Original Article

CLCN3 in mediating the proliferation of human ovarian cancer cells

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Background: Chloride channel-3 (*CLCN3*), a crucial component of the voltage-gated chloride channel family, is implicated in numerous physiological and pathophysiological processes. This study aimed to investigate the characteristics of *CLCN3* in pancancer and its influence on the immune response through the use of a range of databases. Concurrently, we assessed the impact of *CLCN3* on the proliferation of ovarian cancer (OC) cells and explored its potential mechanisms.

Methods: We employed the Tumor Immune Estimation Resource (TIMER) 2.0 and Clinical Proteomic Tumor Analysis Consortium (CPTAC) databases to examine the messenger RNA (mRNA) and the protein expression of *CLCN3* across various cancers. The prognostic significance of *CLCN3* was evaluated using the Gene Expression Profiling Interactive Analysis 2.0 (GEPIA 2.0) database. The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) facilitated the analysis of *CLCN3* promoter methylation levels. The association between *CLCN3* expression and tumor-infiltrating immune cells was investigated using various algorithms. The cBioportal database facilitated the analysis of *CLCN3* mutations and mutation sites across various cancers. The Tumor-Immune System Interactions Database (TISIDB) database was employed to explore the correlation between *CLCN3* expression and immune or molecular subtypes across a variety of cancer types. We collected ovarian tissue samples, encompassing both normal ovarian and OC tissues. The human OC cell lines, SKOV3 cells and OVCAR433 cells, were cultured. *CLCN3* expression was determined via reverse-transcription quantitative polymerase chain reaction (RT-qPCR), while phosphatidylinositol 3-kinase/Akt kinase (*PI3K/AKT*) expression was detected using Western blot. We utilized small interfering RNA (siRNA) technology to suppress CLCN3 expression. The proliferative capacity of SKOV3 and OVCAR433 cells was assessed using the Cell Counting Kit 8 (CCK-8) assay.

Results: *CLCN3* demonstrated an aberrant expression in a number of cancer types and was markedly reduced in OC tissues. Poor prognosis in cervical squamous cell cancer and myeloid leukemia was linked to excessive expression of *CLCN3*. The examination of immune cell infiltration, which included $CD8^+$ T cells, B cells, T regulatory cells, and cancer-associated fibroblast cells, showed a strong association with aberrant *CLCN3* expression. Following the use of siRNA technology, the ability of the ovarian carcinoma cell line SKOV3 and OVCAR433 to proliferate as well as the expression of *PI3K/AKT* both increased.

Conclusions: *CLCN3* is a possible biomarker for immune-related processes and the prognosis of cancer, and the *PI3K/AKT* signaling pathway may affect OC cells' ability to proliferate.

Keywords: Ovarian cancer (OC); chloride channel-3 (*CLCN3*); proliferation; phosphatidylinositol 3-kinase/Akt kinase (*PI3K/AKT*); pancancer

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Introduction

After cervical and endometrial malignancies, ovarian cancer (OC) is the third most prevalent gynecologic carcinoma worldwide (1). In the United States, OC was responsible for 12,810 fatalities and 19,880 recently diagnosed cases in 2022 (2). The absence of certain critical characteristics in the initial stages of OC leads to most patients being diagnosed at an advanced stage (2). Currently, the primary treatment approach for OC involves surgical intervention combined with platinum-based chemotherapy (cisplatin, carboplatin, etc.). Despite receiving appropriate treatment, the majority of patients with OC have an unfavorable outcome due to the recurrence of platinum-resistant disease (3-6). Consequently, finding the hub gene that causes OC is essential for improving the prognosis of those with OC.

Chloride channel-3 (*CLCN3*) is a key member of the voltage-gated chloride channel family (7), which is a $2Cl^-/H^+$ exchanger found on endolysosomal membranes in animals. This anion-proton exchanger affects endocytosis, lysosomal activity, and the ion composition of vesicles (8). It also involved in numerous biological processes in cancer cells, including cell cycle control, cell proliferation, invasion, and migration. According to previous research, human OC cells invade and migrate (9) through the *CLCN3* receptor (10). Additionally, *CLCN3* participates in the amplification of cervical cancer (11) and breast cancer (12). Cisplatin is the primary chemotherapeutic agent used in first-line treatment for OC, and previous research has indicated an association between *CLCN3* and chemoresistance (13-15).

Genes have a significant role in the early detection and prognosis of many malignancies. In this study, in addition to characterizing the association between *CLCN3* and OC, we used multiple bioinformatics platforms to explore the involvement and mechanism of *CLCN3* in 33 different human tumors. In order to identify the significant associations between *CLCN3* expression and mutation status, methylation patterns, and the immunological and molecular subtypes in a variety of tumor types and to highlight the potential significance of *CLCN3* in anticancer immune responses, various analyses and experiments were performed. Moreover, a systematic analysis of the prevalence and predictive value of *CLCN3* was conducted in a variety of tumor types.

The classical phosphatidylinositol 3-kinase/Akt kinase (*PI3K/AKT*) signaling pathway is essential for cell development, proliferation, and apoptosis (16) and has been shown to promote the growth of colorectal cancer cells (17). Consequently, the *PI3K/AKT* signal pathway is thought to play a crucial role in the development and treatment of cancers (18). However, there is still little research on how *CLCN3* and the *PI3K/AKT* signal pathway interact in OC. The aim of this work was thus to clarify the role of *CLCN3* in OC progression via modulating the *PI3K/AKT* signal pathway. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1272/rc).

Highlight box

Key findings

- Chloride channel-3 (*CLCN3*) was found to be aberrantly expressed in many cancer types, and its overexpression was associated with poor prognosis in cervical squamous cell carcinoma and myeloid leukemia. Moreover, immune cell infiltration was shown to be associated with aberrant *CLCN3* expression, and the proliferative capacity and phosphatidylinositol 3-kinase/Akt kinase (*PI3K/AKT*) expression was increased in ovarian cancer (OC) cell lines after knockdown of *CLCN3*.

What is known and what is new?

- *CLCN3* is a $2Cl^-/H^+$ exchanger, which is involved in a variety of physiological and pathophysiological processes, including cell cycle regulation, cell proliferation, invasion, and migration.
- Pancancer analysis of *CLCN3* was performed, and the effect of *CLCN3* on the proliferation of OC through *PI3K/AKT* was confirmed via basic experiments.

What is the implication, and what should change now?

- *CLCN3* may be a biomarker for immune-related processes and cancer prognosis, and the *PI3K/AKT* signaling pathway also has an impact on the proliferative capacity of OC.

Methods

Gene expression analysis

The differential *CLCN3* expression patterns between pancancer and surrounding normal tissues were examined using Tumor Immune Estimation Resource (TIMER) 2.0 (19).

log2 [transcripts per millions (TPM)+1] was used to describe the degree of gene expression. The National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) database (20) was used to examine the protein expression of *CLCN3* in pancarcinoma. Through the use of Gene Expression Profiling Interactive Analysis 2.0 (GEPIA 2.0) (21), the association between *CLCN3* expression and the pathological stage of patients in all malignancies in The Cancer Genome Atlas (TCGA) was examined.

Survival prognosis analysis

The prognostic value of *CLCN3* in terms of overall survival (OS) and disease-free survival (DFS) was evaluated for 33 tumor types through the GEPIA 2.0 database. Additionally, the association of OS and DFS with *CLCN3* genetic alterations was analyzed.

Genetic alteration analysis

CBioportal (22) was used to gather information regarding the frequency of changes, mutation types, and locations in proteins of interest across all TCGA tumors. The prevalence of various methylation types and their corresponding methylation locations in pancarcinoma involving *CLCN3* was also determined. The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) (23) was employed to compare the degree of promoter methylation in *CLCN3* across various cancer types.

Immune cell invasion and expression in molecular and immune subtypes

TIMER 2.0 was used to examine the relationship between *CLCN3* expression and the degree of immune infiltration in several TCGA cancer tissues. For in-depth analysis, B cells, $CD8^+$ T cells, T regulatory cells (Tregs), and cancer-associated fibroblasts (CAFs) were selected. The Tumor-Immune System Interactions Database (TISIDB), a comprehensive database for tumor-immune system interactions, was used for tumor-immune analysis. Associations between *CLCN3* expression and molecular subtypes or the immunology of various cancer types were examined via TISIDB.

Gene enrichment analysis

The BioGRID website (24) was employed to analyze protein-protein interaction networks. The top 100 genes linked with *CLCN3* in all TCGA cancers and normal tissues were retrieved using GEPIA 2.0, and then *CLCN3* and the selected genes were correlated pairwise using Pearson correlation coefficients.

Cell culture

The human OC cell line SKOV3 cells (PRICELLA, Wuhan, China) and OVCAR433 cells (a gift from the University of Science and Technology of China, Hefei, China) were used in this investigation. The cell lines were each cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained at 37 ℃ with a 5% CO_2 atmosphere in a humidified incubator.

Human tissue collection

From 2019 to 2022, fresh tissues were obtained from nine patients with the primary OC diagnosis who did not receive preoperative adjuvant therapy at the First Affiliated Hospital of Anhui Medical University, and nine normal ovarian tissues were obtained from patients undergoing surgery for other gynecological diseases without ovarian involvement at the same hospital.

Liquid nitrogen tanks were used to store all tissue samples obtained after excision and kept at −80 ℃. The matching nearby normal ovarian tissues were obtained from sites about 3 cm distant from the OC tissues. The patients with cancer were grouped into stages IB to IIA according to the 2009 International Federation of Gynecology and Obstetrics (FIGO) classification. None of the patients had been treated with chemoradiotherapy before they underwent radical operation. Written informed consent was provided by all patients. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the First Affiliated Hospital of Anhui Medical University Ethics Committee (No. PJ2023-10-46).

RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Following the instructions provided by the manufacturer, total cellular RNA was retrieved using TRIzol reagent (Sparkjade, Shandong, China), and complement DNA (cDNA) was produced via a reverse-transcription kit (Promega, Madison, WI, USA). Using the LightCycler 480

Table 1 Human gene primers utilized in the research

Genes	Primer sequence (5'-3')
CLCN3	F: TTTATGCCATGGTTGGTGCTGCTG
	R: ATAAATGCCTTCCCTGCCAAAGGC
PI3K	F: CTCTCCTGTGCTGGCTACTGT
	R: GCTCTCGGTTGATTCCAAACT
AKT	F: ACTCATTCCAGACCCACGAC
	R: AGCCCGAAGTCCGTTATCTT
GAPDH	F: GGGAGCCAAAAGGGTCAT
	R: GAGTCCTTCCACGATACCAA

F, forward primer; R, reverse primer.

system (Roche Diagnostics, Basel, Switzerland) and SYBR Green PCR Master Mix (Vazyme Biotech, Nanjing, China), we detected the relative expression of *CLCN3* in OC tissues and normal tissues. *Table 1* contains a list of the primers. Finally, using the ΔΔCt method, we ascertained the relative level of messenger RNA (mRNA) expression.

Western blotting of protein expression

Cells (1.5×10^5 cells per well in six-well plates) were transfected using a combination of small interfering RNA (siRNA) and Lipofectamine2000. Phosphate-buffered saline (PBS) was used to wash the cells three times, and then a 97:1:2 solution of radioimmunoprecipitation assay (RIPA), phenylmethanesulfonyl fluoride (PMSF), and phosphatase inhibitor was added to the plates. The cells were lysed after 30 minutes of ice-cold incubation. The proteins were denatured by a boiling with loading buffer for 10 minutes. Proteins in equal quantities were split using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. After the film was blocked with 5% skim milk at room temperature for 1 hour, the membrane was then incubated with primary antibodies at a dilution of 1:1,000 for a whole night at 4 ℃. The antibodies included *PI3K* [Cell Signaling Technology (CST), Danvers, MA, USA], *AKT* (CST), and glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*; CST). After three washes with tris-buffered saline with Tween20 (TBST), the membranes were treated for 1 hour at room temperature with anti-rabbit secondary antibody (Zen-Bio Inc., Durham, NC, USA), and anti-mouse secondary antibody (Zen-Bio Inc.). With *GAPDH* acting as the internal reference for protein expression, the bands were quantified using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

Cellular proliferation and Cell Counting Kit 8 (CCK-8) assays

SKOV3 and OVCAR433 cells were transfected with negative control (NC) and si-*CLCN3*. In 96-well plates, the transfected cells (4×10^4 cells per well) were seeded. CCK-8 solution (10 L; Sparkjade) was added to each well after transfection for 0, 24, 48, and 72 hours, and the cells were subsequently subjected to a 2-hour incubation period at 37 ℃ in a 5% CO_2 atmosphere. To ascertain the cell proliferation rate, a microplate reader was employed to determine the optical density (OD) at 450 nm.

Statistical analysis

SPSS 16.0 software (IBM Corp., Armonk, NY, USA) was used to conduct statistical analysis on the data from three independent experiments, with the values being expressed as the mean ± standard deviation (SD). For comparing two groups, the *t*-test was used, while for comparing more than two groups, one-way analysis of variance (ANOVA) was used. Statistical significance was defined as a P value 0.05.

Results

TIMER 2.0 was used to evaluate the degree of *CLCN3* expression in pancarcinoma tissues. *Figure 1A* shows that in contrast to nearby normal tissues, tissues from several cancers had much higher *CLCN3* expression, including cholangiocarcinoma (CHOL), liver hepatocellular carcinoma (LIHC), and stomach adenocarcinoma (STAD). In contrast, other tumors, such as breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), rectum adenocarcinoma (READ), and thyroid carcinoma (THCA), exhibited significant downregulation of *CLCN3* expression. Subsequently, through using the CPTAC database, we assessed the protein level expression of *CLCN3*. Overall, it was evident that OC tissues have lower *CLCN3* expression levels as compared to healthy cells. RT-qPCR analysis corroborated these results (*Figure 1B*).

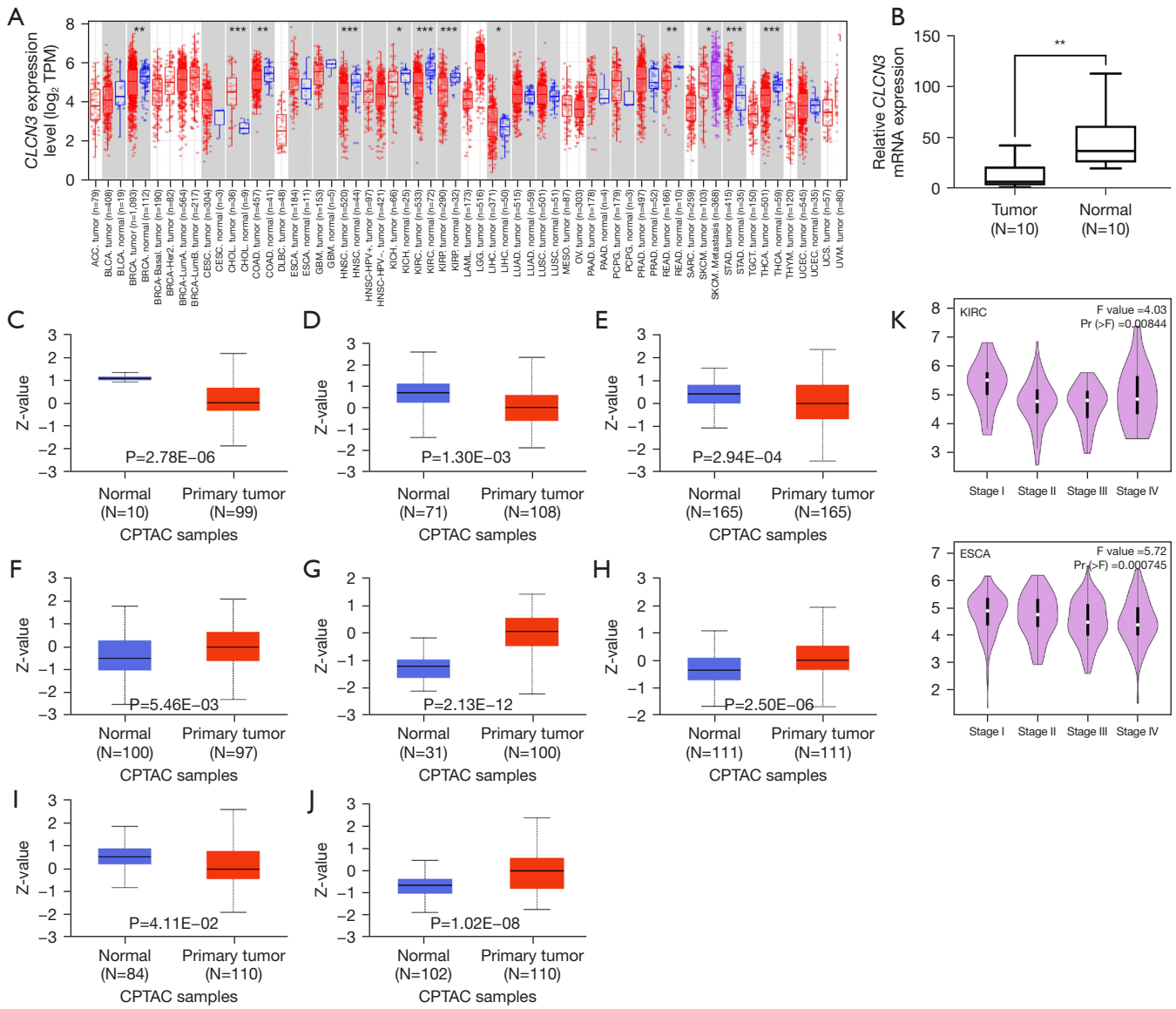

Figure 1 Abnormal expression of *CLCN3* in pancancer. (A) mRNA level of *CLCN3* according to bioinformatics analysis. (B) The relative *CLCN3* protein expression according to RT-qPCR. (C-J) The protein levels of *CLCN3* in glioblastoma multiforme, head and neck squamous carcinoma, hepatocellular carcinoma, colon adenocarcinoma, uterine corpus endometrial carcinoma, lung adenocarcinoma, clear cell renal cell carcinoma, and lung squamous cell carcinoma. (K) The relationship of *CLCN3* expression levels and the pathological stages in KIRC and ESCA. *, P<0.05; **, P<0.01; ***, P<0.001 indicate statistically significant differences. *CLCN3*, chloride channel-3; TPM, transcripts per millions; mRNA, messenger RNA; KIRC, kidney renal clear cell carcinoma; ESCA, esophageal carcinoma; CPTAC, Clinical Proteomic Tumor Analysis Consortium; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

The CPTAC database was then used to evaluate *CLCN3* protein expression, and the results showed that there was a substantial drop in *CLCN3* protein expression in COAD, uterine corpus endometrial carcinoma (UCEC), and lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC), while it was increased in glioblastoma multiforme (GBM), HNSC, LIHC, and KIRC (*Figure 1C-1J*). *CLCN3* expression level and tumor pathological stage were investigated using GEPIA 2.0. The results showed that the staging of esophageal carcinoma (ESCA) and KIRC was

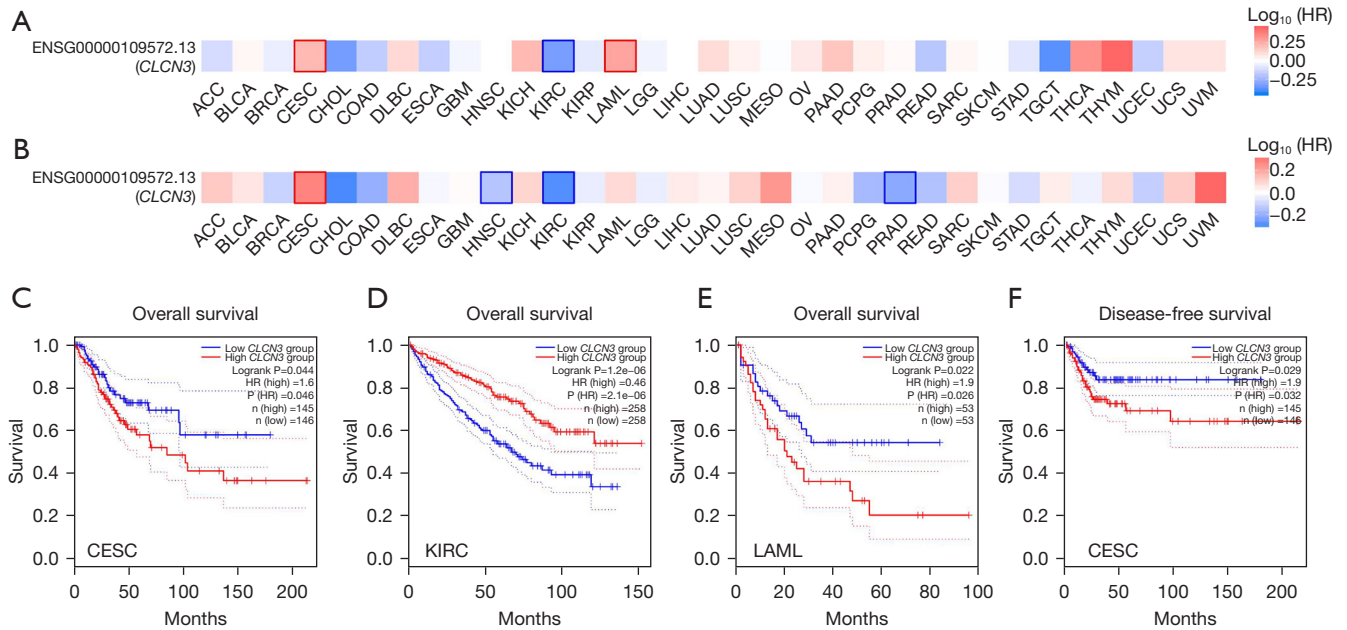

Figure 2 Prognostic value of *CLCN3*. Survival analysis including OS (A) and DFS (B) of *CLCN3* expression in pancancer. GEPIA 2.0 was used to investigate the association of *CLCN3* and patient prognosis in terms of OS (C-E) and DFS (F). A P value <0.05 indicates a statistically significant difference. *CLCN3*, chloride channel-3; HR, hazard ratio; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; KIRC, kidney renal clear cell carcinoma; LAML, acute myeloid leukemia; GEPIA 2.0, Gene Expression Profiling Interactive Analysis 2.0; OS, overall survival; DFS, disease-free survival.

significantly associated with *CLCN3* expression (*Figure 1K*).

Association of CLCN3 gene expression and survival in pancancer

The correlation of *CLCN3* expression with patient prognosis in terms of OS and DFS was evaluated using GEPIA 2.0 (*Figure 2A,2B*). Increased *CLCN3* expression was associated with a worse outcome in patients with cervical squamous cell carcinoma (CESC) and those with endocervical adenocarcinoma according to OS (P=0.046) and DFS (P=0.032) analyses; a similar association was found between increased *CLCN3* expression and OS in patients with acute myeloid leukemia (LAML) (P=0.026). Conversely, in patients with KIRC, a high level of *CLCN3* expression was linked to a positive outcome (P=2.1e−06) (*Figure 2C-2F*). Thus, for a number of malignancies, *CLCN3* could act as a prognostic marker.

CLCN3 gene alterations in pancancer

Using TCGA data via the cBioportal database, we evaluated the *CLCN3* mutation in pancancer. Pancarcinoma analysis revealed a high frequency of *CLCN3* mutations in UCEC (>8%) and a 4% incidence of depth loss in LUSC (*Figure 3A*). Missense and truncation were found to be the primary mutation types of *CLCN3* (*Figure 3B*). For instance, a truncating mutation in the Voltage_CLC (Voltage gated chloride channel) domain, namely the R311Gfs*14 alteration, was detected in 10 cases.

Promoter methylation of CLCN3 gene in pancancer

The formation of tumors is likely related to promoter DNA methylation, which has been shown to play a part in transcriptional suppression (25). UALCAN was used to compare the methylation levels of *CLCN3* in normal and malignant tissues. According to the findings, various cancer types had considerably higher levels of methylation at the *CLCN3* promoter, including ESCA, HNSC, KIRC, LUSC, pancreatic adenocarcinoma (PAAD), and prostate adenocarcinoma (PRAD) (*Figure 4*). Thus, transcription expression of *CLCN3* may be associated with alterations in promoter methylation.

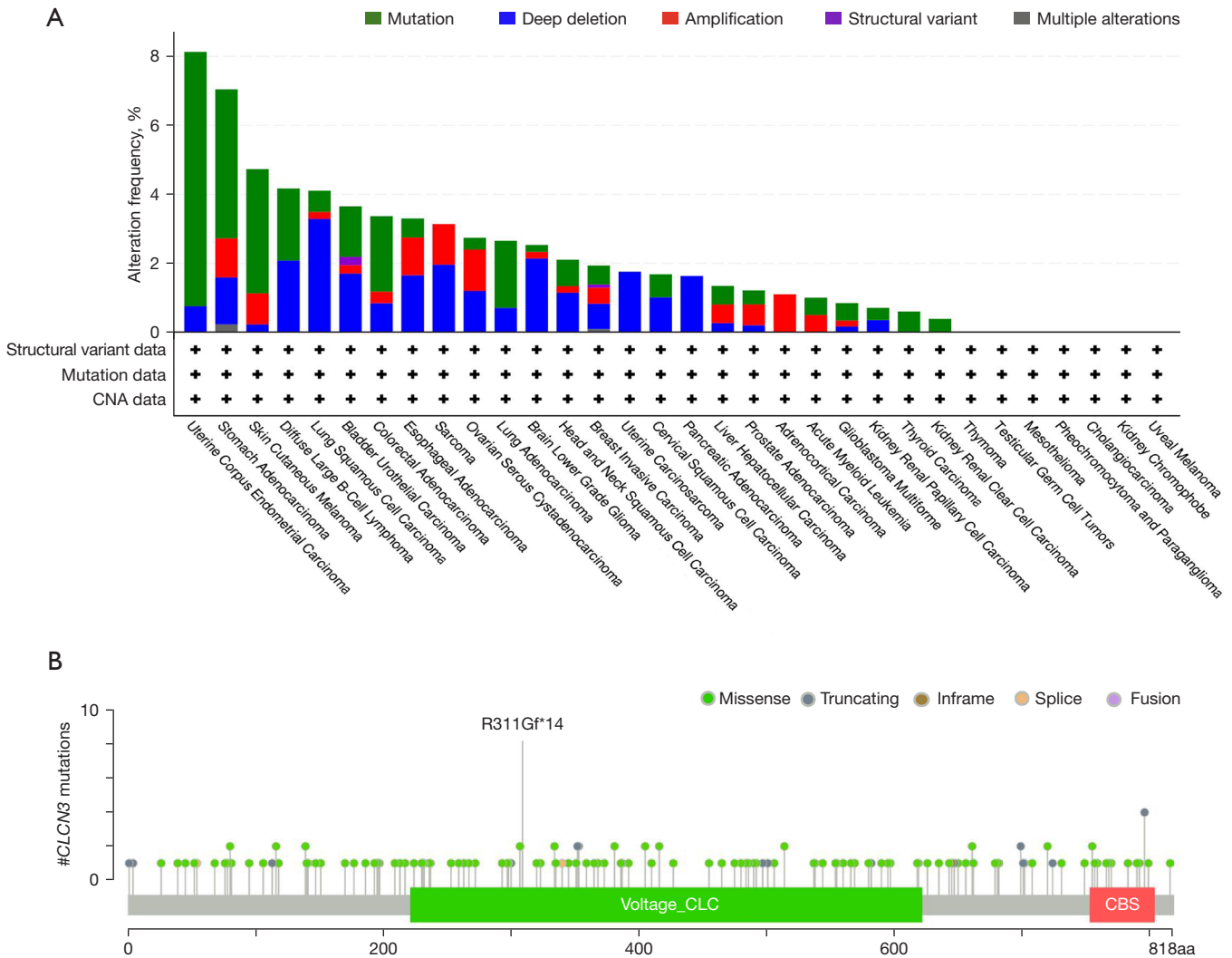

Figure 3 The cBioportal database was used to analyzed the mutation (A) and mutation site (B) of *CLCN3* in pancancer. CNA, copy-number alteration; *CLCN3*, chloride channel-3; Voltage_CLC, Voltage gated chloride channel; CBS, cystathionine-beta-synthase.

CLCN3 gene and immunological infiltration in pancancer

Based on TCGA data, a number of algorithms, including TIMER, EPIC (Experimental Physics and Industrial Control System), MCPCOUNTER (Microenvironment Cell Populations-counter), CIBERSORT (Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts), CIBERSORT-ABS (Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts-absolute value), QUANTISEQ (Quantitative Sequencing), and XCELL, were used to examine the association of immune infiltrating cells and *CLCN3* expression in pancancer. As seen in *Figure 5*, *CLCN3* expression and $CD8^+$ T cell infiltration were positively correlated in KICH, pheochromocytoma and paraganglioma (PCPG), PRAD, uveal melanoma (UVM), and other cancer types. There was also an association between B cells and GBM, KICH, brain lower grade glioma (LGG), PCPG, and PRAD. While ESCA demonstrated an association with Tregs, other cancer types, including CESC, CHOL, HNSC, LIHC, LUAD, LUSC, ovarian serous cystadenocarcinoma (OV), testicular germ cell tumors (TGCT), and thymoma (THYM) were linked to CAFs.

Gene set enrichment analysis of CLCN3 gene in pancancer

Gene set enrichment analysis was conducted to assess the

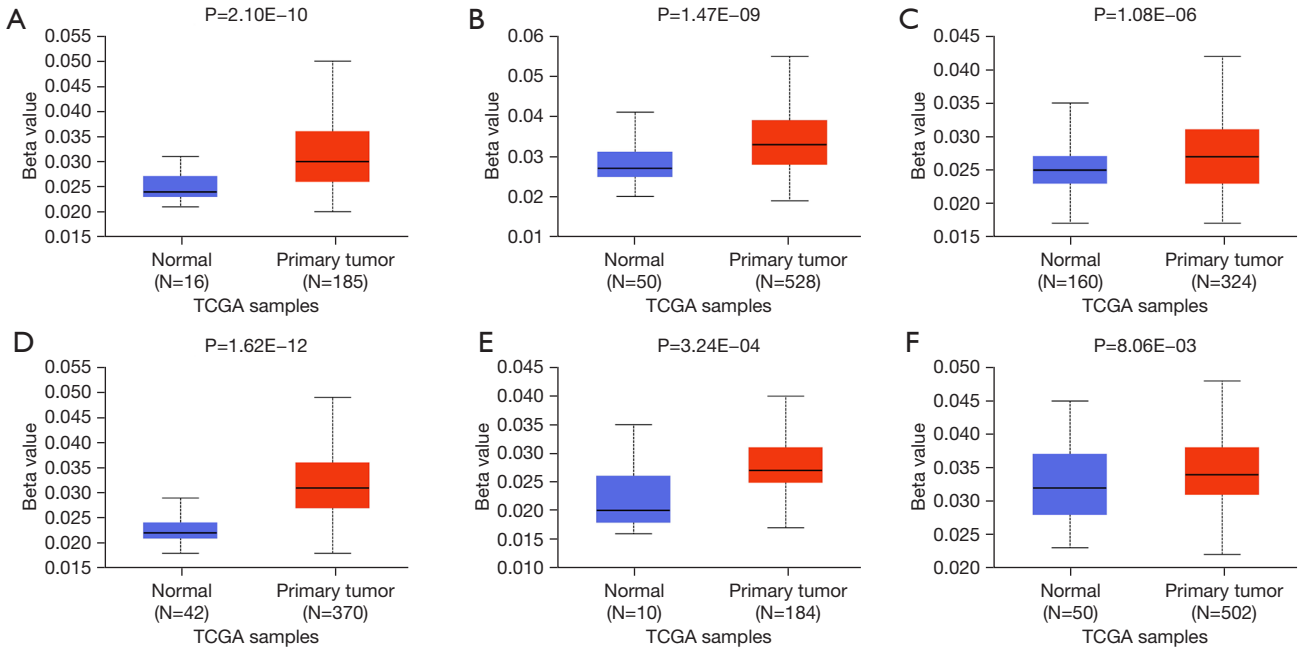

Figure 4 Promoter methylation levels of *CLCN3*. The methylation values of *CLCN3* in normal and tumor tissues, including esophageal carcinoma (A), head and neck squamous cell carcinoma (B), kidney renal clear cell carcinoma (C), lung squamous cell carcinoma (D), pancreatic adenocarcinoma (E), and prostate adenocarcinoma (F) were compared using UALCAN. P<0.05 indicates a statistically significant difference. TCGA, The Cancer Genome Atlas; *CLCN3*, chloride channel-3; UALCAN, University of Alabama at Birmingham Cancer Data Analysis Portal.

potential molecular processes underlying the effect of *CLCN3* on cancer formation and growth. *Figure 6A* shows the 70 molecules that were found to interact with *CLCN3*, as identified in the BioGRID database. Notably, in most cancer types, CCL25, CCL5, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, and TIGIT exhibited a negative association with *CLCN3*. In contrast, across various cancer types, *CLCN3* and CD274 exhibited a highly positive association (*Figure 6B*,*6C*).

Relationship between immune and molecular subtypes and CLCN3 gene expression in pancancer

The expression of *CLCN3* in different molecular and immunological subtypes of human tumors was examined using TISIDB. Six categories were used to classify the immune subtypes: C6 [transforming growth factor beta (TGF-β) dominant], C5 (immunologically quiet), C4 (lymphocyte depleted), C3 (inflammatory), C2 [interferon gamma (IFN-γ) dominant], and C1 (wound healing). Different immunological subtypes in a variety of cancer types were found to be related to *CLCN3* expression, including bladder urothelial carcinoma, BRCA, COAD, ESCA, GBM, HNSC, KICH, KIRC, LGG, LIHC, LUAD, PAAD, PRAD, sarcoma, STAD, TGCT, and UCEC (*Figure 7*). Moreover, within the same cancer type, *CLCN3* expression differed amongst various immunological subgroups. For example, in BLAC, *CLCN3* exhibited high expression in types C1 and C4, while it had a low expression in type C6. Similarly, the *CLCN3* expression level demonstrated a significant association with certain cancer molecular subtypes, including those of BRCA, COAD, LIHC, PCPG, and UCEC (*Figure 8*).

CLCN3 gene inhibited OC proliferation

The SKOV3 and OVCAR433 cell lines were transfected with siRNA directed against *CLCN3* to examine the effect of *CLCN3* on the migration and invasion of OC cells. In SKOV3 and OVCAR433 cells, transfection of si-*CLCN3* resulted in a decrease in *CLCN3* mRNA expression. With the same technique, NC was transfected into the control group. RT-qPCR was used to validate the transfection effectiveness of the *CLCN3* siRNA, indicating that

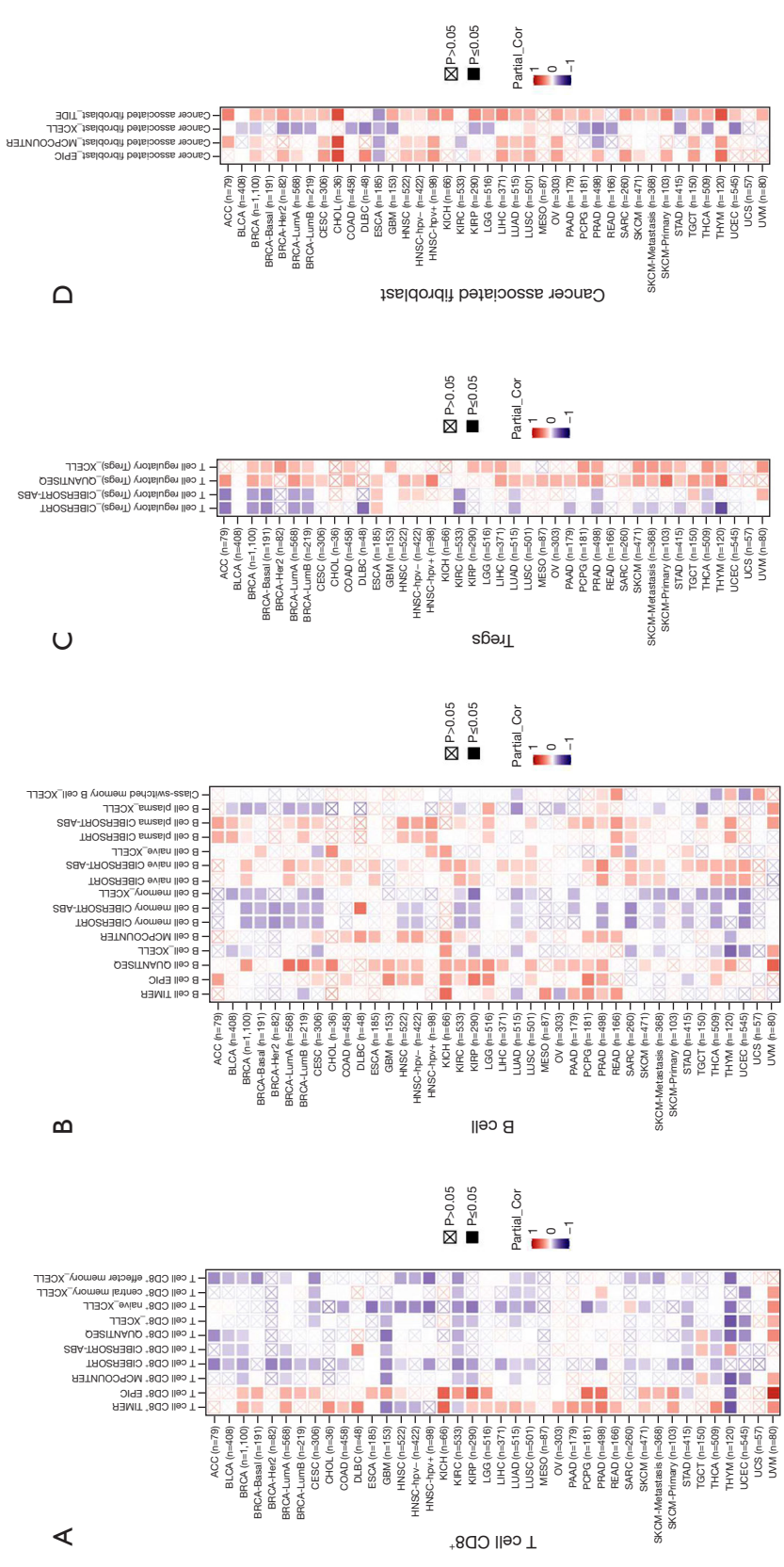

Figure 5 The correlation between *CLCN3* expression and immune cells in pancancer. (A-D) Several algorithms, including TIMER, EPIC, QUANTISEQ, XCELL, MCPCOUNTER, CIBERSORT, CIBERSORT-ABS, and TIDE, were applied to explore the correlation between *CLCN3* expression and immune cells in pancancer. Positive correlations (0–1) are marked with the red color, while negative correlations (–1 to 0) are marked with purple color. P<0.05 indicates a statistically significant difference. Tregs, T regulatory cells; *CLCN3*, chloride channel-3; TIMER, Tumor Immune Estimation Resource; EPIC, Experimental Physics and Industrial Control System; QUANTISEQ, Quantitative Sequencing; MCPCOUNTER, Microenvironment Cell Populations-counter; CIBERSORT, Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts; CIBERSORT-ABS, Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts-absolute value.

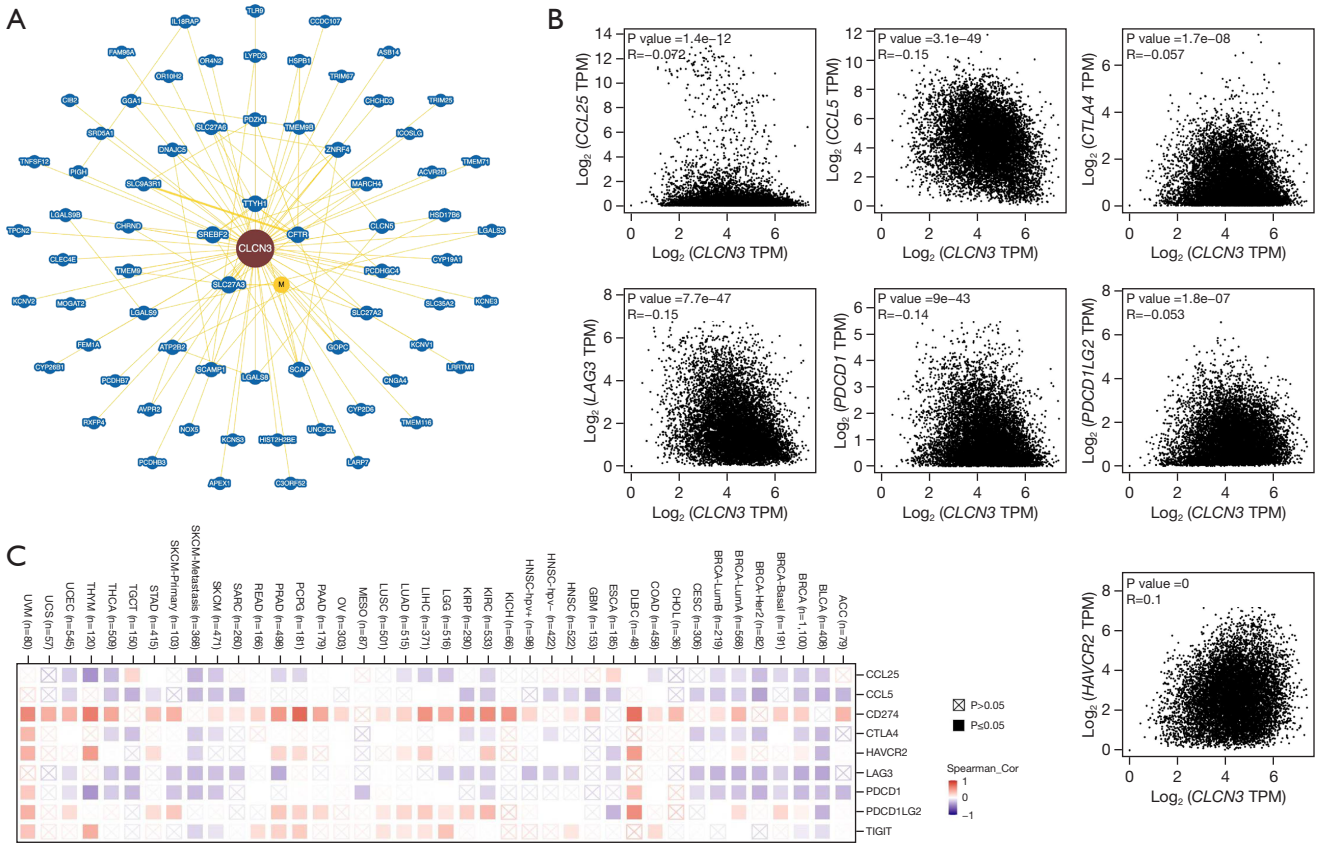

Figure 6 Functional enrichment analysis of *CLCN3*-related genes. (A) Seventy molecules that were found to interact with *CLCN3* were obtained from the BioGRID database. (B,C) GEPIA 2.0 showed that *CCL25*, *CCL5*, *CTLA4*, *HAVCR2*, *LAG3*, *PDCD1*, *PDCD1LG2* and *TIGIT* had a negative correlation with *CLCN3* in most cancer types. *CLCN3*, chloride channel-3; TPM, transcripts per millions; GEPIA 2.0, Gene Expression Profiling Interactive Analysis 2.0.

CLCN3 was successfully knocked down (*Figure 9A*). With the use of the CCK-8 assay, the proliferative ability was assessed. Results showed that in SKOV3 and OVCAR433 cells, NC reduced proliferation, whereas si-*CLCN3* boosted it (*Figure 9B*).

CLCN3 gene knockdown promoted OC proliferation via the PI3K/AKT signaling pathway

In order to ascertain how *CLCN3* influences the proliferation of OC cells, Western blotting was used to examine the association between ClC-3 expression and *PI3K/AKT* protein levels. As expected, in SKOV3 and OVCAR433 cells, *PI3K* and *AKT* protein levels increased when *CLCN3* was knocked down (*Figure 9C,9D*). Meanwhile, RT-qPCR was used to verify the changes of *CLCN3* knockdown on the mRNA levels of *PI3K* and *AKT*, and the results also indicated that the expression of *PI3K* and *AKT* was increased after knockdown of *CLCN3* (*Figure 9E*).

According to earlier research, the growth of breast cancer and lung cancer is aided by *PI3K/AKT* (18,26). According to these findings, *CLCN3* knockdown may increase the *PI3K/AKT* pathway activation, which may promote the development of OC.

Discussion

Compared to other cancers, OC develops insidiously and has no early warning signs, resulting in a lower early diagnosis rate. Despite significant progress made in combination treatment, such as that involving surgical resection and chemoradiotherapy, the treatment effect

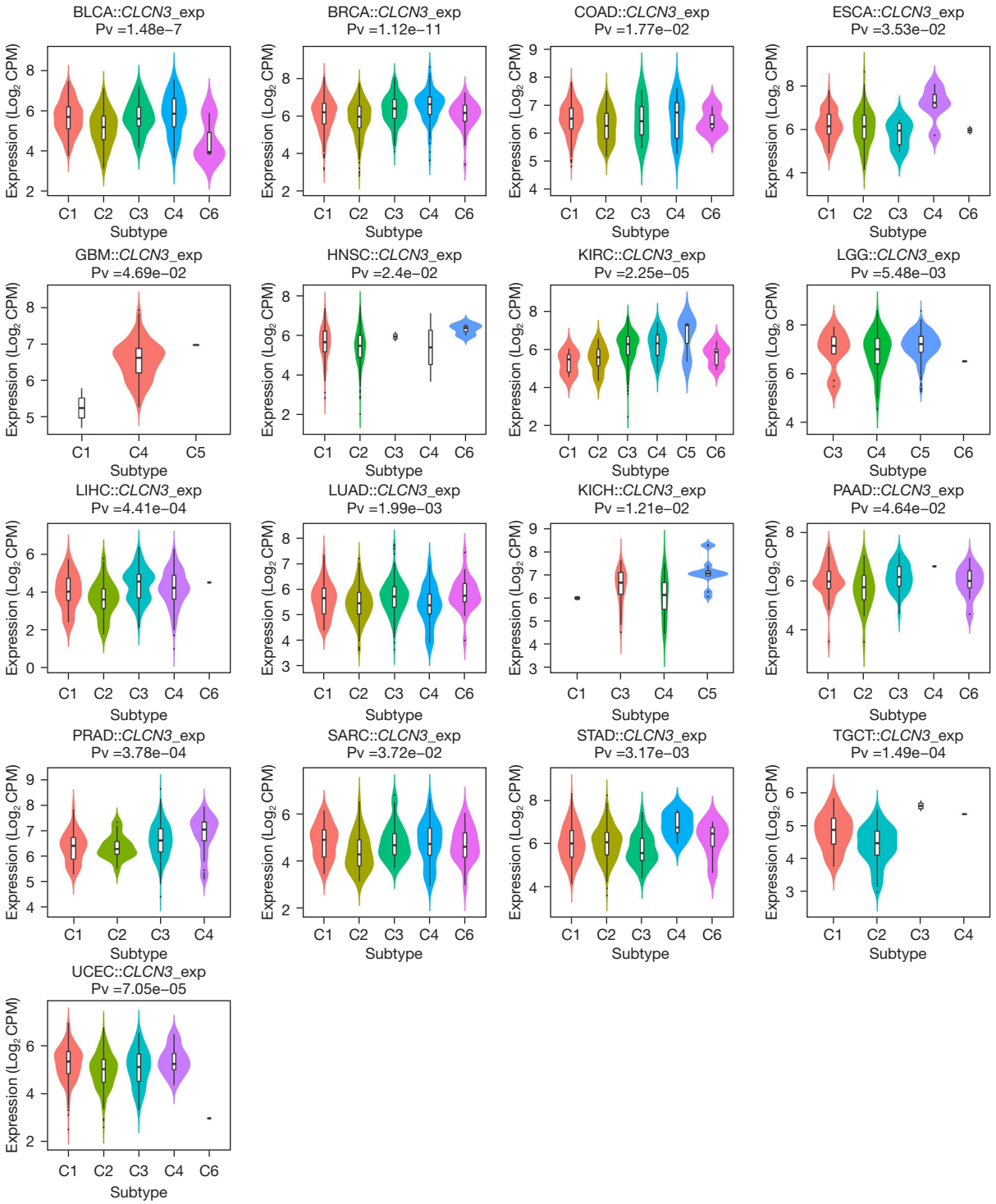

Figure 7 The relationship between *CLCN3* expression and pancancer immune subtypes BLCA, BRCA, COAD, ESCA, GBM, HNSC, KICH, KIRC, LGG, LIHC, LUAD, PAAD, PRAD, SARC, STAD, TGCT, and UCEC. BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; SARC, sarcoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumor; UCEC, uterine corpus endometrial carcinoma; *CLCN3*, chloride channel-3; CPM, counts per million.

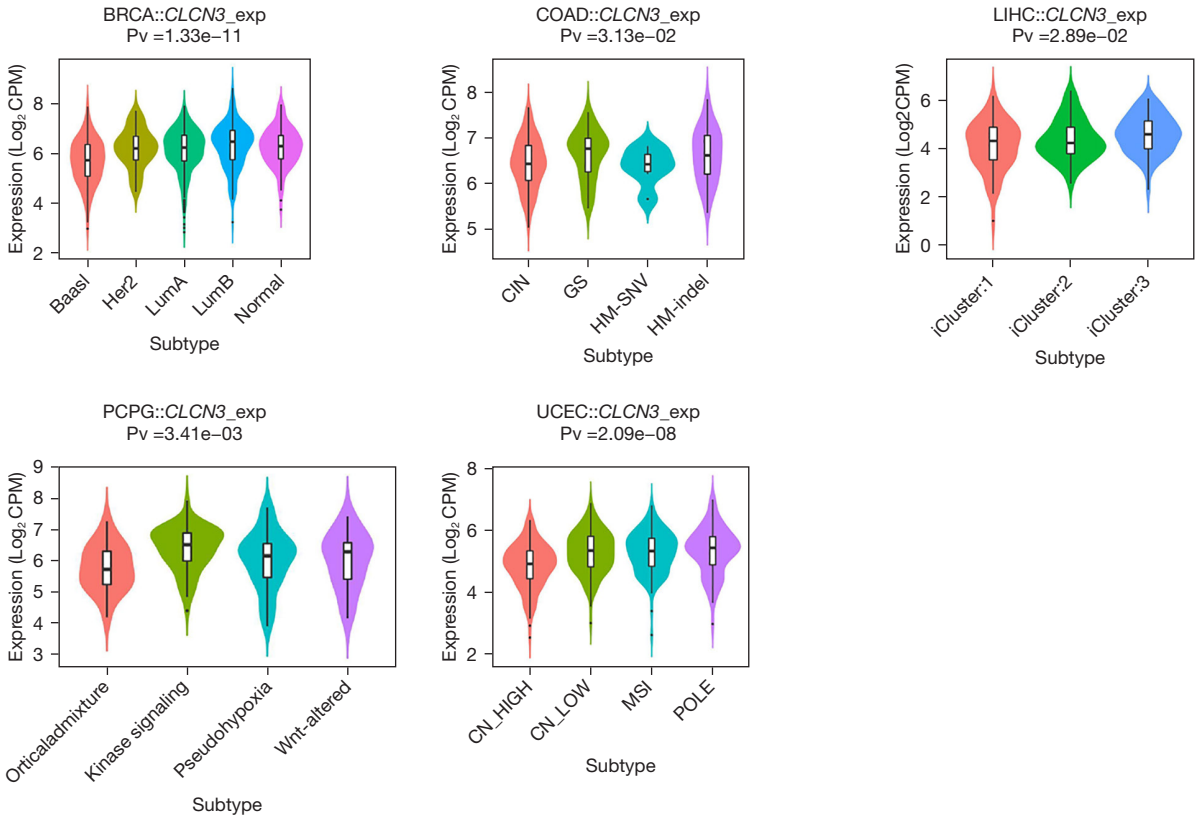

Figure 8 The relationship between *CLCN3* expression and pancancer molecular subtypes in BRCA, COAD, LIHC, PCPG, and UCEC. BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; LIHC, liver hepatocellular carcinoma; PCPG, pheochromocytoma and paraganglioma; UCEC, uterine corpus endometrial carcinoma; *CLCN3*, chloride channel-3; CPM, counts per million; CIN, chromosomal instability; HM-SNV, hypermutated-single-nucleotide variant predominant; CN, copy number; MSI, microsatellite instability; POLE, polymerase epsilon.

remains unsatisfactory for patients with advanced disease. Therefore, the study and identification of key regulatory factors are crucial for early diagnosis, treatment efficacy, and prognosis.

In internal vesicles and the cell membrane, *CLCN3* is an essential $2Cl^-/H^+$ exchanger (8). The link between *ClCN3* expression and the growth, invasion, and migration of nasopharyngeal carcinoma cells, colorectal carcinoma, and cervical squamous cell cancer has been demonstrated in earlier research (7,9,11).

Therefore, we examined *CLCN3* across a variety of cancers. Using the TIMER, GEPIA 2.0, and BioGRID databases, we initially performed a thorough investigation of *CLCN3* expression levels in cancer and healthy tissues among 33 different tumors. We observed a considerable increase in *CLCN3* expression in various tumor tissues, such as BRCA and CHOL. Poor prognosis was linked to overexpression of *CLCN3* in individuals with CESC and LAML. Additionally, we found that overexpression of *CLCN3* predicted poor OS in patients with LAML, poor OS and DFS in patients with CESC, but excellent OS in patients with KIRC. These results suggest that *CLCN3* may be a viable biomarker for estimating the prognosis of patients with tumors.

The tumor microenvironment is a dynamic extracellular ecological system comprising immune cells, stromal cells, cancer cells, and other cell types (27). These cells are essential for the growth, prognosis, immune defense, and therapeutic resistance. The invasion of immune-related cells, such as B cells, $CD8^+$ T cells, Tregs, and CAFs, were highly correlated with the aberrant expression of *CLCN3*. Therefore, *CLCN3* may serve as an immune target for patients with cancer. Additionally, mutations and profound deletions of the *CLCN3* gene have been observed in a number of malignancies. Additionally, there were discernible changes between normal tissues and

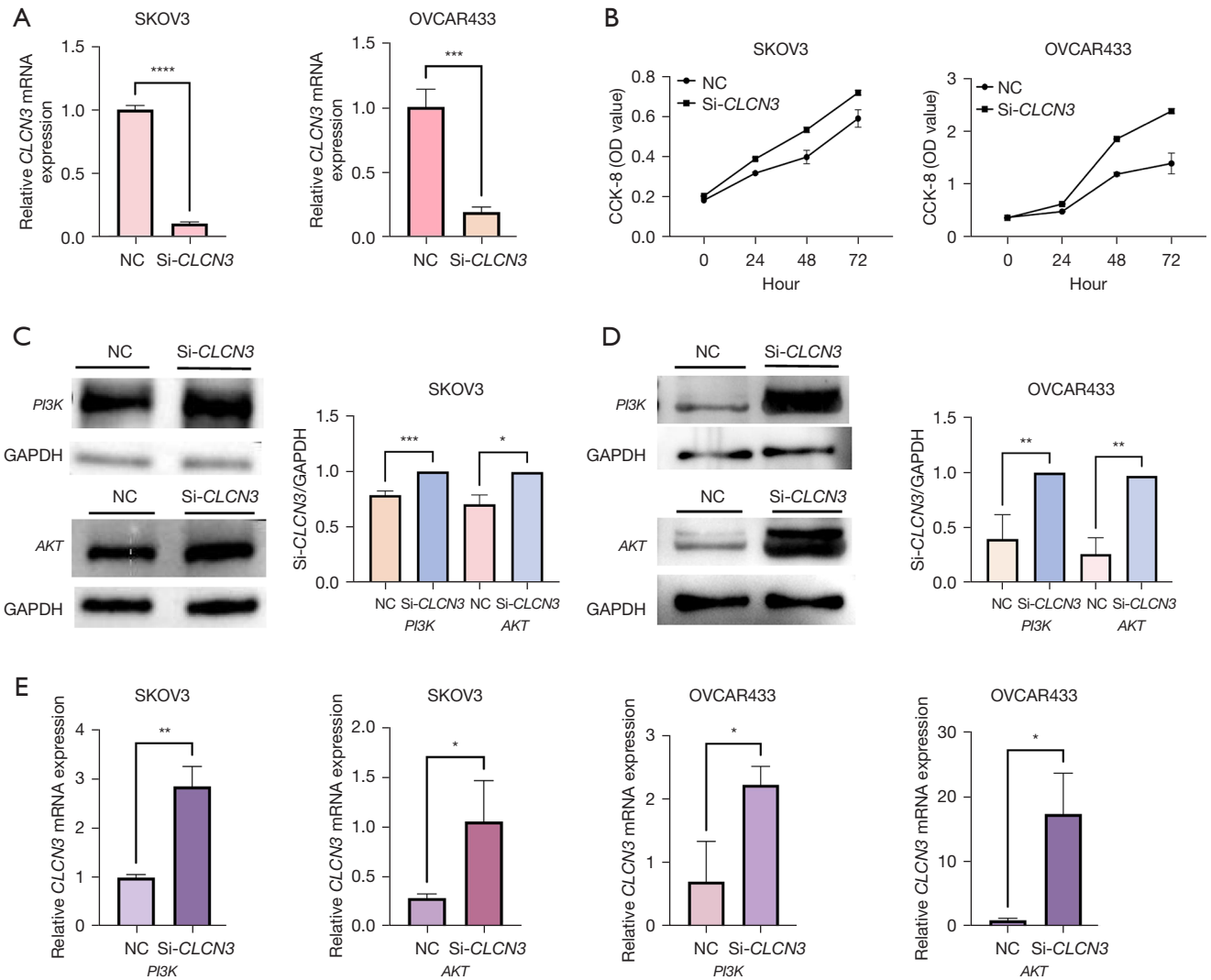

Figure 9 *CLCN3* may mediate the *PI3K/AKT* pathway in *SKOV3* and *OVCAR433* cells. (A) The transfection efficiency of *CLCN3* siRNA was detected via RT-qPCR. (B) CCK-8 assay evaluated the proliferative ability of *SKOV3* and *OVCAR433* cells after knockdown of *CLCN3*. (C-E) Western blotting and RT-qPCR analysis of *PI3K* and *AKT* expression, with *GAPDH* used as the loading control. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, indicate statistically significant differences. NC, negative control; *CLCN3*, chloride channel-3; mRNA, messenger RNA; OD, optical density; PI3K, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; AKT, Akt kinase; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; CCK-8, Cell Counting Kit 8.

tumor in the degree of *CLCN3* methylation. Furthermore, the purpose of this study was to focus on how *CLCN3* affects the proliferation of OC. Initially, using the RT-qPCR study of tumor tissues and cells, we discovered that *CLCN3* was downregulated in OC tissues and cell lines, indicating its potential involvement as a tumor-suppressor gene in cancer pathogenesis. In this work, siRNA-mediated reduction of *CLCN3* led to an increase in the proliferation of OC cells.

Multiple studies have demonstrated that in addition to its role as a $2Cl^-/H^+$ exchanger, *CLCN3* also functions as a regulatory protein that modulates a variety of signaling pathways (9). *PI3K/AKT* signaling is essential for controlling cell proliferation (28). Therefore, the purpose of this study was to determine whether ClC-3 controls the expression of the *PI3K/AKT* signaling pathway and as a result, influences how quickly OC cells proliferate. The findings showed that the knockdown of ClC-3 increased the protein expression

of *PI3K* and *AKT* in OC cells, demonstrating that the downregulation of *CLCN3* encourages the growth of OC cells by activating the *PI3K/AKT* pathway.

Conclusions

Our comprehensive bioinformatics analysis indicated that for those with tumors, *CLCN3* may be used as an immune-related and prognostic biomarker. This study serves as a foundation for additional research into the precise mechanisms underlying the role of *CLCN3* in the pathogenesis and management of various cancers. Additionally, our findings offer a fresh understanding of how *CLCN3* inhibits OC: by altering the *PI3K/AKT* signaling pathway, *CLCN3* prevents the proliferation of OC cells. Therefore, *CLCN3* may be a promising target for preventing the progression of OC.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1272/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1272/coif). All authors report that this work was supported by the Natural Science Research Project of Universities in Anhui Province (No. KJ2019ZD25). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Written informed permission was given by every patient. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the First Affiliated Hospital of Anhui Medical University Ethics Committee (No. PJ2023-10-46).

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