

Original Article

LINC00460 drives clear cell renal cell carcinoma progression via complement/coagulation and p53 pathways: a potential therapeutic target

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Abstract: This study demonstrates that the long noncoding RNA (lncRNA) LINC00460 is significantly overexpressed in clear cell renal cell carcinoma (ccRCC) and is strongly associated with adverse clinical outcomes. Analysis of data from The Cancer Genome Atlas (TCGA), validated by an independent cohort (GSE53757) and quantitative real-time polymerase chain reaction (qRT-PCR), shows that high LINC00460 expression has robust diagnostic value (area under the curve [AUC] = 0.818) and predicts shorter overall survival, highlighting its potential as a prognostic biomarker. Functional enrichment analyses indicate that LINC00460 is involved in key pathways, including complement and coagulation cascades, cytokine-cytokine receptor interactions, extracellular matrix remodeling, and p53 signaling. *In vitro* experiments confirm that silencing LINC00460 in ccRCC cell lines (Caki-2 and ACHN) markedly inhibits tumor cell proliferation, migration, and invasion while promoting apoptosis. Mechanistically, LINC00460 knockdown reduces levels of inflammatory factors (interleukin-6 [IL-6], tumor necrosis factor- α [TNF- α], and C-X-C motif chemokine ligand 8 [CXCL8]) and coagulation-related proteins (C3, SERPINA1, and PLAU), and activates the p53 pathway by upregulating p21 and BAX while downregulating Bcl-2. Genomic analysis reveals higher mutation rates of *BAP1* and *LRP2* in LINC00460-high tumors, and drug repurposing screening identifies cinchonine and iproniazid as candidate therapeutic agents. These findings establish LINC00460 as an oncogenic driver in ccRCC and a promising target for both diagnosis and precision therapy.

Keywords: ccRCC, LINC00460, bioinformatics, targeted therapy

Introduction

Renal cell carcinoma (RCC) represents the most common malignant tumor of the urinary system, accounting for approximately 90% of all kidney cancer cases [1]. Epidemiological data indicate a persistent upward trend in the global incidence of RCC over recent decades [2, 3]. Clinically, clear cell RCC (ccRCC), the predominant histological subtype, is characterized by late-stage diagnosis due to the lack of specific early symptoms and a high propensity for metastasis. Although targeted therapies and immune checkpoint inhibitors have advanced the treatment landscape, the prognosis for patients with metastatic ccRCC remains poor, with high rates of drug resistance and recurrence [4]. Consequently, there is an urgent

unmet clinical need to identify reliable biomarkers for early diagnosis and prognosis, as well as novel therapeutic targets to improve clinical outcomes.

Long noncoding RNAs (lncRNAs), defined as transcripts longer than 200 nucleotides with limited protein-coding potential, have emerged as critical regulators in tumorigenesis and cancer progression [5-7]. Accumulating evidence demonstrates that lncRNAs modulate key biological processes, including cell proliferation, apoptosis, metastasis, and drug resistance, through diverse mechanisms such as chromatin remodeling, transcriptional regulation, and post-transcriptional processing [8-10]. The advent of high-throughput sequencing technologies has facilitated the discovery of cancer-

associated lncRNAs. Public repositories, such as The Cancer Genome Atlas (TCGA) maintained by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI), provide extensive multi-omics data accessible to the research community [8-10]. While traditional studies have leveraged TCGA data to screen for ccRCC-related lncRNA markers and correlate them with patient prognosis [7, 11, 12], many identified candidates lack rigorous experimental validation and mechanistic exploration, limiting their translational potential. The primary motivation for this study was to bridge the gap between large-scale bioinformatic screening and functional experimental validation. While previous studies have implicated LINC00460 in the progression of various malignancies, including breast and colorectal cancers, its specific role, regulatory mechanisms, and clinical significance in ccRCC remain largely unexplored. Merely identifying a correlation is insufficient; understanding the downstream molecular pathways is essential for developing targeted interventions.

Innovatively, this study employs a comprehensive strategy integrating weighted gene co-expression network analysis (WGCNA) of TCGA data with robust *in vitro* and *in vivo* functional assays. Unlike prior reports, we not only confirm LINC00460 as a potent oncogene in ccRCC but also elucidate its novel mechanism of action. Specifically, we demonstrate that LINC00460 drives ccRCC progression by modulating the complement and coagulation cascades and the p53 signaling pathway. Furthermore, leveraging these mechanistic insights, we performed a drug repurposing screen to identify potential small-molecule inhibitors targeting the LINC00460-associated network, offering a new avenue for therapeutic intervention.

The clinical significance of this work is twofold. First, we establish LINC00460 as an independent prognostic biomarker that can stratify ccRCC patients into distinct risk groups, aiding in personalized clinical decision-making. Second, by uncovering the specific signaling axes regulated by LINC00460 and identifying candidate therapeutic compounds, our findings provide a theoretical basis for developing novel precision medicine strategies for ccRCC. Therefore, the objective of this study is to comprehensively evaluate the prognostic value of LINC00460, dissect its functional mechanisms,

and explore its potential as a therapeutic target in ccRCC.

Materials and methods

Data acquisition

We retrieved RNA-seq expression profiles and clinical data of ccRCC and adjacent non-tumor renal tissue samples from the TCGA database (<https://portal.gdc.cancer.gov/>) in January 2020.

Construction of weighted gene co-expression network analysis (WGCNA)

The TCGA dataset, which includes detailed information on ccRCC patients such as Grade, Pathologic_T, N, M, stage, overall survival (os_time), and status, was suitable for WGCNA construction. We used R/Bioconductor's "WGCNA" package to create a gene expression dataset for TCGA and chose the top 25 percent of genes having the highest variance in expression among the tumor samples as the data source for following WGCNA research. Before we picked our favorite power of the soft threshold, which was used for building the scale free network, we used hierarchical clustering to get rid of a few outliers. We also created the adjacency matrix and topological overlap matrix (TOM). We then calculated the difference between 1 and TOM and made use of dynamic tree cutting to create the gene dendrogram and determine module identification. Minimum module size was determined to be 30 and then we did clustering. Modules with a similarity difference of < 0.25 were clustered. To examine the relationship between module characteristic genes and ccRCC clinical phenotypes, we calculated the correlation. Modules containing LINC00460 were selected for further analysis, and both genetic significance (GS) and module membership (MM) were determined to identify the modules associated with ccRCC clinical features.

qRT-PCR validation of the core lncRNA

Three pairs of ccRCC samples and the adjacent non-tumor samples were obtained from The Second Affiliated Hospital of Qiqihar Medical University: The samples were stored in LN2 first before being stored at -80°C. All patients provided written informed consent for using their tissue samples in the present study. The Ethics Committee of The Second Affiliated Hospital,

Qiqihar Medical University has given permission: 202403004-01.

We used the TRIzol reagent (Takara Japan) to obtain total RNA and then synthesized the cDNA using the Prime Script RT Reagent Kit (Takara). We performed qRT-PCR on an ABI Step One Real-time PCR System (Life Technologies, <http://www.Abinspection.com>) and performed experiments using the TB Green Premix Ex Taq (Poly (Takara, TaKara) as described previously [13]. The PCR primers were particularly crafted and built by Sangon Biotech (Shanghai) Co., Ltd. The forward and reverse primer sequences for qRT-PCR amplification of LINC00460 were corrected as follows: Forward: 5'-GGCATTGTAGAAAGACTGAGCG-3'; Reverse: 5'-TAGCATACGAATTTGGGTGGG-3'. The PCR conditions were optimized with an annealing temperature of 60°C, yielding a specific product length of 125 bp. No secondary structures affecting efficiency were observed under these conditions. Statistical analysis was conducted through one-way ANOVA, in which p -value < 0.05 is considered as having statistical meaning.

Differentially expressed gene (DEGs) analysis and functional annotation

To explore the mechanisms behind the varying expression levels of core lncRNAs, we utilized the “limma” package in R software to identify DEGs between distinct expression groups of the core lncRNA in ccRCC samples. Genes with a $|\log_2$ fold change| greater than 1 and a p -value smaller than 1.0×10^{-10} were classified as differentially expressed between the low and high expression groups of the core lncRNA in ccRCC samples (based on the median value). To examine the potential functions of the core lncRNA, functional enrichment analysis was conducted on the DEGs, identifying significantly enriched GO terms and KEGG pathways. Pathways enrichment: Pathways with adjusted $P < 0.05$ were considered enriched. These DEGs interaction was realized with Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and GeneMANIA (<http://genemania.org/>).

Gene set enrichment analysis (GSEA) was performed on the whole-genome ccRCC dataset along with the expression levels of core lncRNAs

GSEA was employed to further investigate the underlying mechanisms between different exp-

ression groups of the core lncRNA in ccRCC samples (using the median value). GSEA was conducted to identify enriched terms potentially associated with KEGG pathways in C2, and in C5, to find gene sets containing genes annotated with the same GO term. Its statistical significance was determined using a p value of < 0.01 and a FDR q value of < 0.05. In addition, it meets all the conditions: the difference in nominal p value is less than 0.05, absolute value of NES (normalized enrichment score) is greater than 1, and FDR is less than 0.25.

Multi experiment matrix (MEM) analysis

MEM is not only a multi experiment gene expression query based on web, but also a visualization tool. It integrates hundreds of publicly available gene expression datasets from the ArrayExpress repository, with distinct datasets featuring diverse tissues, diseases, and conditions. Given a gene as an input, MEM sorts other genes with their similarity in each individual data set. MEM analysis was used to identify the target genes that interact closely with the core lncRNA, aiming to find new ideas for the treatment of ccRCC.

Mutation analysis

For the mutation analysis, the ccRCC sample data were obtained from TCGA in the Mutation Annotation Format (MAF). The MAF file format, established by TCGA, includes essential information related to mutations. And waterfall plots were drawn up by means of the GenVisR bundle inside R.

Cell culture and cell line authentication

Human ccRCC cells (786-O, A498, Caki-1, Caki-2, ACHN), and human immortalized proximal tubule epithelial cell line HK-2 were purchased from ATCC (Manassas, VA, USA) or Chinese Academy of Sciences Cell Bank (Shanghai, China). Culture all cells in the 100 mm dish in DMEM or RPMI - 1640 medium (Gibco, Thermo Fisher Scientific, USA) with 10% FBS (Gibco), 1% Penicillin-streptomycin (Gibco) at 37 degrees Celsius in a humidified incubator with 5% CO₂. The cell lines were regularly tested for mycoplasma contamination and authenticated by STR profiling within 6 months of purchase.

Construction of stable LINC00460-knockdown cell lines

Four distinct short hairpin RNA (shRNA) sequences targeting LINC00460 (shRNA#1-#4)

and a non-targeting control shRNA (NC shRNA) were designed and cloned into the pLKO.1-puro lentiviral vector (Addgene, USA). The lentiviruses were packaged in HEK293T cells using standard protocols with packaging plasmids psPAX2 and pMD2.G. Caki-2 and ACHN cells were infected with the viruses in the presence of 8 µg/mL polybrene (Sigma-Aldrich, USA). Stable cell lines were created using puromycin (2 µg/mL, Sigma-Aldrich) for a period of two weeks. Knockdown efficiency was verified by RT-qPCR as described in 2.3. Four distinct short hairpin RNA (shRNA) sequences targeting LINC00460 were designed: shRNA #1: 5'-GCT-AGACCTAATAGCCAATA-3'; shRNA #2: 5'-GCC-ATCCACTTCAAAGTATTC-3'; shRNA #3: 5'-ACCT-TGGTCCAAACGTTTAAACC-3'; shRNA #4: 5'-GC-TAAGACCTAAGGCAACA-3'.

Functional assays: proliferation, migration, invasion, and apoptosis

Colony formation assay: Cells were plated into 6-well plates at 500 cells/well, and cultured for 10-14 days. Colonies were poured with 4% para-formaldehyde, stained with 0.1% crystal violet, and then counted manually.

Wound healing assay: Cells were seeded into 6wl, grown till 90% confluence. A 200-µL pipette tip was used to create a scratch wound. Image were shot at 0 h, 24 h with an inverted microscope and wound closure rate was figured out.

Transwell invasion assay: Matrigel (Corning, USA) was precoated into the upper chamber of Transwell insert (8-µm pore size). Cells (5×10^4) were plated in the upper chamber in a serum free medium with 10% FBS in the lower chamber as a chemoattractant medium. After 24-48 h incubation time, Hex, id cells on the upper side that did not invade were wiped off with a cotton swab. The stain of crystal violet was fixed in the invaded cells on the lower surface, and the number of the invaded cells was counted under the microscope.

Flow cytometry for apoptosis: Cells were harvested, washed by cold PBS and treated with ANNIXIN V-FITC and PI through Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). The samples were run on a flow cytometer (e.g., BD FACSCanto II).

Western blot analysis

Total protein was extracted from cultured cells using RIPA lysis buffer (removing protease inhibitors and phosphate inhibitor, Beyonint, China). A BCA Protein Assay kit (Thermofisher) was used to analyze the level of proteins. 20-40 µg proteins were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, USA). The membrane was blocked with 10% non-fat milk in TBS and incubated with appropriate primary antibodies at 4°C for 8 hours, including C3, SERPINA1, PLAUI, IL-6, TNF-alpha, CXCL8, Bcl-2, p21 (CDKN1A), BAX and β-actin (all from Cell Signaling Technology USA or Abcam UK; 1:1000). Washed again, membranes were incubated in HRP second antibody (1:5000) at room temperature for 1 hour. Protein bands were viewed with a more sensitive light-producing chemical substance (ECL) detection device (Bio-radios US). The density of protein bands was counted with ImageJ software.

Statistical analysis

Graphpad prism 9.0/R (v4.3.0) was used for statistical analysis Data from ≥ 3 independent experiments were presented as mean $\pm$ SEM. Group comparisons: Two-tailed unpaired t-tests (two groups) or one-way ANOVA with Tukey's post hoc tests (≥ 3 groups) were carried out. The diagnostic performance of the candidate genes was assessed using ROC curve analysis (AUC obtained with MedCalc). Kaplan-Meier survival analysis used log-rank test with median lncRNA expression as cutoff. For bioinformatics, we corrected differentially expressed genes (DEGs) and GSEA for multiple testing (Benjamini-Hochberg, FDR < 0.05 or < 0.25), and made a PPI network in Cytoscape.*P < 0.05 was considered statistically significant.

Results

Data preprocessing

There were, in all, 530 ccRCC samples and 72 matched adjacent normal renal tissues sourced from TCGA. All ccRCC samples had associated survival data, and 365 possessed mutation data. Clinical characteristics include gender, age, pathologic M, N, T, histological grade. Additionally, GSE53757, comprising 72 ccRCC specimens and 72 normal renal control samples, was used for independent validation.

Weighted co-expression network construction and identification of key modules

WGCNA is a very popular analysis method for finding the complex relationship between genes and the appearance characteristics. It creates co-expression networks through gene expression information giving hints about signaling network connected to target trait [14]. In this study, we excluded 2 samples which were outliers in the following analysis leaving a total of 528 samples with survival data for WGCNA (Figure 1A). Next, we chose a soft-threshold power, $\beta = 3$ (scale free $R^2 = 0.88$) to get a scale free network (Figure 1B, 1C). After removing neutral gray modules through merged adaptive tree pruning, eight gene co-expression modules were identified (Figure 1D). A heatmap was used to visualize the topological overlap matrix (TOM) of the 400 selected genes, with the results confirming the independence of each module (Figure 1E). Subsequently, the yellow module was identified as the most strongly correlated with all characteristics in ccRCC and was considered the key module ($R^2 = 0.42$, $P = 1e-24$ with grade; $R^2 = 0.3$, $P = 3e-12$ with pathologic T; $R^2 = 0.22$, $P = 3e-07$ with pathologic N; $R^2 = 0.24$, $P = 2e-08$ with pathologic M; $R^2 = 0.32$, $P = 8e-14$ with pathologic stage; $R^2 = -0.19$, $P = 1e-05$ with survival time; $R^2 = 0.33$, $P = 5e-15$ with survival status; Figure 1F).

Identification of LINC00460

To further screen for lncRNAs closely related to ccRCC, the yellow module was presented for further analysis. We made scatter plots to look at the connection between gene important (GS) and being a member of a specific Module (MM) for the yellow module that had the biggest relationship with grades (correlation = 0.49, $P = 9.2e-10$). This analysis revealed that seven lncRNAs met the criteria of $MM > 0.60$ and $GS > 0.25$ (Figure 2A). To evaluate the diagnostic potential of these seven lncRNAs for ccRCC, ROC curve analysis was conducted, comparing AUC values to assess their sensitivity and specificity in TCGA. The top one lncRNA was LINC00460 according to its AUC value which was 0.818, demonstrating that the lncRNA had the highest diagnostic significance for ccRCC (Figure 2B, 2C). Furthermore, we found that LINC00460 was significantly upregulated in

ccRCC in the GSE53757 dataset (Figure 2D). In addition, qRT-PCR results show that LINC00460 is upregulated in ccRCC, and the relative expression of linc00460 is consistent with the results of microarray hybridization (Figure 2E). Survival analysis points out that high levels of LINC00460 expression is linked to poor survival, and it is likely a risk factor (Figure 2F).

LINC00460 is highly expressed in ccRCC cell lines and its knockdown significantly suppresses malignant phenotypes

To find out the function of LINC00460 in ccRCC, we first looked into the basic expression levels of LINC00460 in a number of ccRCC cell groups. The expression of LINC00460 was upregulated in all 5 ccRCC cell lines (786-O, A498, Caki-1, Caki-2, ACHN) as compared to the immortalized human proximal tubule epithelial cell line HK-2 via RT-qPCR analysis. We noticed that the highest level was observed in Caki-2 and ACHN cells (Figure 3A). Thus, the cancerous effect might be more significant. Next, we transfected Caki-2 and ACHN cells with four different shRNAs against LINC00460 (shRNA#1-#4): RT-QPCR verification showed that the shRNA#2 and shRNA#3 reaped the biggest knockdown ($> 70\%$ reduction, $P < 0.001$), but shRNA#1 and shRNA#4 only displayed little effects (Figure 3B). Therefore, shRNA#2 and shRNA#3 were chosen for all following functional tests. In the colony formation experiments, it was seen that the Caki-2 and ACHN cells had less number of colonies than the Control and NC shRNA group when the knockdown of LINC00460 was done, which was less than 0.01, proving that LINC00460 promotes ccRCC cell division as shown by Figure 3C. Wound healing assays showed that after knocking out LINC00460, it greatly hindered cell migration; there were notably less wound healing rates at 24 hours between the 2 cell lines ($P < 0.01$) (Figure 3D). In Transwell invasion assays we saw this too: LINC00460 knockdown cells exhibited large decrease in invasivity through Matrigel coated membrane ($P < 0.001$) (Figure 3E). Moreover, flow cytometry analysis using Annexin V/PI staining demonstrated that LINC00460 knockdown significantly increased the percentage of apoptotic cells in both Caki-2 and ACHN lines ($P < 0.01$) (Figure 3F), suggesting that LINC00460 exerts anti-apoptotic effects in ccRCC.

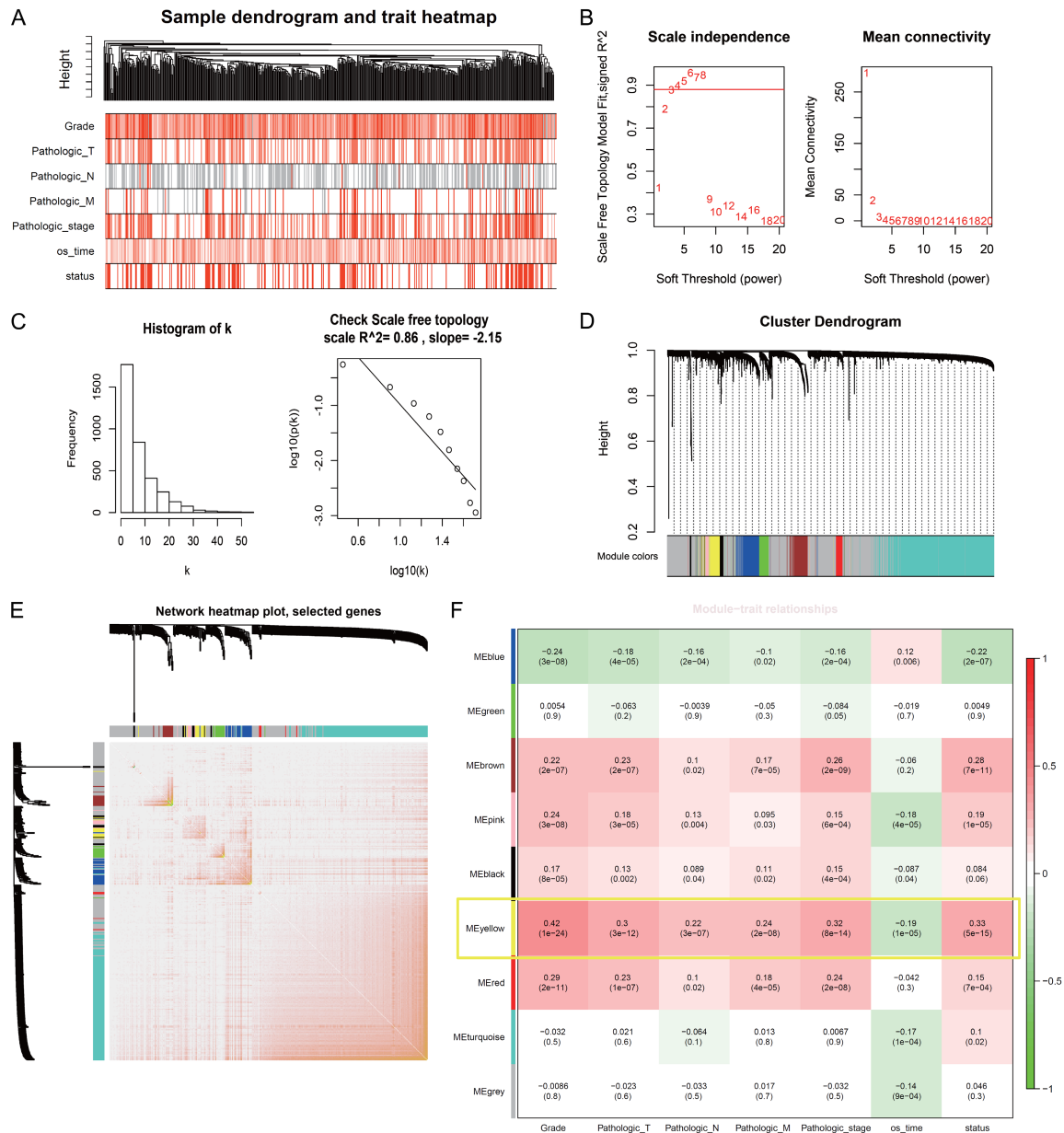


Figure 1. Weighted gene co-expression network analysis (WGCNA) results for ccRCC. (A) Sample dendrogram and trait heatmap showing the clustering of 528 ccRCC samples after outlier removal. Clinical traits are indicated on the right. (B, C) Analysis of scale-free topology fit (left) and mean connectivity (right) for various soft-thresholding powers. A power of $\beta = 3$ (scale-free $R^2 = 0.88$) was selected to construct the scale-free network. (D) Cluster dendrogram of genes showing module identification via dynamic tree cutting with a minimum module size of 30. (E) Heatmap of the topological overlap matrix (TOM) for 400 representative genes, demonstrating the independence of each identified module. (F) Heatmap displaying module-trait relationships. The yellow module showed the strongest positive correlation with clinical traits (e.g., tumor grade: correlation coefficient = 0.42, $P = 1 \times 10^{-24}$). Statistical methods: Correlations in (F) were calculated using Pearson's correlation coefficient.

Integrated functional annotation and pathway analysis of LINC00460 in ccRCC

To elucidate the molecular mechanisms underlying *LINC00460* function in ccRCC, we first stratified samples based on the median expression level of *LINC00460*. This analysis identi-

fied 630 DEGs, comprising 518 upregulated and 112 downregulated genes (Figure 4A; Table S1).

Functional enrichment analysis of these DEGs revealed a strong association with immune-related pathways. Gene Ontology (GO) term

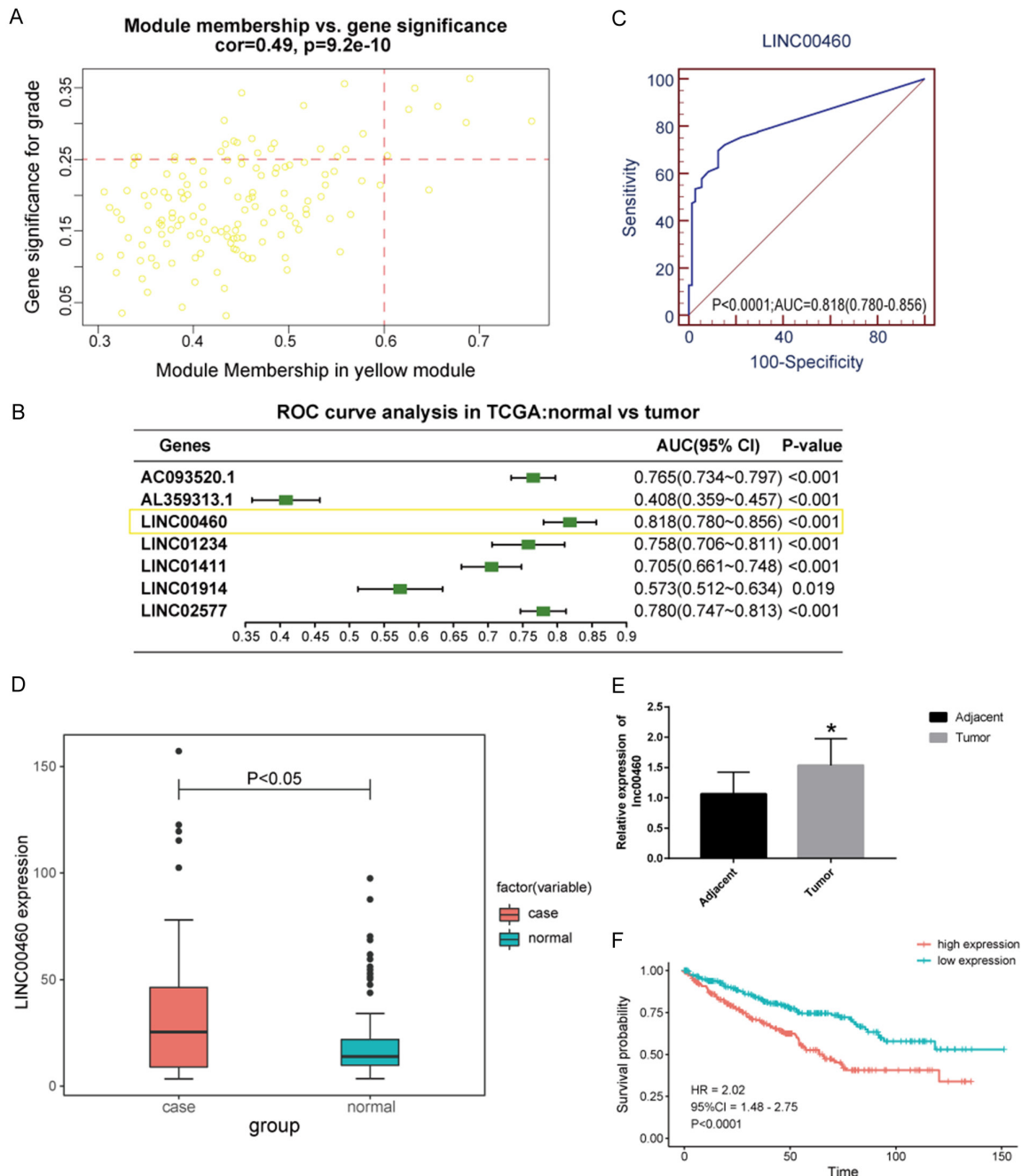


Figure 2. Identification and validation of LINC00460 in ccRCC. (A) Scatter plot of Gene Significance (GS) versus Module Membership (MM) within the yellow module, showing a significant positive correlation (cor = 0.49, $P = 9.2 \times 10^{-10}$). (B) ROC curve analysis evaluating the predictive performance of seven candidate lncRNAs for ccRCC diagnosis in the TCGA dataset. (C) ROC curve specifically for LINC00460, yielding an area under the curve (AUC) of 0.818 (95% CI: 0.780-0.856, $P < 0.0001$). (D) Validation of LINC00460 expression in the GSE53757 dataset, showing significant upregulation in ccRCC tissues compared to normal controls. (E) qRT-PCR validation of LINC00460 expression levels in three pairs of ccRCC tissues and adjacent normal tissues ($P < 0.05$). (F) Kaplan-Meier survival analysis indicating that high LINC00460 expression is significantly associated with poorer overall survival in ccRCC patients (HR = 2.02, 95% CI = 1.48-2.75, $P \leq 0.0001$). Statistical methods: Differences in (D) and (E) were analyzed using an unpaired two-tailed Student's t-test. Survival differences in (F) were assessed using the log-rank test.

analysis highlighted significant enrichment in “Staphylococcus aureus infection” and “com-

plement and coagulation cascades” (Tables S2, S3, S4). Consistently, Kyoto Encyclopedia of

LINC00460 drives ccRCC

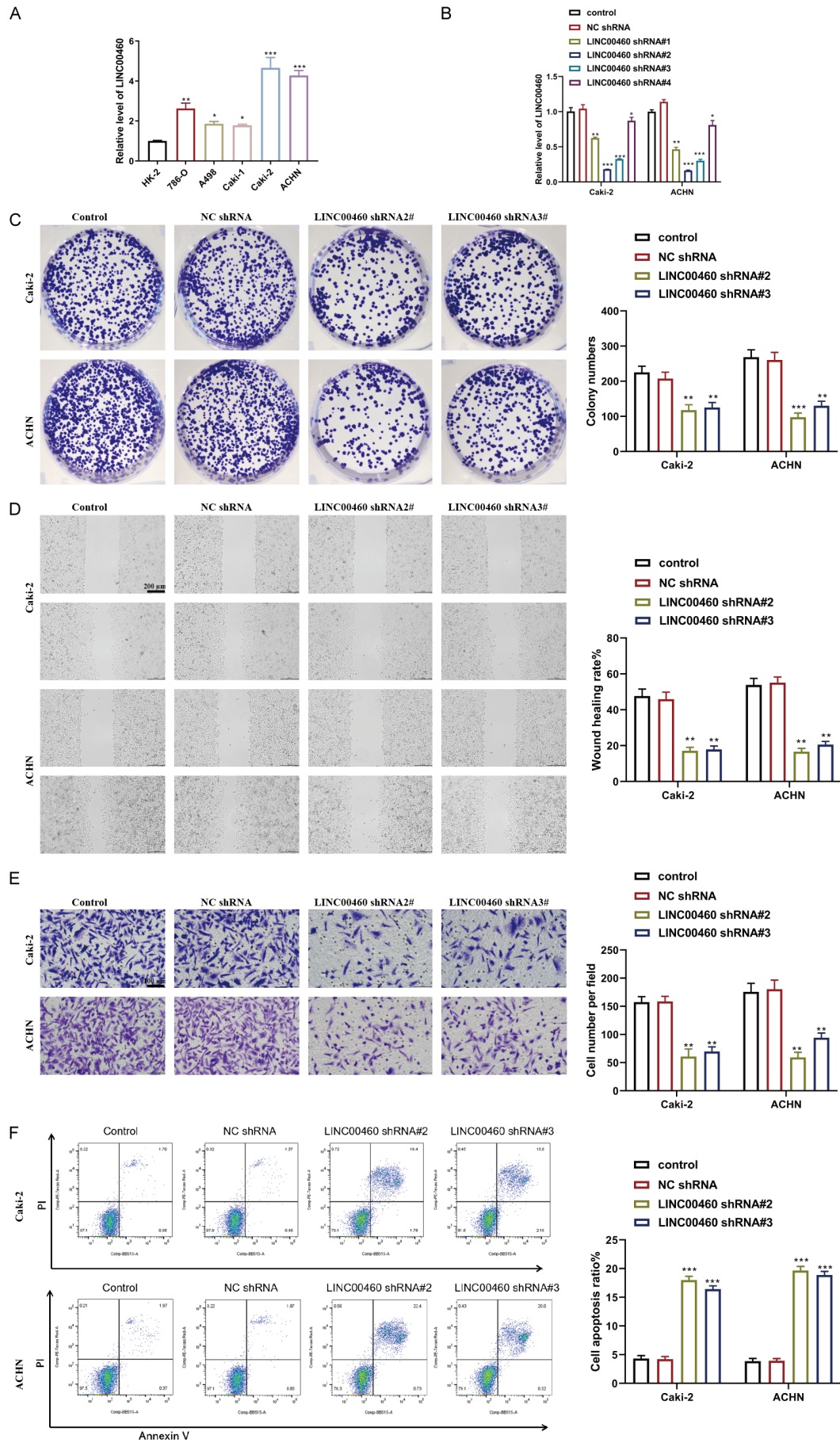


Figure 3. LINC00460 is highly expressed in ccRCC cell lines, and its knockdown suppresses malignant phenotypes. (A) Relative expression levels of LINC00460 in seven ccRCC cell lines (786-O, A498, Caki-1, Caki-2, ACHN, etc.) compared to the normal renal tubular epithelial cell line HK-2, measured by qRT-PCR. (B) Knockdown efficiency of LINC00460 in cells transfected with different shRNAs (shRNA #1-#4) compared to negative control (NC) shRNA, assessed by qRT-PCR. (C) Colony formation assays in Caki-2 and ACHN cells transfected with control, NC shRNA, LINC00460 shRNA #2, or shRNA #3. Representative images and quantification of colony numbers are shown. (D) Wound healing assays evaluating cell migration in Caki-2 and ACHN cells at 0 h and 24 h post-scratch after transfection with indicated shRNAs. Representative images and relative migration rates are presented. (E) Transwell invasion assays determining the invasive capacity of Caki-2 and ACHN cells following LINC00460 knockdown. Representative images and counts of invaded cells are shown. (F) Flow cytometry analysis of apoptosis in Caki-2 and ACHN cells using Annexin V/PI staining after LINC00460 silencing. Representative plots and quantification of apoptotic rates are displayed. Data are presented as mean $\pm$ SEM ($n = 3$ independent experiments). Statistical methods: Comparisons between multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC shRNA.

Genes and Genomes (KEGG) pathway analysis indicated that these DEGs were significantly involved in “inflammatory response” and “acute inflammatory response” (Figure 4B, 4C; Table S5). To further validate these findings at a genome-wide level without arbitrary cutoffs, we performed Gene Set Enrichment Analysis (GSEA) using C2 curated canonical pathways. The results confirmed that high LINC00460 expression was significantly positively correlated with multiple immune and proliferation-related pathways, including “complement and coagulation cascades”, “cytokine-cytokine receptor interaction”, “graft-versus-host disease”, “allograft rejection”, “autoimmune thyroid disease”, “ECM-receptor interaction”, and “DNA replication” (Figure 5A-G). Additionally, GSEA based on C5 gene ontology sets reinforced these observations, showing significant enrichment in humoral immune responses mediated by circulating immunoglobulins and complement activation (Figure 5H-K). Collectively, both DEG-based annotation and GSEA underscore the pivotal role of LINC00460 in modulating immune and inflammatory microenvironments in ccRCC.

To explore the regulatory landscape of these DEGs, protein-protein interaction (PPI) networks and gene-gene interaction maps were constructed using STRING and GeneMANIA, respectively. These networks illustrated the complex co-expression relationships and functional clusters among the LINC00460-associated genes (Figure 4D, 4E). Finally, aiming to identify potential therapeutic strategies targeting LINC00460-driven signatures, we conducted Connectivity Map (CMap) analysis. This screening identified two small-molecule compounds with significant negative connectivity scores, suggesting their potential to reverse

the LINC00460-high expression signature: cinchonine (mean connectivity score = -0.386; $P = 0.0148$; Figure 4F) and iproniazid (mean connectivity score = -0.416; $P = 0.01738$; Figure 4G; Table 1). These findings not only clarify the immune-modulatory functions of LINC00460 but also propose promising candidate drugs for ccRCC treatment.

Identification of the target gene of LINC00460

To further identify potential target genes regulated by LINC00460 and uncover novel therapeutic strategies, we performed MEM analysis. By searching with different probes (1558930_AT and 1563062_AT), the slightly different results were obtained. However, both results showed that LINC00973 was the most closely interacted gene of LINC00460, which was regarded as the target gene of LINC00460 (Figure 6; Tables 2, 3). Studies have shown that LINC00973 enhances the expression of Siglec-15, which is involved in cancer immune suppression in ccRCC. Also, high LINC00973 expression in ccRCC cells significantly inhibited the immune response stimulation of Jurkats cells with Siglec-15 mainly involved in the start and development of ccRCC [15]. The screening of target gene in our research also supported the results of pathway analysis.

Mutation analysis between different groups of LINC00460 expression

To investigate the molecular mechanisms in ccRCC samples with elevated LINC00460 expression, we generated a waterfall plot for further analysis. The high-expression group showed a higher frequency of mutations in BAP1 and LRP2 compared to the low-expression group (Figure 7A, 7B). BAP1 is thought to play a role in

LINC00460 drives ccRCC

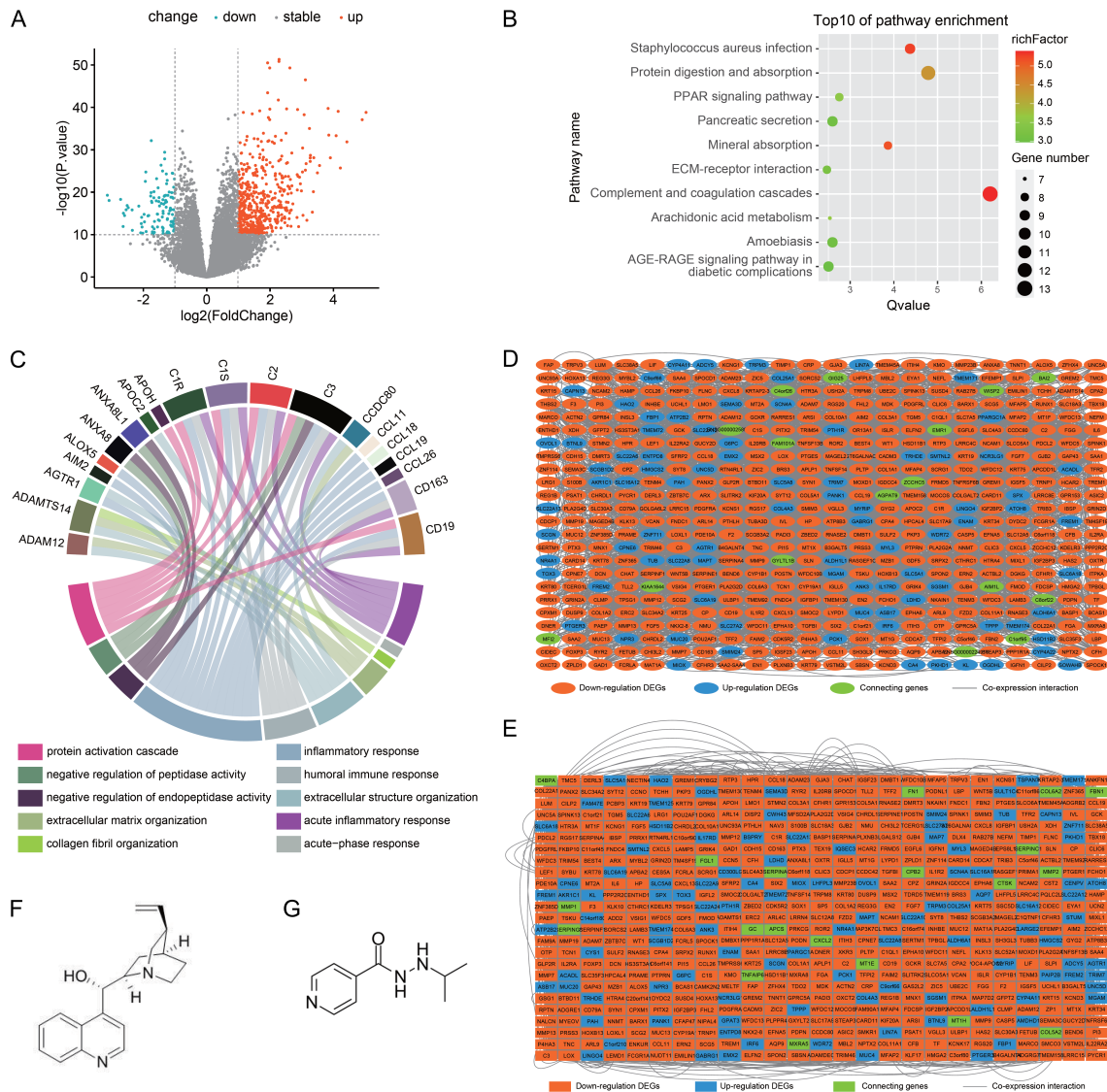


Figure 4. Differentially expressed genes (DEGs) related to LINC00460 and functional annotation. (A) Volcano plot illustrating 630 DEGs (112 downregulated, 518 upregulated) between high and low LINC00460 expression groups (criteria: $|\log_2 \text{fold change}| > 1$, $P < 1.0 \times 10^{-10}$). (B, C) Bar charts displaying the top 10 enriched KEGG pathways (B) and Gene Ontology (GO) terms (C) for the identified DEGs. (D, E) Protein-protein interaction (PPI) networks of DEGs constructed using STRING (D) and GeneMANIA (E) databases. (F, G) Chemical structures of cinchonine (F) and iproniazid (G), identified as potential therapeutic drugs targeting the LINC00460-associated network via CMap analysis. Statistical methods: Enrichment significance in (B) and (C) was determined using hypergeometric tests with Benjamini-Hochberg correction.

transcriptional regulation, cell cycle control, growth, DNA damage response, and chromatin dynamics, functioning as a tumor suppressor. LRP2, which is expressed in various tissues, particularly in absorptive epithelial tissues like the kidney, is involved in cell signaling. These results show that a high LINC00460 expression may contribute to progress of ccRCC by some kind of gene mutation.

LINC00460 regulates the complement and coagulation cascades pathway

Bioinformatic analyses implicated LINC00460 in several key pathways, including the complement and coagulation cascades. Here we see some protein levels on Western blot for a number of these components. As shown in **Figure 8**, knockdown of LINC00460 resulted in a notable

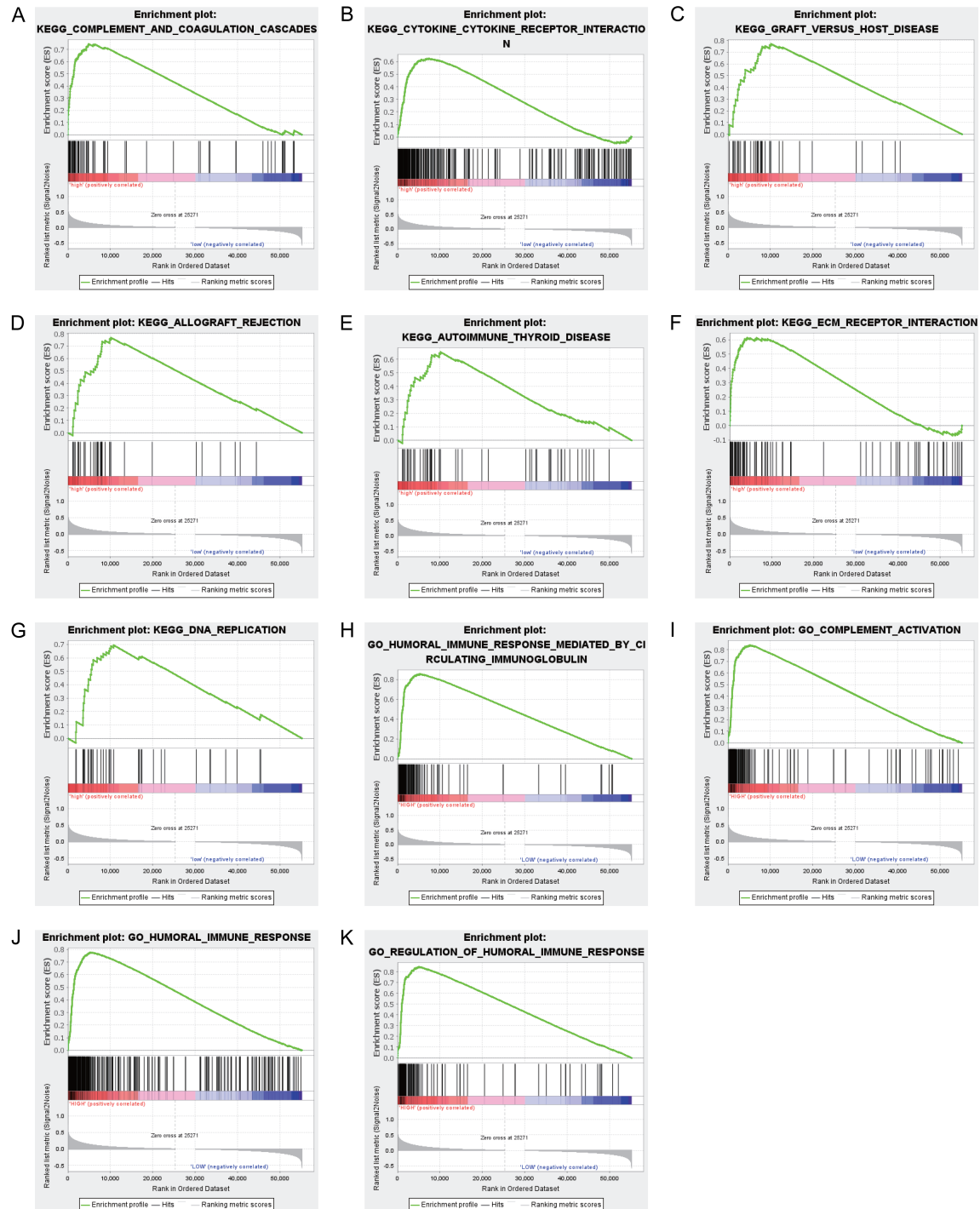


Figure 5. Gene Set Enrichment Analysis (GSEA) results for LINC00460 in ccRCC. (A-G) GSEA plots using the C2 gene set (curated gene sets). High LINC00460 expression was significantly enriched in pathways including “Complement and Coagulation Cascades”, “Cytokine-Cytokine Receptor Interaction”, and “DNA Replication”. (H-K) GSEA plots using the C5 gene set (Gene Ontology terms). Results indicate a strong enrichment of pathways related to humoral immune responses, such as “Complement Activation” and “Immunoglobulin-mediated Immune Response”. Enrichment significance was assessed using the Kolmogorov-Smirnov statistic within the GSEA algorithm; nominal $P < 0.05$ and FDR $q < 0.25$ were considered significant.

decrease of C3, SERPINA1 and PLA2 in both Caki-2 and Achn cells compared to control cell.

LINC00460 promotes the complement and coagulation signaling pathway according to the

Table 1. CMap analysis results

Cmap name	Mean connective score	n	Enrichment	P-value	Specificity	Percent non-null
Cinchonine	-0.386	4	-0.709	0.0148	0.1111	50
Iproniazid	-0.416	5	-0.632	0.01738	0.0376	60

result which can bring about a pro-tumorigenic microenvironment.

LINC00460 modulates cytokine-cytokine receptor interaction signaling

Because cytokine-cytokine receptor interaction pathway has been enriched after our bioinformatic analysis, we investigated the expression levels of some inflammatory mediators. Western blot shows that silence of LINC00460 greatly decreases the levels of protein IL-6, TNF- α , CXCL8 in both cell line (**Figure 9**). Because these kinds of chemicals help form the sickness, new blood lines, and hiding from our scrutineer, their downregulation upon LINC00460 knockdown suggests that LINC00460 fosters an immunosuppressive and pro-inflammatory niche in ccRCC.

LINC00460 influences the p53 signaling pathway despite low TP53 mutation frequency in ccRCC

And p53 signaling can be changed, even though TP53 mutations make up only a small part (roughly 5%) of ccRCC. We can change it using different regulator materials like MDM2 or ARF. Regarding the western blotting analysis of p53 downstream effectors, it's shown that after LINC00460 was knocked down, p21 (CDKN1A) and pro-apoptotic factor BAX were highly expressed and anti-apoptotic factor Bcl-2 was low (**Figure 10**). These changes collectively indicate activation of the p53-mediated tumor-suppressive response following LINC00460 depletion, even in the absence of TP53 mutation. This suggests that LINC00460 may indirectly suppress p53 pathway activity to support ccRCC cell survival and proliferation.

Discussion

In this study, we focused on potential oncogenes which have diagnostic and prognostic values. We identified genes significantly associated with clinical staging and survival of ccRCC by truncating the yellow modules in

WGCNA. We identified LINC00460 with the best diagnostic ability for ccRCC by ROC analysis. Re-analyzed the LINC00460, LINC00460 expression is clearly enhanced in ccRCC, and there exists an evident and strong positive association between it and poor life outcomes. Moreover, it can also be seen that LINC00460 is enriched in the path of coagulation and complement cascades and cytokine-cytokine receptor. In this paper, we noticed target genes that worked together with LINC00460 and treatment drugs that stopped the work of LINC00460 and we also saw gene mutations that could change LINC00460, giving fresh inventive thoughts to cure ccRCC.

LINC00460 is a cancer related biomarker which appears recently. Preceding studies had reported the clinical values of LINC00460 in breast cancer [16], colorectal cancer [17] and its biological function in cancers. Previous studies have shown that LINC00460 expression is significantly upregulated in osteosarcoma (OS) tumor tissues, with higher expression levels being associated with poorer patient prognosis. LINC00460 has also been found to play a critical role in the onset and progression of OS. In vitro studies revealed that silencing LINC00460 in OS cell lines significantly reduces cell proliferation, invasion, and migration. These results showed that down-regulation of LINC00460 can inhibit OS progression in vitro. The above findings also suggest that LINC00460 may exert oncogenic effects in OS and has potential as a biomarker for disease prognosis and progression [18]. We can find the similarity in gastric cancer. LINC00460 has been identified in various cancers, where it promotes tumor development and functions as an oncogene [19]. In our study, the overall survival of ccRCC patients with high expression of LINC00460 in tumor tissues was lower than that of patients with low expression.

Based on genomic wide co-expression analysis result, we can tell that LINC00460 has strong impact on different phenomenon like complement and coagulation cascades, cytokine-cyt-

LINC00460 drives ccRCC

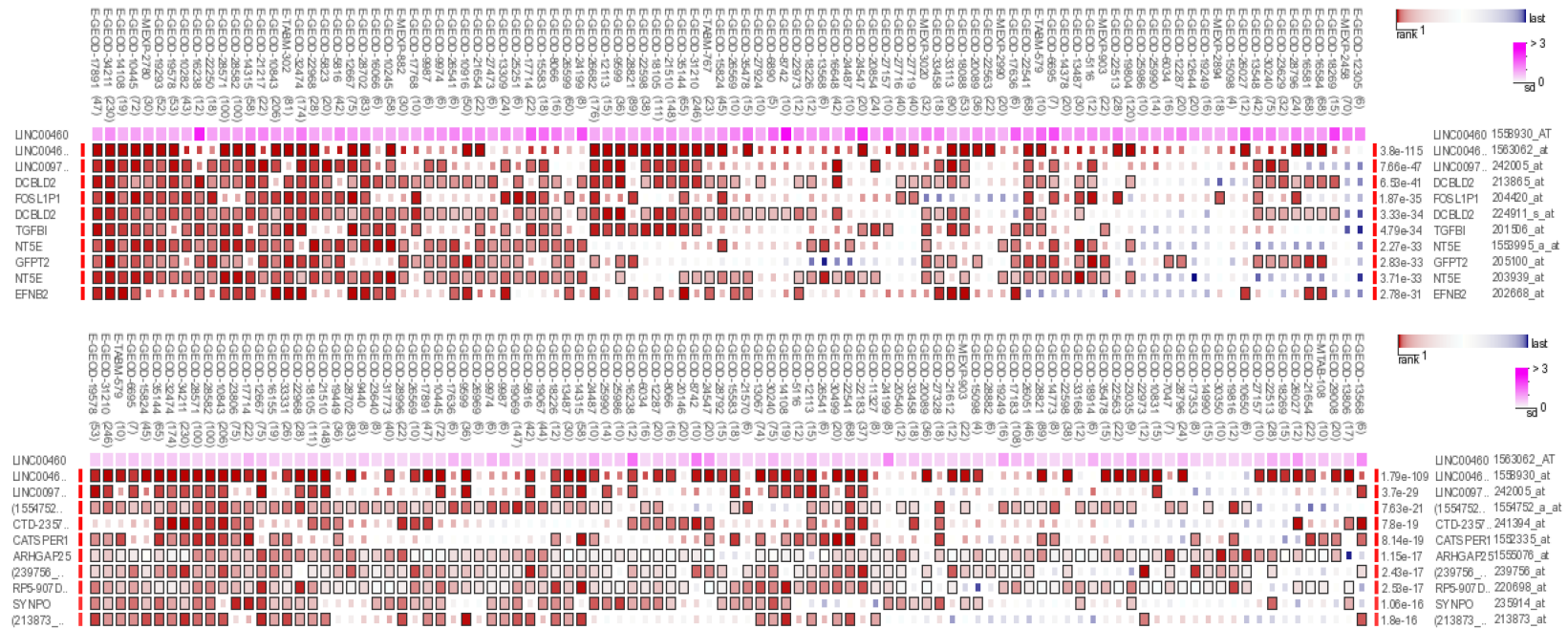


Figure 6. Identification of LINC00460 target genes by Multi-Experiment Matrix (MEM) analysis. Scatter plots showing the correlation of LINC00460 expression with potential target genes based on MEM analysis using probes 1558930_AT and 1563062_AT across multiple microarray datasets. Correlations were calculated using Pearson's correlation coefficient.

Table 2. MEM analysis results using the probe 1558930_AT

Score	Gene name	Probeset id
1.79E-109	LINC00460	1558930_at
3.70E-29	LINC00973	242005_at
7.63E-21	N/A	1554752_a_at
7.80E-19	CTD-2357A8.3	241394_at
8.14E-19	CATSPER1	1552335_at
1.15E-17	ARHGAP25	1555076_at
2.43E-17	N/A	239756_at
2.53E-17	RP5-907D15.4	220698_at
1.06E-16	SYNPO	235914_at
1.80E-16	N/A	213873_at

kines receptor interaction and P53 signaling, which is very important for cell cycle regulation, metabolism, aging, growth, reproduction and tumor suppression, etc. [20, 21]. A defect in p53 signaling pathways plays a critical role in cancer initiation and progression [22]. Cytokine-cytokine receptor interaction processes play important correlation in invasive traits in cancer cells, including pancreatic cancer [23] and ccRCC [24]. Functional enrichment analysis of DEGs in ccRCC patients stratified by LINC00460 expression revealed that these DEGs are involved in extracellular matrix, signaling receptor binding, complement and coagulation cascades, and protein catabolism and absorption. Studies have shown that complement and coagulation cascades can be used as targeted treatment of ccRCC [25]. For the two drugs we identified in this article, previous studies have not determined their function in cancer treatment. Therefore, more clinical trials are required to verify whether cinchonine and iproniazid can be used in the clinical treatment of ccRCC.

Based on the prediction made by these bioinformatic data, the functional cellular experiments also show that knockdown LINC00460 in the ccRCC cell line inhibited the proliferation, migration, and invasion of cells but promoted the apoptosis of cells. Broken down to molecular detail, after getting rid of LINC00460, it also lowered the key factors like C3, SERPINA1 subclasses, and PLAU from complementaries and coagulation part, together with inflammatory stimulators like IL-6, TNF- α , and CXCL8. Concurrently, the p53 signaling pathway is activated upon LINC00460 silencing, as evidenced by

Table 3. MEM analysis results using the probe 1563062_AT

Score	Gene name	Probeset id
3.80E-115	LINC00460	1563062_at
7.66E-47	LINC00973	242005_at
6.53E-41	DCBLD2	213865_at
1.87E-35	FOSL1P1	204420_at
3.33E-34	DCBLD2	224911_s_at
4.79E-34	TGFBI	201506_at
2.27E-33	NT5E	1553995_a_at
2.83E-33	GFPT2	205100_at
3.71E-33	NT5E	203939_at
2.78E-31	EFNB2	202668_at

increased expression of p21 and BAX and decreased levels of the anti-apoptotic protein Bcl-2. These findings provide direct experimental support for the role of LINC00460 as an oncogenic driver in ccRCC through modulation of inflammation, coagulation, and p53-dependent apoptosis.

In this study, we found that LINC00460 was a good prognosis marker for ccRCC patients. In addition, we employed TCGA genome wide RNA sequence data to investigate the role of LINC00460 in the functional analysis of ccRCC tissue using the co-expression analysis and identification of DEGs and further followed by the GO and KEGG pathway enrichment analysis. Also, we used GSE to study the role of LINC00460 in ccRCC. However, our research still has some limitations. First, the analytical results came from a small sample cohort, thus a larger sample cohort was needed for verifying our results. Second, our TCGA-derived analytical cohort lacked comprehensive clinical data, potentially limiting analytical scope. Third, we should still do more in vivo and in vitro experimental verification in this study to verify the correctness of the results enriched in this study. One way to search for the function of molecules in this article is a gene function enrichment method and GSEA method. Our research used a variety of bioinformatics analysis methods with genome-wide RNA sequencing data set, and comprehensively analyzed the clinical significance of LINC00460 in ccRCC, identified targeted drugs and explored its potential mechanisms. Our study also explored the prognostic value of LINC00460 in ccRCC, which provided a potential theoretical idea for the clinical strategy for ccRCC.

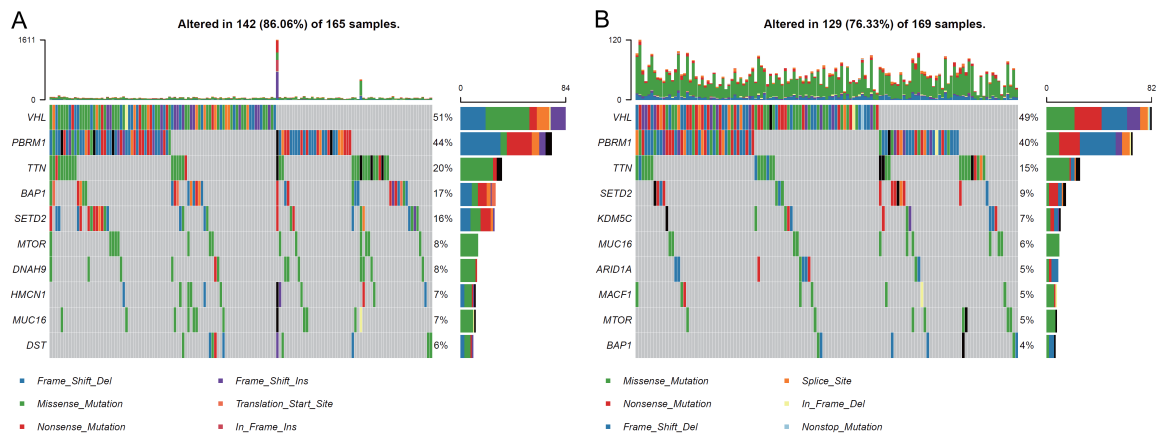


Figure 7. Mutation analysis of ccRCC samples stratified by LINC00460 expression levels. (A, B) Oncoprint waterfall plots displaying the mutational landscape of *BAP1* (A) and *LRP2* (B). Samples with high LINC00460 expression exhibited a higher mutation frequency in these genes compared to the low-expression group. Differences in mutation frequencies were analyzed using Fisher's exact test.

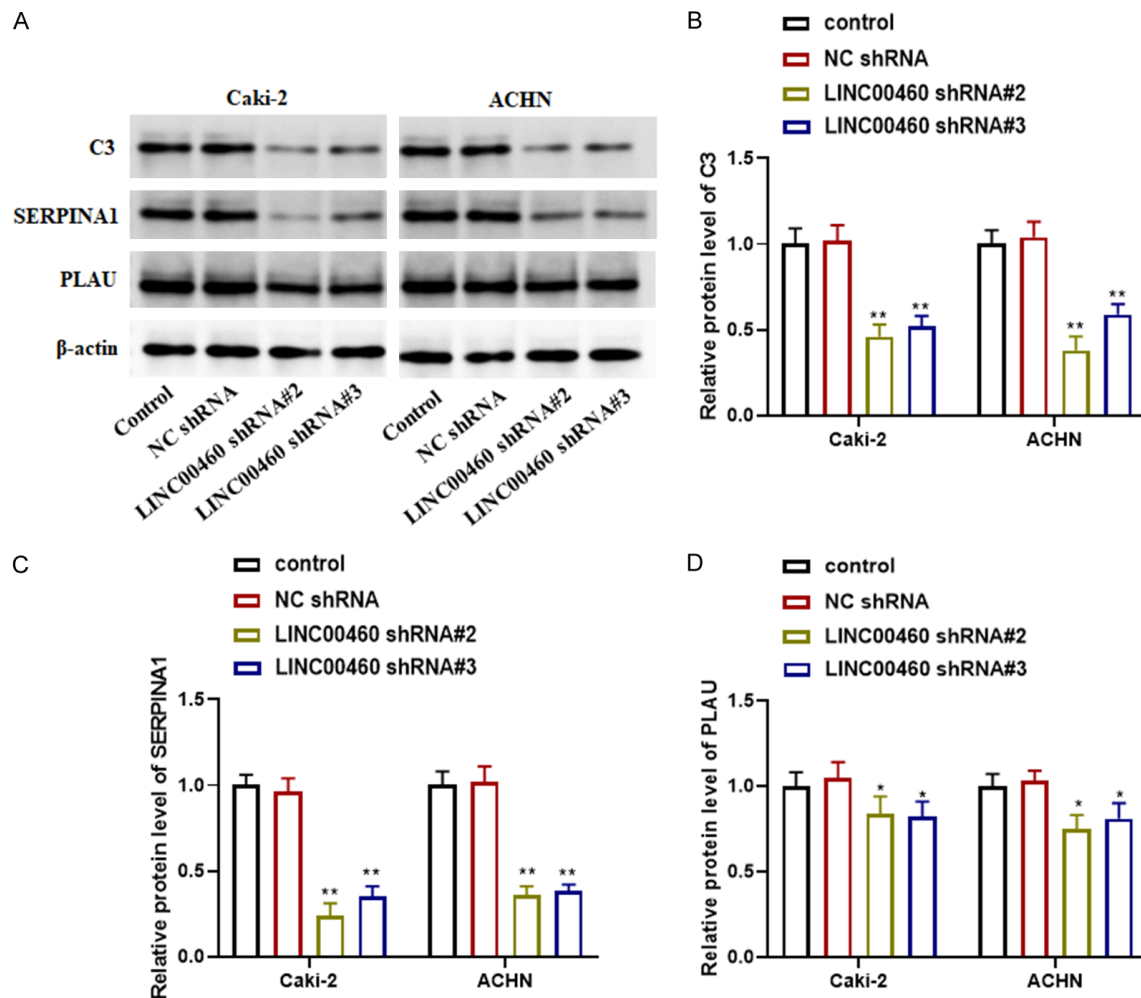


Figure 8. LINC00460 knockdown downregulates key components of the complement and coagulation cascades pathway. (A) Representative Western blot images showing protein levels of C3, SERPINA1, and PLAU in Caki-2 and ACHN cells transfected with Control, NC shRNA, shLINC00460 #2, or shLINC00460 #3. β -actin served as the load-

ing control. (B-D) Quantitative densitometric analysis of C3 (B), SERPINA1 (C), and PLAU (D) protein expression normalized to β -actin. Data are presented as mean $\pm$ SEM ($n = 3$). Statistical methods: Comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC shRNA.

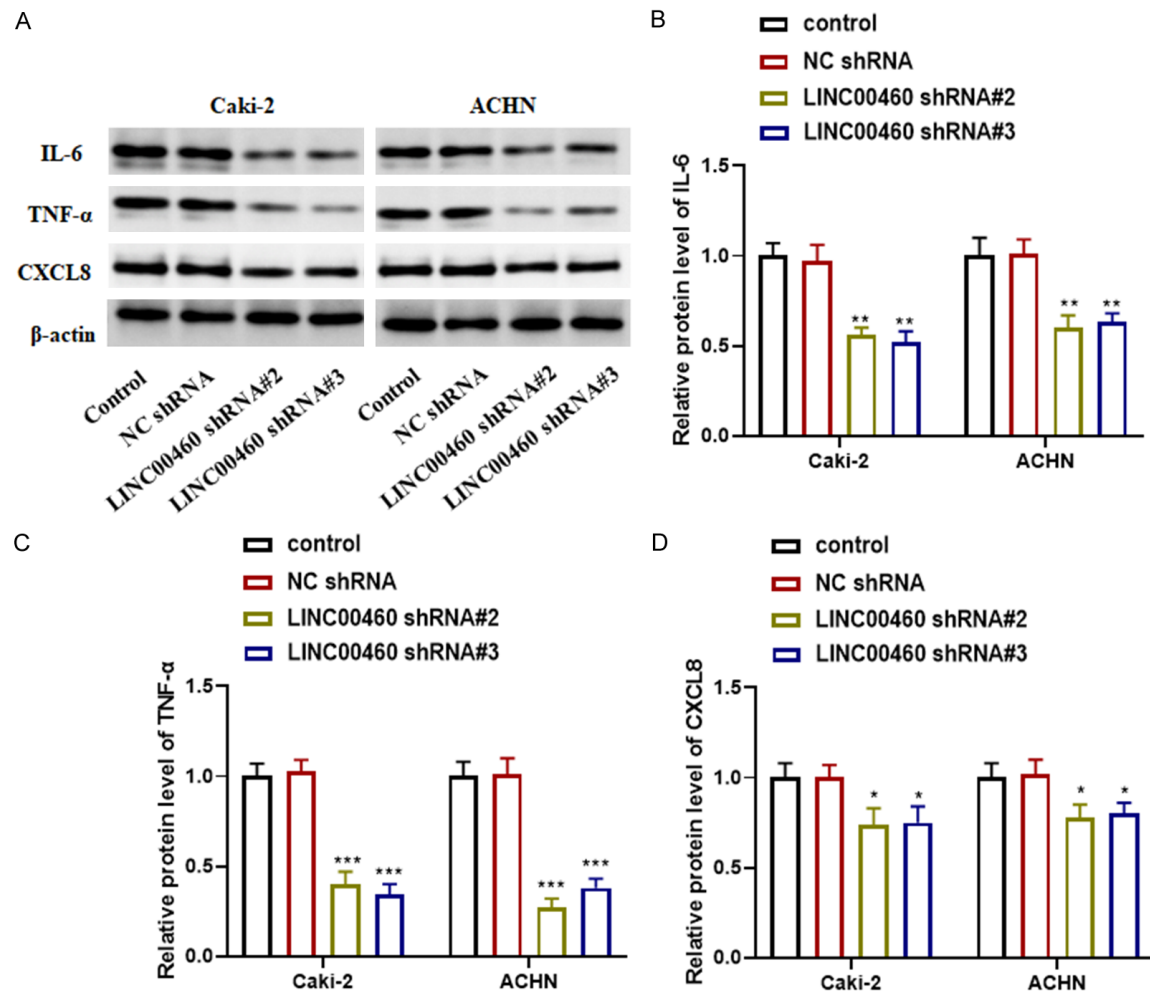


Figure 9. LINC00460 regulates the cytokine-cytokine receptor interaction pathway. (A) Representative Western blot images detecting the expression of pro-inflammatory cytokines IL-6, TNF- α , and CXCL8 in Caki-2 and ACHN cells after LINC00460 knockdown (Control, NC shRNA, shLINC00460 #2, shLINC00460 #3). β -actin was used as the loading control. (B-D) Quantitative analysis of IL-6 (B), TNF- α (C), and CXCL8 (D) protein levels normalized to β -actin. LINC00460 silencing significantly reduced the expression of these tumor-promoting cytokines. Data are presented as mean $\pm$ SEM ($n = 3$). Comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC shRNA.

Conclusion

In conclusion, this paper found that LINC00460 is a good biomarker and illness forecaster for ccRCC. High expression is associated with bad survival and the enrichment is found in the immune and inflammatory gene set as well as part of the immune system like the complement/coagulation cascade, and activity of cyto-

kine signaling path from p53. As a proof, we show in experiments functional improvement after knock down of CCRC cell with LINC00460, grow ability, migrative and invasive were suppressed but pro death process was increased, inflammatory/coagulation factors were suppressed and p53 was activated. In summary, LINC00460 can promote the appearance of ccRCC and it is a possible treatment target.

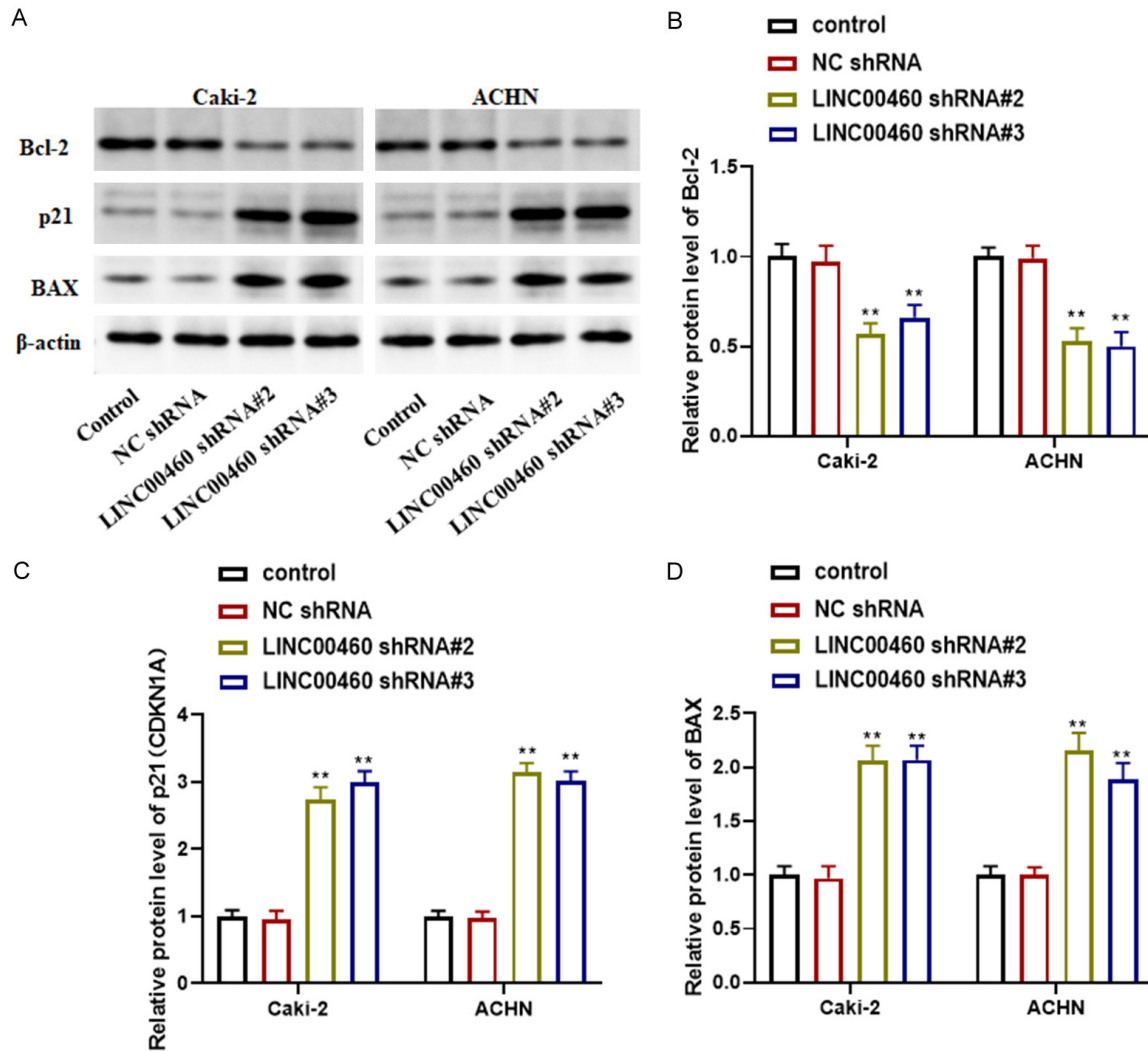


Figure 10. LINC00460 suppresses p53 signaling activity in ccRCC cells. (A) Representative Western blot images analyzing key effectors of the p53 pathway: Bcl-2 (anti-apoptotic), p21 (CDKN1A, cell cycle inhibitor), and BAX (pro-apoptotic) in Caki-2 and ACHN cells with or without LINC00460 knockdown. β -actin served as the loading control. (B-D) Quantitative densitometry of Bcl-2 (B), p21 (C), and BAX (D) expression levels. Silencing LINC00460 resulted in decreased Bcl-2 and increased p21 and BAX expression, indicating the activation of p53-mediated tumor-suppressive responses. Data are presented as mean $\pm$ SEM ($n = 3$). Comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC shRNA.

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Disclosure of conflict of interest

None.

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