Enhanced expression of CXCL1 in renal cell carcinoma facilitates tumor cell malignancy via PI3K/AKT-dependent mechanisms

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Abstract. The expression of CXC motif chemokine ligand 1 (CXCL1) is elevated in numerous types of human cancers, and CXCL1 is also able to act as an autocrine/paracrine factor to accelerate the malignant progression of malignancies. However, at present, there is limited understanding of the role of CXCL1 in renal cell carcinoma (RCC). The present study aimed to investigate the mRNA and protein expression levels of CXCL1 in RCC tissues, which were found to be markedly elevated compared with normal and paracancerous tissues using bioinformatics and immunohistochemical analyses. In addition, high expression of CXCL1 was shown to be closely associated with higher stage, grade, tumor, lymph node and metastasis status of clear cell RCC, as well as with poor prognosis in patients. In addition, the expression level of CXCL1 mRNA was found to be associated with the expression of various tumor-associated genes, including numerous chemokines and their receptors, immune cell recruitment of B cells, $CD4^+$ T cells and neutrophils, as well as microsatellite instability in RCC. Functional studies were also performed, which demonstrated that both exogenous and overexpression of CXCL1 led to a marked enhancement of the proliferative and migratory capacities of RCC cells, whereas downregulation of CXCL1 exerted a suppressive influence on the malignant behaviors of the tumor cells. In addition, western blot analysis experiments revealed that overexpression of CXCL1 in RCC cells was able to regulate the expression levels of phosphoinositide 3-kinase (PI3K)/AKT pathway-associated proteins, including Bcl-2-associated X protein, B-cell lymphoma-2, PI3K and AKT. Finally, the AKT-specific inhibitor GDC-0068 was shown to reverse the promoting effects of CXCL1 on the malignant behaviors of RCC cells. Taken together, the findings of the present study have shown that CXCL1 exhibits high expression patterns in RCC tissues and may serve diverse functions in facilitating various aspects of RCC advancement.

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Introduction

Renal cell carcinoma (RCC) represents one of the most prevalent malignant tumors within the genitourinary system, accounting for ~5% of all newly diagnosed cancers in men and 3% in women (1). Previous statistical data have shown that, in 2019, the United States recorded ~73,820 new diagnoses of RCC, and 14,770 mortalities were attributed to RCC (1). Among the diverse subtypes of RCC, clear cell RCC (ccRCC) has emerged as the predominant form and is widely regarded as one of the most aggressive malignancies in the urinary tract, with an annual global mortality rate of ~90,000 cases (2). Cytokine-based and checkpoint inhibitor immunotherapies have been shown to elicit robust immune responses through distinct mechanisms, thereby having a pivotal role in RCC treatment (3,4). However, despite the major improvements that have been made in terms of understanding tumor initiation and progression, the etiological mechanisms underlying RCC remain poorly understood (5). Due to the high incidence and mortality rates associated with RCC, it is essential to identify predictive biomarkers that impact immune responses in patients diagnosed with this malignancy.

Chemokines, a class of small, secreted proteins, are recognized for their pivotal regulatory roles in mediating immune cell trafficking and lymphatic tissue development (6,7). As the largest subfamily of cytokines, chemokines can be further classified into four primary subgroups according to the arrangement of their two cysteine (C) residues in their protein sequences, namely CC-chemokines, CXC-chemokines,

C-chemokines and CX3C-chemokines (7). Within the tumor microenvironment (TME), chemokines are expressed by a diverse array of cell types, encompassing both tumor cells themselves and other essential components, such as immune cells and stromal cells (8,9). In response to specific chemokines, diverse immune cell subsets migrate to the TME, orchestrating the immune response against tumors in a precisely regulated manner (8,9). Additionally, chemokines directly target non-immune cells within the TME, including tumor, stromal and vascular endothelial cells, which have been shown to modulate tumor cell proliferation, tumor stemness properties and tumor invasion and metastasis (10). Consequently, chemokines exert both direct and indirect effects on tumor immunity, thereby shaping the immunological and biological phenotypes of tumors, and subsequently influencing tumor progression, treatment efficacy and patient prognosis (8,10,11). CXC motif chemokine ligand 1 (CXCL1) belongs to the glutamate-leucine-arginine (ELR) + CXC chemokine subfamily that is distinguished by the conserved amino acid sequence of ELR (12). The role of CXCL1 is mediated through its interaction with its receptor, CXCR2 (13). To date, a burgeoning body of evidence has demonstrated that CXCL1 displays an increased level of expression in a variety of different types of human malignancies, with CXCL1 serving as a potent signal within the TME that promotes malignant progression (13). The present study therefore aimed to investigate the expression patterns of CXCL1 in RCC tissues, also exploring whether CXCL1, as a component of the RCC microenvironment, may contribute to the carcinogenic potential of RCC through exogenous and autocrine mechanisms. The findings of the present study may help to facilitate the discovery of valuable biomarkers and potential therapeutic targets for the clinical diagnosis and molecular targeted therapy of RCC.

Materials and methods

Bioinformatics analysis. The initial CXCL1 RNA sequencing (RNA-seq) data were acquired from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) and Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) databases. The expression of CXCL1 in tumor tissues, as well as its expression in tumor tissues with different pathological parameters, was investigated in the GSE53757 dataset (including RNA-seq data of 72 pairs of normal kidneys and tumor tissues) and the GSE40435 dataset (including 101 pairs of matched adjacent and tumor tissue RNA-seq data) (14,15). The STRING database (https://string-preview.org) was utilized to identify core (hub) genes that are co-expressed with CXCL1, and the expression levels of these hub genes were subsequently examined using TCGA database. Furthermore, the tumor immune estimation resource (TIMER) database (https://cistrome.shinyapps.io/timer/) was used to assess the association between CXCL1 expression and immune cell recruitment. To determine the MSI status, MSI scores were calculated using the program Microsatellite Analysis for Normal-Tumor InStability (MANTIS) with a default threshold of 0.4, defining high MSI (MSI-H) as scores above the threshold, and microsatellite stable (MSS or no apparent MSI) as scores below it.

Clinical samples and immunohistochemical analysis. Paraffin-embedded specimens, which had undergone surgical resection at the First Affiliated Hospital of Jiamusi University (Jiamusi, China) between February 2023 and January 2025 and were subsequently diagnosed as RCC by the Department of Pathology, were selected for the present study. A total of 68 pairs of RCC tissues and their corresponding adjacent non-cancerous tissues, along with complete clinical data, were randomly chosen for inclusion in the present study. The clinical characteristics of these 68 samples are presented in Table I. The study included patients with histologically confirmed renal cell carcinoma who underwent partial or radical nephrectomy and for whom high-quality paired tumor and adjacent non-cancerous tissue samples were available. All participants had complete clinicopathological data (age, sex, stage and grade). Exclusion criteria were prior systemic anti-tumor therapy, previous renal surgery or biopsy on the same kidney, and a concurrent malignancy. In brief, the specimens were fixed in 10% neutral-buffered formalin at room temperature for 24 h, then processed into paraffin blocks and cut into 5-μm thick sections. These sections were deparaffinized with 100% xylene at room temperature and subsequently rehydrated through a graded ethanol series. Antigen retrieval was carried out in a high-pressure cooker using 0.01 M citric-acid buffer at 120˚C for 10 min. To suppress endogenous peroxidase activity, slides were incubated in 3% H_2O_2 in PBS for 30 min at room temperature. Non-specific binding sites were blocked by applying 20% normal goat serum (cat. no. AR0009; Wuhan Boster Biological Technology, Ltd.) and incubated at 37˚C for 1 h. Finally, the sections were incubated overnight at 4˚C with a CXCL1-specific antibody (dilution, 1:100; cat. no. YT2074; ImmunoWay Biotechnology Company), and subsequently processed using a StreptAvidin-Biotin Complex Kit (cat. no. SA1022; Wuhan Boster Biological Technology, Ltd.), in accordance with the manufacturer's instructions. The sections were subjected to routine 3,3'-diaminobenzidine staining and hematoxylin counterstaining at room temperature for 15 min, followed by an assessment of CXCL1 protein expression, as determined under an epifluorescence microscope (Olympus Corporation) by two specified parameters: i) Intensity of staining, ranging from negative-weak (assigned a score of 1) to moderate (score of 2), strong (score of 3) and extremely strong (score of 4); and ii) the proportion of stained cells, categorized as ≤25% (score of 1), >25% to ≤50% (score of 2), >50% to ≤75% (score of 3) and >75% (score of 4). The total score was calculated by generating the sum of the parameters (i) and (ii). A score of ≤4 was indicative of low expression, whereas a score of ≥5 was indicative of high expression. The staining intensity was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Cell lines and cell culture. The human ccRCC cell line 786-O and papillary RCC (pRCC) cell line CAKI-2 were supplied by the American Type Culture Collection. The cell lines were maintained in Gibco RPMI-1640 medium (cat. no. 11875093; Thermo Fisher Scientific, Inc.) supplemented with 10% Gibco fetal bovine serum (FBS; cat. no. A5670201; Thermo Fisher Scientiffc, Inc.) and 1:100 penicillin/streptomycin (cat. no. PYG0016; Wuhan Boster Biological Technology, Ltd.). In the present study, the medium containing FBS

Table I. Clinical characteristics of patients with clear cell renal carcinoma.

Characteristic	n
Sex	
Male	46
Female	22
Age, years	
≥60	21
<60	47
Tumor grade	
Grade 1	31
Grade 2	26
Grade 3	11
Clinical stage	
Stage I	44
Stage II	20
Stage III	4

and penicillin/streptomycin was referred to as complete medium (CM).

Cell transfection. Overexpression and RNA-interference strategies were employed for transfection experiments. Pseudovirus particles containing either empty lentiviral plasmids (cat. no. LPP-NEG-Lv201-100) or overexpression lentiviral plasmids (cat. no. LPP-G0095-Lv201-100) were produced by GeneCopoeia, Inc., whereas small interfering (si)RNAs were synthesized by HyCyte, Inc. The sense and antisense sequences specifically designed for RNA-interference to silence the CXCL1 gene were: 5'-GAUGCUGAACAGUGACAAATT-3' and 5'-UUUGUCACUGUUCAGCAUCTT-3'; 5'-CCAAGAACAUCCAAAGUGUTT-3' and 5'-ACACUUUGGAUGUUCUUGGTT-3'; and 5'-GCUGCUCCUGCUCCUGGUATT-3' and 5'-UACCAGGAGCAGGAGCAGCTT-3'. The negative-control sense and antisense sequence were 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. Cell transfection was carried out according to the supplier's instructions. In the overexpression study, a total of $4x10^4$ cells were seeded into each well of a 24-well plate in 500 μl of serum-free medium. After a 24 h incubation at 37˚C under starvation conditions, lentiviral particles were added directly to the medium at a multiplicity of infection of 10^{-8}. Following a further 5 h incubation at 37˚C, an additional 500 μl of 10% serum-containing medium was added. A total of 24 h later, the entire medium was replaced with fresh complete medium. 72 h after the initial viral infection, puromycin was added to a final concentration of 2 μg/ ml for selection. Finally, downstream experiments were performed after an additional 72 h incubation to allow sufficient phenotypic expression. In the RNA-interference study, 1.25 μl siRNA stock solution was diluted with 50 μl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.), and was gently pipetted 3-5 times. Meanwhile, 1.0 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted with 50 μl Opti-MEM, and was gently pipetted 3-5 times. After standing at room temperature for 5 min, the transfection reagent and siRNA dilution were mixed and gently pipetted 3-5 times. Subsequently, they were left to stand at room temperature for 20 min. Finally, the transfection complex was added to the 24-well cell plate with cells.

ELISA detection assay. Within each compartment of the 6-well dishes, $5x10^5$ cells were grown in 1 ml serum-free medium. Following 24 h culture, the supernatant was isolated via centrifugation at 4˚C, 1,000 x g for 5 min, and a Human CXCL1 ELISA kit (cat. no. EK0722; Wuhan Boster Biological Technology, Ltd.) was employed to determine and quantify the level of CXCL1 in the supernatant, following precisely the manufacturer's instructions. The ELISA experiments were repeated three times.

Cell proliferation detection assay. Concerning the exogenous study, 786-O and CAKI-2 cells were seeded into each well of a 96-well dish at a density of $3x10^3$ cells/well, utilizing CM supplemented with various concentrations (0, 2, 5, 10, 20 and 30 ng/ml) of exogenous CXCL1 (cat. no. 300-11-25UG; PeproTech, Inc.; Thermo Fisher Scientific, Inc.). Concerning the endogenous study, 786-O and CAKI-2 cells with overexpression of CXCL1, 786-O and CAKI-2 cells with decreased expression of CXCL1, along with their corresponding control cells were seeded into each well of a 96-well dish at a density of $3x10^3$ cells/well, and CM served as the growth medium for all cell types. The cells were subsequently incubated for 72 h. Subsequently, 10 μl Cell Counting Kit-8 (CCK-8) reagent (cat. no. AR1160; Wuhan Boster Biological Technology, Ltd.) was added to each well, and the cells were further incubated for 2 h in the incubator. Finally, the optical density (OD) values at a wavelength of 450 nm were read using a microplate reader. The cell proliferation assay experiments were repeated three times.

Cell migration detection assay. The 786-O and CAKI-2 cells were seeded at a density of $1x10^4$ cells/well into the top chambers of Transwell dishes (cat. no. 3428; Corning, Inc.). The top chambers were filled with serum-free medium containing various concentrations of exogenous CXCL1, whereas the bottom chambers were maintained with CM. Concerning the endogenous study, 786-O and CAKI-2 cells with overexpression of CXCL1, 786-O and CAKI-2 cells with decreased expression of CXCL1, along with their respective control cells were seeded at a density of $1x10^4$ cells/well (for overexpression study) or $3x10^4$ cells/well (for siRNA study) into the top chambers of the Transwell dishes. The top chambers were filled with serum-free medium, whereas the bottom chambers contained CM. The cells were subsequently incubated for 48 h, after which the upper surface of the chambers was gently wiped with a cotton swab to remove the non-migrated cells. Following methanol fixation and crystal violet staining at room temperature for 20 min, the chambers were imaged under an inverted microscope in order to count the numbers of migrated cells. The cell migration experiments were repeated three times.

Western blotting assay. Total cellular proteins were extracted and quantified utilizing RIPA protein lysis buffer

(cat. no. P0013B; Beyotime Institute of Biotechnology) and a BCA protein quantitation kit (cat. no. AR0146; Wuhan Boster Biological Technology, Ltd., respectively. Subsequently, 40 μg total protein was subjected to 12% SDS-PAGE and transferred onto a PVDF membrane (cat. no. IPVH00010; MilliporeSigma). The membrane was incubated overnight at 4˚C with primary antibodies against Bcl-2-associated X protein (Bax; cat. no. CY5059; Shanghai Abways Biotechnology Co., Ltd.; 1:1,000), B-cell lymphoma-2 (Bcl-2; cat. no. CY5032; Shanghai Abways Biotechnology Co., Ltd.; 1:1,000), phosphoinositide 3-kinase (PI3K; cat. no. CY5224; Shanghai Abways Biotechnology Co., Ltd.; 1:1,000), AKT (cat. no. AF6261; Affinity Biosciences; 1:1,000) and β-actin (cat. no. TA811000; OriGene Technologies, Inc.; 1:5,000). Subsequently, the membrane was further incubated with horseradish peroxidase-conjugated secondary antibodies at 37˚C for 30 min (cat. no. BA1055, Wuhan Boster Biological Technology, Ltd.) at a dilution of 1:10,000 and ECL reagents (cat. no. 34095; Thermo Fisher Scientific, Inc.), prior to being placed in a Tanon 4200 Fully Automatic Chemiluminescence/Fluorescence Image Analysis System (Tanon Science & Technology Co., Ltd.) for visualization and imaging. Protein quantification was performed with Tanon Image software (Tanon Science & Technology Co., Ltd.). The western blotting experiments were repeated three times.

Statistical analysis. The SPSS statistics software, version 22.0 (IBM Corp.) was employed for statistical analyses. The variabilities in CXCL1 expression among different groups were compared using the Mann-Whitney U test. To assess the prognostic value of CXCL3, both univariate and multivariate Cox regression analyses were performed. Pearson correlation analysis was employed to assess the relationship between CXCL1 expression and immune cell recruitment. In the cellular experiments, comparisons between two groups were performed with an unpaired two-sample t-test. For comparisons among three or more groups, a one-way ANOVA was applied. When the one-way ANOVA revealed a significant overall effect ($P<0.05$), pairwise comparisons were conducted using the Tukey's post hoc test.

Results

Expression of CXCL1 mRNA is upregulated in RCC tissues, and its high expression is associated with the clinical characteristics of patients with RCC. TCGA database analysis revealed that the expression level of CXCL1 mRNA in ccRCC or pRCC tissues was significantly upregulated compared with normal tissues (Fig. 1A). Due to ccRCC being the most prevalent subtype of RCC, subsequent experiments in the present study were focused on ccRCC. The analysis of clinical grade (Fig. 1B) and subtypes (Fig. 1C) revealed that the expression level of CXCL1 in grade 4 ccRCC was significantly higher compared with that in the normal tissues and grades 1 and 2 ccRCC, and the ccB subtype exhibited a notably increased expression level compared with both normal tissue and the ccA subtype. Additionally, in both the GSE53757 dataset and the GSE40435 dataset, the expression levels of CXCL1 mRNA were markedly increased in ccRCC samples compared with the normal tissues (Fig. 1D) or the paracancerous tissues (Fig. 1E). Moreover, clinical staging analysis revealed that patients with stage III/IV ccRCC exhibited significantly higher CXCL1 expression levels compared with those with stage I/II ccRCC (Fig. 1F). Similarly, grade 4 tumors exhibited significantly upregulated CXCL1 expression levels compared with grade 1-3 tumors (Fig. 1G). Furthermore, significant associations were observed between the expression levels of CXCL1 and tumor status (T; Fig. 1H), lymph node status (N; Fig. 1I) and metastasis status (M; Fig. 1J). A prognostic assessment was performed among the different groups utilizing the log-rank test method, which revealed that high CXCL1 expression was significantly associated with shorter overall survival (OS) times, (Fig. 1K), shorter disease-specific survival times (Fig. 1L) and shorter progression-free survival times (Fig. 1M).

CXCL1 expression is associated with the expression of tumor-associated genes, immune cell recruitment and microsatellite instability (MSI) in RCC. STRING database analysis disclosed 10 hub genes that exhibited co-expression with CXCL1 in ccRCC, namely CXCL6, C-C chemokine receptor type 2 (CCR2), CXCR1, CXCL3, CXCL5, C-C motif chemokine 11 (CCL11), CXCR2, atypical chemokine receptor 1 (ACKR1), CXCL2 and CXCR4. TCGA database was employed to assess the expression levels of these 10 key genes, and the results demonstrated a significant reduction in the expression of CCL11, and a notable increase in the expression levels of CCR2, CXCR1, CXCL3, CXCL5, CXCR2, ACKR1, CXCL2 and CXCR4 (Fig. 2A). A protein-interaction network analysis that was subsequently constructed based on the CXCL1 gene revealed that numerous chemokines and their receptors exhibited strong interactions with CXCL1 (Fig. 2B). Correlation analysis indicated that, excluding CCL11, CXCL1 exhibited a significant positive correlation with the expression of the other nine genes (Fig. 2C). TIMER database analysis unveiled a negative correlation between CXCL1 expression and the infiltration of B cells, whereas a positive correlation was observed with the recruitment of $CD4^+$ T cells and neutrophils (Fig. 2D). The findings obtained from MSI status study demonstrated a positive correlation between CXCL1 expression and both the number of mutations (Fig. 2E) and MSI scores (Fig. 2F) in patients with RCC. Furthermore, patients in the MSI group exhibited significantly higher CXCL1 expression levels compared with those in the MSS group (Fig. 2G).

Expression of CXCL1 protein is upregulated in RCC tissues, and its elevated expression is associated with the clinicopathological features of patients with RCC. To further elucidate the clinical significance of CXCL1 in RCC, immunohistochemical experiments were utilized to assess the expression of the CXCL1 protein. The results obtained demonstrated that the expression of CXCL1 was significantly upregulated in RCC tissues compared with paracancerous tissues (Fig. 3A-D). In addition, the study of the clinicopathological characteristics of patients with RCC revealed that the CXCL1 protein level in patients with grade 2-3 RCC was significantly higher compared with that in patients with grade 1. In addition, the CXCL1 protein expression levels in patients with stage II/III cancer was significantly higher compared with that in patients with stage I. However, the present study did not find any association

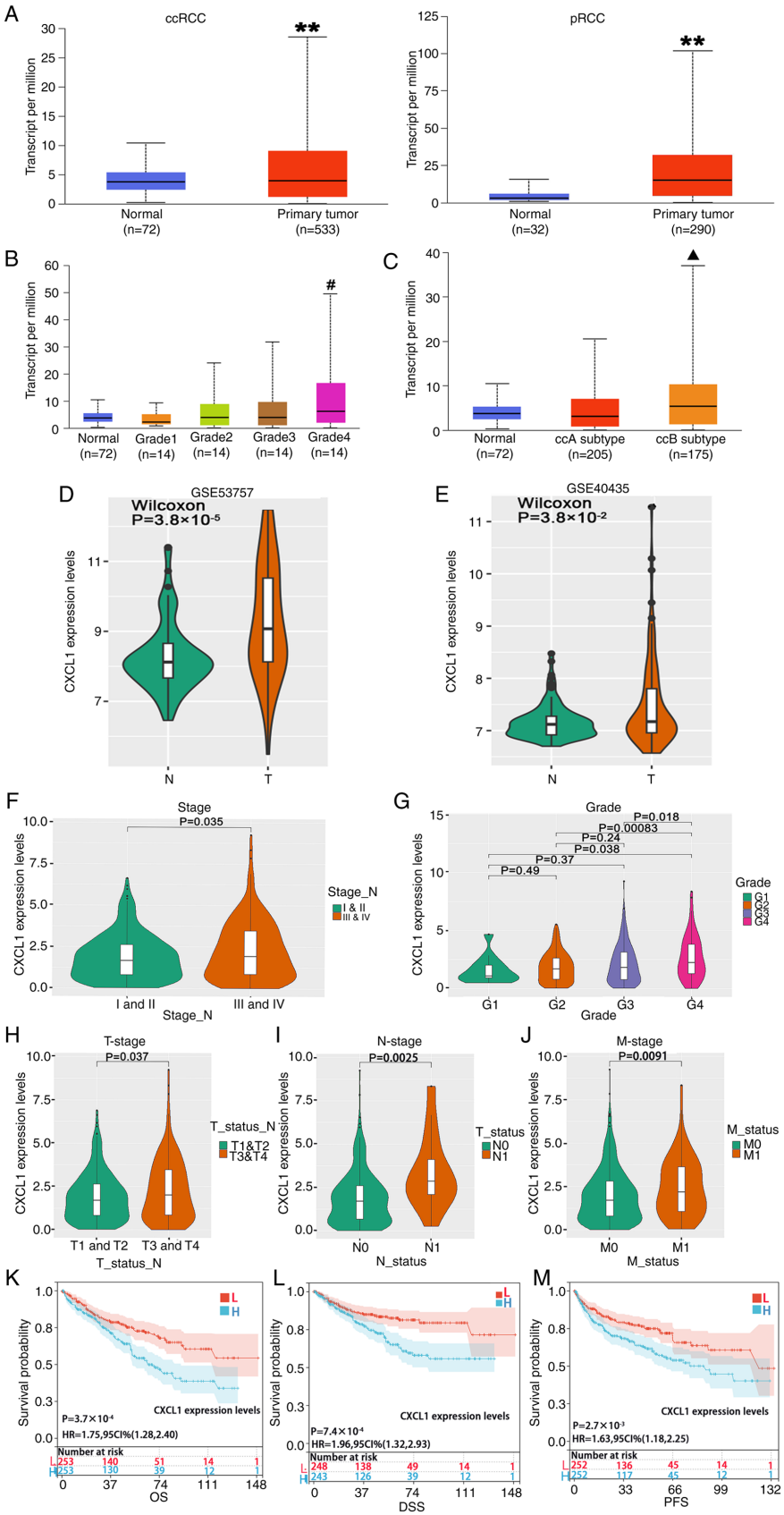

Figure 1. Increased expression of CXCL1 mRNA levels is associated with the clinical characteristics of patients with RCC. (A) The expression profiles of CXCL1 mRNA in normal tissues compared with ccRCC or pRCC tissues. The different levels of expression of CXCL1 mRNA within (B) different clinical grades and (C) subtypes of ccRCC tissues. Expression levels of CXCL1 mRNA in the (D) GSE53757 and the (E) GSE40435 datasets. The different levels of expression of CXCL1 mRNA within different (F) clinical grades and (G) stages of ccRCC tissues. Analysis of the expression level of CXCL1 within the clinical (H) T-status, (I) N-status and (J) M-status categories in ccRCC tissues. (K-M) A prognostic assessment was performed among the various groups. **P<0.01 vs. normal; #P<0.01 vs. normal, grade 1 and 2; ▲P<0.01 vs. normal and ccA subtype. CXCL1, CXC motif chemokine ligand 1; RCC, renal cell carcinoma; ccRCC, clear cell RCC; pRCC, papillary RCC; T, tumor; N, lymph node; M, metastasis; OS, overall survival; DSS, disease-specific survival; PFS, progression-free survival.

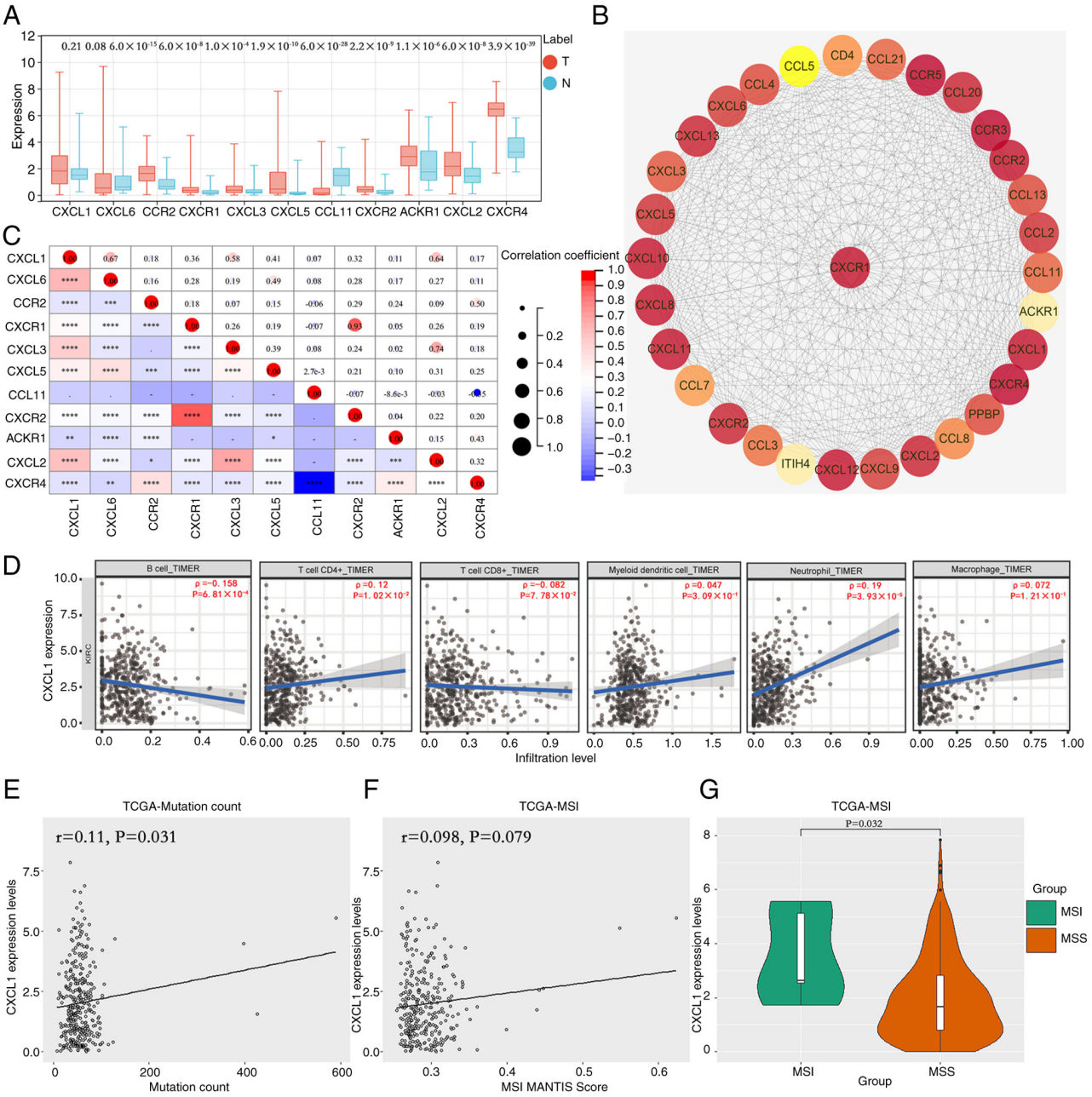

Figure 2. Expression levels of CXCL1 mRNA are correlated with the expression of genes implicated in tumorigenesis, recruitment of immune cells and presence of MSI in renal cell carcinoma. (A) Identification of 10 hub genes exhibiting co-expression with CXCL1. (B) Analysis of the protein-interaction network involving CXCL1. (C) Analysis of the correlation between CXCL1 and the aforementioned 10 hub genes. (D) Analysis of the correlation between CXCL1 expression and immune cell recruitment. The correlation between CXCL1 expression and the (E) number of mutations is presented, as well as (F) MSI scores. (G) The expression levels of CXCL1 in the MSI group compared with the MSS group. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. CXCL, CXC motif chemokine ligand; ACKR1, atypical chemokine receptor 1; CCR, C-C chemokine receptor type 2; MSI, microsatellite instability; MSS, microsatellite stable or no apparent MSI; TIMER, tumor immune estimation resource; TCGA, The Cancer Genome Atlas; T, tumor; N, normal.

between the expression level of CXCL1 protein and the age or the sex of the patients (Table II).

Exogenous CXCL1 contributes to the malignant behaviors of RCC cells. To assess whether CXCL1 is involved in the tumor biological behavior of RCC cells through paracrine or endocrine pathways, relevant studies were performed using exogenous CXCL1. CCK-8 assay analysis revealed that the proliferation rates of 786-O and CAKI-2 cells treated with exogenous CXCL1 at concentrations of 10, 20 and 30 ng/ml (for 786-O cells) or 20 and 30 ng/ml (for CAKI-2 cells) were significantly increased after 72 h in comparison with the 0 mg/ml treatment group (Fig. 4A and B). Furthermore, the Transwell assay experiments also demonstrated that the migratory capabilities of the 786-O cells treated with 5, 10, 20 and 30 ng/ml exogenous CXCL3 and the CAKI-2 cells treated with 10, 20 or 30 ng/ml exogenous CXCL3 after 48 h were significantly enhanced (Fig. 4C-F).

High expression of CXCL1 facilitates the malignant behaviors of RCC cells. To clarify the involvement of endogenous CXCL1 in the biological behavior of RCC progression through an

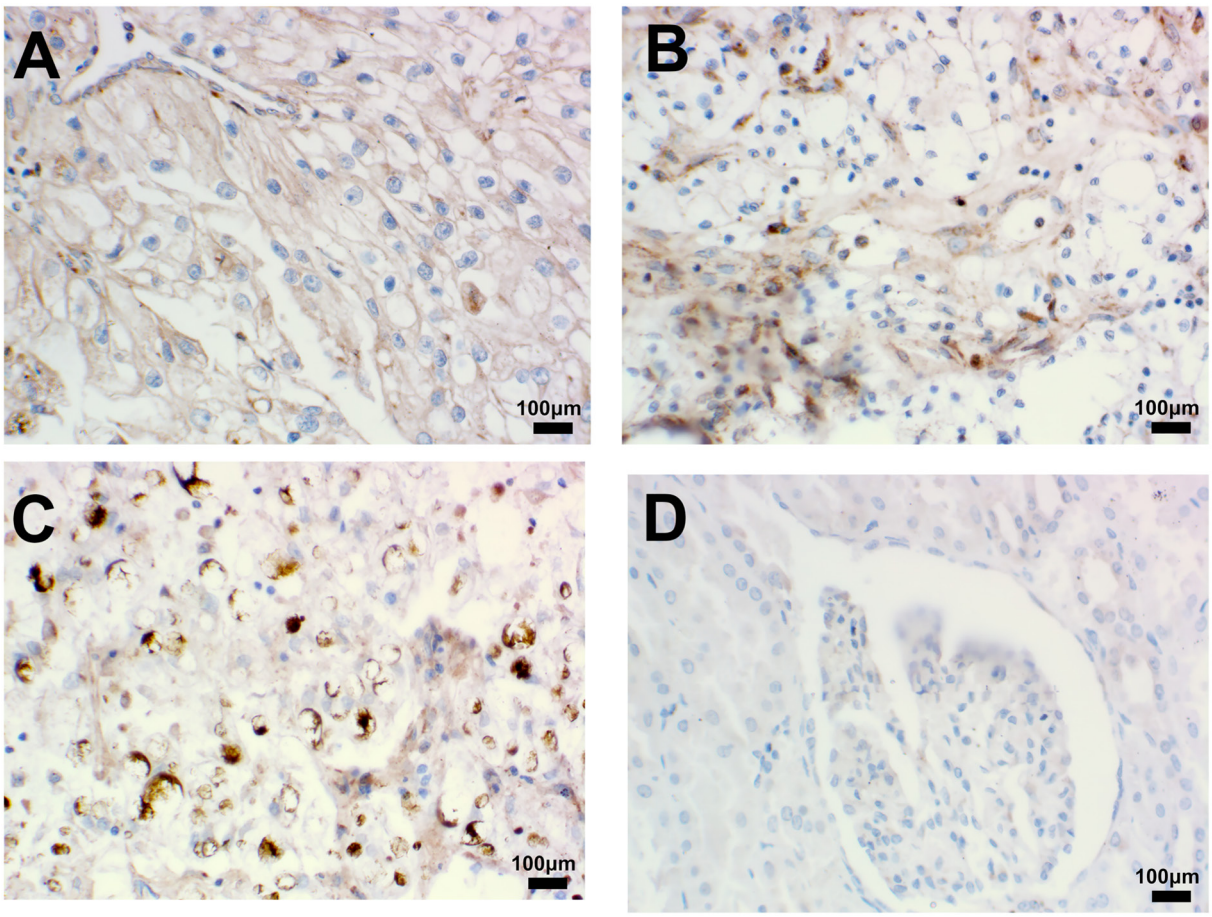

Figure 3. Protein levels of CXCL1 expression are markedly elevated in the tissues of patients with RCC. Representative images, illustrating strong-intensity staining of CXCL1 in (A) grade I, (B) grade II and (C) grade III RCC tissues. (D) Representative image, illustrating weak-intensity staining of CXCL1 in paracancerous tissues. CXCL1, CXC motif chemokine ligand 1; RCC, renal cell carcinoma.

autocrine pathway, lentiviral transfection was used to establish RCC cell lines with a high expression level of CXCL1. ELISA assay revealed that, in comparison with their respective control cells, the secretory levels of CXCL1 protein were significantly increased in the 786-O and CAKI-2 cells expressing a high level of CXCL1 (Fig. 5A and B). Subsequent CCK-8 and Transwell assays demonstrated that the overexpression of CXCL1 led to significant enhancements of the proliferative and migratory capabilities of both 786-O and CAKI-2 cells (Fig. 5C-H).

Low expression of CXCL1 impedes the malignant behaviors of RCC cells. Similarly, in the interference studies, ELISA assay was used to quantify the secretory protein levels of CXCL1 in the supernatant, thereby confirming the successful establishment of 786-O and CAKI-2 cells that downregulated CXCL1 at low levels (Fig. 6A and B). In contrast to the results observed in the CXCL1 overexpression study, the decreased expression of CXCL1 led to a marked reduction in both the cell proliferative and migratory capabilities of the 786-O and CAKI-2 cells (Fig. 6C-H).

Overexpression of CXCL1 regulates the expression of PI3K/AKT-associated proteins in RCC cells. Finally, western blotting assays were performed to investigate the potential underlying mechanisms of CXCL1-mediated malignant progression in RCC cells with high CXCL1 expression. The results showed that, in both 786-O and CAKI-2 cells, overexpression of CXCL1 led to a marked downregulation of the expression Bax, as well as an upregulation of the expression of PI3K, AKT and Bcl-2 proteins (Fig. 7A-D).

Inhibiting AKT reverses the promoting effect of a high expression of CXCL1 on the malignant behaviors of RCC cells. To determine whether the PI3K/AKT pathway regulates the influence of CXCL1 on the malignant behaviors of RCC cells, a further experiment was performed, wherein the AKT-specific inhibitor GDC-0068 was introduced into the cell culture medium to study 786-O and CAKI-2 cells with CXCL1 overexpression and their corresponding empty vector-transfected cells. The CCK-8 assay results revealed that, following GDC-0068 treatment, no significant differences were observed in the proliferative capability between the overexpression and control cells (Fig. 8A and B). Similarly, Transwell (Fig. 8C-F) assays showed that GDC-0068 could reverse the promoting effects of CXCL1 on migration in both cell lines.

Discussion

CXCL1, also known as melanoma growth-stimulatory activity/growth-regulated oncogene a, is a polypeptide that

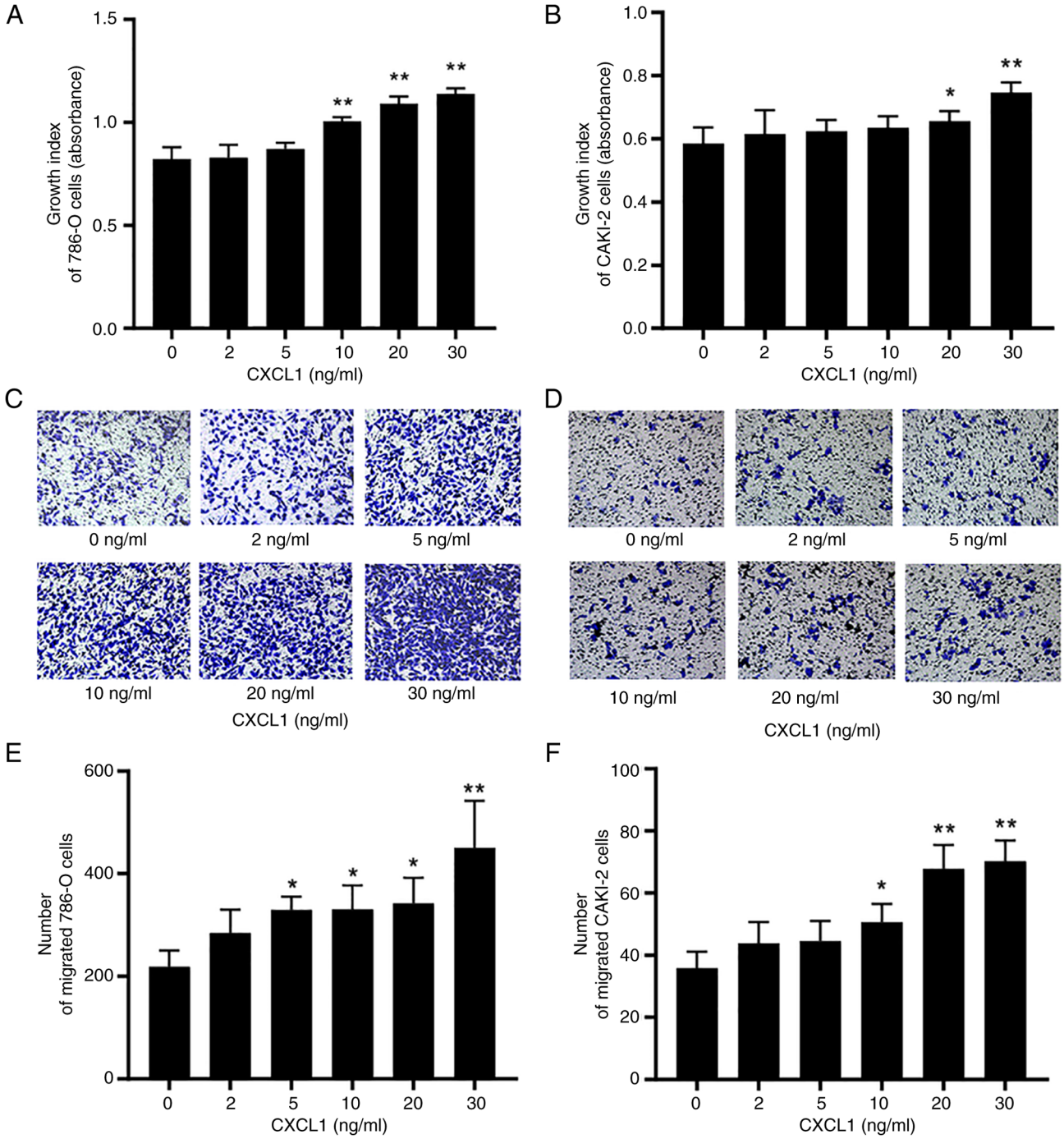

Figure 4. Exogenous CXCL1 treatment enhances the malignant phenotype of renal cell carcinoma cells. Assessment of cell proliferation using a Cell Counting Kit-8 assay in (A) 786-O and (B) CAKI-2 cells. Representative images from Transwell assays with (C) 786-O and (D) CAKI-2 cells (magnification, x100). Assessment of cell migration using a Transwell assay in (E) 786-O and (F) CAKI-2 cells. *P<0.05; **P<0.01 vs. 0 ng/ml. CXCL1, CXC motif chemokine ligand 1.

was originally isolated from human melanoma cells exhibiting chemotactic properties (16). It has been proposed that CXCL1 has the capability to induce migration of immune cells, notably neutrophils, towards sites of inflammation or infection, thereby participating in immune responses and inflammatory reactions (17). To date, accumulating evidence has demonstrated that CXCL1 exhibits upregulated expression in numerous types of human tumor, and its upregulation has been shown to be notably associated with advanced clinical stages, higher grades, distant metastasis and poor prognosis (13,18). For example, evidence has been presented to suggest that the expression level of CXCL1 is markedly upregulated in uterine cervical cancer tissues, and elevated levels of CXCL1 were found to be notably associated with advanced clinical stage and decreased survival probabilities (19). Furthermore, previous studies have revealed a notable association between the expression of CXCL1 and the histological types of tumors. For example, in the case of breast cancer, the expression levels of CXCL1 were found to be markedly increased in tissues of basal-like breast cancer, mesenchymal-like triple-negative

Table II. Expression of CXCL1 in renal cell carcinoma and their adjacent tissues.

Characteristic	CXCL1 expression (mean ± SD)	P-value
Tissue		<0.0001
Adjacent non-cancerous tissues	4.485±1.440	
Cancerous tissues	5.485±1.275	
Sex		0.3836
Male	5.391±1.325	
Female	5.682±1.171	
Age, years		0.9690
≥60	5.489±1.333	
<60	5.476±1.167	
Tumor grade		0.0340
Grade 1	5.129±1.176	
Grade2-3	5.784±1.294	
Clinical stage		0.0384
Stage I	5.250±1.296	
Stage II/III	5.917±1.139	

CXCL1, CXC motif chemokine ligand 1.

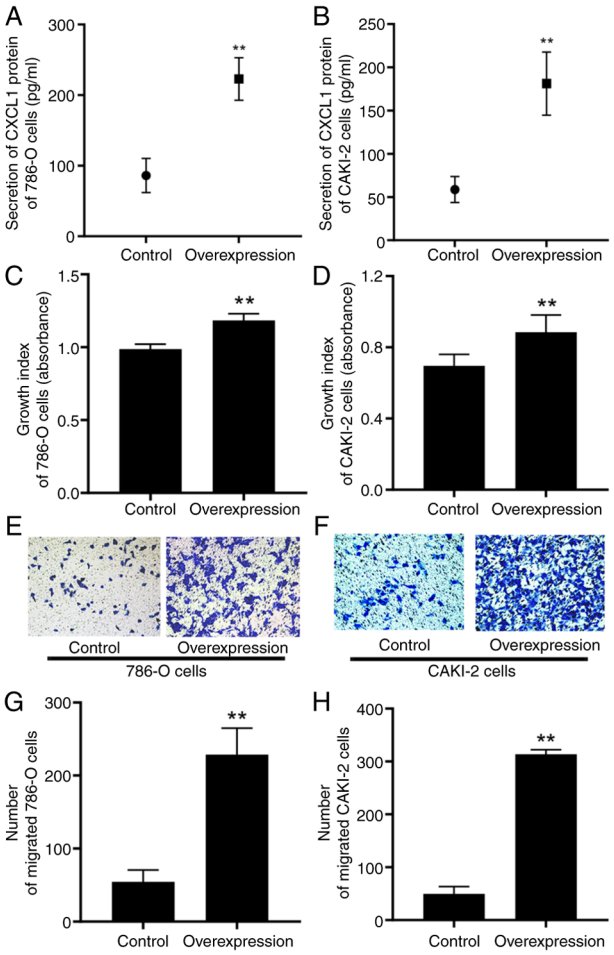

Figure 5. Overexpression of CXCL1 stimulates the malignant phenotype of renal cell carcinoma cells. Evaluation of CXCL3 expression in the supernatant from cell medium using ELISA assay in (A) 786-O and (B) CAKI-2 cells. Assessment of cell proliferation using a Cell Counting Kit-8 assay with (C) 786-O and (D) CAKI-2 cells. Representative images from Transwell assay experiments with (E) 786-O and (F) CAKI-2 cells (magnification, x100). Assessment of cell migration using Transwell assay with (G) 786-O and (H) CAKI-2 cells. **P<0.01 vs. control. CXCL1, CXC motif chemokine ligand 1.

breast cancer and basal-like triple-negative breast cancer compared with normal tissues (13). Additionally, a high expression of CXCL1 was observed in inflammatory breast cancer as compared with other subtypes of breast cancer (13).

Consistent with the aforementioned findings, the present study demonstrated a notable upregulation of CXCL1 mRNA and protein expression in RCC tissues compared with normal and paracancerous tissues. Notably, high expression of CXCL1 was strongly associated with advanced stage, higher grade, unfavorable TNM status and a worse clinical outcome for patients. Moreover, the findings presented in the current study demonstrated markedly increased expression of CXCL1 in the ccB subtype of ccRCC compared with both normal tissues and the ccA subtype. ccRCC comprises two subtypes defined by distinct gene expression profiles: The ccA subtype is characterized by the overexpression of genes associated with hypoxia, angiogenesis, as well as fatty acid and organic acid metabolism, whereas the unfavorable-outcome ccB subtype features the overexpression of genes that regulate epithelial-to-mesenchymal transition, cell cycle progression and wound healing (20). Consequently, the results from the tissue subtype analysis in the present study also suggest an association between elevated CXCL1 expression and the malignancy of ccRCC.

The TME consists not only of tumor cells, but also stromal cells, immune cells, vasculature, extracellular matrix and soluble factors, in which the chemokines form a dynamic system that fulfills a pivotal role in tumor progression (10). It is now widely accepted that the chemokine system is involved in tumor development via multiple direct and indirect mechanisms that modulate immune cell infiltration, angiogenesis, as well as tumor cell proliferation, invasion, metastasis and stem-like properties (21). The present study on co-expression hub gene and protein-interaction network analysis revealed that numerous chemokines and their receptors, including CXCL3, CXCL5, CXCL8, CXCR1 and CXCR2, exhibit co-expression and interactive associations with CXCL1 in RCC. Similarly, CXCL3, CXCL5 and CXCL8 (along with CXCL1) belong to the ELR + CXC chemokine subfamily, and their functions are all mediated by the same CXC receptor, CXCR2. A number of studies have demonstrated that CXCL3, CXCL5 and CXCL8 serve crucial roles in the oncogenic potential of various types of tumor (22-25). For example, the investigation of CXCL3 in prostate cancer in the present study demonstrated that CXCL3 is highly expressed in tumor tissues, and both the exogenous administration and overexpression of CXCL3 have been shown to promote tumor proliferation and migratory capabilities through the ERK/AKT signaling pathway (26,27).

Within the TME, both tumor cells and tumor-associated cells are able to release a series of chemokines that regulate the infiltration and activation of diverse immune cell types, ultimately influencing the balance between tumor-promoting

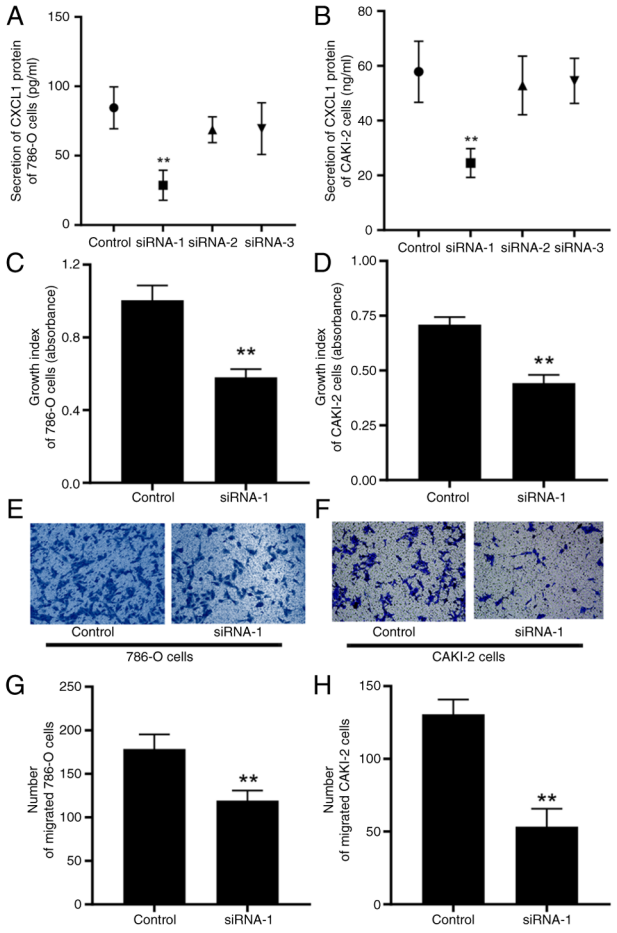

Figure 6. Low expression of CXCL1 suppresses the malignant phenotype of renal cell carcinoma cells. Evaluation of CXCL3 expression in the supernatant from cell medium using ELISA assay with (A) 786-O and (B) CAKI-2 cells. Assessment of cell proliferation using a Cell Counting Kit-8 assay with (C) 786-O and (D) CAKI-2 cells. Representative images from Transwell assay experiments (magnification, x100) with (E) 786-O and (F) CAKI-2 cells. Assessment of cell migration using Transwell assay with (G) 786-O and (H) CAKI-2 cells. **P<0.1 01 vs. control. CXCL1, CXC motif chemokine ligand 1; siRNA, small interfering RNA.

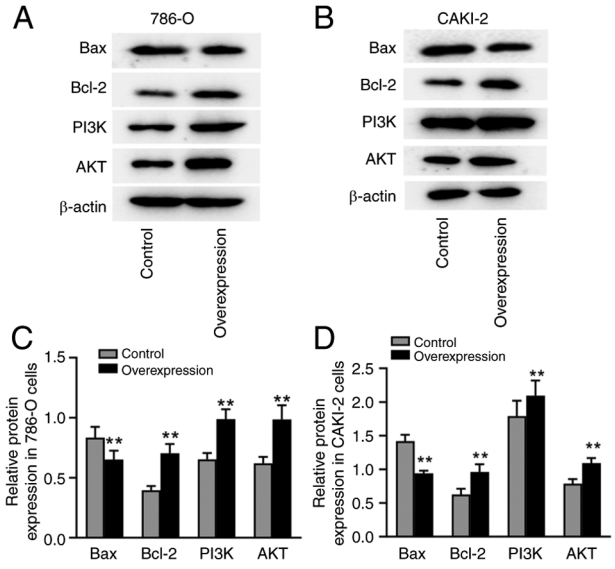

Figure 7. Overexpression of CXCL1 modulates the expression of PI3K/AKT pathway-associated proteins in renal cell carcinoma cells. Representative images from western blotting assays in (A) 786-O and (B) CAKI-2 cells. Semi-quantification of the protein expression levels of Bax, Bcl-2, PI3K and AKT in (C) 786-O and (D) CAKI-2 cells. **P<0.01 vs. control. CXCL1, CXC motif chemokine ligand 1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; PI3K, phosphoinositide 3-kinase.

and tumor-suppressing activities (8). Among the recruited immune cell population, several exhibit antitumor activities, including CD8$^+$ T cells, type 1 T helper (TH1) cells, multifunctional TH17 cells and natural killer cells. Additionally, the recruited antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs), contribute to tumor regression through activating and amplifying local immune effector cells (8). On the other hand, other recruited immune cells, including myeloid-derived suppressor cells, regulatory T cells, IL-22$^+$ CD4$^+$ TH22 cells, IL-22$^+$ innate lymphoid cells and plasmacytoid DCs, have been demonstrated to accelerate tumor progression through fostering angiogenesis, suppressing antitumor immune responses and maintaining tumor stemness (8). In the present study, a negative correlation between CXCL1 expression and B cell infiltration was demonstrated in RCC, whereas a positive correlation was observed with the attraction of CD4$^+$ T cells and neutrophils. It is considered that B cells, possessing the capacity to generate antibodies, serve an important role as crucial mediators in humoral immunity (28). Multiple chemokine/receptor systems, including CCL21/CCR7, CXCL12/CXCR4 and CXCL13/CXCR5, are implicated in the recruitment of B cells to the TME (29). A previous study has shown that B cells exhibit antitumor activity through a multitude of mechanisms, including the direct killing of tumor cells, the production of specific antibodies against tumor antigens, acting as APCs to induce T-cell activation and memory T-cell development, and facilitating the immune responses of CD4$^+$ and CD8$^+$ T cells (21). Similarly, although previous studies have established that neutrophils display antitumor properties through direct cell toxicity, antibody-dependent cellular cytotoxicity and the presentation of specific antigens (30), tumor-associated neutrophils notably present tumor-promoting activities (31).

Subsequently, the functional experiments performed in the present study showed that both the exogenous administration and overexpression of CXCL1 led to a marked augmentation of the proliferative and migratory abilities of RCC cells, whereas decreased expression of CXCL1 had an inhibitory effect on these tumorigenic behaviors. CXCL1 has been recognized as an important autocrine/paracrine molecule in the TME for supporting tumor progression. For example, silencing CXCL1 has been shown to markedly reduce tumor proliferation through effectively inducing apoptosis in hepatocellular carcinoma cells, suggesting that autocrine signaling networks may serve a role in CXCL1-associated tumor biology (32). Tumor cells with higher CXCL1 levels have also been shown to trigger the infiltration of tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) into the TME. CXCL1 derived from TAMs and CAFs serves a crucial role in regulating intercellular adhesion and crosstalk between tumor cells and these stromal cells, further enhancing tumor proliferation in a paracrine manner (33). Ultimately, the findings of the present study have revealed that the high expression

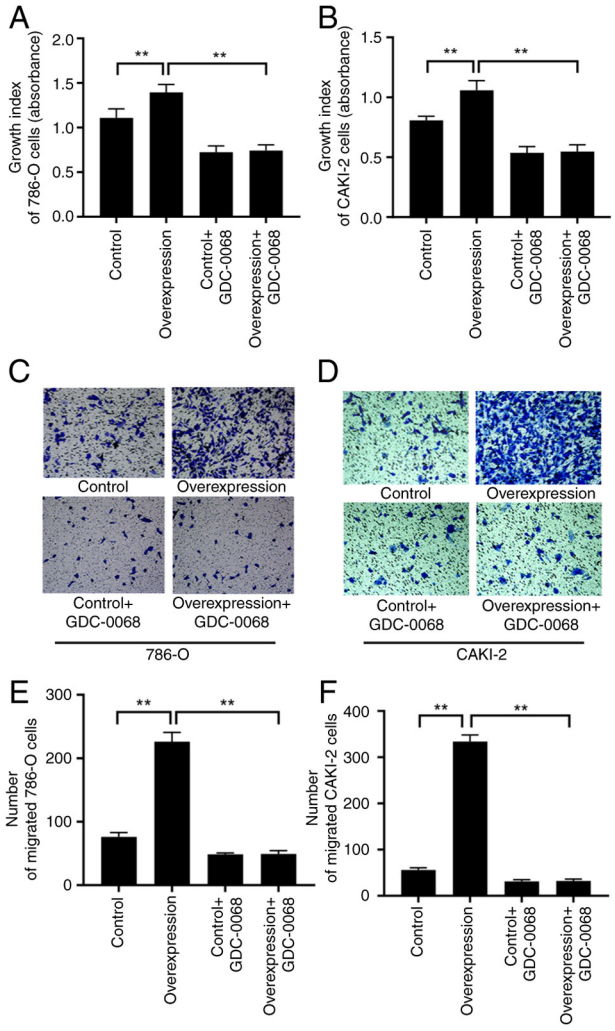

Figure 8. Blocking AKT reverses the promoting effect of overexpression of CXCL1 on the malignant behaviors of renal cell carcinoma cells. Assessment of cell proliferation using a Cell Counting Kit-8 assay in (A) 786-O and (B) CAKI-2 cells. (Representative images from Transwell assay experiments (magnification, x100) with (C) 786-O and (D) CAKI-2 cells. Assessment of cell migration using Transwell assay with (E) 786-O and (F) CAKI-2 cells. (magnification, x100). **P<0.01 compared with overexpression. CXCL1, CXC motif chemokine ligand 1.

of CXCL1 in RCC cells exerts a strong modulatory role on the expression patterns of proteins associated with the PI3K signaling pathway, including Bax, Bcl-2, PI3K and AKT. The PI3K pathway is a crucial signaling cascade within the body, and an abundance of evidence has shown that the activation of the PI3K signaling pathway is one of the most prevalent events in human cancers, being heavily implicated in the development and progression of tumors through its role in regulating cell growth, survival, proliferation and migration (34).

In conclusion, the present study demonstrated that CXCL1 is upregulated in RCC, and this upregulation is positively associated with various clinicopathological factors, as well as tumor-associated chemokine expression and immune cell recruitment. Furthermore, functional studies indicated that overexpression of CXCL1 contributes to the malignant behaviors of RCC cells, which are partly mediated via the PI3K/AKT signaling pathway. However, although the present study has preliminarily elucidated the role of CXCL1 in RCC, the authors acknowledge that these findings require validation through performing *in vivo* experiments in the future, and this represents a key limitation of the current research. Nevertheless, in spite of this limitation, the present study has not only deepened the understanding of the mechanisms underlying RCC, but has also offered crucial insights into, and potential approaches for, the diagnosis and treatment of this disease, highlighting its notable clinical and translational implications.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HQ carried out the experimental design and edited the manuscript. SG and CL performed the cellular and molecular biology experiments. HX performed the bioinformatic analysis. SG drafted the initial manuscript. CL carried out immunohistochemical experiments and statistical analyses. HZ and YW performed cellular experiments. BW carried out molecular biology experiments. SG and HZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of School of Clinical Medicine, Jiamusi University (approval no. 202315; ethical approval period from 2023 to 2025), and was performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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