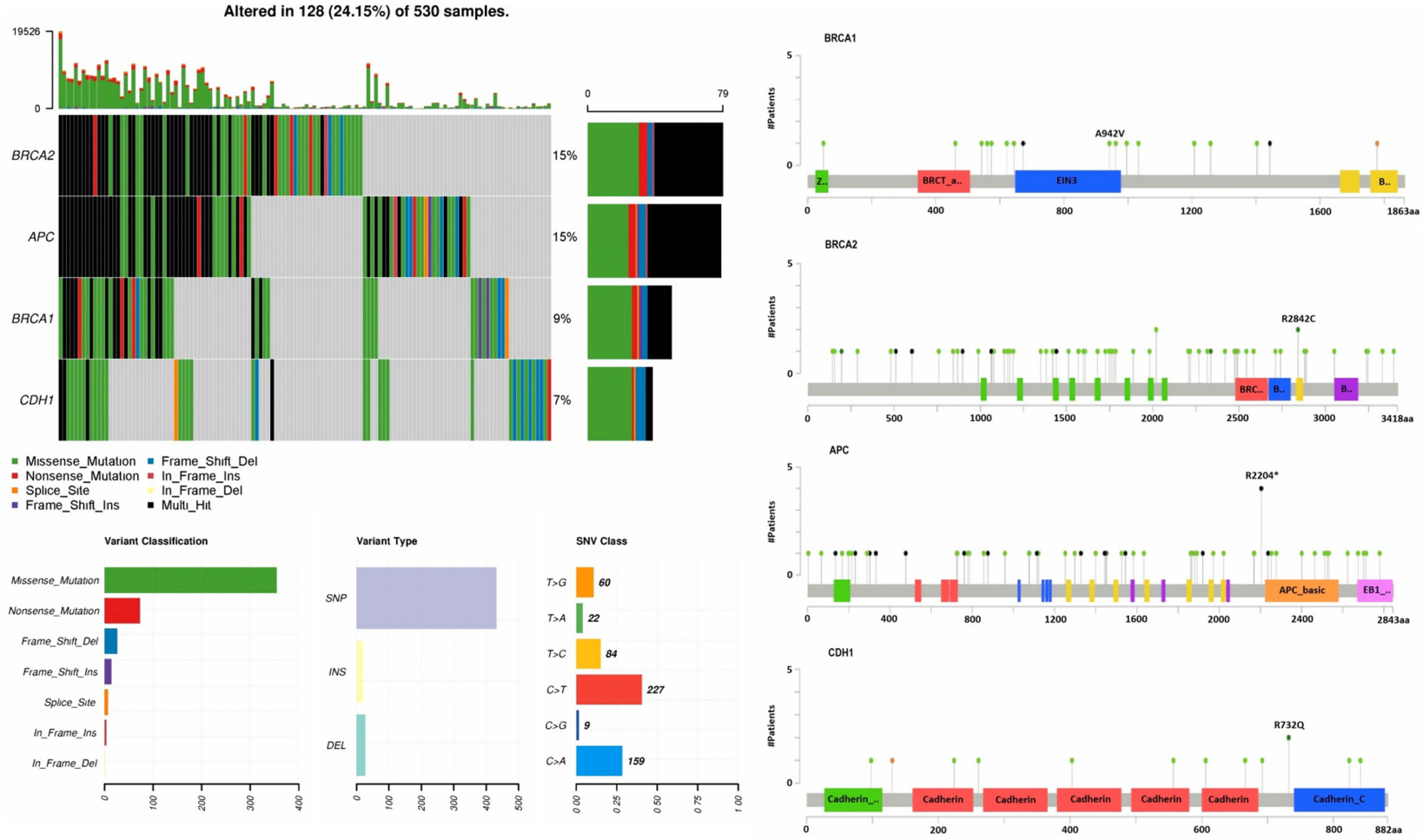

Figure 6. Mutational analysis outcomes of BRCA1, BRCA2, APC, and CDH1 genes across TCGA UCEC samples using cBioPortal platform.

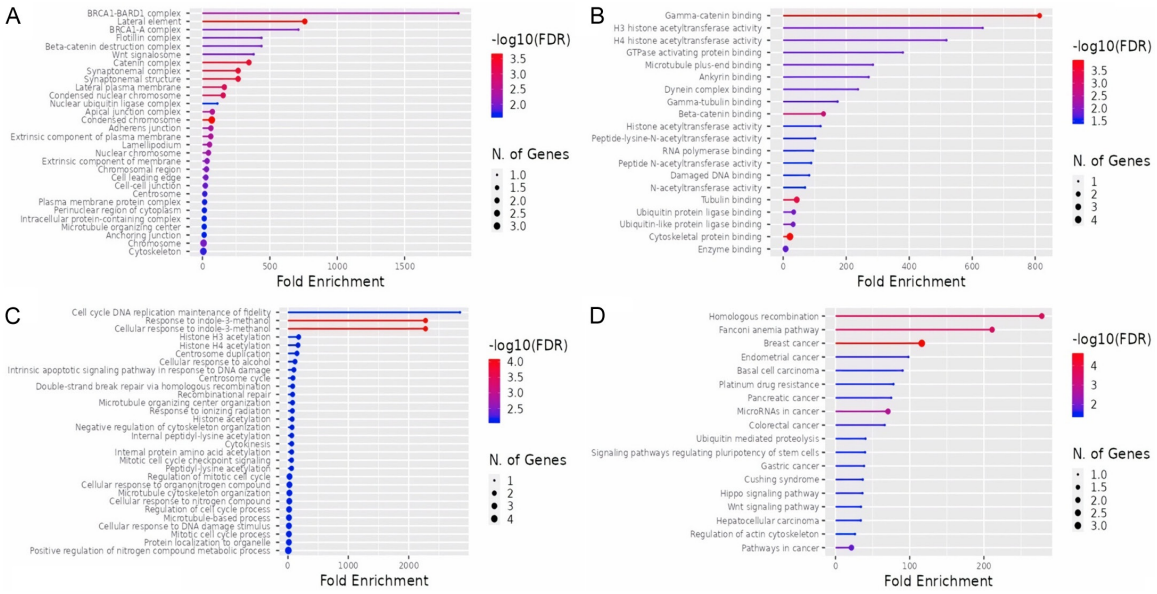

Figure 7. GO and KEGG analyses of BRCA1, BRCA2, APC, and CDH1 genes via metascape. (A) BRCA1, BRCA2, APC, and CDH1 genes-related CC terms, (B) BRCA1, BRCA2, APC, and CDH1 genes-related MF terms, (C) BRCA1, BRCA2, APC, and CDH1 genes-related BP terms, and (D) BRCA1, BRCA2, APC, and CDH1 genes-related KEGG terms. A significance threshold of $P<0.05$ was applied to indicate significant outcomes.

Table 3. DrugBank-based BRCA1, BRCA2, APC, and CDH1 associated drugs

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	BRCA1	Dasatinib	Decrease expression of BRCA1 mRNA	A21899	Approved
		Doxorubicin		A21498	
		Bortezomib		A21448	
		Cyclosporine		A20661	
2	BRCA2	Resveratrol	Decrease expression of BRCA2 mRNA	A23854	Approved
		Lucanthone		A23132	
		Estradiol		A21155	
		Cyclosporine		A20661	
3	APC	Curcumin	Decrease expression of APC mRNA	A21794	Approved
		Quercetin		A23741	
		Tretinoin		A24376	
4	CDH1	Cyclosporine	Decrease expression of CDH1 mRNA	A20661	Approved
		Resveratrol		A23854	

gests their potential association of the observed pathogenic mutations with the enhanced gene activity. Previous studies also suggested that pathogenic mutations can lead to the abnormal expression (up-regulation or down-regulation) variations in the mutated genes in cancer samples [50, 51].

Intriguingly, our investigation of DNA methylation patterns in the promoters of mutated genes (BRCA1, BRCA2, APC, and CDH1) using targeted bisulfite sequencing revealed hypomethylation in the mutated UCEC samples relative to non-mutated samples. This observation underscores the involvement of epigenetic mechanisms in altered gene expression of BRCA1, BRCA2, APC, and CDH1. Similar to our results, previous research has indeed indicated that mutated genes often exhibit hypomethylation in cancer patients. This phenomenon was observed in various cancer types, including breast cancer, colorectal cancer and gastric cancer [52, 53].

In addition to this, KEGG enrichment analysis of the mutated genes (BRCA1, BRCA2, APC, and

CDH1) provides valuable insights into the functional consequences of these pathogenic mutations. Specifically, the disruption of essential DNA repair pathways, such as the homologous recombination pathway, underscores the impact of these mutations on the genomic stability of UCEC cells. The homologous recombination pathway plays a critical role in repairing DNA double-strand breaks and maintaining genomic integrity [54, 55]. The observed disruption of this pathway suggests that the identified mutations in BRCA1, BRCA2, APC, and CDH1 genes may compromise the cells' ability to effectively repair DNA damage, potentially contributing to UCEC development and progression. This finding aligns with previous research linking pathogenic mutations in DNA repair genes to increased cancer susceptibility and genomic instability [56, 57].

Another important contribution of this study is the identification of potential drugs (Dasatinib, Doxorubicin, Bortezomib, Cyclosporine, Resveratrol, Lucanthone, Estradiol, Curcumin, Quercetin, Tretinoin) capable of modulating mutated BRCA1, BRCA2, APC, and CDH1expression regulation.

Dasatinib is a tyrosine kinase inhibitor, Doxorubicin is an anthracycline, Bortezomib is a proteasome inhibitor, and Cyclosporine is an immunosuppressant [58-61]. These drugs are chemotherapy agents, which established interactions with DNA and potentially influence the BRCA1 expression, highlighting their relevance for cancer therapy [58-61]. Resveratrol, known for its antioxidant properties, and Lucanthone, originally studied for its antischistosomal effects, exhibit promise in cancer therapeutic research due their impact of DNA repair pathways by modulating BRCA2 expression [62, 63]. Estradiol, a form of estrogen, is another important chemotherapeutic drug because of its potential interaction with BRCA2 for inducing cell death [64]. Curcumin is a natural compound found in turmeric, Quercetin is a flavonoid with antioxidant and anti-inflammatory properties, and Tretinoin is a derivative of vitamin A, have demonstrated anti-cancer properties, including effects on various signaling pathways. With respect to treating UCEC, there are evidences indicating Curcumin, Quercetin, and Tretinoin abilities to modulate APC function and Wnt signaling [65-67]. Cyclosporine is known to exert indirect influences on CDH1 expression by modulating cellular processes involved in adhesion and migration [68]. Resveratrol on the other hand exhibits antioxidant and anti-inflammatory effects and has been implicated in influencing CDH1 expression, potentially impacting cancer treatment [69]. The exploration of these drugs as part of personalized therapeutic strategies could offer new avenues for the management of UCEC patients with pathogenic mutations in BRCA1, BRCA2, APC, and CDH1 genes, further advancing precision medicine approaches in cancer treatment.

Conclusion

Overall, our study enhances our understanding of UCEC susceptibility and progression in Pakistani patients by considering both genetic and epigenetic factors. The identified mutations have potential implications for prognosis and therapeutic strategies, offering avenues for personalized treatment approaches in UCEC patients. Further research is needed to elucidate the precise mechanisms underlying these findings and translate them into clinical applications.

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Disclosure of conflict of interest

None.

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