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Abnormal expression of CEBPB promotes the progression of renal cell carcinoma through regulating the generation of IL-6

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ABSTRACT

Background: The CCAAT/enhancer-binding protein beta (CEBPB), a transcription factor regulating immune and inflammatory responses, has been implicated in the pathogenesis of various malignancies. However, its specific regulatory mechanism in renal cell carcinoma (RCC) remains poorly understood.
Methods: The expression of CEBPB was detected in RCC cells and tissues using qRT-PCR, western blotting and immunohistochemistry. ELISA assay was used to detect the immune factors regulated by CEBPB in supernatants. Additionally, western blotting was employed to measure the phosphorylation level of STAT3 and the expression levels of its downstream target genes.
Results: CEBPB was found to be overexpressed in both RCC tissues and cell lines, and its higher expression was associated with a lower survival rate. In RCC cells, CEBPB enhances the expression of IL6, consequently promoting the phosphorylation of STAT3 and the expression of its downstream target genes. This mechanism ultimately facilitates tumor progression.
Conclusions: The dysregulated expression of CEBPB facilitates RCC progression through the IL6/STAT3 pathway. CEBPB is a potential diagnostic markers and a novel effective therapeutic target for RCC patients.

1. Introduction

Renal cell carcinoma (RCC) is one of the most common and lethal forms of cancer worldwide, accounting for 85–90% of adult renal malignancies [1]. Currently, surgical resection remains the primary treatment, with partial or radical nephrectomy being options for patients with localized or early RCC [2]. Nevertheless, approximately 30% of first-diagnosed RCC patients present with distant metastases [3]. Advanced-stage RCC patients experience metastasis, accounting for approximately 90% of kidney cancer-related deaths, and their 5-year survival rate is less than 10% [4,5]. Therefore, it is imperative to understand the molecular mechanisms underlying the occurrence and metastasis of RCC to improve its diagnosis and treatment.

The CCAAT/enhancer-binding protein beta (CEBPB) gene is situated in a syntenic region on human chromosome 20q13.13 [6]. As a leucine-zipper transcription factor, CEBPB induces acute inflammatory genes in the liver and facilitates adipocyte differentiation [7,8]. Moreover, studies have reported that the deletion of CEBPB from bone marrow and hematopoietic cells prevented inflammation of adipose tissue and macrophage infiltration [9]. Recently, accumulating evidence has emerged linking CEBPB to malignant tumors [10,11]. However, the understanding of CEBPB in RCC remains limited, and investigating the regulatory mechanisms of CEBPB is crucial

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for comprehending its role in tumor development and genesis.

Mounting evidence indicates that the upregulation of inflammatory mediators can enhance tumor proliferation, invasion, and angiogenesis [12,13]. Interleukin-6 (IL6), a multipotent cytokine, plays a pivotal role in various pathophysiological processes associated with cancer pathology [14]. IL6 activates downstream Janus kinases (JAKs) by binding to the IL6R/gp130 receptor complex expressed on target cells, which induces the activation of the STAT3 signal transducer through phosphorylation at Tyrosine 705 [15]. Additionally, the IL6/STAT3 signaling system strongly influences tumor-infiltrating immune cells in the tumor microenvironment [16]. In RCC, the expression level of IL6 is related to tumor recurrence and long-term prognosis of RCC patients, making it a potential diagnostic marker for RCC [17,18]. Moreover, the IL6 signaling pathway can serve as a target for targeted therapy of RCC [19,20]. Studies have found that CEBPB is required for the transcription of the IL-6 gene, as it regulates the expression of IL6 by directly binding to the IL6 promoter [21,22]. However, the precise mechanism underlying the interplay between CEBPB and IL6 in RCC development remains uncertain, and further research is warranted to elucidate this relationship.

In this research, we evaluated the expression level of CEBPB in RCC tissues and investigated its impact on three distinct RCC cell lines (786-O, 769-P and ACHN). Our findings indicate that silencing CEBPB observably repressed the proliferation, invasion and metastasis of RCC cells. Furthermore, our research highlights that CEBPB can activate STAT3 positively through upregulating IL6, suggesting its potential as an intriguing target for the diagnosis and treatment of RCC.

2. Materials and methods

2.1. Patient tissue specimens and cell lines

All 58 specimens were collected from patients who underwent nephrectomy at the First Affiliated Hospital of Zhengzhou University. Many experienced pathologists confirmed the histological and pathological diagnosis based on the 2004 World Health Organization Consensus Classification and Staging System for RCC. The clinicopathological characteristics of the patients are presented in Table 1. Two cell lines, 786-O and 769-P, were cultured in RPMI 1640 Medium (Gibco, USA), while two other cell lines, HK2 and ACHN, were cultured in Minimal Essential Medium (MEM) Medium (Gibco, USA). Both culture media were supplemented with 10% fetal bovine serum (Gibco, Australia origin) and 1% penicillin-streptomycin (Gibco).

2.2. RNA extraction and PCR assay

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by the synthesis of complementary DNA using random primers with the HiScript III RT SuperMix (Vazyme Biotech, Nanjing, China). Subsequently, quantitative real-time PCR (qRT-PCR) was conducted with cDNA as the template, utilizing specific primers (Supplementary Table 2) and SYBR Green Master Mix (Vazyme Biotech). The ΔΔCt method was employed to analyze all data, which were then normalized to GAPDH expression.

2.3. shRNA and plasmid transfection

Three shRNAs targeting CEBPB (shCEBPB) and IL6-overexpression plasmids were obtained from GenePharma (Shanghai, China). Transfection was conducted using Lipofectamine 3000 (Life Technologies), as specified by the manufacturer. Establish stable transfected cell lines by screening with purinomycin for 4 weeks. The sequence of shRNAs targeting CEBPB is shown in Supplementary Table 1.

Table 1
Correlation between CEBPB mRNA expression and clinicopathological factors in 58 patients with RCC.

Parameters	Group	Cases	CEBPB expression				P Value
			Low	%	High	%	
Gender	Male	31	9	15.52	22	37.93	0.9702
	Female	27	10	17.24	17	29.31	
Age(years)	<65	26	8	13.79	18	31.03	0.5975
	≥65	32	11	18.97	21	36.21	
Tumor stage	T1-T2	40	17	29.31	23	39.66	0.0185*
	T3-T4	18	2	3.45	16	27.59	
Tumor size	<4.0 cm	37	16	27.59	21	36.21	0.0239*
	≥4.0 cm	21	3	5.17	18	31.03	
Grade	I-II	34	11	18.97	23	39.66	0.7925
	III-IV	24	8	13.79	16	27.59	
Lymph node metastasis	Absent	38	16	27.59	22	37.93	0.0366*
	Present	20	3	5.17	17	29.31	
Distant metastasis	Absent	46	15	25.86	31	53.45	0.4771
	Present	12	4	6.90	8	13.79	

$P < 0.05$ represents statistical significance (Chi-square test).

2.4. Western blot analysis

Equal lysates were separated by 12% SDS-PAGE gels. Then, we incubated the membranes with antibodies at 4 °C for 12 h after blocking them in Tris-buffered saline containing 10% skim milk for 1 h at room temperature. The next day membranes were incubated for 1 h with the appropriate fluorescent-dye conjugated secondary antibodies. The Odyssey® CLX Infrared Imaging System (Gene Company Limited, Shanghai, China) was used to obtain all images. Antibodies against CEBPB (Cat No. 23431-1-AP), STAT3 (Cat No. 10253-2-AP), pSTAT3 (Cat No. 39595), MMP9 (Cat No. 27306-1-AP), c-myc (Cat No. 10828-1-AP) and β-actin (Cat No. 81115-1-RR) were purchased from Proteintech Group (Wuhan, China). Li-Cor Biosciences provided the fluorescent-dye conjugated secondary antibodies.

2.5. Immunohistochemistry (IHC) analysis

IHC was carried out on RCC and adjacent normal tissue sections obtained from patients to evaluate the CEBPB protein level. We obtained the images with an Olympus FSX100 microscope. The integrated optical density of immunostaining images was calculated to analyze protein expression levels as described [23].

2.6. Cell counting Kit-8 (CCK-8) assay

Cells were cultured in a 96-well plate until they reached 60% confluence. At 0, 24, 48, and 72 h, each well received 10 mL of CCK-8 solution (Servicebio, Wuhan, China). Subsequently, absorbance at 450 nm was measured using Spectrometers (Thermo Fisher Scientific, MA, USA).

2.7. EdU assay

According with the manufacturer's instructions (RiboBio, Guangzhou, China), we incubated transfected RCC cells with EdU (100 mmol/L) in 96-well plates for 2 h. Subsequently, the cells were fixed with 4% formaldehyde for 30 min. After permeabilization with 0.5% Triton-X, the cells were incubated with Apollo staining solution for an additional 30 min. Photomicrographs of the cells were captured and analyzed using a fluorescent microscope (Olympus). The proliferation activity was assessed by calculating the ratio of EdU-stained cells to Hoechst-stained cells.

2.8. Wound-healing assay

Cells were seeded at an appropriate density and incubated until cell confluence reached 90% in 6-well culture plates. At 0 and 24 h after injury in RPMI 1640 medium with 2% FBS, wound margins were photographed with microscope (Olympus). Relative cell migration is calculated through the distance between advancing edges of cells normalizing against the control value at 0 h.

2.9. Tumor xenografts

Animal experiments were approved by the Animal Care Committee of the First Affiliated Hospital of Zhengzhou University. Four-week-old male nude mice from the Institute of Zoology (Beijing, China) were randomly divided into two groups (n = 5 per group). The stable transfected 786-O cells were subcutaneously injected into the right flanks of the nude mice (2×10^6, 200 μL). The volumes of tumors were measured at 1, 2, 3 and 4 weeks and measured as the length × width^2 × 0.5.

2.10. Trans-well matrigel invasion assay

Trans-wells were pre-coated with 30 μl of 3 × diluted matrix matrigel (Bd Biosciences Pharmingen, San Diego, CA, USA) for 30 min. Cell suspensions (1×10^5/well) of homogenous single cells were seeded into the top chambers in serum-free medium, and the low chamber was added with 10% serum medium. After incubating at 37 °C for 24 h, invaded cells were stained with crystal violet and counted in five randomly chosen fields under the microscope (Olympus).

2.11. ELISA assay

ELISA kit (Proteintech Group, Wuhan, China) was used to detected the IL-6, IL8, TNF-α IL-1-β and MCP-1 protein in supernatants according to the manufacturer's instructions. Firstly, we collected the culture supernatants and centrifuged them for 15 min at 300 g. Then, we determined the amount of the proteins in the supernatant and normalized them to the number of cells.

2.12. Bioinformatics analysis

RNA-sequencing expression (level 3) profiles were obtained from The Cancer Genome Atlas (TCGA, https://portal.gdc.com). Allmicroarray datasets (GSE14762, GSE14378, GSE180925, GSE65637, GSE168845, GSE65639, GSE15641) were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. The two-gene correlation map is realized by the

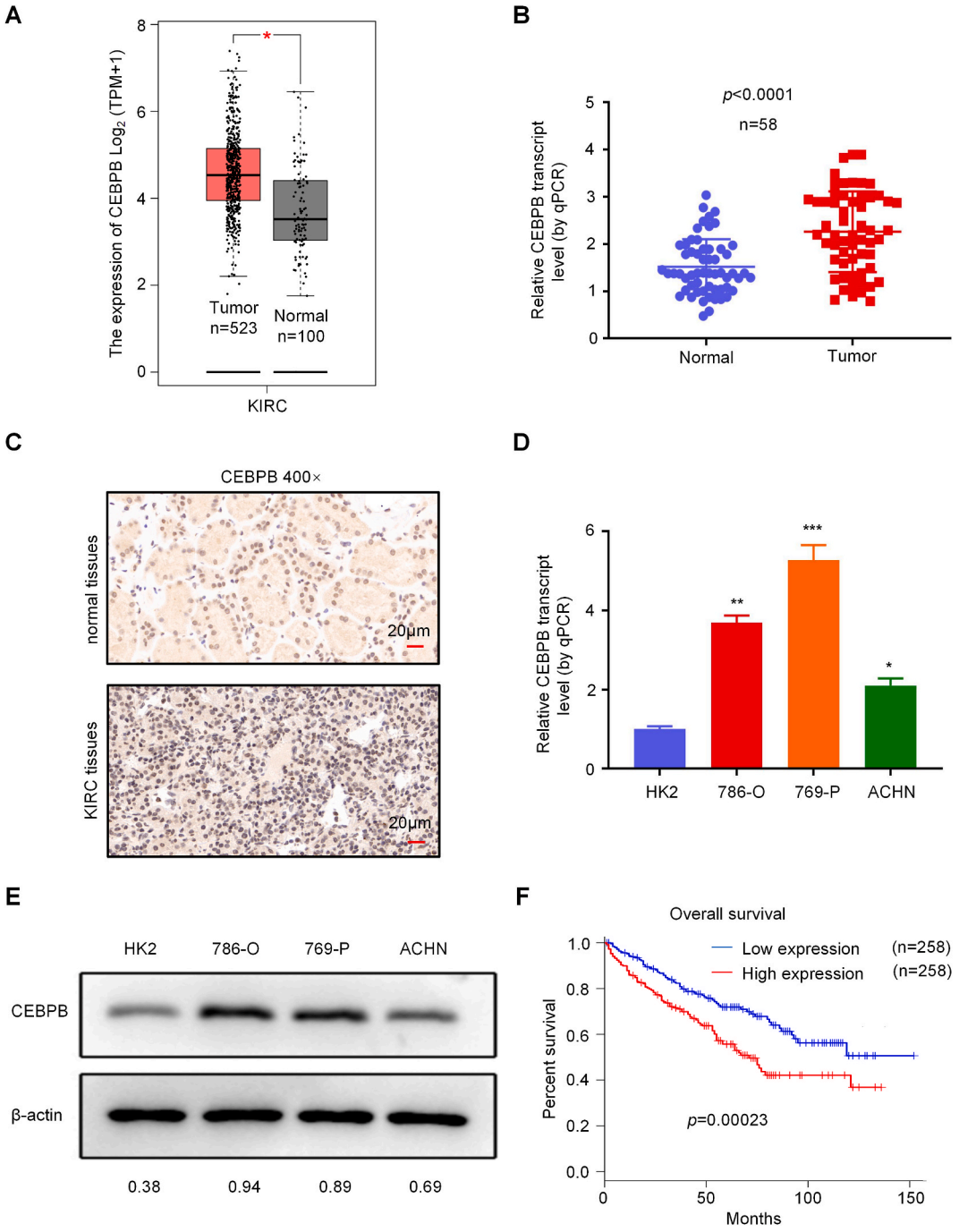

Fig. 1. CEBPB is overexpressed in RCC tissues and cell lines. (A) The mRNA expression levels of CEBPB in kidney renal clear cell carcinoma (KIRC) and normal tissues from the GEPIA database. (B) qRT-PCR analysis of CEBPB mRNA levels in RCC tissues and adjacent normal tissues from our hospital. GAPDH was used as a loading control. (C) Immunohistochemistry assay was used to detect the expression levels of CEBPB protein in RCC tissues and adjacent normal tissues. The tumor sample shown here is stage T3 with grade II and its pathological type is renal clear cell carcinoma. (D, E) The mRNA (D) and protein (E) levels of CEBPB in HK2, 786-O, 769-P and ACHN cells were analyzed by qRT-PCR and western blotting. GAPDH or β-actin was used as a loading control. (F) A Kaplan-Meier survival plot for 516 renal cancer cases from the TCGA database showed a difference between patients with high and low CEBPB expression. (http://gepia.cancer-pku.cn/, n = 516, $p = 0.00023$, log-rank test). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

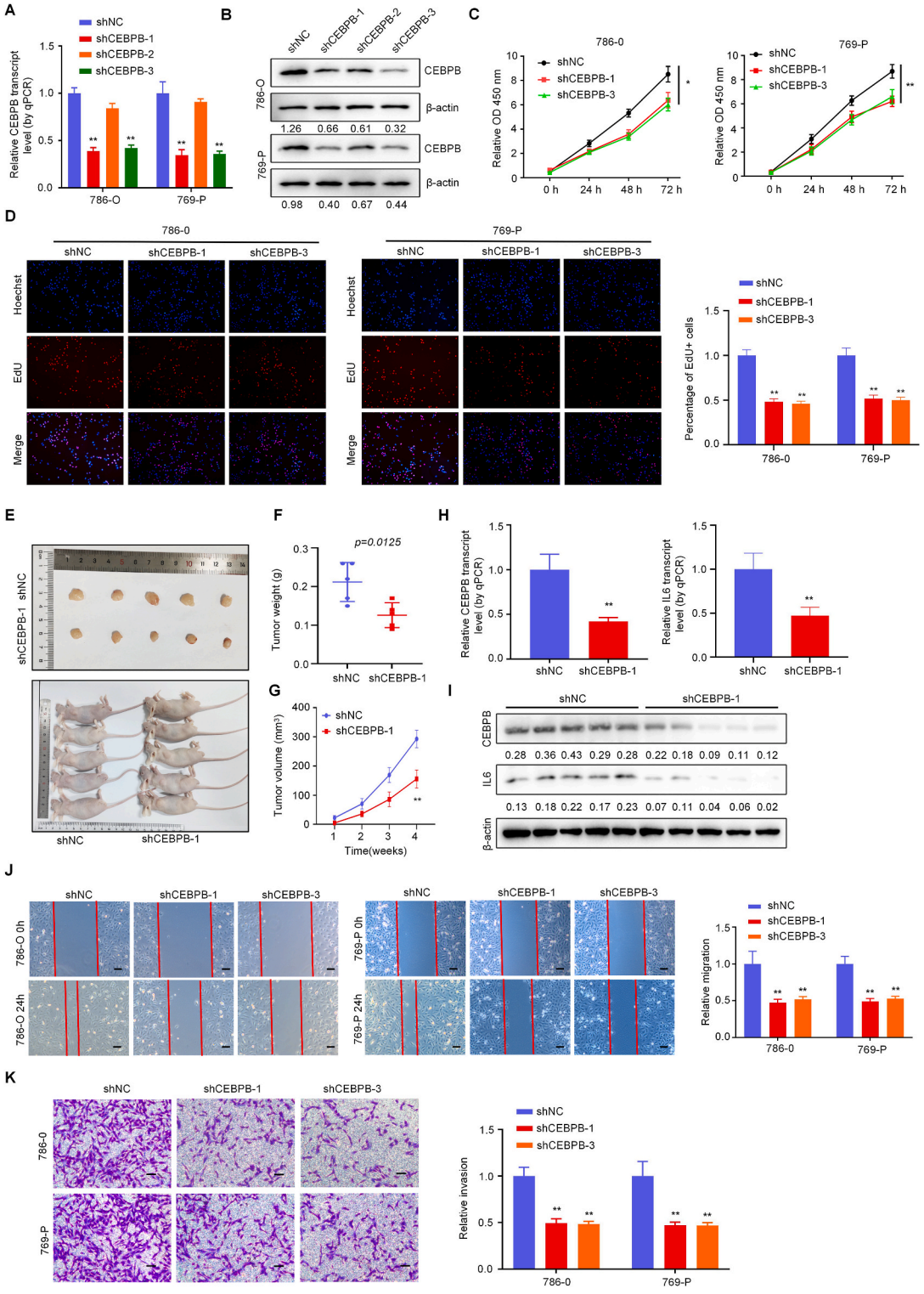

Fig. 2. Knockdown of CEBPB inhibits RCC cells proliferation, migration and invasion. (A, B) The efficiency of CEBPB silencing in 786-O and 769-P cells was detected by qRT-PCR (A) and western blotting assay (B). GAPDH or β-actin was used as a loading control. (C) The viability of the cells was assessed using the CCK-8 assay. (D) EdU assay showed that knockdown of CEBPB repressed the proliferation of 786-O and 769-P cells. (E) Tumors collected from mice were exhibited after one month of hypodermic injection. (F) The weights of xenograft tumors were measured and analyzed after one month of hypodermic injection. (G) The volumes of tumors were measured at 1–4 weeks after hypodermic injection and the growth rates of xenograft tumors. (H) The mRNA expression levels of CEBPB and IL-6 were detected by qRT-PCR in xenograft tumors. (I) The protein expression

levels of CEBPB and IL-6 were detected by western blotting assay in xenograft tumors. (J) In wound healing assay, the migration of RCC cells transfected with shCEBPB was significantly decreased (scale bar, 20 μm). (K) Cell invasion abilities were evaluated by matrigel invasion assay (scale bar, 20 μm). *$p < 0.05$; **$p < 0.01$ (Student's t-test).

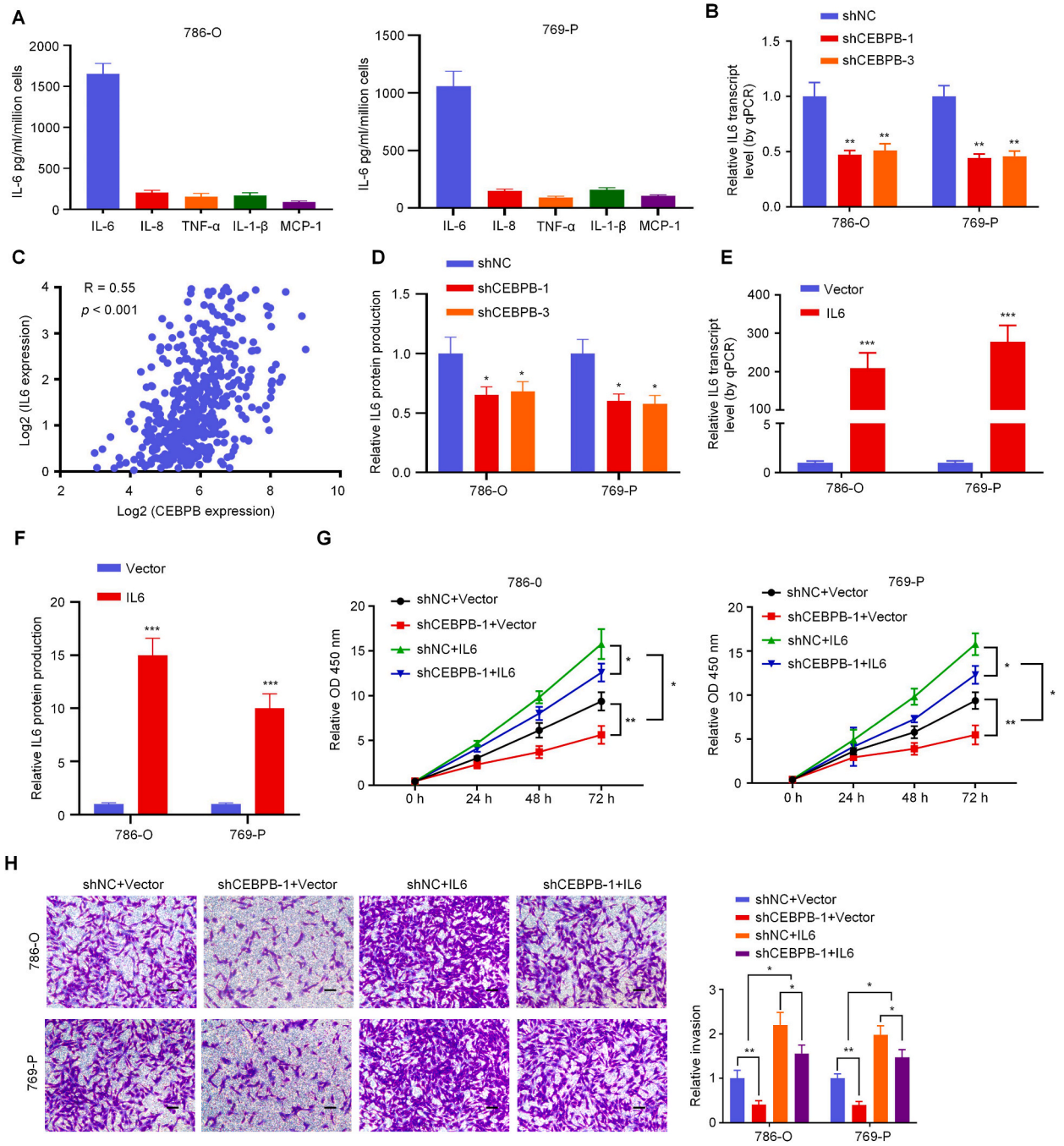

Fig. 3. Knockdown of CEBPB inhibits RCC cells proliferation, migration and invasion by repressing the expression of IL6. (A) ELISA analysis of IL-6 protein production in supernatants of 786-O and 769-P cells. (B) qRT-PCR evaluated the mRNA expression of IL6. (C) Spearman analyzed the expression correlation of CEBPB and IL6 genes. Data obtained from TCGA database. (D) After transfection with shCEBPB or shNC, IL6 protein production were determined by ELISA. (E, F) The efficiency of IL6 overexpression in 786-O and 769-P cells was detected by qRT-PCR (E) or ELISA (F). (G) Cell viabilities in shNC + Vector group, shCEBPB-1 + Vector group, shNC + IL6 group and shCEBPB-1 + IL6 group were evaluated by CCK-8 assays. (H) Cell invasion abilities in shNC + Vector group, shCEBPB-1 + Vector group, shNC + IL6 group and shCEBPB-1 + IL6 group were evaluated by matrigel invasion assay (scale bar, 20 μm). **$p < 0.01$; ***$p < 0.001$ (Student's t-test or Spearman's correlation analysis).

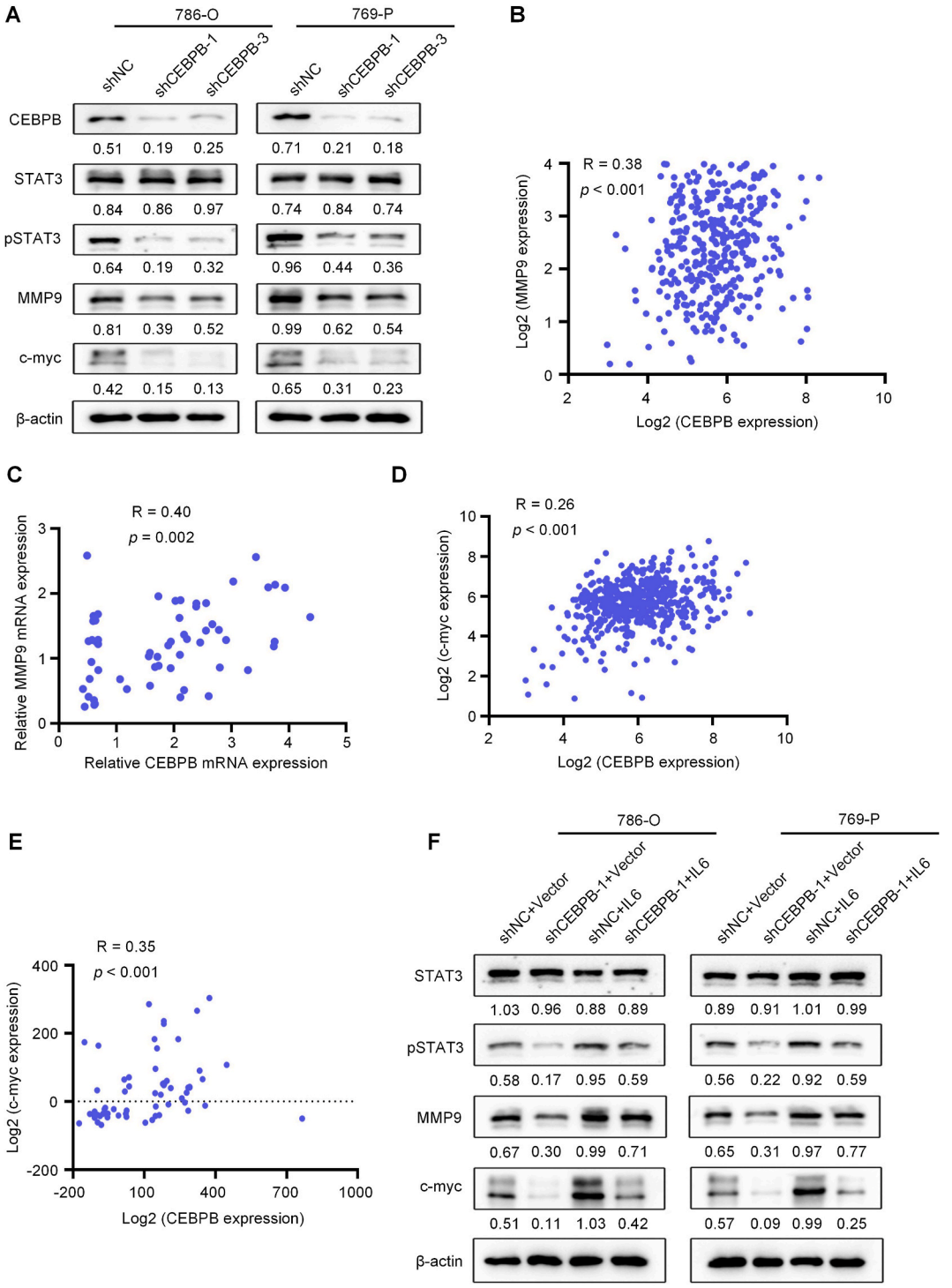

Fig. 4. Knockdown of CEBPB inhibits RCC cells progression through IL6/STAT3 pathway. (A) In 786-O and 769-P cells, western blotting was used to determine the phosphorylation level of STAT3 and the expression of MMP9 and c-myc. (B, C) Spearman rank correlation coefficient analyzed the expression correlation of CEBPB and MMP9 mRNA. Data obtained from TCGA database (B) and our hospital (C). (D, E) Spearman rank correlation coefficient analyzed the expression correlation of CEBPB and c-myc mRNA. Data obtained from TCGA database (D) and GEO database (E). (F) Western blotting was used to examine the phosphorylation level of STAT3 and the expression of MMP9 and c-myc after co-transfection with shCEBPB-1 and IL6.

R software package ggstatsplot.

2.13. Statistical analysis

The data were analyzed by GraphPad Prism 7.0 (La Jolla, USA) and presented as means ± standard error of the mean (SEM) from three independent replicates. Differences among groups were evaluated by two-tailed Student's t-test. The chi-squared test was used to investigate the association between CEBPB expression and clinicopathological characteristics. Log-rank tests were used to compare Kaplan-Meier survival curves. Spearman's correlation analysis was used to describe the correlation between quantitative variables without a normal distribution. $p < 0.05$ was considered significant.

3. Results

3.1. CEBPB is overexpressed in RCC tissues and cell lines

The results obtained from both the GEPIA dataset (Fig. 1A) and our hospital demonstrated a significant increase in CEBPB expression in RCC tumor tissues (Fig. 1B and C). Moreover, we observed a positive correlation between CEBPB upregulation in RCC patients and various clinical indicators, including RCC clinical stage, tumor size, and lymphatic metastasis (Table 1). Additionally, higher levels of both CEBPB mRNA and protein were detected in the RCC cell lines ACHN, 786-O, and 769-P, as compared to the HK2 cell line (Fig. 1D and E). Furthermore, based on the Kaplan-Meier survival plots of 516 RCC cases in the TCGA database, patients with higher CEBPB expression exhibited poorer survival chances (Fig. 1F). These findings suggest that CEBPB may play a role in promoting the progression of RCC.

3.2. Knockdown of CEBPB inhibits RCC cells proliferation, migration and invasion

To investigate the function of CEBPB in RCC cells, we assessed the efficiency of one control negative shRNA and three shCEBPB-shRNAs using qRT-PCR and western blotting (Fig. 2A, B and S1A, B). Among them, shCEBPB-1 and shCEBPB-3 showed the most pronounced knockdown effects, and thus, we selected them for further studies. The CCK-8 assay results revealed that knockdown of CEBPB significantly reduced the growth capacity of 786-O, 769-P and ACHN cells transfected with shCEBPB (Fig. 2C and S1C). Furthermore, the EdU assay demonstrated a significant decrease in the growth rate of 786-O and 769-P cells upon CEBPB knockdown (Fig. 2D). Consistently, we observed that CEBPB knockdown repressed the proliferation of 786-O cells *in vivo* (Fig. 2E–I).

Furthermore, compared to shNC-transfected 786-O, 769-P and ACHN cells, the wound healing assays showed a significantly slower wound healing rate in cells transfected with shCEBPB (Fig. 2J and S1D). Additionally, the *trans*-well invasion assay demonstrated that CEBPB knockdown remarkably reduced the invasion of 786-O, 769-P and ACHN cells (Fig. 2K and S1E). Taken together, these findings indicate that silencing of CEBPB can suppress the biological activity of 786-O, 769-P and ACHN cells, pointing towards a pro-tumor role for CEBPB in RCC progression.

3.3. Knockdown of CEBPB inhibits RCC cells proliferation, migration and invasion by repressing the expression of IL6

To elucidate the molecular mechanism underlying the inhibitory effect of CEBPB on the biological activity of RCC cells, we examined several inflammatory cytokines, including IL-6, IL-8, TNF-α, IL-1β and MCP-1, regulated by CEBPB. Notably, we observed that IL-6 exhibited the highest level in the supernatants of RCC cells (Fig. 3A). Moreover, knockdown of CEBPB led to a reduction in IL-6 mRNA expression in RCC cells, and there was a positive correlation between CEBPB mRNA levels and IL-6 mRNA levels in RCC tissues (Fig. 3B, C and)S2A. Consistently, we found that knockdown of CEBPB significantly reduced the IL-6 protein levels in the supernatants of RCC cells (Fig. 3D and S2B).

We further investigated the involvement of IL-6 in the shCEBPB-induced inhibition of tumor progression. Vector pcDNA-IL6 was constructed by inserting the coding oligonucleotides of IL6 into pcDNA3.1 vector for overexpression of IL6, and the efficiency of overexpression was measured using qRT-PCR and ELISA in 786-O and 769-P cells. (Fig. 3E and F). Remarkably, we observed that overexpression of IL-6 promoted the proliferation and metastasis of RCC cells (Fig. 3G and H). Moreover, the proliferation and metastasis inhibition induced by shCEBPB were partially reversed by the overexpression of IL-6 (Fig. 3G, H and S2C, D). These compelling findings strongly suggest that the suppression of RCC cells' proliferation, migration, and invasion upon knockdown of CEBPB is mediated by the downregulation of IL-6 expression.

3.4. Knockdown of CEBPB inhibits RCC cells proliferation, migration and invasion through IL6/STAT3 pathway

We then proceeded to investigate whether CEBPB inhibits the proliferation, migration, and invasion of 786-O and 769-P cells through the IL-6/STAT3 pathway. Our results from western blot analysis demonstrated that knockdown of CEBPB led to a decrease in the phosphorylation of STAT3 and the expression of downstream target genes of STAT3, such as MMP9 and c-myc (Fig. 4A). Notably, consistent data obtained from multiple sources including TCGA, GEO, and our hospital's dataset indicated that CEBPB mRNA levels positively correlated with MMP9 and c-myc mRNA levels in RCC tissues (Fig. 4B–E). Additionally, the overexpression of IL-6 partially reversed the reduced protein expression of MMP9 and c-myc induced by the inhibition of CEBPB (Fig. 4F and S2E). These compelling observations strongly suggest that knockdown of CEBPB inhibits the proliferation, migration, and invasion of RCC cells through the IL-

6/STAT3 pathway.

4. Discussion

It has been reported that CEBPB exhibits opposing functions in different types of cells. While certain solid cancers like breast cancer [24], ovarian cancer [25], and colorectal cancer [26] show increased expression of CEBPB, squamous cell carcinomas exhibit reduced expression [27]. In our study, we demonstrate that CEBPB is overexpressed in RCC tissues and cell lines. As a transcription factor, CEBPB regulates the expression of various cytokines, including IL-6, TNF-α, IL-8, and MCP-1 [15]. There is increasing evidence that CEBPB plays a role in regulating numerous tumor-related biological activities through the modulation of these cytokines [13,15]. For instance, in triple-negative breast cancer, CEBPB regulates the expression of G-CSF and GM-CSF, leading to the development of myeloid-derived suppressor cells (MDSCs) [11]. Our study further elucidates that CEBPB promotes the proliferation, migration, and invasion of RCC cells through the IL6/STAT3 signaling pathway, providing additional evidence for the involvement of CEBPB in tumor immunity.

Numerous studies have demonstrated the diverse impact of IL6 on cancer cell behavior, encompassing migration [28], invasion, proliferation, apoptosis [29], angiogenesis, and differentiation [30]. In various cancer types, elevated levels of IL6 have been associated with poor prognosis [31]. Within the tumor microenvironment, tumor cells secrete high levels of IL6, which act as an autocrine growth factor through several mechanisms, such as blocking DC differentiation and maturation, inhibiting NKT cell activation, and promoting anergy in NK cells [32]. Upon activation, IL6 primarily triggers the JAK/STAT3 signaling pathway [33,34], contributing to cancer progression by promoting proliferation and EMT via c-myc and MMP9 [35]. In RCC, overstimulation of STAT3 has also been observed and may be induced by IL-6 [36]. In our research, we found that knockdown of CEBPB inhibits the phosphorylation of STAT3, and overexpression of IL6 partly reverses the decreased phosphorylation of pSTAT3 induced by CEBPB inhibition. These results indicate that CEBPB may promote the progression of RCC through the IL6/STAT3 pathway. However, it is worth noting that the IL6/IL6R/gp130 complex can also activate the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways [37,38]. IL-6 may activate multiple pathways simultaneously [20]. In future research, we will further explore whether IL6 activates other pathways involved in the regulation of CEBPB on the proliferation, invasion, and metastasis of renal cell carcinoma.

In conclusion, our study reveals that CEBPB expression is significantly upregulated in RCC tissues, and its high expression is closely associated with adverse clinical indicators, such as RCC pathologic grade, clinical stage, and lymphatic metastasis. Moreover, we demonstrate that knocking down CEBPB leads to a suppression of proliferation, migration, and invasion in 786-O and 769-P cells by reducing IL6 expression in RCC cells. Importantly, our findings highlight the pivotal role of CEBPB in driving RCC progression through the IL6/STAT3 pathway.

Ethics approval and consent to participate

This study was reviewed and approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University, with the approval number: 2019-148.

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Availability of data and materials

All data related to the results of this study are available within the article.

Author contribution statement

Yaoqiang Ren: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Baoping Qiao: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Wenke Guo: Performed the experiments; Analyzed and interpreted the data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20175.

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