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CPA4 overexpression correlates with poor prognosis and tumor progression in endometrial cancer

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Abstract

Background The rise in endometrial cancer rates globally calls for advanced diagnostic methods and new biomarkers. CPA4, known for its role in cancer development, has not yet been studied in relation to endometrial cancer, making it a promising research avenue.

Methods We analyzed CPA4's mRNA expression using data from TCGA and GEO databases and validated these findings with 116 clinical samples through immunohistochemical analysis. The Ishikawa and Hec-1-A cell lines were used to examine CPA4's functionality. In addition, we conducted correlation analysis, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Set Enrichment Analysis (GSEA), and survival analysis to understand CPA4's role in endometrial cancer prognosis. A nomogram model was developed for clinical prognostic predictions.

Results CPA4 is significantly overexpressed in endometrial cancer, correlating with tumor progression and poor prognosis. Overexpression is linked to crucial functions, such as mitosis and cell cycle. Reducing CPA4 in cell lines inhibited tumor growth and spread. Kaplan–Meier plots and Cox regression analysis confirmed CPA4's significance in prognosis, with our predictive model showing high accuracy.

Conclusions CPA4 emerges as a vital biomarker for diagnosing and prognosing endometrial cancer, presenting a novel pathway for research and clinical application. The study highlights its potential as a clinical tool, paving the way for improved patient management and treatment strategies in endometrial cancer.

Keywords Endometrial cancer, Bioinformatics, CPA4, Prognosis, Biomarkers

Introduction

Endometrial cancer, the most common gynecological tumor, accounts for at least 400,000 female deaths annually [1, 2]. In contrast to other gynecological cancers, endometrial cancer is witnessing a rising trend in incidence and mortality rates globally, with an alarming shift towards younger ages [3, 4]. While there are several types of endometrial cancer, the majority of cases are diagnosed early. However, high-grade variants show a tendency to recur and have poor prognoses [5, 6]. Current detection largely relies on symptom presentation, a method with low specificity and no dedicated screening

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tests. This highlights the critical need for innovative biomarkers in screening, diagnosing, and predicting outcomes in endometrial cancer [7, 8]. There's significant room for advancement in managing this disease, underscoring an area ripe for research exploration.

Carboxypeptidase A4 (CPA4), also known as CPA3, is a member of the zinc-containing metallo-carboxypeptidase family [9], located on chromosome 7q32 in humans. Predominantly expressed from maternal alleles in various fetal tissues [10], CPA4 is linked to hormone regulation and is thought to play a role in cell growth and differentiation [11]. It has been closely associated with the development and progression of numerous cancers [12], including prostate, pancreatic [13], stomach [14], liver [15], colon [16], lung [17], bladder [18], and breast cancer [19]. These associations suggest a potential role in the proliferation, metastasis, and invasion of tumor cells. However, no studies to date have established a link between CPA4 and the development or progression of endometrial cancer.

In this study, we leveraged the Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) to analyze the expression of CPA4 and its correlation with clinical features. We further validated its expression using immunohistochemistry on 116 endometrial cancer clinical specimens. In addition, a protein–protein interaction (PPI) network was constructed using CPA4 and its associated differentially expressed genes, alongside Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) to predict CPA4's role in facilitating the onset and progression of endometrial cancer. Utilizing online database data, we developed a nomogram tool aimed at providing clinicians with a new method to predict the prognosis of endometrial cancer patients, thereby aiding in the formulation of more effective treatment strategies. Finally, we used endometrial cancer cell lines Ishikawa and Hec-1-A to validate the function of CPA4 and elucidate the molecular mechanisms by which CPA4 promotes the progression of endometrial cancer.

Materials and methods

Expression data acquisition and processing

For our study, RNAseq data from 33 tumor samples were sourced from the TCGA database (https://portal.gdc.cancer.gov). These data were meticulously organized and extracted in TPM (Transcripts Per Million) format, then transformed using a log2 (value + 1) calculation for enhanced clarity. To augment our data set, we harnessed the GEOquery package to obtain RNAseq data from the GSE17025 data set in the GEO database. This additional data underwent a second normalization process using the normalizeBetweenArrays function in the powerful limma package. [20, 21]. Throughout our meticulous data processing phase, we tackled the challenge of probe multiplicity: probes linked to multiple molecules were removed, ensuring data set integrity. Furthermore, when encountering multiple probes for the same molecule, we strategically selected the probe with the highest signal value to maintain data precision. All data processing and analytical procedures were expertly carried out using R software, version 4.2.1.

Differential expression analysis of genes and diagnostic ROC curve drawing

To conduct statistical analysis on RNAseq data, the stats package and car package were employed. The selection of the statistical method was carried out using the Wilcoxon signed-rank test. Subsequently, a box diagram illustrating the variances in CPA4 gene expression across pan cancer, the UCEC project, and GSE17025 was generated. To assess the diagnostic effectiveness of CPA4 in endometrial cancer, ROC analysis was undertaken using the pROC package, and the resulting ROC curve was plotted. The visualization of these aforementioned outcomes was facilitated by the usage of the ggplot2 utility.

Specimens

The research undertaken received the green light from the Ethical Review Committee of the School of Nursing at Jilin University, Changchun, China. This study entailed the collection of paraffin-embedded specimens from 116 patients diagnosed with endometrial cancer between December 2012 and December 2019, alongside 15 normal controls, for comparative analysis. The follow-up protocol was meticulously adhered to, thereby ascertaining the overall survival time of these UCEC patients. Participation in the research was preceded by informed consent from all patients. Criteria for patient inclusion were rigorously defined as follows: (i) initial diagnosis of UCEC, with treatment comprising standard surgery and/or radiotherapy, calibrated according to FIGO staging and the patient's specific pathology type; (ii) confirmation of the tumor diagnosis by an experienced gynecologic pathologist; (iii) postoperative pathology outcomes, meticulously evaluated by a seasoned gynecologic pathologist in alignment with the FIGO staging criteria (2009 version); and (iv) availability of complete follow-up data. Exclusion criteria were clearly delineated: (i) a personal history of other cancers; (ii) preoperative exposure to radiotherapy, chemotherapy, or hormonal therapy; and (iii) presence of secondary uterine tumors. The comprehensive clinical and pathological data utilized were sourced from the case database of the Second Hospital of Jilin University, as detailed in Table 1.

Table 1 Baseline characteristics of 116 patients

Characteristic	Low expression of CPA4	High expression of CPA4	*p*
n	58	58	
Tumor invasion (%), *n* (%)			0.316
< 50	43 (37.1%)	37 (31.9%)	
> = 50	15 (12.9%)	21 (18.1%)	
Histological type, *n* (%)			0.756
Endometrioid	51 (44%)	49 (42.2%)	
Mixed	6 (5.2%)	6 (5.2%)	
Serous	1 (0.9%)	3 (2.6%)	
Histologic grade, *n* (%)[a]			**0.008**
G1	28 (24.8%)	16 (14.2%)	
G2	20 (17.7%)	37 (32.7%)	
G3	8 (7.1%)	4 (3.5%)	
Lymphatic metastasis, *n* (%)[a]			1.000
No	37 (40.2%)	41 (44.6%)	
Yes	7 (7.6%)	7 (7.6%)	
FIGO grade, *n* (%)[b]			0.062
Stage I	45 (38.8%)	36 (31%)	
Stage II	5 (4.3%)	8 (6.9%)	
Stage III	6 (5.2%)	14 (12.1%)	
Stage IV	2 (1.7%)	0 (0%)	
Age, *n* (%)			1.000
< 55	27 (23.3%)	26 (22.4%)	
> = 55	31 (26.7%)	32 (27.6%)	
Radiation therapy, *n* (%)			0.401
No	45 (38.8%)	40 (34.5%)	
Yes	13 (11.2%)	18 (15.5%)	
Menopause status, *n* (%)			0.116
No	15 (12.9%)	24 (20.7%)	
Yes	43 (37.1%)	34 (29.3%)	
Diabetes, *n* (%)			0.806
No	47 (40.5%)	49 (42.2%)	
Yes	11 (9.5%)	9 (7.8%)	
Ki67, median (IQR)	47.5 (30, 70)	60 (46.25, 70)	**0.031**
AOD, median (IQR)	0.2 (0.18, 0.21)	0.25 (0.23, 0.27)	< 0.001

[a] Data incomplete as some record data were lost

[b] FIGO, International Federation of Gynecology and Obstetric

Immunohistochemistry

In this experiment, we meticulously replicated the immunohistochemistry (IHC) staining technique described in a prior study [22]. The process began with the sample undergoing a 24-h fixation in 10% formalin at room temperature, followed by embedding in paraffin wax, resulting in sections of 3 μm thickness. These sections were then immersed in EDTA retrieval buffer (Wuhan Bost Biotechnology Co., LTD, catalog number AR0023) for 20 min at room temperature. To prevent nonspecific binding, we applied 5% bovine serum albumin (Boster Biotech, product code AR1006). The primary antibody used was rabbit anti-CPA4 (Proteintech, product code: 268,241-AP; dilution: 1:100). This was followed by incubation with the secondary antibody, Goat anti-Rabbit IgG H&L (HRP) (Servicebio, product code GB23204; dilution: 1:200), at 37 °C for 30 min. Visualization of the reaction products was achieved using 3,3' diaminobenzene (Boster Biological Technology, Inc.) as a chromogen, and sections were counterstained with 0.1% hematoxylin (Boster Biological Technology, Inc.) at room temperature for 2 min. Tissue images were captured under an optical microscope (AE2000, Motic) at a 200× magnification. The density of positively stained cells was quantified

using Image J software, with results expressed as average optical density (AOD) values. Notably, the immunohistochemical staining assessments were independently conducted by two experienced pathologists from the Second Hospital of Jilin University, under double-blind conditions, ensuring unbiased evaluation.

Relationship between CPA4 expression and prognosis of patients with endometrial carcinoma

We utilized the Stats package to conduct a thorough statistical examination of the CPA4 expression data and associated clinical information, culminating in a detailed baseline information table. To test the proportional hazards hypothesis, we employed the survival package, followed by Cox regression analysis. Univariate samples that met the preliminary inclusion criterion of $p < 0.1$ were subsequently selected for multivariate Cox regression analysis, leading to the construction of a comprehensive model. The survminer and ggplot2 packages were instrumental in visually representing the results, enabling us to generate Overall Survival (OS), Progress Free Interval (PFI), and Kaplan–Meier (KM) survival curves for the disease under study. To further assess the predictive value of CPA4 expression in determining patient prognosis in endometrial cancer cases, we applied the timeROC package. This facilitated the generation of time-dependent ROC curves, providing valuable insights into the prognostic capabilities of CPA4 expression. In addition, we utilized the rms package to develop a nomogram correlation model and to conduct Calibration analysis, enhancing the precision of our prognostic assessments. It is pertinent to note that the prognostic data leveraged in our analysis were sourced from an article published in the journal 'Cell', ensuring the reliability and relevance of our research [23].

Single gene difference analysis and single gene correlation analysis

Samples were stratified into high and low expression groups based on CPA4 molecule levels. The raw Counts matrix from the chosen public data sets underwent differential analysis using the DESeq2 package, in line with established protocols [24, 25]. The results of this differential analysis were elegantly visualized using the ggplot2 package, which facilitated the creation of volcano plots for single-gene differential analysis. For these plots, we set the logFC (log fold change) threshold at 1 and the p value threshold at 0.05.

Further exploration involved analyzing the identified differential genes using the STRING database. This allowed us to construct a comprehensive protein–protein interaction network (PPI). We then imported these results into Cytoscape software and employed the MCODE plug-in to pinpoint HUB genes within the differential gene pool. These HUB genes were crucial in generating a heat map that illustrated co-expression patterns associated with CPA4.

To deepen our understanding of CPA4's function, we conducted a correlation analysis between CPA4 and other molecules. This analysis utilized Pearson correlation analysis, supplemented by the BH method for p value correction. Ultimately, a total of 41 genes exhibiting the strongest correlations were selected. These genes were pivotal in generating heat maps that depicted their intricate co-expression patterns.

Functional enrichment analysis

Functional enrichment analysis was tailored specifically for the human species (Homo sapiens). To facilitate this, gene IDs obtained from the single-gene differential analysis were converted using the org.Hs.eg.db package. We then conducted a comprehensive GO versus KEGG enrichment analysis utilizing the clusterProfiler package. The z-score value for each enriched entry was accurately calculated using the GOplot package, which utilized the Log|FC| (logarithm of the absolute fold change) value of the analyzed molecule [26, 27]. Moreover, a thorough Gene Set Enrichment Analysis (GSEA) was carried out on all genes, again employing the clusterProfiler package for this purpose. For this analysis, we selected the h.all.v7.2.symbols.gmt [Hallmarks] gene set from the msigdbr package as our reference gene set, ensuring a robust and relevant analytical framework [28].

Cell culture and stably transfected cell line development

The Ishikawa and HEC-1-A human UCEC cell lines, integral to our study, were procured from iCell Bioscience Inc., headquartered in Shanghai. We cultivated the Ishikawa cells in MEM (iCell, product code iCell-0012), enriched with 10% fetal bovine serum (TransGen, product code FS301-02), 1% nonessential amino acids (iCell, product code iCell-01000), and 1% penicillin–streptomycin (Solarbio, product code P1400). Conversely, the HEC-1-A cells thrived in McCoy's 5A medium (iCell, product code iCell-0011), supplemented similarly with 10% fetal bovine serum and 1% penicillin–streptomycin. Both cell lines were meticulously maintained in a humidified atmosphere with 5% CO2 at a constant temperature of 37 degrees Celsius.

For the construction of the pLKO.1–Scramble and pLKO.1–shCPA4 plasmids, we utilized the lentiviral vector plasmid pLKO.1–Puro (Hunan Fenghui Biotechnology Co., Ltd., product code FH1717). The specific interference sequences for shCPA4 (5′-CCAAAG AACAUCUGAGAUGTT-3′) and shScramble (5'-GTA TAAGTCAACTGTTGAC-3') were carefully selected.

The lentiviral particles were produced employing a three-plasmid packaging system, combining the vector plasmids with packaging plasmids PMD2.G (Fenghui, product code BR037) and psPAX2 (Fenghui, product code BR036), in conjunction with LipofectamineTM 3000 transfection reagent (ThermoFisher, product code L3000150). The resulting complex was introduced into HEK-293 T cells (iCell, product code iCell-h237). Post 48 and 72 h, the medium was harvested and filtered through a 0.22 μm filter, then stored at 4 °C for up to 1 week.

To establish transfected cell lines, we seeded 300,000 Ishikawa and HEC-1-A cells into separate wells of a 6-well plate. After 24 h, we added 1 ml of medium containing the lentivirus to each well. A further 48 h later, the medium was replaced. Cells expressing the puromycin resistance gene, a result of viral infection, were subsequently isolated using 2 μg/mL puromycin. This selection process was continued for a week, culminating in the collection and analysis of the cells.

Real-time PCR

For the isolation of total RNA from both fresh frozen tissue and stably transfected cells, we employed the EasyPure RNA Kit (TransGen, product code ER101-01), following the protocol specified by the manufacturer. Subsequent cDNA synthesis was conducted using the cDNA Synthesis Kit (TransGen, product code AT311-02). To quantify the transcription levels of CPA4, we performed real-time PCR analysis using the SYBR Green qPCR reagent (TransGen, product code AQ132), with GAPDH serving as the internal reference gene.

The specific primers for the CPA4 gene were as follows: forward, 5′-CTGGACGGCAAGGAAGATTGT-3′ and reverse, 5′-GACCGCGTCTTCCTCCATAA-3′. For the GAPDH gene, the primers used were: forward, 5'-GAA GGTGAAGGTCGGAGTC-3', and reverse, 5'-GAAGAT GGTGATGGGATTTC-3'.

PCR amplification was adeptly carried out using the ABI-Q3 instrument from Thermo Fisher Scientific, Inc., under the following conditions: an initial denaturation at 94 °C for 30 s, followed by 45 cycles of 94 °C for 5 s, 51 °C for 15 s, and 72 °C for 10 s. The mRNA expression levels were determined using the 2-ΔΔCq method, ensuring accurate quantification [29].

Cell counting Kit-8 (CCK-8) assay

In this rigorous study, we employed 96-well plates to culture 3,000 cells per well, encompassing a range of cell types, including Ishikawa, HEC-1-A, Ishikawa–shScramble, HEC-1-A–shScramble, Ishikawa–shCPA4, and HEC-1-A–shCPA4. Over designated time intervals of 24, 48, and 96 h, we added 10 μl of CCK-8 reagent (Bioss, product number BA00208) to each well. Following incubation at a controlled temperature of 37 degrees Celsius for 1.5 h, the absorbance in each well was precisely quantified at 450 nm using a microplate reader (Detie, Inc., model E0226).

Invasion assay

In this segment of our study, the invasive potential of the UCEC cells, as previously mentioned, was evaluated using a transwell chamber (Labselect, product code 14,342). In alignment with the manufacturer's guidelines, we pre-coated the chamber with Matrigel (BD Biosciences, product code 356,234) to simulate the extracellular matrix. For the assay, 3×10^4 cells were seeded into the upper chamber in 100μL of serum-free MEM or McCoy's 5A medium. The lower chamber was filled with 600μL of media containing 10% FBS to serve as a chemoattractant. After incubating the cells for 30 h at 37 °C, non-invasive cells remaining on the upper surface were gently wiped away with a cotton swab. The cells that had migrated through the Matrigel and reached the lower surface were then stained using 10% Giemsa solution. We captured images of these stained cells using an optical microscope (Motic, model AE2000) for further analysis.

Wound-healing assay

The wound-healing assay was employed to assess the migratory capacity of the previously mentioned UCEC cells. For this assay, 3×10^5 cells per well were cultured in a six-well plate. To simulate a wound, we created a linear scratch using a 200 μl pipette tip. The wells were then gently rinsed with PBS to remove dislodged cells. The migration of cells into the wound area was monitored and photographed at 24- and 48-h intervals using a digital camera attached to an optical microscope (Motic Corporation). Consistency in data collection was ensured by capturing all micrographs under identical magnification settings.

Colony formation assay

In this part of our study, HEC-1-A and Ishikawa cells were carefully seeded into 6-well plates at a density of 100 cells per well. Over a period of 10 days, these cells were observed to form characteristic colonies. Following this incubation period, the cells were fixed with methanol and stained with 10% Giemsa solution (Biotopped, China) to enhance visibility of the colonies. The quantification of these visible colonies was then carried out, providing an assessment of the colony-forming ability of the cells. To ensure reliability and reproducibility of our results, each experiment was performed in triplicate.

Apoptosis assay and cell cycle assay

In this study, we established cell cultures in 6-well plates at a density of 3×10^5 cells per well. For the apoptosis assay, cells were treated with a combination of Annexin V-FITC and PI in 100 µL of Annexin V binding buffer. This mixture was incubated for 15 min at room temperature, shielded from light. Following the incubation, 400 µL of binding buffer was added to each sample. These samples were then analyzed using a BD FACS Calibur flow cytometer.

The cell cycle assay entailed fixing the cells in a pre-chilled solution of 70% ethanol, serving as a cell fixative, for 1 h at 20 °C. This step was followed by a 30-min incubation in a staining solution containing propidium iodide, RNase-A, and Triton X-100. Similar to the apoptosis assay, the stained samples were analyzed using the BD FACS Calibur. The resultant data was meticulously analyzed using Modfit LT 5 software for detailed cell cycle profiling.

Statistical analysis

In our study, the statistical analyses were based on the mean values derived from three independent experiments, each accompanied by their respective standard deviations (SD). We utilized SPSS software, version 23.0, and R, version 3.6.3, for these analyses. To discern differences between various groups, we employed a range of statistical tests including one-factor analysis of variance (ANOVA) followed by Dunnett's post hoc test, the Kruskal–Wallis test, or the Student's *t* test, as appropriate. A *p* value of less than 0.05 was considered statistically significant in our evaluations.

All the raw code used for the analyses in this study is available in the supplementary materials (Supplementary Material 1) for transparency and reproducibility.

Results

Differential expression analysis of CPA4 in pan-cancer and endometrial cancer

Recent RNA-seq data from the TCGA and GEO databases were analyzed to assess differential expression in pan-cancer samples. The findings, detailed in Supplementary Fig. 1 and Fig. 1A, B, reveal that in non-paired samples, CPA4 expression was significantly higher in various cancers—including BLCA, CHOL, COAD, GBM, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, READ, STAD, THCA, and UCEC—compared to normal tissues. In contrast, CPA4 showed significantly lower expression in BRCA, KICH, PAAD, and PRAD. For detailed statistical data, refer to Supplementary Table 1.

In paired samples, CPA4 expression was markedly higher in CHOL, COAD, HNSC, KIRC, KIRP, LUSC, READ, THCA, and UCEC, while it was significantly lower in KICH and PRAD, compared to normal tissues. Comprehensive statistical analysis is available in Supplementary Table 2. These findings underscore that CPA4 is consistently overexpressed in endometrial cancer compared to normal tissue, in both paired and non-paired samples.

In addition, the expression data of CPA4 from the study GSE17025, which included 91 tumor samples and 12 normal samples, were utilized to validate these findings. The results confirmed significant overexpression of CPA4 in endometrial cancer (Fig. 1C). The constructed Receiver Operating Characteristic (ROC) model yielded an Area Under the Curve (AUC) of 0.836, signifying high accuracy of these results (Fig. 1D).

Analysis of the clinical relevance of CPA4 expression

In this study, immunohistochemistry was employed to assess the Average Optical Density (AOD) values of CPA4 in 116 endometrial cancer clinical specimens and 15 normal tissue samples. As depicted in Fig. 2A, CPA4

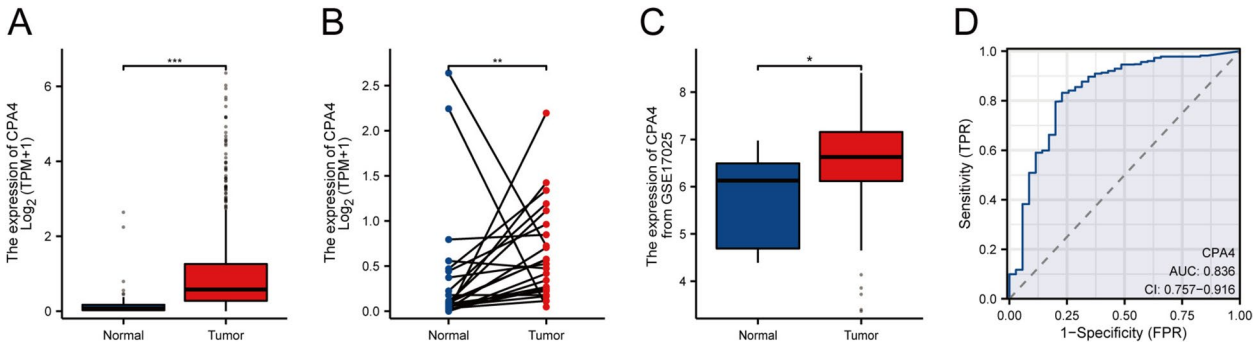

Fig. 1 Differential expression of CPA4 in endometrial carcinoma. **A** Analysis of CPA4 differential expression in unpaired samples. **B** Analysis of CPA4 differential expression in paired samples. **C** Analysis of CPA4 differential expression using data from data set GSE17025. **D** ROC curve for CPA4 as a biomarker

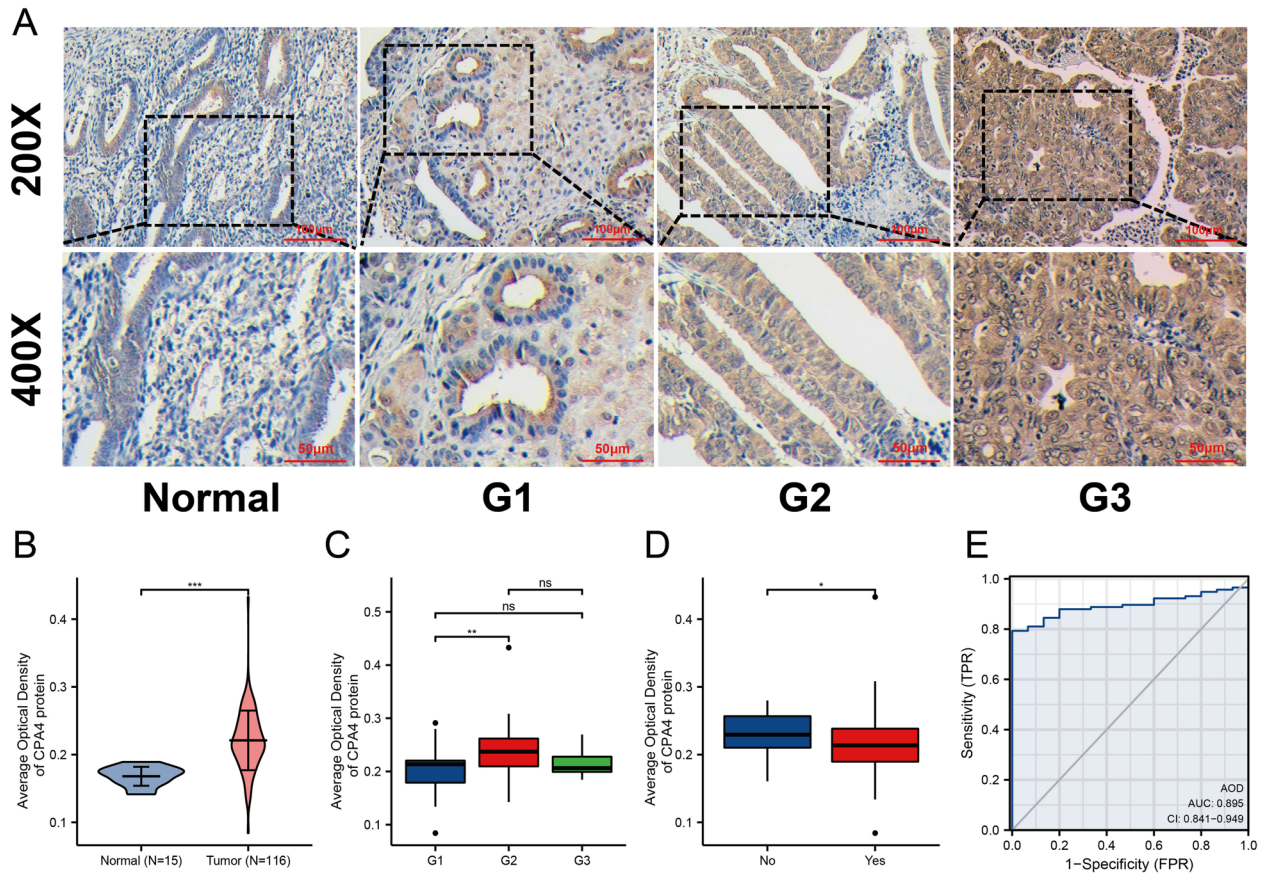

Fig. 2 Expression and clinical correlation of CPA4 in endometrial carcinoma clinical samples. **A** Immunohistochemical staining images depicting CPA4 in endometrial carcinoma patients with varying histologic grades. **B** Comparative analysis of CPA4 immunohistochemistry in 116 endometrial carcinoma clinical specimens versus 15 normal endometrial tissue samples. **C**, **D** Comparative assessment of CPA4 protein expression levels in samples with differing clinical characteristics: **C** histologic grade and **D** lymphatic metastasis. **E** Diagnostic ROC curve for CPA4. Significance levels denoted as: ns (not significant), $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

expression varied across tissues with different degrees of differentiation. The expression levels of CPA4 protein in the 116 endometrial cancer samples and 15 normal samples are presented in Fig. 2B. This data indicates that CPA4 protein expression is lower in normal tissues and significantly elevated in tumor tissues. Figure 2C, D demonstrates the variation in CPA4 expression in patients with different histological grades and lymphatic metastasis. The baseline characteristics of these 116 patients are detailed in Table 1. The findings suggest that the expression of CPA4 correlates not only with histological grading but also with the expression of Ki67, a nuclear proliferation antigen. ROC curve for the CPA4 protein expression data is shown in Fig. 2E, with an AUC of 0.895, suggesting a potential close association between CPA4 expression and tumor development and progression.

Survival prognosis analysis of CPA4 expression

To validate the immunohistochemical results, we analyzed the clinical relevance of CPA4 expression in endometrial cancer patients using data from the TCGA database. The results were consistent with the immunohistochemistry findings, indicating that CPA4 expression correlates with different histological grades and levels of lymphatic infiltration in patients, as shown in Fig. 3A, B. In addition, baseline data of TCGA patients presented in Table 2 demonstrate a correlation between CPA4 expression, histological grade, and age in endometrial cancer patients. A ROC analysis (Fig. 3C) was conducted to examine the relationship between CPA4 expression and patient prognosis. Findings revealed 1-year AUC = 0.520, 3-year AUC = 0.601, and 5-year AUC = 0.603, suggesting that high expression of CPA4 is a risk factor for patients. Survival analysis using data from 116 clinical samples, as shown in Fig. 3D, indicates that high CPA4

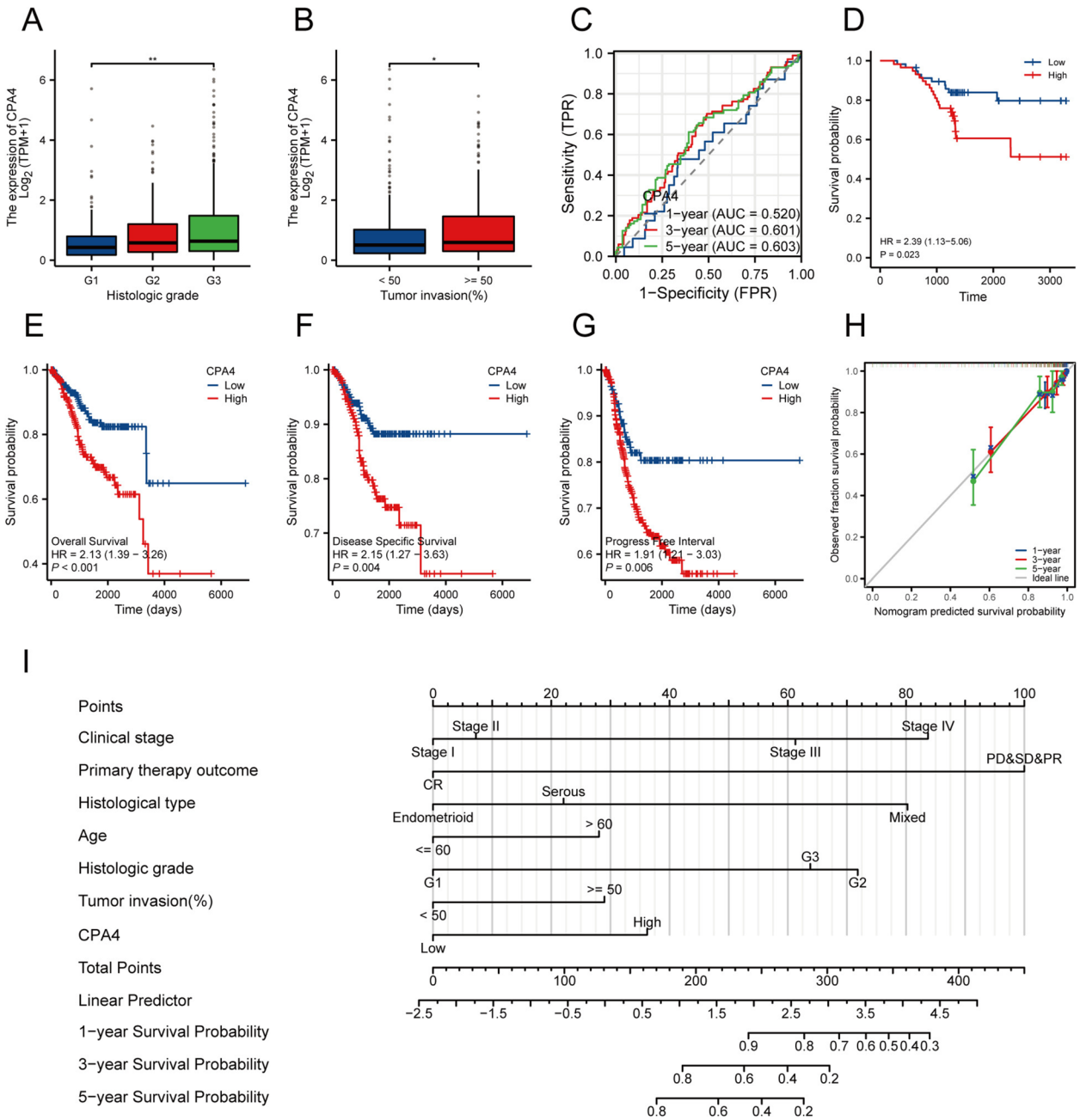

Fig. 3 Survival prognostic analysis and CPA4 expression correlation. **A**–**C** Differential analysis results of CPA4 expression: **A** histologic grade, **B** tumor invasion, and **C** diagnostic ROC curve for CPA4. **D** Kaplan–Meier curves showing CPA4 expression and its relation to different clinicopathological factors in patient subgroups. **E**–**G** Kaplan–Meier curves correlating CPA4 expression with overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI). **H**, **I** Development and validation of a CPA4-based nomogram: **H** calibration graph, **I** nomogram model. Significance levels: ns (not significant, $p \geq 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

expression is predictive of poor prognosis in patients. This is further corroborated by survival prognostic analysis using TCGA data for endometrial cancer patients, which shows that high CPA4 expression is associated with overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI). The elevated expression of CPA4 in these tumors suggests a potential close association with tumor progression, as depicted in Fig. 4E–G.

Table 2 Baseline characteristics of TCGA patients

Characteristics	Low expression of CPA4	High expression of CPA4	*P* value
n	184	155	
Clinical stage, *n* (%)			0.178
Stage I	125 (36.9%)	89 (26.3%)	
Stage II	12 (3.5%)	18 (5.3%)	
Stage III	38 (11.2%)	40 (11.8%)	
Stage IV	9 (2.7%)	8 (2.4%)	
Primary therapy outcome, *n* (%)[a]			0.345
PD&SD&PR	17 (5%)	10 (2.9%)	
CR	167 (49.3%)	145 (42.8%)	
Age, *n* (%)			0.018
< =60	59 (17.4%)	69 (20.4%)	
>60	125 (36.9%)	86 (25.4%)	
Histological type, n (%)			0.153
Endometrioid	145 (42.8%)	114 (33.6%)	
Serous	34 (10%)	30 (8.8%)	
Mixed	5 (1.5%)	11 (3.2%)	
Histologic grade, *n* (%)			0.014
G1	53 (15.6%)	24 (7.1%)	
G2	40 (11.8%)	41 (12.1%)	
G3	91 (26.8%)	90 (26.5%)	
Tumor invasion (%), *n* (%)			0.497
<50	110 (32.4%)	87 (25.7%)	
> =50	74 (21.8%)	68 (20.1%)	
Menopause status, *n* (%)			0.349
Pre	17 (5%)	8 (2.4%)	
Peri	7 (2.1%)	7 (2.1%)	
Post	160 (47.2%)	140 (41.3%)	
Diabetes, *n* (%)			0.595
No	133 (39.2%)	116 (34.2%)	
Yes	51 (15%)	39 (11.5%)	
BMI, *n* (%)			0.260
< =30	65 (19.2%)	64 (18.9%)	
>30	119 (35.1%)	91 (26.8%)	

[a] PD, Progressive Disease; SD, Stable Disease; PR, Partial Response; CR, Complete Response

Construction and validation of a CPA4-based nomogram for prognostic prediction in endometrial cancer

To enhance the prognostic prediction of endometrial cancer patients, this study has developed an easily interpretable nomogram, serving as a tool for clinical assessment of prognosis in endometrial cancer. Initially, we employed both univariate and multivariate Cox regression analyses to examine the relationship between various clinicopathological factors, including CPA4 expression, and patient prognosis. These analyses, detailed in Fig. 4, identified that both univariate and multivariate results consistently indicated that high expression of CPA4 adversely affects patient prognosis. This finding also suggests that CPA4 could be a novel target for tumor therapy.

Our constructed nomogram, as shown in Fig. 3I, utilizes factors such as clinical staging, primary therapy outcome, histological type, age, histological grade, tumor infiltration depth, and CPA4 expression for prediction purposes. To validate the accuracy of this predictive model, we generated a calibration chart, depicted in Fig. 3H. The model demonstrated a concordance index (C-index) of 0.823 (range: 0.795–0.851), indicating its precise predictive capability. The calibration line's close proximity to the diagonal line further signifies the model's robust calibration performance.

To further validate the molecular efficacy of CPA4 as a clinical prognostic predictor, we conducted both univariate and multivariate Cox regression analyses using various clinical data collected from our own patient samples. The results are presented in Supplementary Table 3. In the multivariate Cox regression analysis, we identified FIGO stage, histological type, and Ki67 as significant factors influencing patient prognosis. Based on these variables, we developed a prognostic nomogram model. The accuracy of this model in predicting 1-year, 3-year, and 5-year survival probabilities was assessed using calibration curves (Supplementary Fig. 2). The results indicate that the model demonstrates strong predictive performance for clinical application.

Comprehensive analysis of single-gene differential expression and correlation with CPA4

The results of the single-gene differential analysis are depicted in the volcano plot in Fig. 5A. A total of 730 genes met the threshold of $|\text{LogFC}|>1$ and $\text{p.adj}<0.05$, with 539 genes being upregulated and 191 genes downregulated. These 730 genes were input into the STRING database to construct a differential protein interaction network, identifying 16 HUB genes (CASP14, SPRR2E, SPRR2B, SPRR2G, SPRR2F, SPRR2A, SPRR2D, SPRR3, SPRR1B, S100A7, IVL, SPRR1A, RPTN, CDSN, PI3, LOR).

The PPI network of these HUB genes, which are primarily proteins related to the terminal differentiation of keratinocytes or keratins, is illustrated in Fig. 2B. Based on these genes, we created a gene co-expression heatmap with CPA4, as shown in Fig. 5D. Finally, a single-gene correlation analysis was conducted for CPA4, selecting the top 41 genes with the strongest correlation. The heatmap depicting these correlations with CPA4 is presented in Fig. 2C.

By analyzing the differential expression of 41 genes associated with CPA4 expression in endometrial cancer,

A

Characteristics	Total(N)	HR(95% CI) Univariate analysis	P value Univariate analysis
Clinical stage	553		< 0.001
Stage I	342	Reference	
Stage II	52	1.738 (0.833 − 3.626)	0.14
Stage III	130	3.084 (1.911 − 4.977)	< 0.001
Stage IV	29	8.082 (4.497 − 14.524)	< 0.001
Primary therapy outcome	482		< 0.001
PD&SD&PR	38	Reference	
CR	444	0.129 (0.078 − 0.214)	< 0.001
Histological type	553		< 0.001
Endometrioid	411	Reference	
Mixed	24	2.428 (1.040 − 5.672)	0.04
Serous	118	2.674 (1.744 − 4.100)	< 0.001
Age	551		0.007
<= 60	207	Reference	
> 60	344	1.850 (1.162 − 2.944)	0.009
BMI	520		0.861
<= 30	211	Reference	
> 30	309	0.963 (0.634 − 1.464)	0.861
Histologic grade	542		< 0.001
G1	99	Reference	
G2	121	7.111 (1.616 − 31.297)	0.009
G3	322	13.303 (3.262 − 54.242)	< 0.001
Tumor invasion(%)	475		< 0.001
< 50	261	Reference	
>= 50	214	2.825 (1.752 − 4.554)	< 0.001
Menopause status	506		0.508
Pre	35	Reference	
Peri	17	0.338 (0.042 − 2.745)	0.31
Post	454	0.842 (0.388 − 1.826)	0.664
Diabetes	453		0.524
No	329	Reference	
Yes	124	1.167 (0.728 − 1.871)	0.52
CPA4	553		< 0.001
Low	276	Reference	
High	277	2.126 (1.387 − 3.260)	< 0.001

B

Characteristics	Total(N)	HR(95% CI) Multivariate analysis	P value Multivariate analysis
Clinical stage	553		
Stage I	342	Reference	
Stage II	52	1.136 (0.377 − 3.422)	0.82
Stage III	130	2.950 (1.565 − 5.560)	< 0.001
Stage IV	29	4.383 (1.895 − 10.138)	< 0.001
Primary therapy outcome	482		
PD&SD&PR	38	Reference	
CR	444	0.171 (0.089 − 0.331)	< 0.001
Histological type	553		
Endometrioid	411	Reference	
Mixed	24	4.123 (1.458 − 11.659)	0.008
Serous	118	1.476 (0.776 − 2.810)	0.235
Age	551		
<= 60	207	Reference	
> 60	344	1.641 (0.899 − 2.996)	0.107
Histologic grade	542		
G1	99	Reference	
G2	121	3.562 (0.774 − 16.397)	0.103
G3	322	3.092 (0.691 − 13.842)	0.14
Tumor invasion(%)	475		
< 50	261	Reference	
>= 50	214	1.668 (0.901 − 3.090)	0.104
CPA4	553		
Low	276	Reference	
High	277	1.895 (1.073 − 3.347)	0.028

Fig. 4 Cox regression analysis outcomes for clinicopathological factors and CPA4 expression. **A** Univariate Cox analysis results. **B** Multivariate Cox analysis results

we identified significant differences in the expression levels of these genes between endometrial cancer tissues and normal endometrial tissues. Specifically, genes such as PADI1, KRT16, KRT17, and S100A9 were significantly upregulated in endometrial cancer tissues, while NDRG1, IL36RN, and MMP1 were notably downregulated (Supplementary Fig. 3).

Further prognostic analysis of these associated genes using online data sets revealed that the expression levels of certain genes are correlated with clinical outcomes.

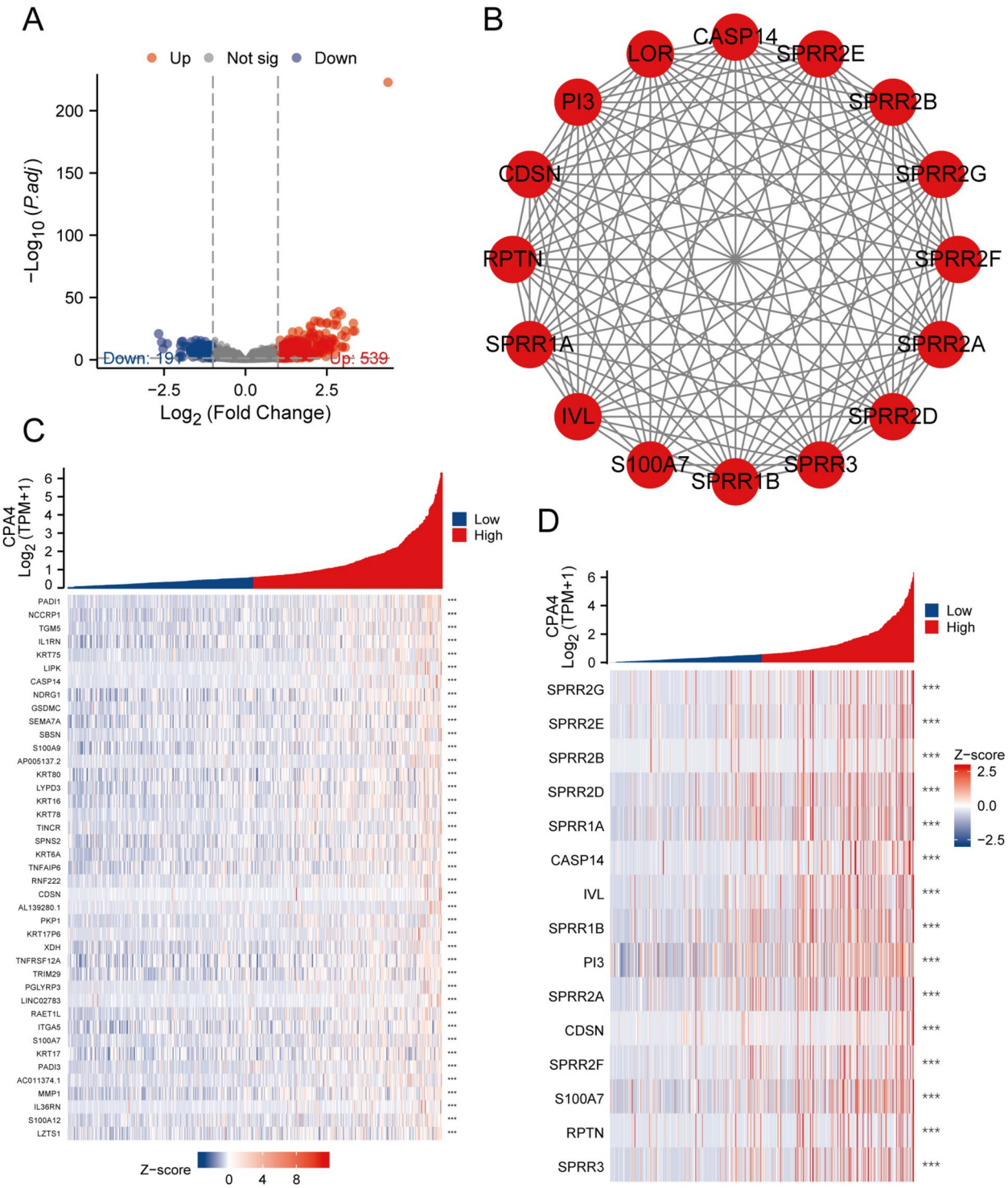

Fig. 5 Single gene differential analysis and correlation with CPA4. **A** Volcano plot illustrating differential expression analysis of CPA4. **B** Protein–protein interaction (PPI) network of HUB genes. **C** Heatmap of the top 41 genes correlated with CPA4. **D** Heatmap showing coexpression of HUB genes and CPA4

For instance, high expression of S100A9 and IL36RN was associated with poorer prognosis, whereas low expression of NDRG1 and MMP1 was similarly linked to unfavorable outcomes. These findings suggest that these genes may play crucial roles in the progression and prognosis of endometrial cancer (Supplementary Figs. 4, 5).

Functional enrichment analysis of CPA4 in endometrial carcinoma

CPA4 and its differentially expressed genes were subjected to GO and KEGG functional enrichment analyses. The GO analysis revealed significant enrichment in several aspects. Under 'Biological Processes,' the genes are involved in keratinization, keratinocyte differentiation, organization of intermediate filaments, and intermediate filament-based processes. Regarding 'Molecular Functions,' pathways such as G-protein coupled receptor binding, serine-type endopeptidase inhibitor activity, peptidase regulator activity, structural constituent of cytoskeleton, interleukin-1 receptor binding, and metalloendopeptidase activity were notably enriched. In the 'Cellular Components' category, pathways such as keratin fibers intermediate filaments, intermediate filament cytoskeleton, motile cilia, and axoneme were enriched, as shown in Fig. 6A and Table 3.

The KEGG functional enrichment analysis indicated significant enrichment in pathways, such as Staphylococcus aureus infection, olfactory transduction, estrogen signaling pathway, complement and coagulation cascades, and neuroactive ligand–receptor interaction, as shown in Fig. 6B, C, and Table 3. Finally, GSEA functional enrichment analysis was used to predict the role of CPA4 in the progression of endometrial carcinoma. It revealed that CPA4 is associated with processes, such as allograft rejection, oxidative phosphorylation, Kras signaling pathway, epithelial–mesenchymal transition, and hypoxia, as illustrated in Fig. 6D–F.

Impact of CPA4 downregulation on proliferation, invasion, and metastasis in endometrial carcinoma cells

We investigated the effects of CPA4 downregulation using HEC-1-A and Ishikawa cell lines. As demonstrated in Fig. 7A, there was a marked decrease in CPA4 expression. Cell proliferation rates, assessed via CCK-8 assays, showed a significant reduction in both cell lines, as illustrated in Fig. 7B, C. Wound healing assays further revealed that cells with reduced CPA4 exhibited notably weaker metastatic capabilities over time compared to the control group, as shown in Fig. 7D through Fig. 7D. In addition, colony formation and Transwell assays indicated a substantial decrease in the invasive and proliferative abilities of cells with CPA4 knockdown, as depicted in Fig. 7G–J. Finally, flow cytometry analysis of apoptosis levels and cell cycle changes in both cell lines indicated increased apoptosis and a higher number of cells in the G2M phase following CPA4 downregulation, as shown in Fig. 7K–N.

Discussion

The increasing incidence and mortality rates of endometrial cancer highlight the critical need for new molecular biomarkers [30–32]. These biomarkers are essential for early diagnosis, accurate prognosis, and the development of effective treatment strategies. Advances in molecular classification, particularly in identifying distinct subtypes, are revolutionizing the approach to endometrial cancer care. This progress, combined with emerging blood-based biomarkers, significantly enhances early detection, enables more precise prognostic assessments, and supports the implementation of targeted therapies [33–36]. Broadening the search for and application of these molecular markers is pivotal in advancing personalized medicine, leading to more customized and efficacious treatments, ultimately improving outcomes and the quality of life for patients with endometrial cancer.

Recent analyses have confirmed CPA4 as a potential biomarker for early cancer diagnosis. In bladder cancer, CPA4 overexpression correlates with poor survival outcomes, similar to findings in endometrial cancer. The link between CPA4 expression and immune cell infiltration in tumors, as evidenced in bladder cancer, may also extend to endometrial cancer, considering the immune system's role in cancer development and progression [18].

The Human Protein Atlas indicates that CPA4 is an oncogene that is enhanced in certain cancers, with its expression significantly correlated with various malignant tumor characteristics, such as size, staging, lymph node metastasis, depth of invasion, and distant metastasis [14, 16, 17]. Studies have demonstrated that high expression of CPA4 is significantly associated with the TNM staging, lymph node metastasis, and distant metastasis of pancreatic cancer [13]. In gastric cancer research, CPA4 expression has been found to be positively correlated with Ki67, a proliferation marker, and negatively correlated with p53, a tumor suppressor protein, suggesting that CPA4 may exert its effects by influencing these key factors in tumor progression. CPA4 expression in bladder cancer [18] has also been linked to the infiltration of various immune cells, including Th1 cells, Th2 cells, T-cell exhaustion, and tumor-associated macrophages (TAMs), indicating that CPA4 may play a role by affecting the infiltration of immune cells in the tumor microenvironment.

Studies in other cancer types, such as breast cancer, have found serum and mRNA levels of CPA4 to be associated with disease aggressiveness and progression,

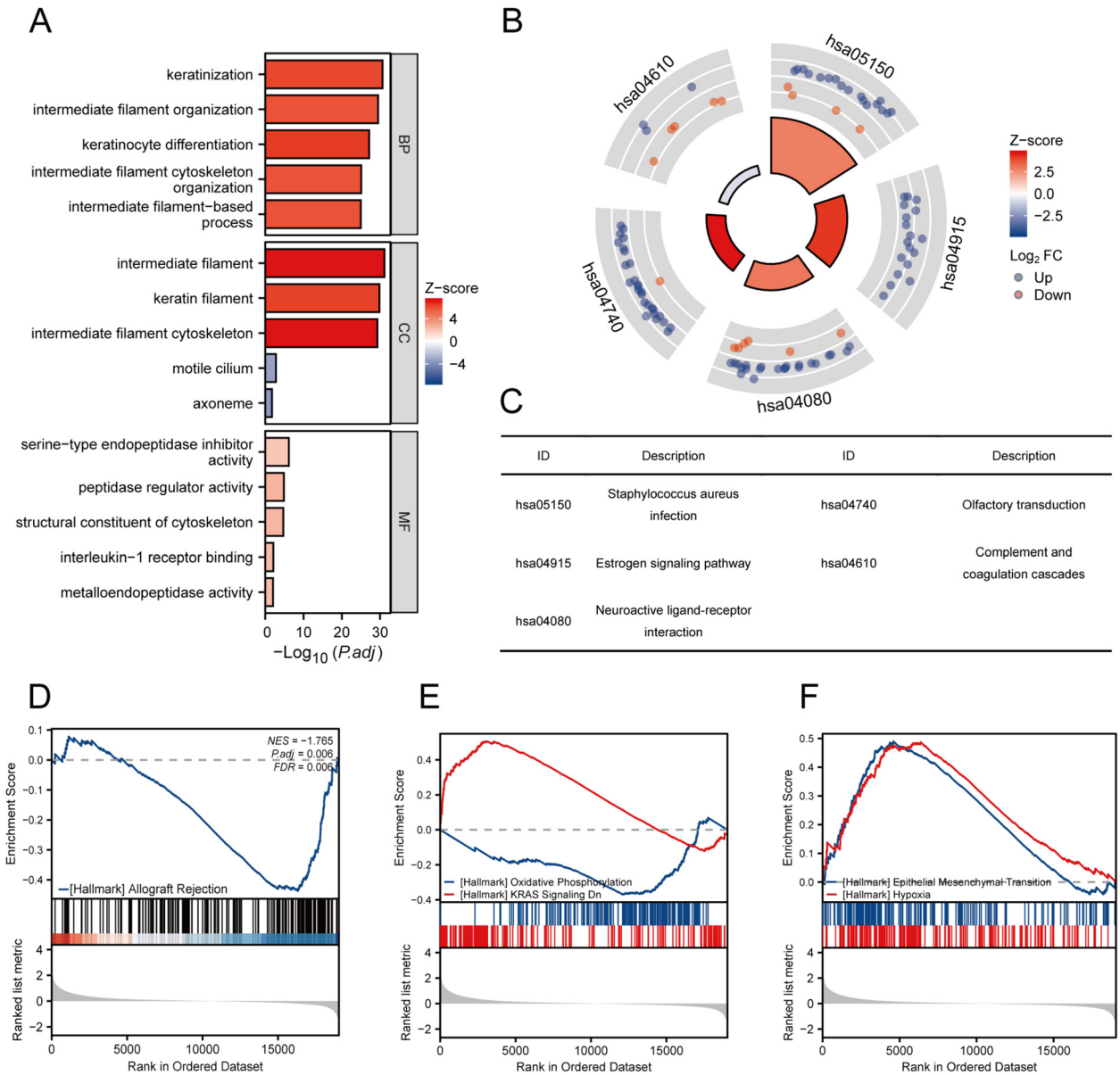

Fig. 6 CPA4 and related differentially expressed genes in UCEC: functional enrichment analysis. **A** GO analysis outcomes. **B**, **C** KEGG analysis results, with corresponding category names for each KEGG identifier. **D**–**F** GSEA analysis findings

highlighting its potential as a diagnostic and prognostic marker [19]. The expression of CPA4 in the endometrium has been documented, with specific data on its RNA expression levels and cell-type specificity, which may inform its expression patterns in endometrial cancer. The observed correlation between CPA4 expression and Ki67, a marker of proliferation, could suggest a role in tumor growth and histological grading, which may be explored further in endometrial cancer.

Given the complexity of cancer biology, these findings should be contextualized within the larger framework of tumor microenvironment interactions and genetic variability among patients. The integration of CPA4 expression data with clinical outcomes and molecular profiling in endometrial cancer could provide insights into its potential as a therapeutic target or a component of prognostic models.

To strengthen the discussion, further research could focus on the functional role of CPA4 in endometrial cancer, exploring how its expression affects tumor behavior and response to therapy, and how it might interact with known pathways of endometrial carcinogenesis.

Table 3 GO and KEGG analysis

Ontology	ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	zscore
BP[a]	GO:0031424	Keratinization	38/582	85/18800	7.28e-35	2.3e-31	6.164414
BP	GO:0045109	Intermediate filament organization	34/582	68/18800	2e-33	3.16e-30	5.830952
BP	GO:0030216	Keratinocyte differentiation	46/582	167/18800	6.92e-31	7.29e-28	6.487446
BP	GO:0045104	Intermediate filament cytoskeleton organization	34/582	88/18800	1.08e-28	8.5e-26	5.830952
BP	GO:0045103	Intermediate filament-based process	34/582	89/18800	1.69e-28	1.07e-25	5.830952
CC[a]	GO:0005882	Intermediate filament	56/644	216/19594	1.93e-34	7.02e-32	7.483315
CC	GO:0045095	Keratin filament	40/644	102/19594	7.93e-33	1.44e-30	6.324555
CC	GO:0045111	Intermediate filament cytoskeleton	58/644	257/19594	4.35e-32	5.26e-30	7.615773
CC	GO:0031514	Motile cilium	21/644	227/19594	2.07e-05	0.0015	− 3.273268
CC	GO:0005930	Axoneme	13/644	131/19594	0.0004	0.0167	− 3.605551
MF[a]	GO:0004867	Serine-type endopeptidase inhibitor activity	18/598	98/18410	2.3e-09	6.54e-07	1.885618
MF	GO:0061134	Peptidase regulator activity	25/598	230/18410	1.36e-07	1.3e-05	2.600000
MF	GO:0005200	Structural constituent of cytoskeleton	16/598	104/18410	2.36e-07	1.94e-05	2.500000
MF	GO:0005149	Interleukin-1 receptor binding	5/598	17/18410	0.0002	0.0076	2.236068
MF	GO:0004222	Metalloendopeptidase activity	12/598	111/18410	0.0003	0.0088	2.309401
KEGG	hsa05150	Staphylococcus aureus infection	21/213	96/8164	2.92e-14	6.68e-12	2.8368326
KEGG	hsa04915	Estrogen signaling pathway	18/213	138/8164	1.56e-08	1.79e-06	4.2426407
KEGG	hsa04080	Neuroactive ligand–receptor interaction	28/213	362/8164	2e-07	1.53e-05	3.0237158
KEGG	hsa04740	Olfactory transduction	28/213	439/8164	9.23e-06	0.0005	4.9135381
KEGG	hsa04610	Complement and coagulation cascades	8/213	85/8164	0.0016	0.0740	− 0.7071068

[a] CC, Cellular component; BP, Biological process; MF, Molecular function

To elucidate the role of CPA4 in tumorigenesis and its association with the 16 hub genes identified in single-gene differential analysis and correlation analysis, an integrative approach is warranted. These hub genes include S100A7, IVL, members of the SPRR family, CASP14, RPTN, CDSN, PI3, and LOR. The comprehensive analysis of literature indicates a multifaceted role of CPA4 in cancer, particularly through its association with hub genes involved in keratinocyte differentiation and function. The exploration of these hub genes and their relationship to cancer can provide insights into the potential functions of CPA4 in endometrial cancer.

S100A7, also known as psoriasin, is associated with tumor progression and has been suggested as a potential diagnostic and prognostic biomarker for esophageal cancer [37, 38]. Its role in promoting migration, invasion, and metastasis of human cancer cells highlights its involvement in aggressive cancer behavior [38]. Moreover, it has been implicated in breast cancer progression, indicating its relevance across different types of cancers [39, 40].

The IVL gene, which codes for involucrin, a protein involved in the formation of the cell envelope in keratinocytes, shows a pattern of downregulation in oral malignancies and is associated with poor differentiation and reduced overall survival [41]. This loss of expression might be indicative of a compromised epithelial barrier, which could be relevant to the progression of endometrial cancer.

The SPRR family genes are associated with increased epithelial proliferation and malignant processes [42]. Specifically, SPRR1B has been identified as a significant prognostic gene in lung adenocarcinoma, indicating its role in cancer prognosis [43]. The involvement of these genes in colorectal tumors and their upregulation in lung squamous carcinoma further underscore their importance in tumorigenesis [44, 45].

(See figure on next page.)

Fig. 7 Effects of CPA4 knockdown in UCEC cell lines on tumoral behaviors. **A** CPA4 expression levels in Ishikawa and Hec-1-A cells post-transfection with shCPA4, evaluated via qRT-PCR. **B**, **C** Proliferation of Ishikawa and Hec-1-A cells assessed by CCK-8 assays. **D**–**F** Metastatic potential of Ishikawa and Hec-1-A cells determined through wound-healing assays. **G**, **I** Colony formation ability of the cell lines analyzed via colony-formation assays. **H**, **J** Migration capabilities of Ishikawa and Hec-1-A cells evaluated using Transwell assays. **K**–**N** Apoptosis and cell cycle progression in the cell lines measured by flow cytometry. Significance levels are denoted as: $p \geq 0.05$; * for $p < 0.05$; ** for $p < 0.01$

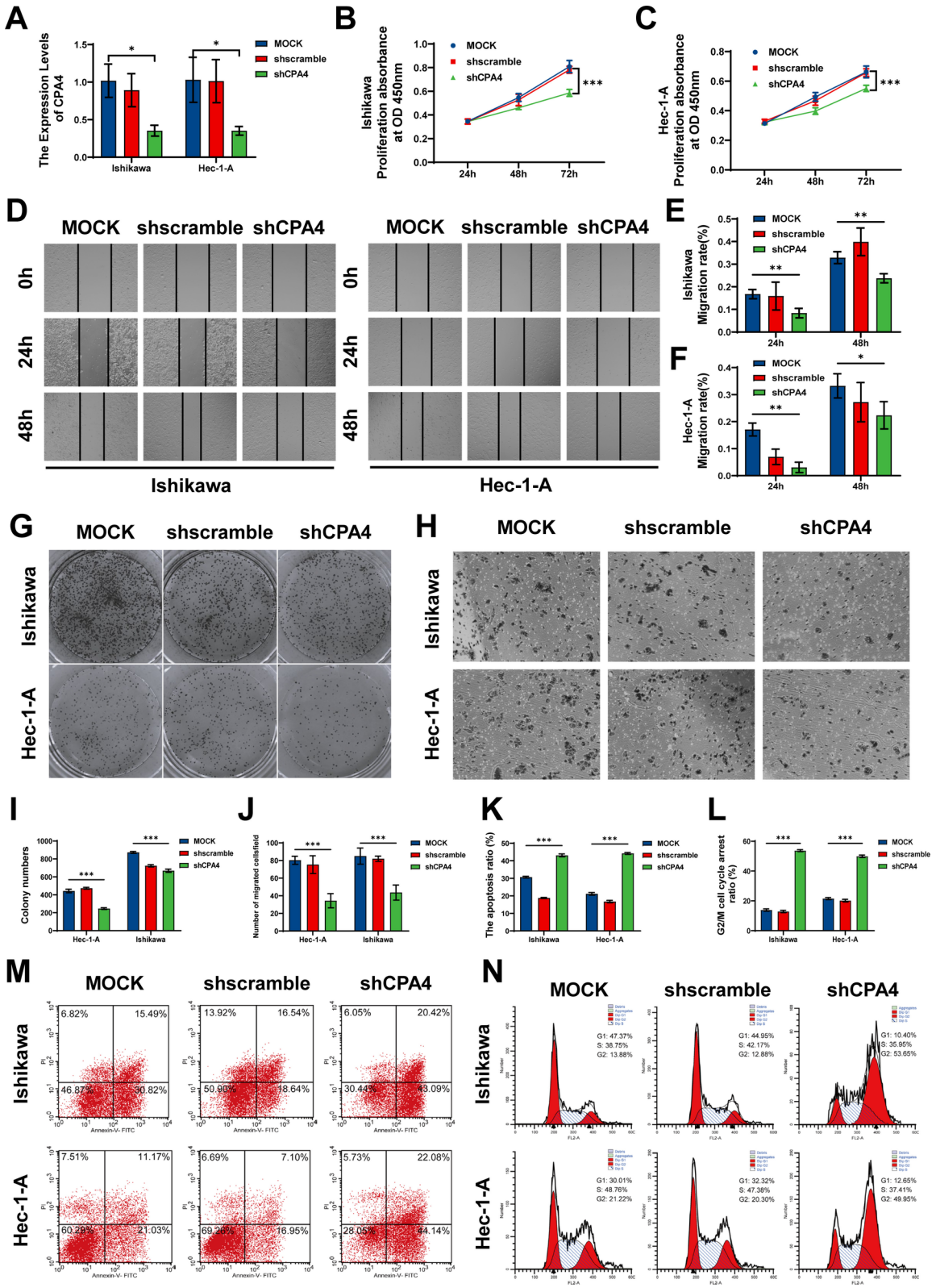

Fig. 7 (See legend on previous page.)

CASP14, a member of the caspase family, is notably higher in triple-negative breast cancer (TNBC) tissues compared to other subtypes, linking it to cancer aggressiveness [46]. It has been implicated in brain metastatic breast cancer and is associated with cancer progression in ovarian and colon cancer [47, 48].

The collective findings from these genes suggest that CPA4, through its interaction with them, may be involved in cell differentiation, immune regulation, and the epithelial-to-mesenchymal transition, all of which are pivotal in cancer progression. Therefore, by understanding the functions of these hub genes, we can predict the potential role of CPA4 in endometrial cancer, particularly in its capacity to affect cell proliferation, differentiation, and possibly immune evasion.

The above synthesis indicates that CPA4, in conjunction with its associated hub genes, may be a key player in the complex molecular pathways that contribute to the development and progression of endometrial cancer. It could potentially serve as a biomarker for cancer aggressiveness and a target for therapeutic intervention. Future research should focus on elucidating the precise molecular mechanisms by which CPA4 and these hub genes contribute to endometrial carcinogenesis and on verifying these findings in clinical settings.

Through a differential expression analysis focused on CPA4, we identified 41 genes associated with CPA4 expression, including KRT16, KRT17, and KRT80, which belong to the keratin family. The high expression of these keratin genes in endometrial cancer tissues may be linked to phenotypic changes in cancer cells and an increase in tumor invasiveness. Alterations in keratin expression can potentially affect the stability of the cytoskeleton and cell–matrix interactions, thereby promoting cancer cell migration and metastasis.

MMP1, a matrix metalloproteinase, is well-known for its critical role in tumor invasion and metastasis [49]. Reduced expression of MMP1 may indicate a decreased requirement for extracellular matrix degradation by tumor cells [50], which could, in turn, limit their invasive capabilities. Conversely, the downregulation of NDRG1 [51]might be associated with cellular stress responses and changes in the tumor microenvironment. IL36RN, an immune-related gene, is highly expressed and may reflect immune dysregulation within the tumor microenvironment, further influencing cancer progression [52]. PADI1 and PADI3 are involved in post-translational protein modification, and their aberrant expression could impact the biological behavior of cancer cells [53]. In addition, LZTS1 and TRIM29 may contribute to the development of endometrial cancer by regulating cell cycle and apoptosis [54].

It can be speculated that the changes in CPA4 expression may influence the occurrence and development of endometrial cancer by regulating the expression of the aforementioned genes. These gene alterations may lead to the remodeling of the tumor microenvironment, enhancing the survival capability of tumor cells and promoting tumor progression. In addition, the immune-related characteristics of these genes may also suggest a possible association between CPA4 and tumor immune evasion. Therefore, in-depth research on the specific mechanisms of action of these genes and CPA4 in endometrial cancer could potentially provide new therapeutic targets for this disease.

The functional enrichment analyses of CPA4 and its differentially expressed genes in the context of endometrial cancer progression have brought to light the significance of several biological processes and molecular functions. The keratinization process, which involves the production of the protective protein keratin, though generally a protective mechanism for the skin, can abnormally contribute to the development of cancers, such as squamous cell carcinoma. This aberrant keratinization indicates a potential role for CPA4 in epithelial tumorigenesis, given keratins' active involvement in cancer cell invasion, metastasis, and treatment responsiveness [55].

Further examination reveals that CPA4-associated genes are actively involved in G-protein coupled receptor (GPCR) binding, an integral part of cellular signaling that governs tumorigenesis including proliferation, invasion, and survival [56–58]. The dysregulation of GPCRs is a common feature in various cancers, implicating CPA4 in the modulation of these receptors, possibly influencing cancer stem cell maintenance and progression [59].

The estrogen signaling pathway's enrichment suggests that CPA4 may also play a role in hormone-dependent cellular processes. Estrogens are known to modify the DNA damage response and DNA repair mechanisms, leading to cancer progression and chemoresistance [60–62]. Given the prominence of estrogen signaling in endometrial cancer, CPA4 could influence disease progression by interacting with this pathway [63].

Complement and coagulation cascades, part of the innate immune response, are also implicated in cancer progression, with these pathways potentially correlating with chemosensitivity and patient survival [64]. The involvement of CPA4 in these cascades indicates a potential impact on the inflammatory response and tumor microenvironment, which are crucial for cancer development and metastasis [65, 66].

Moreover, the KRAS signaling pathway, which is central to cell growth, differentiation, and survival, is often dysregulated in cancer due to mutations [67, 68]. CPA4's association with this pathway suggests it may influence

the tumorigenesis process, particularly since KRAS mutations lead to uncontrolled cell proliferation and are present in a significant fraction of cancers [69, 70].

Finally, the epithelial-to-mesenchymal transition (EMT) process, which is facilitated by CPA4-related pathways, is critical in cancer progression as it enables tumor cells to disseminate, invade, and form metastases [71, 72]. The association with EMT also highlights CPA4's possible contribution to the aggressive behavior of endometrial cancer cells, including their chemoresistance and poor prognosis.

In summary, CPA4 is poised at the intersection of multiple signaling pathways that are critical for the progression of endometrial cancer. These include the regulation of epithelial cell function, immune system interactions, hormonal responses, and the cell's invasive and metastatic capabilities. As a result, CPA4 emerges as a potential biomarker for cancer aggressiveness and a promising target for therapeutic intervention. Future research should focus on elucidating CPA4's molecular mechanisms within these pathways to provide new insights into endometrial cancer's molecular underpinnings and pave the way for novel therapeutic strategies.

Utilizing multivariate Cox regression results, a predictive tool in the form of a nomogram plot was constructed to analyze the model's accuracy. The survival prediction plots for 1, 3, and 5 years showed a good correlation with actual values, suggesting that this model may emerge as a novel and valuable method for prognosis estimation. The Cox regression analysis of clinical samples and the validation of the prognostic nomogram model further support the prognostic value of CPA4 in endometrial cancer. Although other variables, such as FIGO stage and Ki67 index, also demonstrated significant prognostic relevance, the expression level of CPA4 remained an independent prognostic marker. This finding strengthens the hypothesis that CPA4 could be a potential therapeutic target in endometrial cancer. Previous extensive research on molecular biomarkers for endometrial cancer has proposed the use of both protein-coding RNAs and non-coding RNAs as predictors for the incidence and prognosis of the disease [73, 74]. Studies have indicated that genes such as PTEN, KNL1, PPP2R1A, PPP1R14B, L1CAM, and CTNNB1 are associated with the occurrence, progression, and prognosis of UCEC [75]. These findings support the feasibility of CPA4 as a potential molecular marker to aid in the diagnosis and prognostic assessment of endometrial cancer.

Furthermore, although specific inhibitors targeting CPA4 are still in the early stages of research, several studies have laid a critical foundation for their development. Kayashima et al. were the first to reveal the imprinted gene characteristics of CPA4 [10], while Pallarès et al. and Covaleda et al. further elucidated the interactions between CPA4 and its endogenous inhibitors [70, 71]. These studies provide the structural basis for future efforts in screening and designing CPA4 inhibitors. In addition, research by Kim et al. demonstrated that natural products or plant extracts, such as CPA4-1, exhibit inhibitory effects in animal models, offering a new direction for future therapeutic research [72]. Future studies should focus on screening more effective CPA4-specific inhibitors and validating their efficacy across different cancer types.

CPA4 plays a role in various signaling pathways across different types of tumors. In lung cancer cells [76], circCPA4 can regulate the expression of TGF-β2 by acting as a sponge for miR-214-3p. In colorectal cancer, CPA4 promotes the growth of CRC cells by activating the STAT3 and ERK pathways [11]. Shao et al. found that CPA4 promotes the progression of pancreatic cancer by activating the PI3K/Akt/mTOR signaling pathway, suggesting that drugs targeting this pathway, such as everolimus and temsirolimus, might indirectly inhibit the function of CPA4 [12]. In non-small cell lung cancer (NSCLC), the downregulation of CPA4 inhibits the activation of AKT, subsequently reducing the expression of c-MYC. This change leads to G1 phase arrest in tumor cells, inhibits cell proliferation, and promotes the suppression of tumors by inducing apoptosis [77].

In the last, the downregulation of CPA4 via shRNA in HEC-1-A and Ishikawa cell lines led to decreased cell viability, migration, invasion capabilities, an increase in apoptotic cells, and a cell cycle arrest predominantly in the G2/M phase. These cellular experiment outcomes further corroborate the close relationship between CPA4 and cancer cell proliferation, invasion, and metastasis, positioning CPA4 as a potential molecular target for cancer therapy.

In light of recent literature, such as the comprehensive analysis of CPA4 as a poor prognostic biomarker correlated with immune cell infiltration in bladder cancer [18], it is evident that CPA4's overexpression is associated with shorter overall survival and may influence immune response via markers related to T cell exhaustion. These findings enhance the proposition of CPA4 as a valuable biomarker for the diagnosis and prognosis of endometrial cancer, supporting its potential as a target for therapeutic interventions. The investigation into CPA4's role in endometrial cancer progression is a significant step towards understanding the molecular underpinnings of this malignancy and devising new strategies for its management.

In addition, pathway enrichment analysis identified several key signaling pathways, such as PI3K/Akt, MAPK, and TGF-β, which have been extensively reported to be

associated with tumor proliferation, migration, and invasion [12]. In cellular experiments, inhibition of CPA4 resulted in decreased cell proliferation, migration, and invasion, potentially linked to the downregulation of the PI3K/Akt and MAPK signaling pathways. Furthermore, the role of the TGF-β signaling pathway in epithelial–mesenchymal transition (EMT) aligns with previous findings, suggesting that CPA4 inhibition may suppress the EMT process by affecting the TGF-β pathway, thereby reducing the invasive capacity of tumor cells.

These results indicate that CPA4 may influence the progression of endometrial cancer through multiple signaling pathways. However, current research is primarily based on database analysis and functional experiments, lacking further mechanistic validation, especially in vivo studies, which may introduce bias. Future research should focus on verifying the specific roles of these pathways, particularly through in vivo experiments, to explore their functions within the tumor microenvironment.

Future studies should emphasize the use of larger sample sizes and more in-depth pathway enrichment analyses to comprehensively explore the molecular mechanisms of CPA4 in endometrial cancer. Given CPA4's association with multiple key signaling pathways, such as the G-protein-coupled receptor (GPCR) signaling pathway, estrogen signaling pathway, and EMT process, subsequent research should further validate the specific roles of these pathways in CPA4-mediated tumor progression.

Moreover, future research could integrate animal models and clinical samples to evaluate the potential of targeting CPA4-related pathways in the treatment of endometrial cancer. This approach will help clarify the feasibility of CPA4 as a therapeutic target and provide a foundation for personalized treatment strategies.

In summary, the elevated expression of CPA4 is closely associated with the progression, metastasis, and invasion of endometrial cancer, as well as the suppression of immune responses. This suggests that CPA4 could serve as a potential diagnostic and therapeutic biomarker and an independent risk factor for prognosis. Despite its recognized role in cancer, the molecular mechanisms underlying CPA4's function remain poorly understood, particularly in the context of endometrial cancer. Therefore, future research should focus on elucidating the specific biological functions and pathways through which CPA4 contributes to endometrial cancer progression.

Current studies primarily describe the correlation between CPA4 expression and cancer-related phenomena through bioinformatics and cell-based experiments, but the underlying mechanisms have not been thoroughly explored. Specifically, CPA4 may promote cancer cell proliferation, invasion, and metastasis by modulating multiple key signaling pathways. First, CPA4 may regulate cellular behavior via the G-protein-coupled receptor (GPCR) signaling pathway. GPCRs play a crucial role in tumorigenesis, particularly in cancer cell proliferation, migration, and immune evasion. Future studies should investigate whether CPA4 directly or indirectly modulates GPCR activity, influencing cancer cell growth and immune response.

In addition, the enrichment of the estrogen signaling pathway suggests that CPA4 may be involved in hormone-dependent cellular processes. Previous research has shown that estrogen can affect DNA damage repair mechanisms and induce chemoresistance, thereby promoting cancer progression. Therefore, further investigation is needed to determine whether CPA4 interacts with estrogen receptors, modulating DNA repair processes or influencing chemotherapy resistance in tumor cells.

Regarding CPA4's involvement in epithelial-to-mesenchymal transition (EMT), previous studies suggest that EMT is critical for tumor cell migration and invasion. CPA4 may influence this process through the regulation of the TGF-β signaling pathway. Future experiments should explore whether CPA4 facilitates EMT via the TGF-β pathway or other related mechanisms, thereby promoting cancer cell invasion and metastasis.

Although current research has identified CPA4's association with several key signaling pathways, in-depth experimental validation, especially in vivo studies, is lacking. Thus, future research should focus on verifying the molecular mechanisms of CPA4 using animal models and clinical samples. Moreover, larger-scale studies incorporating multifactorial diagnostic models are needed to minimize sample bias and enhance the accuracy of prognostic assessments.

In future investigations, the development of specific CPA4 inhibitors should be a key priority. By leveraging CPA4's structural and functional characteristics, high-throughput screening and structure-based optimization strategies could lead to the identification of effective CPA4 inhibitors. Such inhibitors could provide novel therapeutic options for endometrial cancer and other CPA4-associated malignancies, ultimately contributing to personalized treatment strategies, including targeted therapies and immune checkpoint inhibitors.

Abbreviations

UCEC	Uterine corpus endometrial carcinoma
CPA4	Carboxypeptidase A4
TCGA	The cancer genome atlas
GEO	Gene Expression Omnibus
PPI	Protein–protein interaction
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
GSEA	Gene set enrichment analysis
FIGO	International Federation of Gynecology and Obstetrics
DEGs	Differentially expressed genes
OS	Overall survival

DSS	Disease-specific survival
PFI	Progress free interval
AOD	Average optical density
CCK-8	Cell counting kit-8
ANOVA	One-factor analysis of variance
IHC	Immunohistochemical

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40001-025-02293-0.

Supplementary material 1: Figure S1. Differential Expression of CPA4 in pan-cancer.Analysis of CPA4 differential expression in unpaired pan-samples.Analysis of CPA4 differential expression in paired pan-samples. Adrenocortical Cancer; Bladder Cancer; Breast Cancer; Cervical Cancer; Bile Duct Cancer; Colon Cancer; Colon and Rectal Cancer; Large B-cell Lymphoma; Esophageal Cancer; FFPE Pilot Phase II; Glioblastoma; lower grade glioma and glioblastoma; Head and Neck Cancer; Kidney Chromophobe; Kidney Clear Cell Carcinoma; Kidney Papillary Cell Carcinoma; Acute Myeloid Leukemia; Lower Grade Glioma; Liver Cancer; Lung Adenocarcinoma; Lung Cancer; Lung Squamous Cell Carcinoma; Mesothelioma; Ovarian Cancer; Pancreatic Cancer; Pan-Cancer; Pheochromocytoma & Paraganglioma; Prostate Cancer; Rectal Cancer; Sarcoma; Melanoma; Stomach Cancer; Testicular Cancer; Thyroid Cancer; Thymoma; Endometrioid Cancer; Uterine Carcinosarcoma; Ocular melanomas.

Supplementary material 2: Figure S2**.** Clinical prediction model. Prognostic nomogram model constructed based on these features.calibration curve.

Supplementary material 3: Figure S3. Differential expression of CPA4-related genes in endometrial cancer.

Supplementary material 4: Figures S4. Analysis of CPA 4-related genes for endometrial cancer prognosis.

Supplementary material 5: Figures S5. Analysis of CPA 4-related genes for endometrial cancer prognosis.

Supplementary material 6: Supplementary Material 1. Complete code related to the computational process and model building.

Supplementary material 7: Supplementary Material 2. Ethical documents.

Supplementary material 8.

Supplementary material 9.

Supplementary material 10.

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Author contributions
A.B.C wrote the main manuscript text D.E participated in data collection and form induction F.G participated in the preparation of the pictures and table summary H.I Revise and improve the whole article All the authors reviewed the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of the School of Nursing, Jilin University (Changchun, China). The patients were informed and agreed to participate in the UCEC-relative study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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