M2 Macrophages Secrete CXCL13 to Promote Renal Cell Carcinoma Migration, Invasion, and EMT

Yingwei Xie
Sun Yat-Sen Memorial Hospital
Zhiliang Chen
Sun Yat-Sen Memorial Hospital
Qiyu Zhong
Sun Yat-Sen Memorial Hospital
Zaosong Zheng
Southern Medical University Nanfang Hospital
Yuqing Chen
Sun Yat-Sen Memorial Hospital
Wentai Shangguan
Sun Yat-Sen Memorial Hospital
Yishan Zhang
Sun Yat-Sen Memorial Hospital
Jingying Yang
Sun Yat-Sen Memorial Hospital
Dingjun Zhu
Sun Yat-Sen Memorial Hospital
Wenlian Xie (xiewl@mail.sysu.edu.cn)
Sun Yat-Sen Memorial Hospital https://orcid.org/0000-0001-5222-0767

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Abstract

Objective: M2 macrophages are associated with a poor prognosis in a variety of malignancies. There are, however, few relevant investigations in clear cell renal cell carcinoma (ccRCC).

Methods: The expression of M2 macrophages in ccRCC tissues was first discovered using immunohistochemistry in this study. Then, M2 macrophages were created in vitro to see how they affected the proliferation, migration, invasion, and EMT of ccRCC cells. Using qPCR and prognostic analysis identifies important chemokine. Antibody neutralization tests confirmed the chemokine's involvement and function. Pathway inhibitors confirmed the main pathway of M2 macrophages in ccRCC. Finally, qPCR and IHC were used to confirm the expression of chemokine receptors in ccRCC tissues.

Results: The presence of M2 macrophages was linked to a poor outcome in ccRCC. M2 macrophages enhanced the proliferation, migration, invasion, and EMT of ccRCC lines in vitro. CXCL13 was identified as the main chemokine by prognostic analysis and qPCR tests. CXCL13 neutralizing antibodies can inhibit the stimulation of M2 macrophages in ccRCC lines' proliferation, migration, invasion, and EMT. M2 macrophages and CXCL13 may activate the Akt pathway in ccRCC lines, and Akt inhibitors decrease ccRCC lines proliferation, migration, invasion, and EMT. CXCR5 expression is a poor prognostic factor for renal cell carcinoma, according to qPCR and immunohistochemistry. In vivo experiments further proved that CXCL13 secreted by M2 macrophages can promote tumor proliferation.

Conclusion: M2 macrophages in the immunological milieu secrete CXCL13, which promotes ccRCC proliferation, migration, invasion, and EMT. Our findings contribute to a better understanding of the function of the tumor microenvironment in the incidence and progression of ccRCC, and they may point to novel therapeutic targets for ccRCC.

Background

Renal cell carcinoma (RCC) accounts for around 2–3% of all malignant tumors worldwide, and its prevalence is rising. Metastatic renal cell carcinoma accounts for 25–30% of all RCC and has an exceedingly poor prognosis [1]. In 2020, there will be roughly 430,000 new instances of RCC found worldwide, with 179,000 fatalities as a result of RCC [2]. Clear cell renal cell carcinoma (ccRCC) is the most prevalent form of RCC, accounting for about 70–80% of all cases [3]. As a result, determining the mechanism and molecular indicators of ccRCC metastasis is critical to improving the prognosis of ccRCC patients.

RCC is an immunogenic tumor, and immunotherapy has long been an essential part of RCC treatment [4]. The tumor microenvironment (TME), which is composed of mesenchymal cells and immune cells that have been recruited, has immunosuppressive and tumor-promoting effects [5]. Tumor-associated macrophages (TAM) are among the most numerous cells in TME. M2 macrophages, the major

component, have been recognized as a poor prognostic factor for a variety of malignancies, including RCC [5–7]. However, the mechanism through which TAM contributes to tumor development is unknown. M2 macrophages in TAM have been demonstrated in several studies to have a role in tumor growth, invasion, and metastasis through chemokines [8, 9]. However, research on TAM's function in ccRCC is currently limited.

Chemokine ligand 13 (CXCL13) belongs to the CXC chemokine family, and chemokine receptor 5 is its particular receptor (CXCR5). In a variety of malignant tumors, CXCL13 interacts with its receptor CXCR5 to promote carcinogenesis, development, and metastasis [10]. CXCL13 has previously been shown to be a poor prognostic factor for ccRCC and to enhance the proliferation of ccRCC cells [11]. We also discovered that M2 macrophages may release a lot of CXCL13, which promotes the proliferation, invasion, and metastasis of ccRCC.

Methods

Patient tissue sample

This study was approved by the Sun Yat-sen Memorial Hospital Ethics Committee. From September 2011 to March 2021, our hospital (SYSMH) collected 190 ccRCC tissues and their matching adjacent tissues. The 190 ccRCC patients included 111 men and 79 women of age 21–89 years (average age: 53.2 years). Written informed consent was obtained from all participants. The patient inclusion criteria included clear cell renal cell carcinoma. Exclusion criteria included patients who did not receive any anti-tumor therapy before surgical resection. The pathologist of our hospital confirmed the ccRCC tissues and the adjacent non-tumor tissues.

Database

The database information used in this study is TCGA (https://portal.gdc.cancer.gov/). Since the data was sourced from an open public database, there was no need to obtain research approval from the ethics committee.

Reagents and antibodies

Recombinant human CXCL13 (300-47-50) was purchased from PeproTech (New Jersey, USA). PMA was obtained from Sigma (Darmstadt, Germany). The following antibodies are used for western blotting (WB), neutralization assay, flow cytometry, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (IHC): CD11b and CD206 (BD Biosciences, San Diego, USA); CXCL13 ELISA (ab179881), rabbit anti-CXCL13 (Ab272874), rabbit anti-CXCR5 (ab203212), mouse anti-GAPDH (ab8245), rabbit anti-AKT (9272; CST); rabbit anti-p-AKT (Ser473; 4060, CST), rabbit anti-E-cadherin

(3195; CST), rabbit anti-N-cadherin (13116; CST), and rabbit anti-vimentin (5741; CST). Akt inhibitor A3149 was purchased from APExBIO (Houston, USA).

Cell culture

The human ccRCC cell lines Caki-1, ACHN, and human THP-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS; Hyclone Technologies) and
1% penicillin and streptomycin. All cell lines are maintained in a humid environment at 37°C and under a 5% CO_2 atmosphere.

THP-1 cells induced differentiation

The THP-1 cells were collected and inoculated into 6-well plates and then induced with PMA (100 ng/mL). M0 macrophages were obtained after culturing for 48 h. The Caki-1 and ACHN cells were seeded in the upper cavity of a Boyden chamber (0.4-μm pore size; Corning, USA) and co-cultured with M0 macrophages for 4 days to obtain M2 macrophages, after which flow cytometry was performed to determine whether the induction was successful.

Conditioned medium (CM)

After the macrophages are induced to differentiate, and then the macrophages were washed with PBS. Then, a fresh medium was added to the cells, followed by incubation for another 24 h. The medium supernatant was harvested and filtered, after which the complete medium was added at a ratio of 30% to produce the corresponding conditioned medium (M0-CM and M2-CM).

Co-cultivation program

The ccRCC cells and THP-1-derived macrophages were co-cultured in a Boyden chamber. M0 and M2 macrophages were seeded in the upper chamber, and the Caki-1 and ACHN cells were seeded in the lower chamber. Next, recombinant human CXCL13, CXCL13-neutralizing antibody, or AKT inhibitor A3149 were added to the lower chamber and incubated for12 h for co-cultivation. The RCC cells in the lower chamber were finally collected for PCR or WB.

RNA extraction and quantitative PCR (RT-qPCR).

TRIzol (Thermo Fisher Scientific) was used to extract the total RNA from cultured cells or tissues. With reference to the manufacturer's protocol, total RNA was reverse-transcribed into cDNA

using the Prime Kit (Takara Biotechnology). The ABI7500 real-time PCR system (Thermo Fisher Scientific) was used to perform PCR with SYBR premixed real-time fluorescent quantitative PCR reagents (Taojiu Biotechnology). GAPDH was used as an internal control. The comparative 2-ΔΔCq method was used for relative quantification. The primer sequences of the analyzed genes are shown in Appendix 1.

Western blotting analysis

The total protein was separated from the cells with RIPA lysis buffer, separated by polyacrylamide gel, and then transferred onto the PVDF membrane. After blocking with 5% BSA solution, the membrane was incubated with primary antibodies (including E-cadherin, N-cadherin, Vimentin, AKT, and p-AKT) overnight. This membrane was then incubated with HRP-conjugated anti-rabbit antibody or anti-mouse antibody for 1 h at room temperature. The super signal WestFemto Maximum Sensitive Substrate (Thermo Fisher Science) was used to detect protein bands and the images were captured.

MTS determination

Cell proliferation was measured by the MTS assay. Cells (1×10^3; 100 μL per well) were seeded into 96-well plates. Different groups of cells were added with different (fresh) conditioned media (PBS, M0-CM, and M2-CM) and the corresponding additives (recombinant human CXCL13, neutralizing antibody of CXCL13, or AKT inhibitor A3149) daily. Next, 20% MTS solution was added to each well, followed by incubation at 37°C in the dark for 2 h. The optical density of the cells was then measured at 492 nm. The measurements were performed at 0, 24, 48, 72, 96, and 120 h to generate growth curves.

Wound healing assay

The cells were seeded in 12-well plates and cultured to produce a confluent monolayer. By using a 200-μL pipetting tip, the wound area was scratched and then collected tissues were washed thrice in PBS to remove debris. The washed cells were placed in a 12-well Boyden chamber and then inoculated with M0 and M2 macrophages in the upper chamber and FBS-free medium and additives (recombinant human CXCL13, neutralizing antibody to CXCL13, or AKT inhibitor) in the lower chamber. The wound closure was observed under an inverted microscope and the images were captured during 0–12 h.

Migration and intrusion detection

In the migration test, 1×10^5 cells were suspended in 200-μL of serum-free medium per well and seeded in the upper chamber of a 24-well Boyden chamber. In the invasion test, 1×10^5 cells suspended in 200 μL of serum-free medium were inoculated into the upper chamber of a Matrigel-coated cell. At the beginning of the cell migration assay, 1×10^4 M0 and M2 macrophages were seeded in the lower

compartment. Next, recombinant CXCL13, anti-CXCL13 neutralizing antibody, and Akt inhibitor were added to the lower compartment. After 48 h or 72h of incubation, the membrane was fixed with 4% paraformaldehyde and then stained with crystal violet solution. At least 5 randomly selected areas were counted at a magnification of 100X.

ELISA

According to the manufacturer's instructions, the secretion of human CXCL13 in the supernatant of M0 and M2 macrophages was detected. After color development, the absorbance at 450 nm was measured on a microplate reader.

Flow cytometer

According to the manufacturer's instructions, the digested cells were washed with PBS and incubated with flow cytometry antibodies CD11b and CD206 for 20 min in the dark, followed by washing with PBS and resuspension on the machine. Finally, the CellQuest software version 7.5.3 (FACS Vantage-SE, BD Immunocytometry Systems, San Diego, CA) was employed to analyze the cells by multicolor flow cytometry.

Immunochemistry

The ccRCC and adjacent tissues were fixed with formalin, and the paraffin-embedded tumor tissues and the adjacent sections were stained with anti-CD206 antibody and anti-CXCR5 antibody, respectively. CD206 was used as a marker for M2 macrophages. The stained cells were counted in each field of view, and each slice had a field of view of at least 400 times. The average number of positive cells from the 5 highest field measurements was used for subsequent data analysis. The expression of the target protein was evaluated by the ratio and intensity of positive cells.

Xenotransplantation experiment

All animal experiments were performed following the protocol approved by the Animal Care and Use Institutional Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University (Guangzhou, China). In order to study the effect of CXCL13 on tumor growth, 5-week-old female BALB/c nude mice were randomly divided into negative and CXCL13 groups (n = 5 in both groups). Approximately 5.0×10^6 Caki-1 cells were injected subcutaneously into the upper back of nude mice. When the xenograft was palpable (approximately 0.5-cm in diameter), 0.1 mg/kg of PBS or CXCL13 was injected weekly for 4 consecutive weeks [9]. The following formula was used to calculate the tumor volume (V) every 3 days: $V = (W^2 \times L)/2$. Finally, mice were sacrificed (place the mice in the euthanasia chamber, and then introduce 100% CO2 gas at a flow rate 25% of chamber volume per minutefor 4 min.).

Statistical analysis

All statistical calculations were performed using the SPSS 20.0 (IBM Corp.) and R software. Quantitative data and categorical data were analyzed by Student's *t*-test and Fisher's exact test, respectively. One-way analysis of variance and Tukey's post-test were performed for statistical analysis on more than 2 groups. The cumulative survival rate was calculated by Kaplan–Meier analysis and log-rank test. $P < 0.05$ was considered to indicate a statically significant difference.

Results

Increased M2 macrophage infiltration is linked to a poor outcome in patients with cRCC.

To see if M2 macrophage infiltration is linked to ccRCC tumorigenesis and progression, we utilized CD206 protein as an M2 macrophage marker and assessed M2 macrophage infiltration in 55 ccRCC samples using IHC (Fig. 1). The degree of M2 macrophage infiltration was positively correlated with the pathological grade and T stage of ccRCC patients, according to the findings (table 1). These findings suggest that M2 macrophages play a crucial role in ccRCC tumorigenesis and development.

Table 1

Associations of M2 macrophage infiltration with clinicopathological ccRCC features

Characteristics		Number	CD206		*P*-value
			Positive	Negative	
Gender	Male	30	8	22	0.456
	Female	25	9	16	
Age	≤60	39	10	29	0.187
	60	16	7	9	
Grade	G1-2	35	6	29	0.003*
	G3-4	20	11	9	
T stage	T1-2	45	11	34	0.028*
	T3-4	10	6	4	
*Results were considered statistically significant at $P < 0.05$					

M2 macrophages generated from THP-1 induce EMT in ccRCC cells

To better understand the function of M2 macrophages in ccRCC. THP-1 was initially converted into M0 macrophages using PMA, and then co-cultured with ccRCC cell lines Caki-1 and ACHN to produce M2 macrophages, which were detected by flow cytometry (Fig. 2A-B), and CD206 positive rates increased substantially, demonstrating that macrophages and ccRCC cell lines may differentiate into M2 macrophages when co-cultured. Through M2 macrophage conditioned medium and co-culture, we discovered that M2 macrophage can enhance Caki-1 and ACHN proliferation, migration, and invasion (Fig. 2C-E).

We investigated the expression of EMT markers in ccRCC since M2 macrophages can enhance the migration and invasion of ccRCC cell lines. Co-culture with M2 macrophages resulted in a substantial reduction in E-cadherin expression in Caki-1 and ACHN cells, whereas N-cadherin and vimentin expression increased at the same time (Fig. 2F). In conclusion, these findings suggest that M2 macrophages may enhance ccRCC cell proliferation, migration, and invasion via EMT.

Differential expression of chemokines in macrophages and RCC tissues

Many chemokines are known to be secreted by M2 macrophages to modulate their function in tumor spreading. As a result, we first assessed the effect of different chemokines on the prognosis of ccRCC. The TCGA database was used to evaluate the expression of different chemokines in ccRCC, and the results are given in Appendix 2. Then, using univariate prognostic analysis, we investigated the effect of different chemokines on the prognosis of ccRCC. The findings revealed that 15 chemokines were strongly associated with the prognosis of ccRCC (Fig. 3A). Using qPCR, we found a variation in Chemokine expression between M2 macrophages and M0 macrophages. The findings revealed that the expression of 7 chemokines rose substantially (Fig. 3B). Finally, we used the intersection of the Venn diagram to identify the three essential chemokines CCL7, CCL8, and CXCL13 (Fig. 3C). We chose CXCL13 with the highest expression difference for ELISA experiments, and the results confirmed that M2 macrophages secreted a large amount of CXCL13 (Fig. 3D).

CXCL13 is an important cytokine for M2 macrophages in promoting EMT in ccRCC

Based on the data above, we tried to investigate if CXCL13 is required for M2 macrophages to induce ccRCC EMT. As a result, we utilized anti-CXCL13 antibodies to neutralize CXCL13 function in the conditioned medium and co-culture supernatant. We found that anti-CXCL13 antibodies can decrease M2 macrophages proliferation, migration, and invasiveness to ccRCC cells (Fig. 4A-C). Furthermore, we

discovered that CXCL13 can induce a substantial reduction in E-cadherin expression while increasing N-cadherin and vimentin expression. CXCL13 neutralization significantly reversed the down-regulation of epithelial markers and the up-regulation of mesenchymal markers caused by ccRCC cells and M2 macrophages co-culture (Fig. 4D).

M2 macrophages secreted CXCL13 to promotes EMT of ccRCC cells via Akt signaling pathway

Previous research has shown that CXCL13 can enhance the proliferation of ccRCC cells via the PI3K-Akt pathway (11). We utilized a specific AKT inhibitor (A3149) in this work and discovered that A3149 can inhibit the stimulation of M2 macrophages on the proliferation, migration, and invasion of ccRCC cells (Fig. 5A-C). A3149 also inhibited the expression of phosphorylated Akt (p-Akt) and EMT in ccRCC cells co-cultured with M2 macrophages or treated with CXCL13 (Fig. 5D). These results indicate that CXCL13 produced by M2 macrophages may enhance ccRCC cell proliferation, migration, invasion, and EMT via Akt signaling pathway.

CXCL13 receptor CXCR5 is a poor prognostic factor for ccRCC

CXCR5 is the sole CXCL13 receptor. We initially investigated the connection between CXCR5 mRNA expression and the clinicopathological characteristics and prognosis of ccRCC using our hospital's (SYSMH) and TCGA databases. The findings revealed that increased CXCR5 expression was associated with a higher pathogenic grade and stage. The patients with high CXCR5 expression had a substantially worse outcome than patients with low expression (Fig. 6A, B). IHC was then used to examine the expression of the CXCR5 protein in RCC tissues. The findings revealed that there was a substantial difference in CXCR5 staining between cancer and surrounding normal tissues (Fig. 6C). In 90 ccRCC tissues, there are substantial variations in the clinicopathological features of CXCR5 positive and negative patients (Table 2). These clinical findings on CXCR5 demonstrate that the prognosis of ccRCC patients is poor, which is consistent with the in vitro results and supports the critical function of the CXCL13-CXCR5 axis in the development of ccRCC.

Table 2

Correlation between CXCR5 expression and clinical characteristics of ccRCC patients

Characteristics		Number	CXCR5		*P*-value
			Positive	Negative	
Gender	Male	55	27	28	0.829
	Female	35	18	17	
Age	≤60	60	29	31	0.989
	60	30	16	14	
Grade	G1-2	56	23	33	0.030*
	G3-4	34	22	12	
T stage	T1-2	80	37	43	0.044*
	T3-4	10	8	2	
*Results were considered statistically significant at $P < 0.05$					

CXCL13 promotes the growth of ccRCC in vivo

Caki-1 cells were placed beneath the skin of nude mice to examine the role of CXCL13 in vivo. The nude mice were randomly split into two groups: experimental and control. Every week, the experimental group received an intratumor injection of CXCL13, and the tumor volume was assessed. After four weeks, the tumor was removed. CXCL13 greatly increased tumor volume when compared to the control group (Fig. 7A-C). These findings suggest that CXCL13 can considerably enhance ccRCC proliferation in vivo, which is consistent with the in vitro findings.

Discussion

Macrophages are immune cells that are part of the human immune system's humoral immunity and play a key role in autoimmunity, inflammatory response, and tumor immunity [12]. Macrophages are a varied collection of cells with high plasticity and heterogeneity. Most macrophages are classified into two phenotypes based on their activation status. M1 type and replacement activated macrophages(M2 type) are two types of classically activated macrophages [13]. Tumor-associated macrophages are macrophages that infiltrate TME (TAMs).TAMs are the most predominant infiltrating leukocyte group in most advanced solid tumors. TAM is now well acknowledged to primarily exhibit the kind of M2 macrophages that promote tumor development [13–15]. We co-cultured macrophages with tumor cells in this study to obtain M2 macrophages. The findings demonstrate that induced differentiation of M2 macrophages can promote the proliferation, migration, and metastasis of renal cancer cell lines. And, using qPCR and ELISA, we demonstrated that M2 macrophages play a key role in the chemokine CXCL13.

CXCR5 is the sole CXCL13 receptor.CXCR5 was discovered to be a poor predictive factor for ccRCC in this research.

According to recent research, significant TAMs infiltration is related to a poor prognosis of different malignancies [16]. TAMs have an impact on nearly every aspect of tumor biology. It has the potential to induce angiogenesis, tumor progression, invasion, and metastasis. This influence on tumor development at various stages demonstrates their functional diversity. M2 macrophages have been reported to enhance the invasion and metastasis of gallbladder cancer [8], breast cancer [9], and head and neck squamous cell carcinoma [17] via the chemokine CCL18. M2 macrophages have also been reported to stimulate tumor angiogenesis via CCL-2 and CXCL8, as well as destroy the surrounding tissue matrix by producing tissue enzymes, therefore establishing a prerequisite regulation for cancer cell metastasis [18–19]. TAM plays a complex function in tumor immunosuppression. TAM generates many immunosuppressive factors and chemokines, including IL-6, IL-10, TNF-α, TGF-β, etc., which decrease antigen presentation and impair T cell activity, allowing tumor cells to elude the body's immunological surveillance [20]. We observed that M2 macrophages may enhance the proliferation, migration, invasion, and EMT of ccRCC through CXCL13 in our study. This is consistent with the finding M2 macrophage infiltration is a poor prognostic factor for RCC in the majority of studies [21].

The CXCL13/CXCR5 axis has been investigated in several tumors, although the associated consequence varies. According to research, the CXCL13/CXCR5 axis promotes colon cancer incidence, progression, and metastasis by secreting MMP13 and activating the PI3K/AKT pathway [22]. It can also suppress tumor immunity via the STAT3 signaling pathway, increasing colon cancer development and metastasis [23]. According to research, CXCL13 is an androgen-responsive gene. CAFs release CXCL13 in prostate cancer and have a role in androgen-independent prostate cancer development [24]. CXCL13, on the other hand, has been shown in certain studies to prevent the occurrence and growth of malignant tumors. Upregulation of CXCL13 expression in estrogen receptor-negative and human epidermal growth factor-positive breast tumors predicts a favorable prognosis. The Tfh cell subset capable of secreting CXCL13 converts Treg-mediated immunosuppression into adaptive anti-tumor immune activity. The essential component and has the potential to play a long-term function in preventing the development of breast tumor cells [25–27]. CXCL13 research in RCC is mostly aimed at promoting tumor growth and evading immunity. CXCL13 + CD8 + T cell infiltration in tumors has been linked to poor clinical outcomes in ccRCC patients, according to research. CXCL13 + CD8 + T cell abundance is an independent prognostic factor and potential immunotherapy target for ccRCC treatment [28]. Previously, we discovered that elevated CXCL13 expression in serum is a poor prognostic marker for ccRCC. The addition of CXCL13 to ccRCC cell lines can increase cell line growth [11]. In this study, we found that M2 macrophages in the tumor tissue microenvironment produce high levels of CXCL13 and that by attaching to the CXCR5 receptor on tumor cells, they promote the proliferation, migration, invasion, and EMT. Furthermore, CXCR5 is the sole receptor for CXCL13.CXCR5 expression was shown to be strongly linked with high clinic pathological

features in the TCGA database and data from our hospital. CXCR5 was found to be a poor predictive factor for renal cancer in a prognostic study.

The significance and mechanism of TAM in tumorigenesis and development have become evident as research has progressed. We anticipate that targeted TAM is a very promising research area in the field of malignant tumor treatment and that it will likely become a novel technique of treating malignancies. There are now three major TAM therapies: inhibiting macrophage recruitment, depleting TAM, and promoting TAM anti-tumor transformation. For example, by blocking the most common CCL-2 and CCR-2 signaling pathways, macrophages recruitment and infiltration can be decreased, and tumor development can be delayed [29]. Some mannose-modified nanoliposome carriers and PLGA nanoparticle carriers selectively target the highly expressed mannose receptor (CD206) in M2 macrophages, which can accurately destroy M2 macrophages while having a low unfavorable effect on the body [30–31]. The experimental results of Li et al. [32] show that the activation of PTEN and NHERF-1 may impede the development of TME macrophages other than M1 to M2. Whether the targeted TAM treatment may be coupled with currently utilized radiation and chemotherapy, anti-tumor blood vessels, small molecule targeted drugs, immunotherapy, and other treatments is deserving of further investigation.

Several studies have now revealed the potential advantages of targeting the CXCL13/CXCR5 pathway in different malignancies. Although no small molecule inhibitors that can directly target CXCL13 or CXCR5 have been discovered in pharmacology. However, recent gene knockdown/suppression findings including the neutralization of overexpressed CXCL13 and/or CXCR5 activities have shown the therapeutic potential/validation of this pathway in many kinds of human cancers [22,33–35]. At the moment, various novel approaches are being employed to inhibit CXCL13/CXCR5 signaling in the tumor microenvironment. Recently, it was revealed that drug-loaded nanoparticles had promising outcomes in cancer therapy [36]. Silica nanoparticles loaded with snake venom may cause apoptosis and limit proliferation in human prostate cancer cells [37], as well as dramatically lower the levels of numerous chemokines (including CXCL13) and their receptors (including CXCR5). Simultaneously, chemokine-mediated migration lowers the risk of prostate cancer and breast cancer [38].

Conclusions

In conclusion, our findings suggest that M2 macrophages play a crucial role in the genesis and progression of ccRCC. M2 macrophages can enhance ccRCC cell proliferation, migration, invasion, and EMT via CXCL13 in an Akt-dependent manner. These findings contribute to our understanding of the immune micro environment's function in ccRCC and may aid in the identification of possible novel therapeutic targets for ccRCC.

Abbreviations

RCC: renal cell carcinoma; ccRCC: clear cell renal cell carcinoma; TME: tumor microenvironment; TAM: tumor-associated macrophages; EMT: epithelial–mesenchymal transition; ; TCGA: The Cancer Genome

Atlas; RT-qPCR: reverse-transcription quantitative PCR; WB: western blotting; ELISA: enzyme-linked immunosorbent assay; IHC: immunohistochemistry; Akt: Protein kinase B; CM: conditioned medium;

Declarations

Acknowledgements

Not applicable

Authors' contributions

Wenlian Xie and Dingjun conceived of the design of the study. Zhiliang Chen, Qiyu Zhong, and Yingwei Xie performed the most experiments, analyzed the data and drafted the manuscripts. Zaosong Zheng, Wentai Shangguan, Yuqing Chen, Jingying Yang and Yishan Zhang contributed to the collecting of clinical tissues, in vivo experiments, and material support. All of the authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The use of human tissues was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University, and all patients or their relatives signed the informed consent. All experimental procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee (IACUC), Sun Yat-Sen University, and all eforts were made to minimize animal sufering, according to the NIH Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author details

[1] Department of Urology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, 510120, China. [2] Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, 510120, China. [3] Guangdong Provincial Clinical Research Center for Urological Diseases [4] Department of Pathology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, 510120, China. [5] Department of Urology, Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China.

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Figures

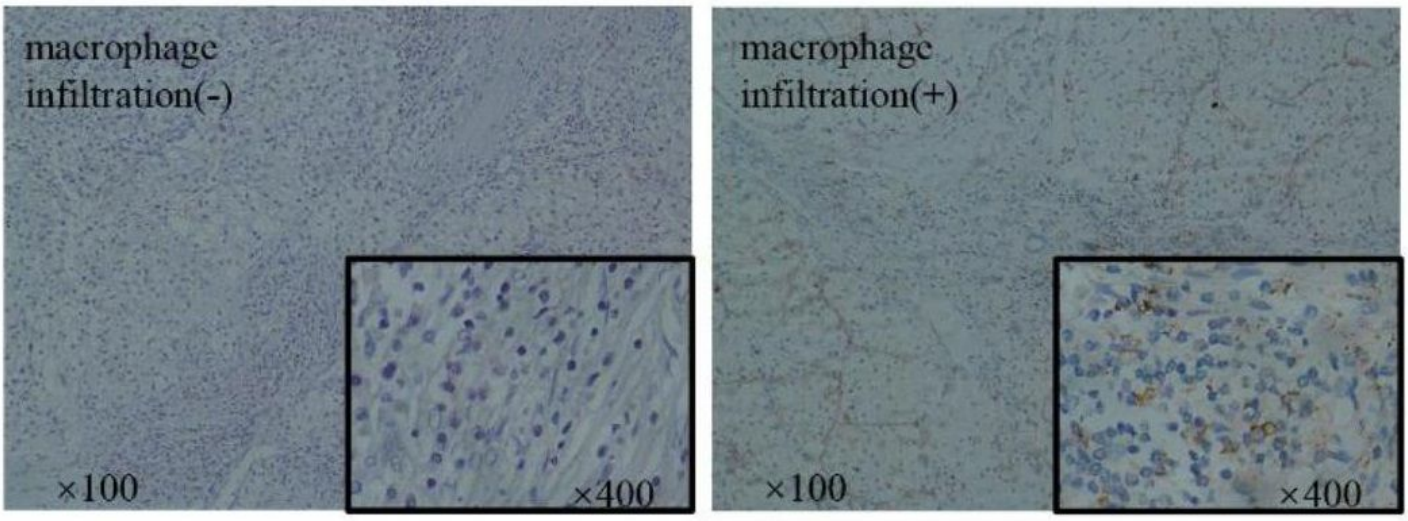

Figure 1

M2 macrophage infiltration is increased in ccRCC. Immunohistochemical staining of CD206 protein (M2 macrophage marker) in ccRCC tissue.

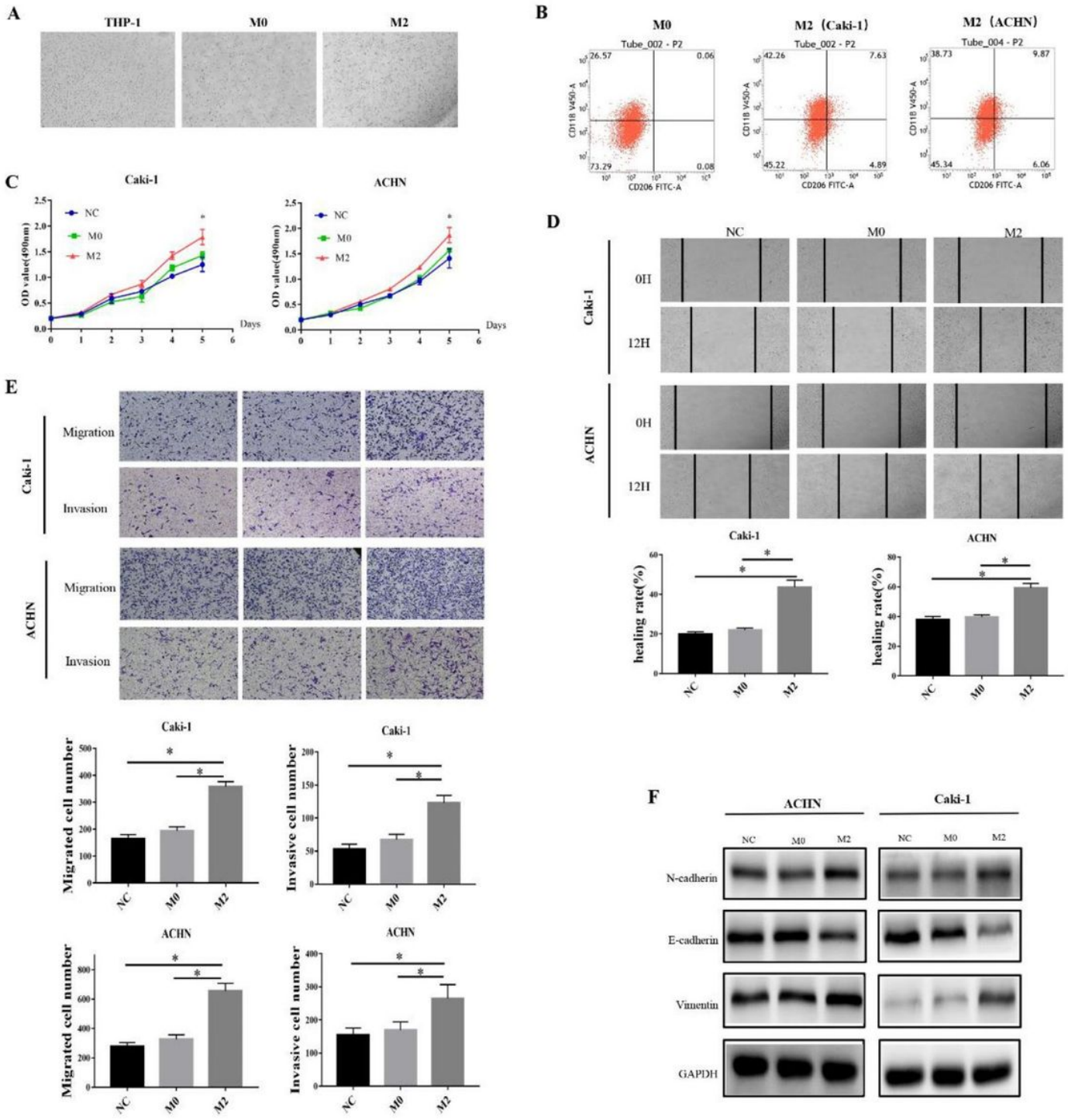

Figure 2

THP-1-derived M2 macrophages promote EMT in ccRCC cells. A Cell morphology of THP-1, M0 and M2 cells. B Detect the expression of CD11b and CD206 in M0 and M2 macrophages by flow cytometry. C MTS detects the effect of M2 macrophages on the proliferation of ACHN and Caki-1. D Scratch healing test to detect the effect of M2 macrophages on the migration of ACHN and Caki-1. E Transwell chamber experiment to detect the influence of M2 macrophages on the migration and invasion of ACHN and Caki-1. F WB experiment to detect the effect of M2 macrophages on ACHN and Caki-1 EMT. (*P□0.05)

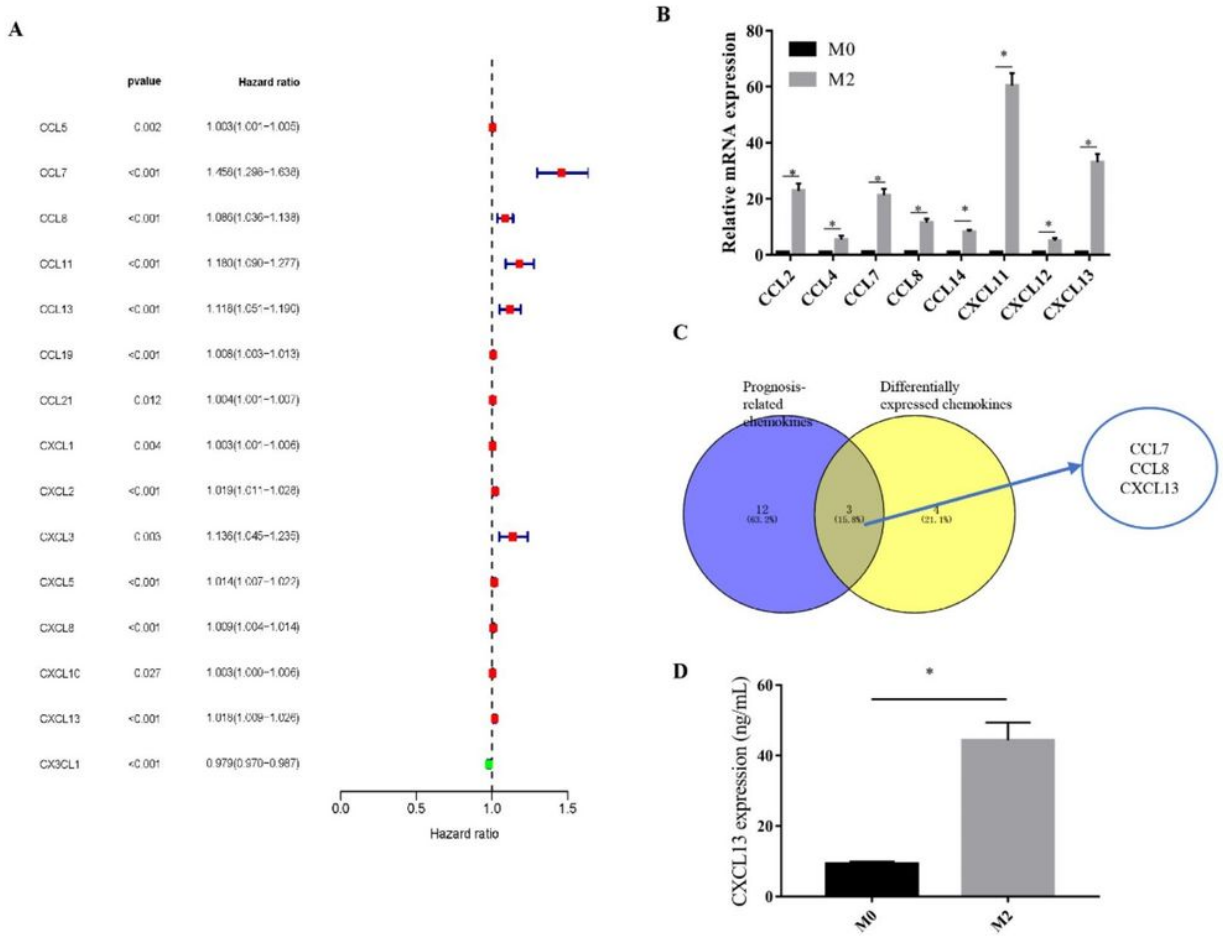

Figure 3

CXCL13 is a key chemokine secreted by M2 macrophages. A Univariate prognostic analysis of chemokines on the prognosis of ccRCC. B M2 macrophages are highly expressed chemokines compared to M0 macrophages. C Venn diagram takes the intersection of diagram A and diagram B. D ELISA to detect the expression of CXCL13 in the supernatant of M2 macrophages. (*P□0.05)

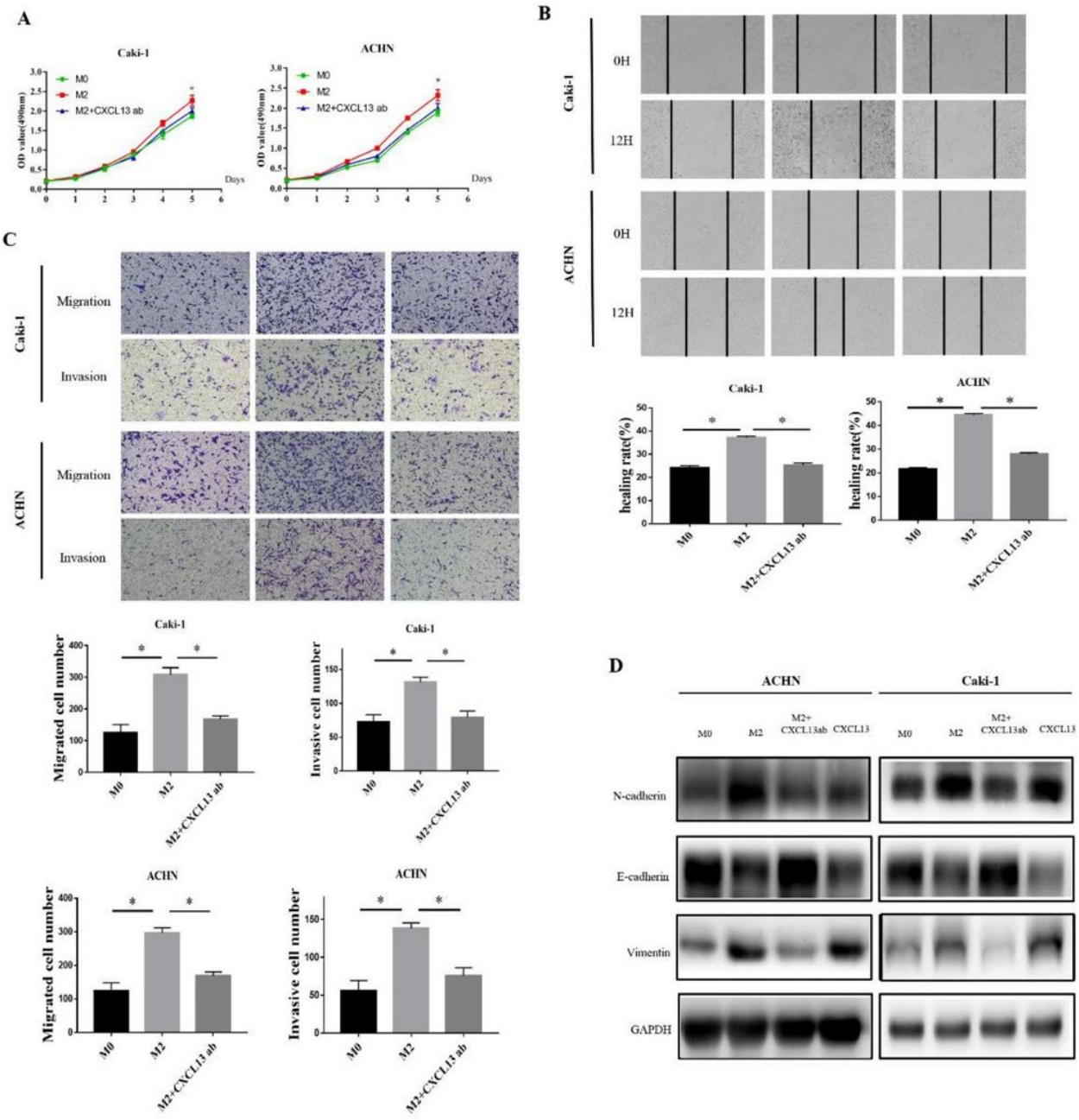

Figure 4

M2 macrophages secrete CXCL13 to promote EMT in ccRCC cells. A In the presence or absence of anti-CXCL13 antibodies, MTS detects the effect of M2 macrophages on the proliferation of ACHN and Caki-1. B In the presence or absence of anti-CXCL13 antibodies, the scratch healing test detects the effect of M2 macrophages on the migration of ACHN and Caki-1. C In the presence or absence of anti-CXCL13 antibodies, the Transwell chamber test detects the effect of M2 macrophages on the migration and invasion of ACHN and Caki-1. D In the presence or absence of anti-CXCL13 antibodies, the WB test detects the effect of M2 macrophages on ACHN and Caki-1EMT. (*P□0.05)

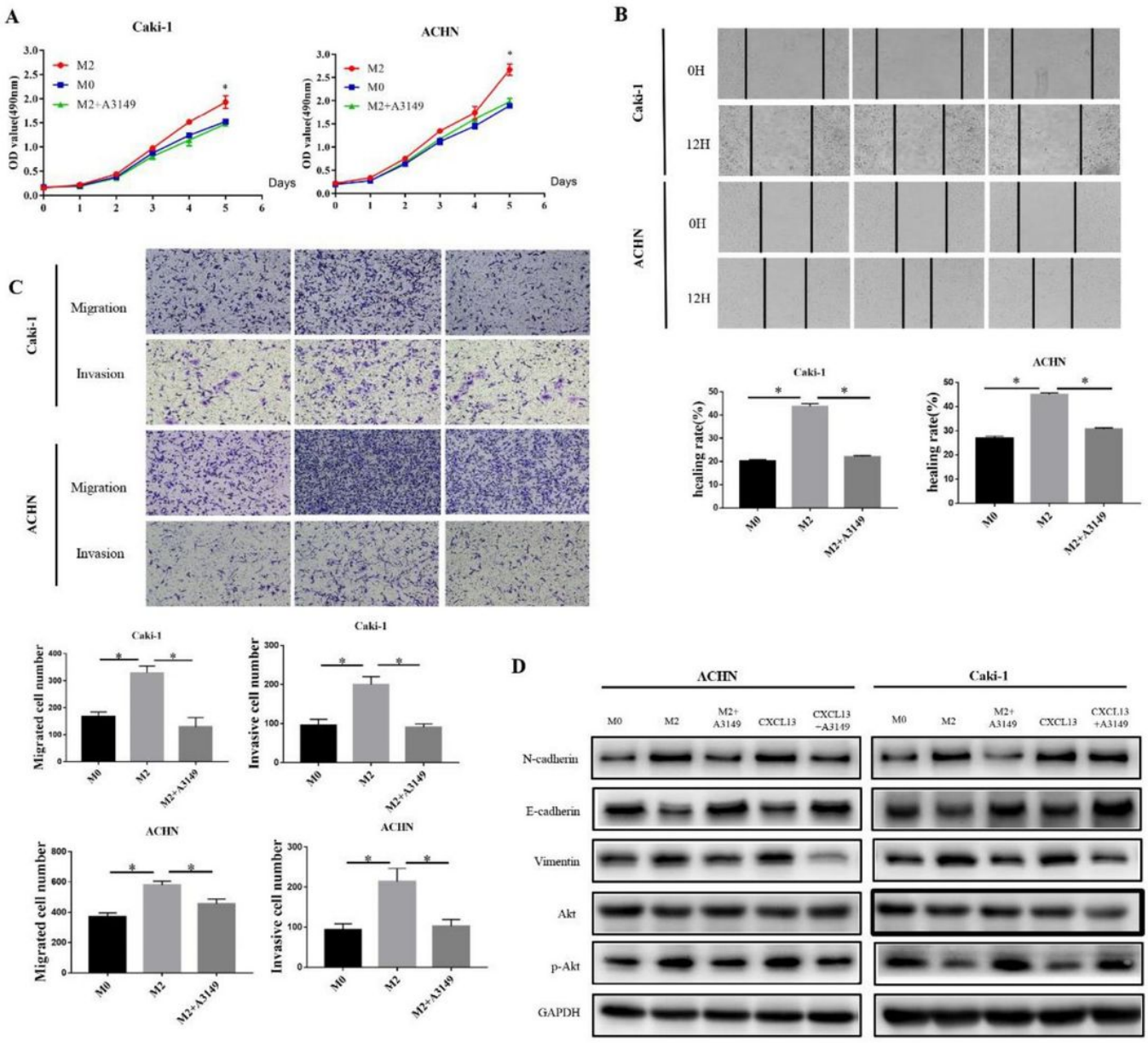

Figure 5

M2 macrophage secretion of CXCL13 promotes EMT in ccRCC cells via the Akt signaling pathway. A In the presence or absence of Akt inhibitors, MTS detects the effect of M2 macrophages on the proliferation of ACHN and Caki-1. B In the presence or absence of Akt inhibitors, the scratch healing test detects the effect of M2 macrophages on the migration of ACHN and Caki-1. C In the presence or absence of Akt inhibitors, the Transwell chamber test detects the effect of M2 macrophages on the migration and invasion of ACHN and Caki-1. D In the presence or absence of Akt inhibitors, the WB test detects the effects of M2 macrophages on ACHN and Caki-1EMT. (*P□0.05)

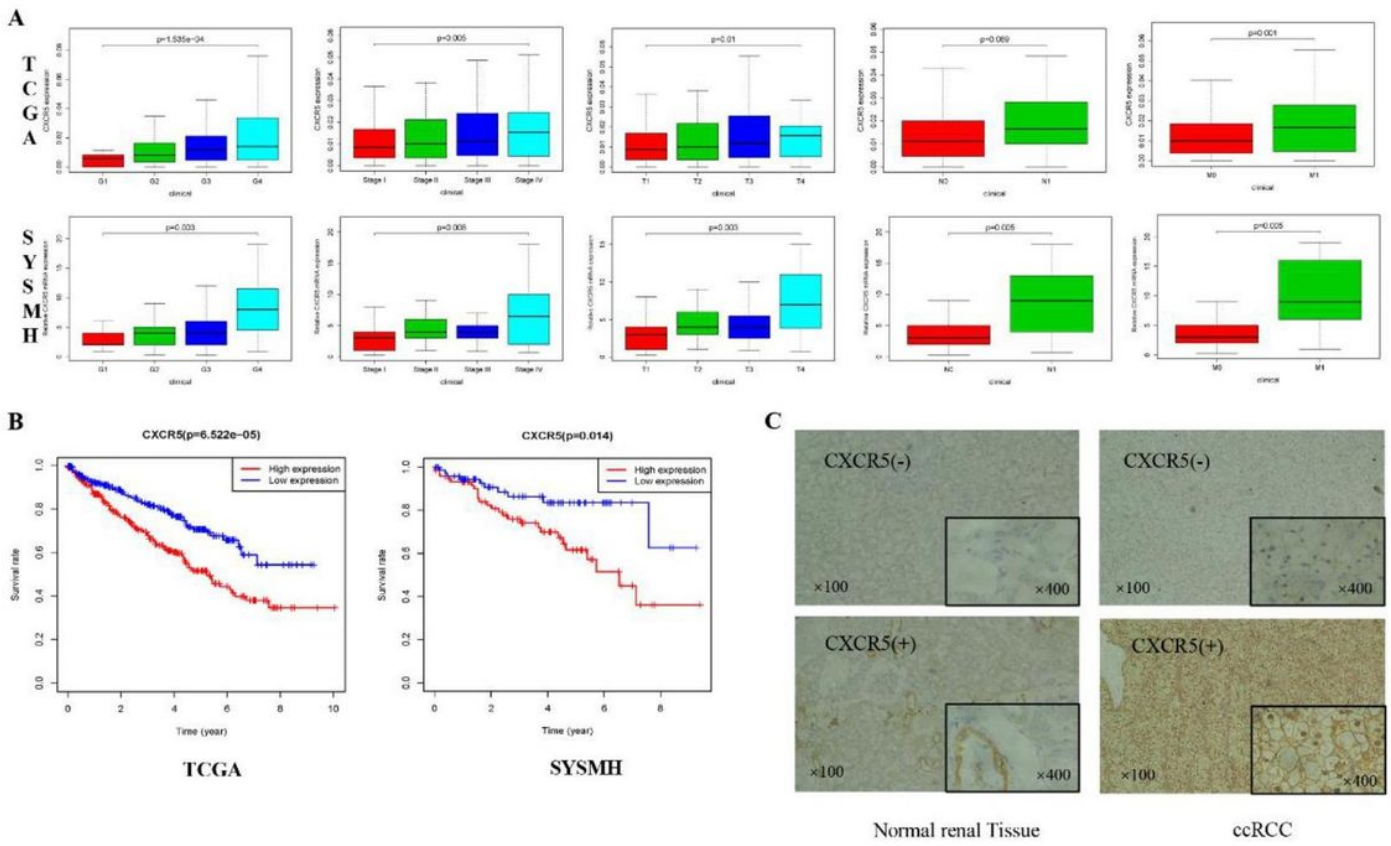

Figure 6

qPCR and Immunohistochemical to detect the expression of CXCR5 in ccRCC tissues. A TCGA database and our hospital database detect the relationship between CXCR5mRNA expression and ccRCC clinicopathological characteristics. B TCGA database and our hospital database to detect the relationship between CXCR5mRNA expression and ccRCC prognosis. C Immunohistochemical staining of CXCR5 protein in ccRCC tissues and adjacent tissues.

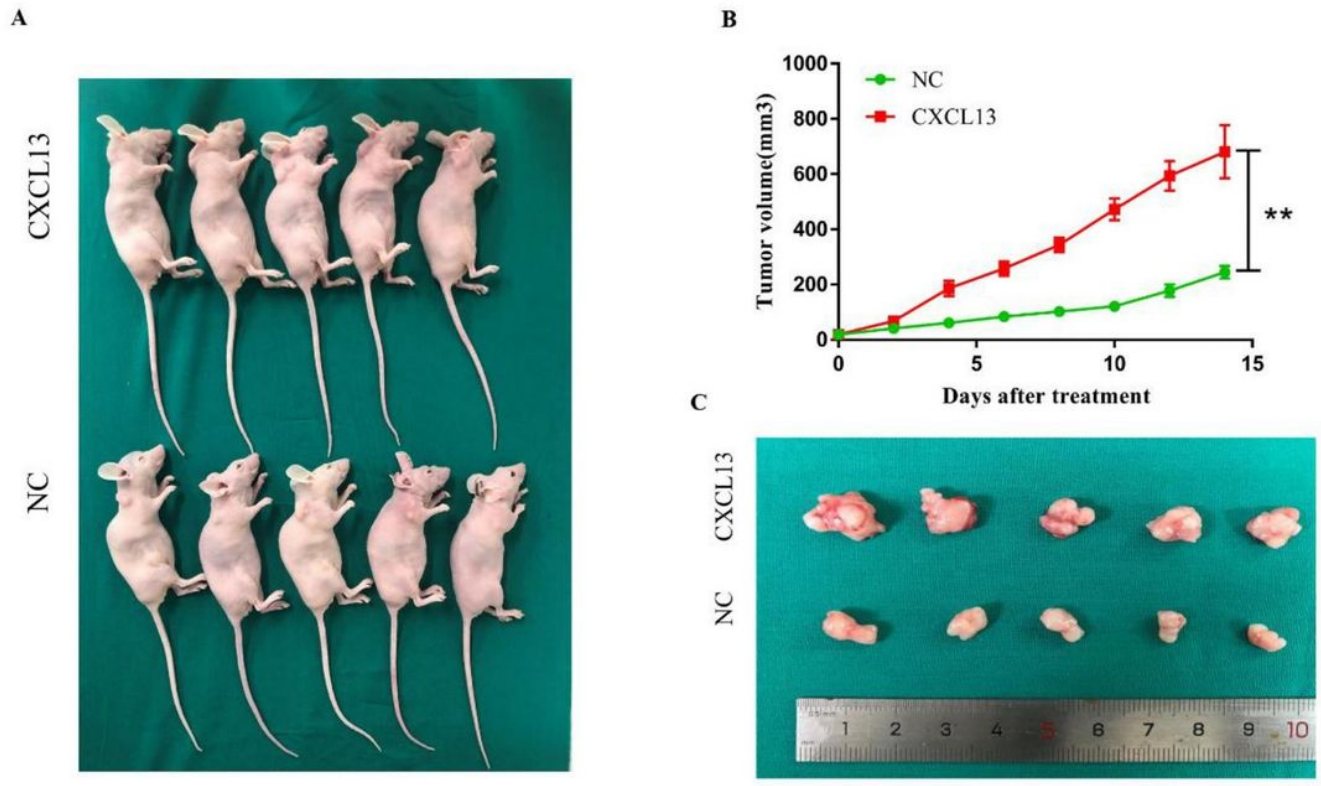

Figure 7

The effect of CXCL13 on tumor proliferation in vivo. A Xenograft tumor models show that CXCL13 promotes the proliferation of ccRCC in vivo. B-C CXCL13 promotes the growth of tumor volume in vivo. (**P□0.01)

Supplementary Files

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- Excel1.xlsx
- Excel2.xlsx