

β -Catenin promotes cell proliferation, migration, and invasion but induces apoptosis in renal cell carcinoma

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Abstract: β -Catenin (*CTNNB1* gene coding protein) is a component of the Wnt signaling pathway that has been shown to play an important role in the formation of certain cancers. Abnormal accumulation of *CTNNB1* contributes to most cancers. This research studied the involvement of β -catenin in renal cell carcinoma (RCC) cell proliferation, apoptosis, migration, and invasion. Proliferation, cell cycle, and apoptosis were analyzed by using Cell Counting Kit-8 and by flow cytometry. Migration and invasion assays were measured by transwell analysis. Real-time polymerase chain reaction and Western blot analysis were used to detect the expression of *CTNNB1*, ICAM-1, VCAM-1, CXCR4, and CCL18 in RCC cell lines. It was found that *CTNNB1* knockdown inhibited cell proliferation, migration, and invasion and induced apoptosis of A-498 cells. *CTNNB1* overexpression promoted cell proliferation, migration, and invasion and inhibited apoptosis of 786-O cells. Moreover, knockdown of *CTNNB1* decreased the levels of ICAM-1, VCAM-1, CXCR4, and CCL18 expression, but *CTNNB1* overexpression increased the expression of ICAM-1, VCAM-1, CXCR4, and CCL18. Further in vivo tumor formation study in nude mice indicated that inhibition of *CTNNB1* delayed the progress of tumor formation through inhibiting PCNA and Ki67 expression. These results indicate that *CTNNB1* could act as an oncogene and may serve as a promising therapeutic strategy for RCC.

Keywords: kidney cancer, oncogene, β -catenin, survival time, tumor migration-related protein

Introduction

Renal cell carcinoma (RCC), the most common type of adult kidney malignancy, accounts for 80% of all primary renal neoplasms and is composed of 2%–3% of all malignancies. The occurrence of RCC has increased in recent years; worldwide, ~100,000 patients die of disease every year.¹ More than one-third of RCC patients present at advanced stages, and nearly half of the patients who experience curative therapies such as surgical resection suffer recurrence in the subsequent follow-up.^{2,3} Although the rate of detection of incidental RCC has increased with improved diagnostic techniques, metastatic lesions are still found at diagnosis in 30% of patients with RCC.⁴ Therefore, it is essential to find the molecular mechanism of tumor cell metastasis in primary lesions, which can improve the treatment and prognosis.

The canonical Wnt/ β -catenin signaling pathway plays an important role in the embryonic development and cellular homeostasis and modulates cell growth and self-renewal in stem cells and progenitor cells.^{5,6} In the presence of Wnt signaling, β -catenin is accumulated in the cytosol, translocated into the nucleus, and forms a complex with transcription factors to activate target genes.^{7,8} Dysregulated β -catenin signaling is a common feature of many malignant tumors, including prostate, melanoma,

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myeloma, liver, colon, breast, and lung cancer.^{7,9-14} Activation of *CTNNB1* promotes proliferation, migration, and invasion in glioma¹⁵ and oral squamous carcinoma.¹⁶

This study investigates the role of *CTNNB1* in the pathobiology of RCC and shows that downregulation of this protein can effectively reduce proliferation, migration, and invasion of RCC cells. Taken together, these findings provide the evidence linking *CTNNB1* signaling to tumor progression in human RCC.

Materials and methods

Cell lines

Normal kidney cell line (HK-2) and RCC cell lines (A-498, GRC-1, 786-O, and ACHN) were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco minimum essential medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) with the supplements of 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies), 100× penicillin-streptomycin solution (Invitrogen Life Technologies), and incubated in an incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) set to 37°C, 100% humidity, and 5% CO₂.

Construction and infection

Oligonucleotide encoding shRNA-targeted human *CTNNB1* mRNA and a scramble shRNA were both obtained from Sangon Biotech Inc. (Beijing, China). *CTNNB1* cassette was amplified from the expression vector pMD18-T-CTNNB1 (Sangon Biotech Inc.). The recombinant pLKO.1-EGFP-CTNNB1-shRNA vector expressing CTNNB1-shRNA and pLKO.1-EGFP-CTNNB1 vector expressing *CTNNB1* were digested with restriction enzymes, and the fragment containing CTNNB1-shRNA and *CTNNB1* cassette was harvested from gel extraction by agarose gel electrophoresis. The infection of A498, 786-O, and ACHN cell lines by using of lentiviral expressing CTNNB1-shRNA and *CTNNB1* were prepared as previously described.¹⁷ The recombinant pLKO.1-EGFP-scramble shRNA (NC-shRNA) and black pLKO.1-EGFP (NC-vector) were used as the negative control.

Cell proliferation assay

A498, 786-O, and ACHN cells were seeded in a 96-well plate (1×10⁵ cells/well) and cultured at 37°C for indicated times. Cell proliferation was detected by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan). After cells were infected 0, 12, 48, and 72 h, CCK-8 reagent was added to each group of wells at 1:10 (v/v) per 100 µL medium and incubated for 1 h at 37°C. Measurement was

carried out in absorbance at 450 nm with a microplate reader according to the manufacturer's instruction.

Flow cytometry assay

For cell cycle assay, A498, 786-O, and ACHN cells were collected and fixed with ice-cold ethanol overnight. Then the cells were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS containing 500 ng/µL RNase A and incubated at 37°C for 30 min. All the samples were analyzed through flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) with the staining of propidium iodide (PI) and 0.1% TritonX-100. For cell apoptosis assay, A498 and 786-O cells were collected and washed twice with ice-cold PBS. And then 195 µL Annexin V-APC-binding buffer was used to resuspend the cells, followed by the addition of 5 µL Annexin V-FITC in each tube for 20 min, and 5 µL PI was added for another 10 min to incubate on ice. Finally, each sample was resuspended to a volume of 500 µL with 1× binding buffer and was analyzed through flow cytometry within 30 min.

In vitro transwell assay

For cell invasion, Transwell chamber (BD Biosciences) coated with Matrigel (BD Biosciences) was performed as described in the manufacturer's protocol. After infection for 48 h, 1×10⁵ cells in 500 µL serum-free DMEM were seeded into the upper well of the chamber. The lower chamber was filled with 750 µL DMEM containing 10% fetal bovine serum (FBS). Cells were incubated at 37°C for 48 h. Later, the cells that were able to pass through the filter were fixed with 4% paraformaldehyde and stained by crystal violet solution for 30 min. Cells on the top surface of the insert were removed with a cotton swab and counted under a microscope in five fields (×200). Similar procedure of cell invasion was also performed for cell migration transwell assay, except that the transwell membranes were not precoated with Matrigel.

Reverse transcription and real-time polymerase chain reaction (PCR)

Total RNA was extracted from normal kidney cells and four kidney cancer cell lines using Trizol reagent (Life Technologies) and detected by agarose gel electrophoresis according to the manufacturer's instructions. As the template for PCRs, cDNA was synthesized from 5 µg of RNA using AMV reverse transcriptase (Fermentas, Waltham, MA, USA). Real-time quantitative PCR was performed using SYBR® Green 10× Supermix (Takara, Tokyo, Japan) in a total volume of 25 µL and on Roche Light Cycler® 480II

System (Roche Diagnostics Ltd., Basel, Switzerland). Primer sequences were listed as follows: *CTNNB1*, forward: 5'-ATGACTCGAGCTCAGAGGGT-3' and reverse: 5'-ATTGCACGTGTGGCAAGTTC-3'; *GAPDH*, forward: 5'-CTCCTCCTGGCCTCGCTGT-3' and reverse: 5'-GCTGTACCTTCACCGTTCC-3'; *CXCR4*, forward: 5'-GCTGTTGGCTGAAAAGGTGG-3' and reverse: 5'-ATCTGCCTCACTGACGTTGG-3'; *CCL18*, forward: 5'-ATTCTGAAACCAGCCCCCAG-3' and reverse: 5'-CCCTCAGGCATTGAGCTTCA-3'; *ICAM-1*, forward: 5'-CCCTGGAGCCCTTGAAGAAG-3' and reverse: 5'-AGATGACAACCTTCTCGGCA-3'; *VCAM-1*, forward: 5'-CAGGACCACGAGAAGCTGTT-3' and reverse: 5'-GATCTCCTTGGCATCCTCGG-3'. *GAPDH* was used as control of the input RNA level. The gene expression was calculated using the $\Delta\Delta C_t$ method.

Western blot

Total protein was extracted from cells using ice-cold radio immunoprecipitation assay buffer (RIPA; Beyotime Institute of Biotechnology, Shanghai, China) containing 0.01% protease and phosphatase inhibitor (Sigma, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged 12,000× g at 4°C for 10 min and the proteins in supernatant were separated by 10%–15% SDS gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Shanghai, China), followed by blocking with 5% BSA in Tris-buffered saline with Tween 20 (TBST) (Amresco, Solon, OH, USA) and incubating with rabbit monoclonal antibodies anti- β -catenin (cat. no. ab6302; 1:4,000), anti-CXCR4 (cat. no. ab13854; 1:500), anti-CCL18 (cat. no. ab104867; 1:1,000), anti-GAPDH (cat. no. 5174; 1:1,500); mouse polyclonal antibody anti-ICAM-1 (cat. no. ab20; 1:500); rabbit polyclonal antibody anti-VCAM-1 (cat. no. ab174279; 1:5,000). All the primary antibodies were purchased from Abcam (Cambridge, MA, USA) except for anti-GAPDH (Cell Signaling Technology, Inc., Danvers, MA, USA). Blots were then incubated for 1 h at 37°C with goat anti-mouse or anti-rabbit secondary antibody (cat. nos. A0208 and A0216; 1:1,000; Beyotime Institute of Biotechnology), and intensities were measured using enhanced chemiluminescence (Thermo Fisher Scientific). GAPDH antibody was used as an internal control. The blotting bands were quantified with ImageJ software.

In vivo experiments

For tumor growth assay, A-498 cells infected with NC-shRNA or CTNNB1-shRNA were trypsinized, washed, and

resuspended in DMEM without FBS. Twelve male athymic nude mice (SLAC laboratory animal Center, Shanghai, China) were randomly divided into 2 groups (6 mice/group), and 2×10^6 A-498 cells were subcutaneously injected into the right armpit of the mice. The tumor size was determined every 3–4 days after the tumor is formed (around 1–2 weeks). At 45 days after injection, the mice were euthanized and the excised tumor tissues were formalin-fixed, paraffin-embedded, sectioned, and then analyzed with β -catenin, PCNA, or Ki67 immunohistochemistry, and the tumors were weighted on a digital balance. All animal-handling procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Ninth Edition, 2010). The animal use protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital, China Medical University (Shenyang, China).

Immunohistochemistry

Tissue sections were initial treatment for deparaffinization and hydration and then heated in EDTA (pH 8.0) and antigen-retrieved in 10 mm citrate buffer for 5 min at 100°C. The reaction of β -catenin, PCNA, or Ki67 antibody (Abcam) took place for 1 h at room temperature, followed by incubation by biotin-labeled secondary antibodies, and the slides were then developed using 3,3'-diaminobenzidine (DAB) solution and counterstained with hematoxylin staining (BASO, Shanghai, China). Immunohistochemical signals were calculated with the positive staining cells under a microscope (Olympus Corporation) with a magnification of $\times 200$.

Statistical analysis

Results were shown as mean \pm standard deviation. Data were analyzed by using the unpaired, two-tailed Student's *t*-test with Graphpad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). All assays were performed in triplicate and repeated at least three times. All statistical analyses were two-sided and differences below $P < 0.05$ were considered statistically significant.

Results

Upregulation of CTNNB1 associates with poor survival of RCC

To study the biological role of CTNNB1 in RCC tumor tissues, real-time PCR was first used to detect the expression levels of CTNNB1 in 90 clear cell RCC patient tissues and their adjacent normal tissues. As shown in Figure 1A, CTNNB1 expression level was higher in clear cell RCC tissues compared with

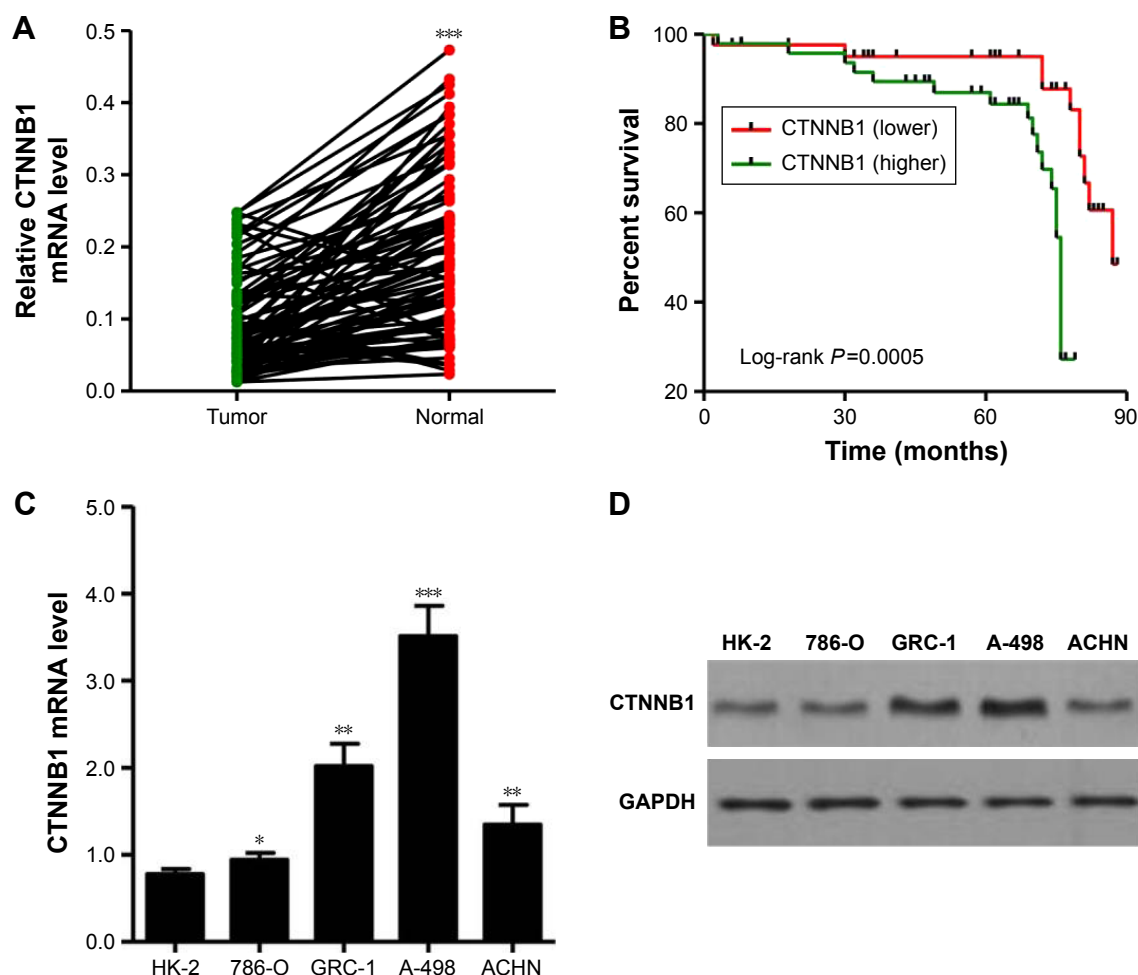


Figure 1 Correlation between *CTNNB1* expression and survival time of patients with clear cell RCC. **(A)** The expression level of *CTNNB1* was detected by real-time PCR in 90 clear cell RCC tissues and their adjacent normal tissues. **(B)** The survival time of 90 clear cell RCC patients showed that a higher *CTNNB1* expression level was correlated with poor survival. **(C)** mRNA expressions of *CTNNB1* and *GAPDH* were determined by real-time PCR using a *CTNNB1*-specific primer set. **(D)** β -catenin protein expression was analyzed by Western blot using anti-*CTNNB1* antibody. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with tumor tissues.

Abbreviations: RCC, renal cell carcinoma; PCR, polymerase chain reaction.

adjacent normal tissue control. Then, the correlation between *CTNNB1* expression and clinical pathological features of the patients with clear cell RCC was investigated. Examination of the correlation between *CTNNB1* expression and clinical pathological features showed that increased *CTNNB1* expression was correlated with advanced stage, metastases, mutant von Hippel-Lindau (VHL) tumor suppressor, and poor survival (Table 1 and Figure 1B).

CTNNB1 is overexpressed in RCC cell lines

mRNA and protein expressions of *CTNNB1* were examined in a normal kidney cell (HK-2) and four RCC cell lines (A-498, GRC-1, 786-O, and ACHN). Real-time PCR and Western blot assay observed that four RCC cell lines showed higher mRNA and protein levels of *CTNNB1* compared with normal HK-2 cells (Figure 1C and D). Among that, A-498 cells are with the highest mRNA and protein levels

Table 1 Relationship between *CTNNB1* and clinical characteristics of clear cell RCC patients

Factor	Characteristic	<i>CTNNB1</i> expression (N)		P-values
		Lower	Higher	
Gender	Male	20	25	1.241
	Female	19	26	
Age (years)	<59	24	30	0.877
	≥ 59	16	20	
Stage	I	6	4	0.0129*
	II	12	19	
	III	19	30	
Metastases	Yes	10	23	0.0018**
	No	31	26	
Grade	I	7	3	0.216
	2	33	22	
	3	3	18	
	4	2	2	
VHL	Wild	23	15	0.0016**
	Mutant	16	36	

Notes: * $P<0.05$, ** $P<0.01$, chi-square test.

Abbreviations: RCC, renal cell carcinoma; VHL, von Hippel-Lindau.

of *CTNNB1* and 786-O cells are with the lowest mRNA and protein levels of *CTNNB1*, compared with three other RCC cells. These two cell lines were therefore used for subsequent experiments.

Effect of *CTNNB1* on A-498 and 786-O cell proliferation

To explore the biological significance of *CTNNB1* in the tumorigenesis of RCC, *CTNNB1* knockdown in A-498 cells and *CTNNB1* overexpression in 786-O cells were introduced, respectively. Real-time PCR and Western blot assay at 48 h after infection showed that *CTNNB1* knockdown in A-498 cells significantly decreased the mRNA and protein levels of *CTNNB1* (Figure 2A and B), whereas overexpression

of *CTNNB1* in 786-O (Figure 2C and D) and ACHN cells (Figure S1A and B) significantly increased the mRNA and protein levels of *CTNNB1*. Moreover, immunohistochemistry staining was performed at 48 h after treatment with pLKO.1-EGFP-*CTNNB1* in ACHN cells. We found that *CTNNB1* translocated from the cytoplasm in the ACHN cells infected with the negative control vector to the nucleus in ACHN cells infected with pLKO.1-EGFP-*CTNNB1* (Figure S2). The cell proliferation of A-498 and 786-O cells was assessed using CCK-8 assay. In A-498 cells with pLKO.1-EGFP-*CTNNB1*-shRNA infection, cell proliferation was significantly decreased by 24.8%, 29.7%, and 28.2% at 12, 24, and 48 h, respectively (Figure 2E). In 786-O and ACHN cells with pLKO.1-EGFP-*CTNNB1* infection, cell

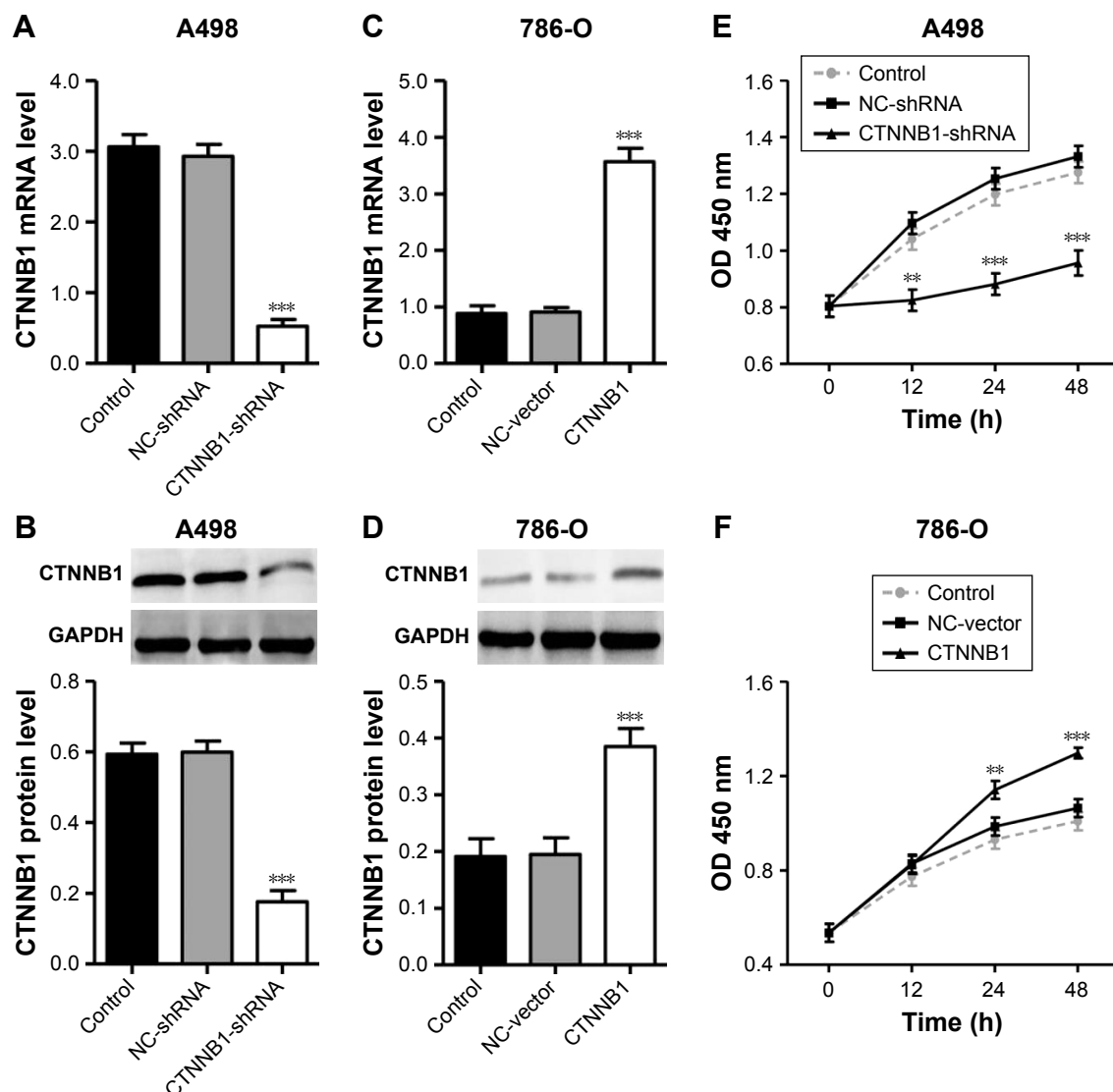


Figure 2 Effect of *CTNNB1* on cell proliferation. After treatment of A498 (A and B) and 786-O (C and D) cells with pLKO.1-EGFP-*CTNNB1*-shRNA vector (CTNNB1-shRNA) or pLKO.1-EGFP-*CTNNB1* vector (CTNNB1), expression of *CTNNB1* assay was analyzed by real-time PCR and Western blot, and cell proliferation (E and F) was performed, as described in "Materials and Methods." Representative pictures of three independent studies are shown. Data are presented as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$ compared with control groups.

Abbreviations: PCR, polymerase chain reaction; SD, standard deviation; OD, optical density; NC, negative control.

proliferation was significantly increased by 22.7% and 11.6% at 24 h, and 22.0% and 13.3% at 48 h, respectively (Figures 2F and S1C).

Effect of *CTNNB1* on A-498 and 786-O cell cycle and apoptosis

In order to further investigate the role of *CTNNB1* in tumor cell growth, the cell cycle of A-498 and 786-O was measured

using flow cytometry. Our data indicated that the cell number of G0/G1 phase was notably increased in *CTNNB1*-silenced A-498 cells, accompanied with decreased cell number of G2/M phase (Figure 3A). However, the cell numbers of G0/G1 and G2/M phases in *CTNNB1*-overexpressed 786-O and ACHN cells were against that in *CTNNB1*-silenced A-498 cells (Figures 3B and S1D). Negative control vector-infected A-498 (NC-shRNA), 786-O cells (NC-vector), and ACHN

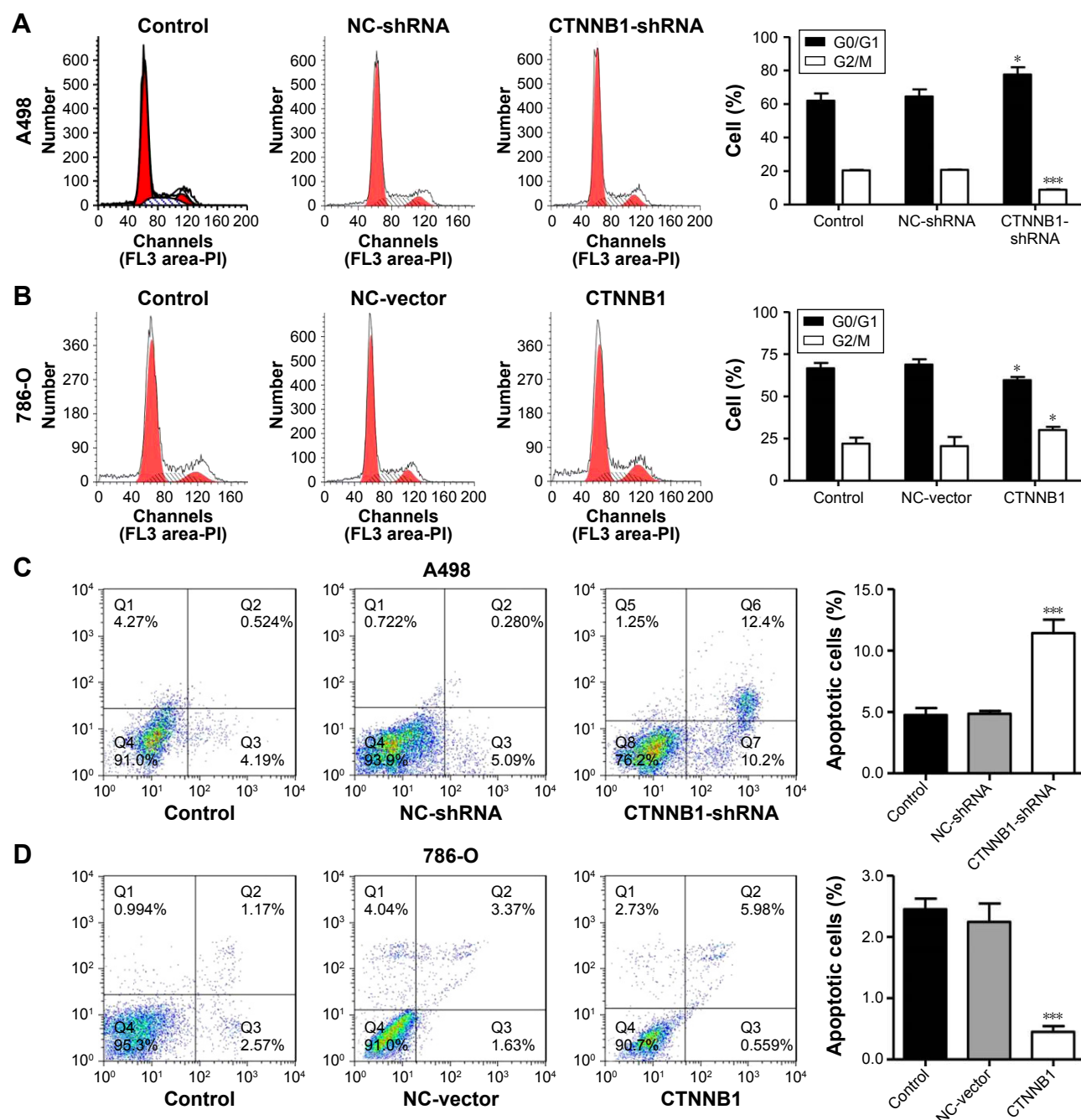


Figure 3 Effect of *CTNNB1* on cell cycle and apoptosis. After treatment of A498 and 786-O cells with pLKO.1-EGFP-*CTNNB1*-shRNA vector (*CTNNB1*-shRNA) or pLKO.1-EGFP-*CTNNB1* vector (*CTNNB1*), cell cycle (**A** and **B**) and cell apoptosis (**C** and **D**) were performed, as described in "Materials and methods." Representative pictures of three independent studies are shown. Data are presented as mean \pm SD. * $P < 0.05$, *** $P < 0.001$ compared with control groups.

Abbreviations: SD, standard deviation; NC, negative control.

(NC-vector) had no cell cycle changes in G0/G1, S, and G2/M phases compared with corresponding control cells.

To figure out the effects of *CTNNB1* on cell apoptosis of A-498 and 786-O cells, an Annexin V-FITC/PI staining and flow cytometry assay were performed. As shown in Figure 3C, it was revealed that *CTNNB1* downregulation in A-498 cells significantly increased cell apoptosis by 2-fold compared with control cells. While, *CTNNB1* upregulation in 786-O and ACHN cells showed a significant decrease in apoptotic rate by 78.2% and 82.8%, respectively (Figures 3D and S1E) compared with control cells. Taken together, these results demonstrate that *CTNNB1* promotes cell proliferation and suppresses apoptosis of RCC cells in vitro.

Effect of *CTNNB1* on A-498 and 786-O cell migration and invasion

Evidences have shown that decrease in cell-cell and/or cell-matrix adhesion correlates with tumor invasion and metastasis. To investigate the effect of *CTNNB1* in migration and invasion, A-498 and 786-O cells were cultured in a Boyden chamber. After 48 h of incubation, knockdown of *CTNNB1* in A-498 cells significantly reduced the cell migration by 60.7% compared with control cells (Figure 4A). Meanwhile, *CTNNB1* upregulation in 786-O and ACHN cells observed significant increase in cell migration by 45.3% and 68.7% compared with control cells (Figures 4B and S1F). As shown in Figure 4C, the invasion assay found that *CTNNB1* downregulation in A-498 cells also markedly suppressed cell invasion by 63.9% compared with control cells. While, *CTNNB1* upregulation in 786-O and ACHN cells observed significant increase in cell invasion by 49.3% and 92.6% compared with control cells (Figure 4D and S1G).

Effect of *CTNNB1* on CXCR4, CCL18, ICAM-1, and VCAM-1 expression

To further explore the mechanism of *CTNNB1* regulating cell migration and invasion of kidney cancer cell lines, two cell migration molecular markers, ICAM-1 and VCAM-1, and cytokines CXCR4 and CCL18, were predicated to be *CTNNB1* targets. As shown in Figure 5A and B, the mRNA and protein levels of ICAM-1, VCAM-1, CXCR4, and CCL18 in A-498 cells were remarkably suppressed by *CTNNB1* shRNA compared with control cells. However, the mRNA and protein levels of ICAM-1, VCAM-1, CXCR4, and CCL18 in 786-O cells were remarkably increased by *CTNNB1* overexpression compared with control cells (Figure 5C and D).

Effect of *CTNNB1* on tumor growth of A-498 cells in nude mice

To further examine the effects of *CTNNB1* on tumor growth in vivo, A-498 cells infected with NC-shRNA or *CTNNB1*-shRNA were subcutaneously injected into the right armpit of nude mice. High expression of β -catenin (Figure 6A and D), PCNA (Figure 6B and D), and Ki67 (Figure 6C and D) was found in the xenograft from the nude mice with NC-shRNA cell injection, but their expressions decreased by 78.3%, 68.6%, and 77.2% in that injected with *CTNNB1*-shRNA cells, respectively. Moreover, *CTNNB1*-shRNA tumors grew slower in mice, whereas NC-shRNA tumors grew fast in mice (Figure 6E). Tumor weights in *CTNNB1*-shRNA mice were notably decreased to 31.1% 45 days after injection compared with NC-shRNA group (Figure 6F). These data suggest that knockdown of *CTNNB1* inhibits tumor growth in nude mice.

Discussion

In the last few decades, the incidence of RCC was increased and more than half of the patients whose survival time was poor had clinical limitations.^{2,18} Numerous candidate RCC biomarkers have been identified to date, while neither the expression pattern of these genes nor its localization and function in the tumor tissues has been investigated. At this point, identifying specific genetic markers associated with tumor metastasis may be useful to evaluate the disease progression. This study shows that *CTNNB1* is overexpressed in RCC cell lines compared with renal tubular epithelial cell line HK-2 and is essential survival factor for RCC cells. In addition, these data also showed that *CTNNB1* is a key factor associated with apoptosis, migration, and invasion of RCC cells. Lastly, it was indicated that *CTNNB1* downregulation inhibits xenograft tumor growth in vivo.

In several types of cancers, including RCC, an enhanced activation of the Wnt signaling pathway is a major etiological factor.¹⁹ Although the number of papers focused on the investigation of Wnt/ β -catenin pathway in renal carcinogenesis is small, the reported results are relevant.^{20,21} In the present study, *CTNNB1* expression was high in clear cell RCC patients and in those associated with advanced stage, metastases, VHL expression and prognosis. In line with our findings that the mutation of VHL gene is observed in ~60% of sporadic clear cell RCCs²² and loss of pVHL leads to combined de-repression of HIF- β -catenin with repercussions on Wnt/ β -catenin pathway,²³ suggesting VHL status could have an influence on β -catenin. Although the role of *CTNNB1* has been elucidated for

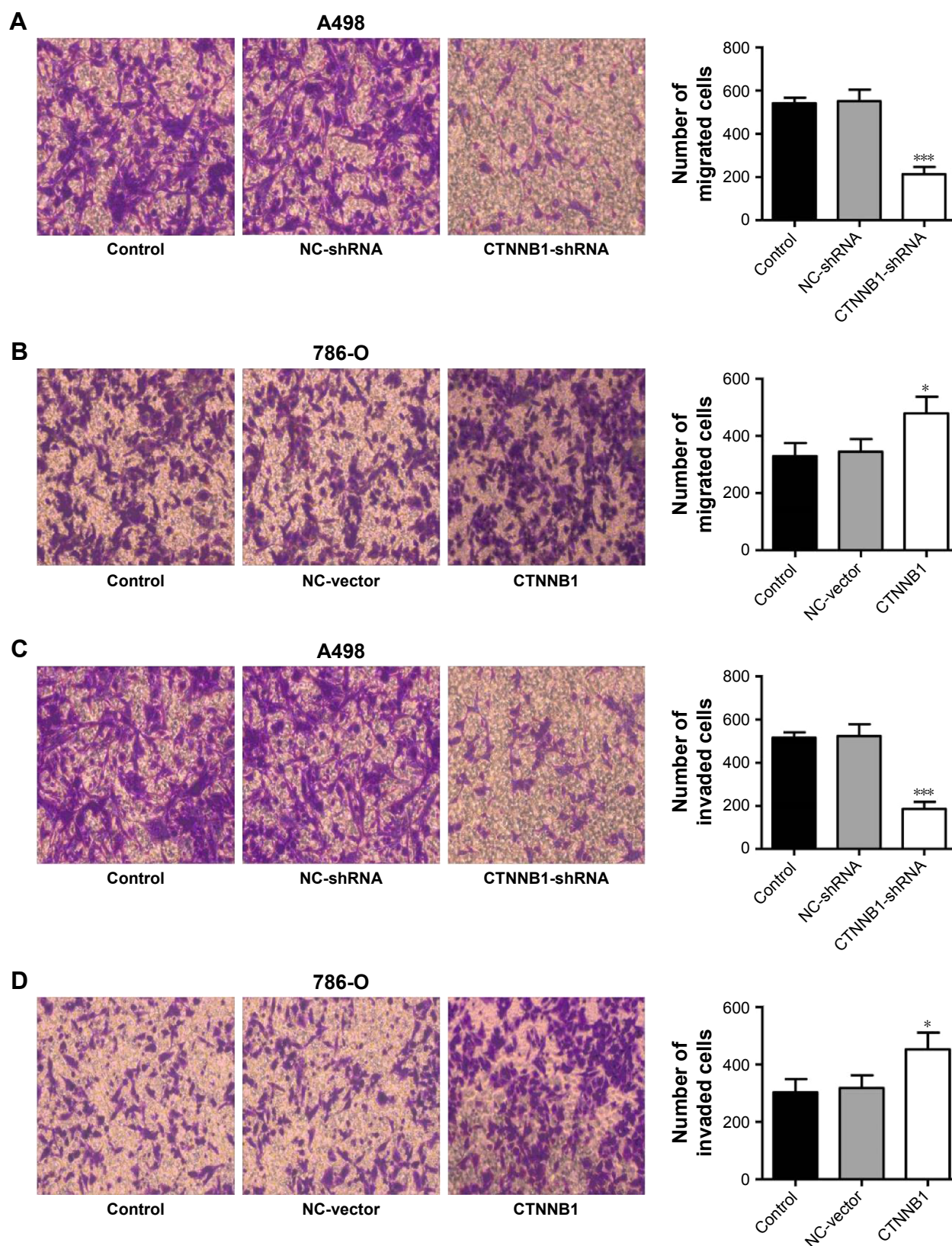


Figure 4 Effect of *CTNNB1* on cell migration and invasion. After treatment of A498 and 786-O cells with pLKO.1-EGFP-*CTNNB1*-shRNA vector (*CTNNB1*-shRNA) or pLKO.1-EGFP-*CTNNB1* vector (*CTNNB1*), cell migration (**A** and **B**) and invasion (**C** and **D**) were performed, as described in "Materials and methods." Representative pictures of three independent studies are shown. Magnification, $\times 200$. Data are presented as mean \pm SD. * $P < 0.05$, *** $P < 0.001$ compared with control group.

Abbreviations: SD, standard deviation; NC, negative control.

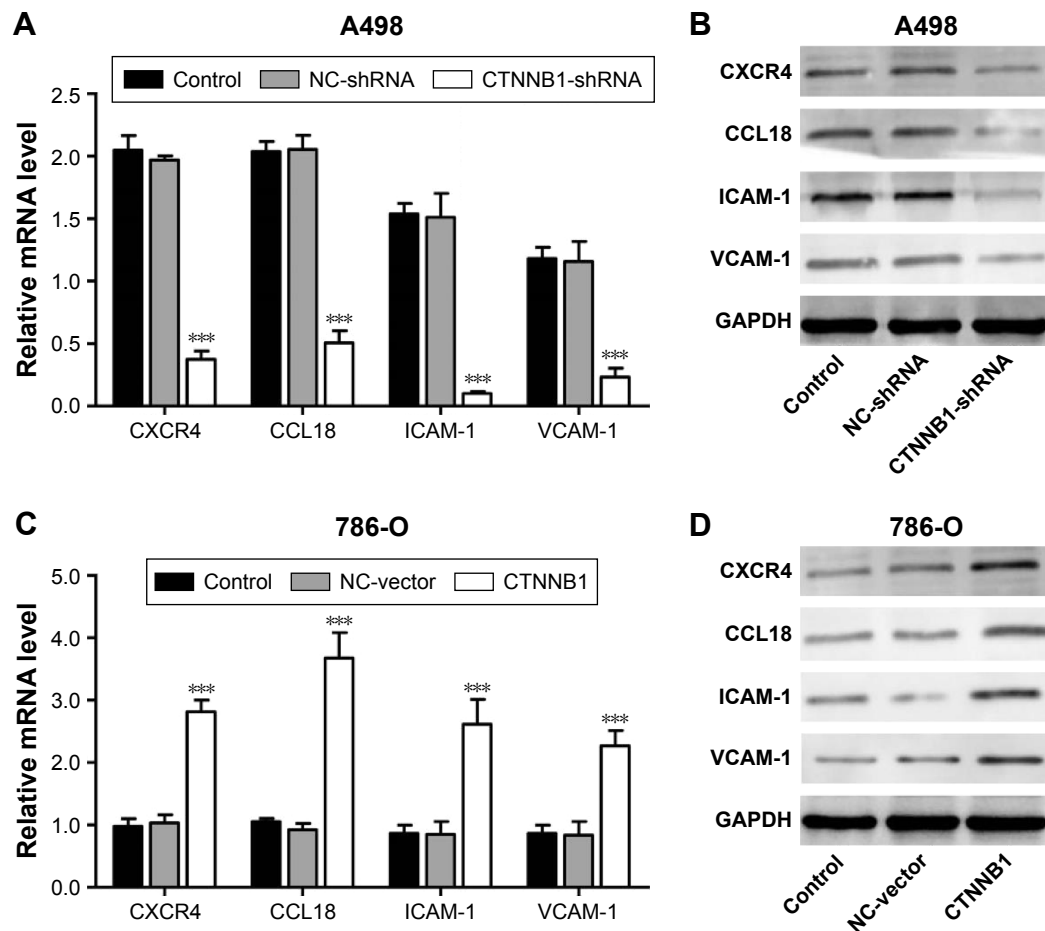


Figure 5 Effect of *CTNNB1* on protein expressions. After treatment of A498 (**A** and **B**) and 786-O (**C** and **D**) with pLKO.1-EGFP-*CTNNB1*-shRNA vector (*CTNNB1*-shRNA) or pLKO.1-EGFP-*CTNNB1* vector (*CTNNB1*), the expressions of CXCR4, CCL18, ICAM-1, and VCAM-1 were measured by real-time PCR and Western blot, as described in "Materials and methods." Representative pictures of three independent studies are shown. Data are presented as mean \pm SD. *** $P < 0.001$ compared with control groups.

Abbreviations: PCR, polymerase chain reaction; SD, standard deviation; NC, negative control.

many types of human cancer, its biological significance in RCC has not been examined in detail. *CTNNB1* is widely expressed in the entire renal cancer cell lines being tested, demonstrating an oncogene feature of *CTNNB1* in RCC. To test this hypothesis, the function of *CTNNB1* in the cell proliferation, cell cycle, apoptosis, cell migration and migration was assessed, using RCC cell lines infected with pLKO.1-EGFP-*CTNNB1*-shRNA vector expressing *CTNNB1*-shRNA and pLKO.1-EGFP-*CTNNB1* vector expressing *CTNNB1*, respectively. Our results showed that *CTNNB1* knockdown significantly inhibited cell proliferation in vitro. However, overexpression of *CTNNB1* promoted the proliferation of RCC cell lines significantly. The tumor growth in nude mice was also investigated using RCC cell lines stably infected with pLKO.1-EGFP-scramble shRNA or pLKO.1-EGFP-*CTNNB1*-shRNA. The inhibitory effect of *CTNNB1* knockdown on renal tumor growth

in mice was observed, which was evidenced by decreased expression of PCNA and Ki67. An increased nuclear translocation and activity of β -catenin was proposed to promote the proliferation of melanoma²⁴ and deteriorate the survival of melanoma patients.²⁵ In addition, our data showed G2/M phase blockage and increased apoptotic cells in *CTNNB1*-upregulated cells compared with empty vector cells. However, *CTNNB1* overexpression observed increased cell number of G0/G1 phase and decreased cell number of G2/M phase. Pu et al²⁶ reported that downregulation of *CTNNB1* by RNA interference in U251 human glioma cells suppressed cell proliferation and invasion and induced cell apoptosis. Our results are consistent with this study. It has also been reported that *CTNNB1* modulates early and late apoptotic progression in other cancer cells;^{27,28} hence, the present study did not conclusively prove the mechanisms of *CTNNB1* function in RCC cell lines because

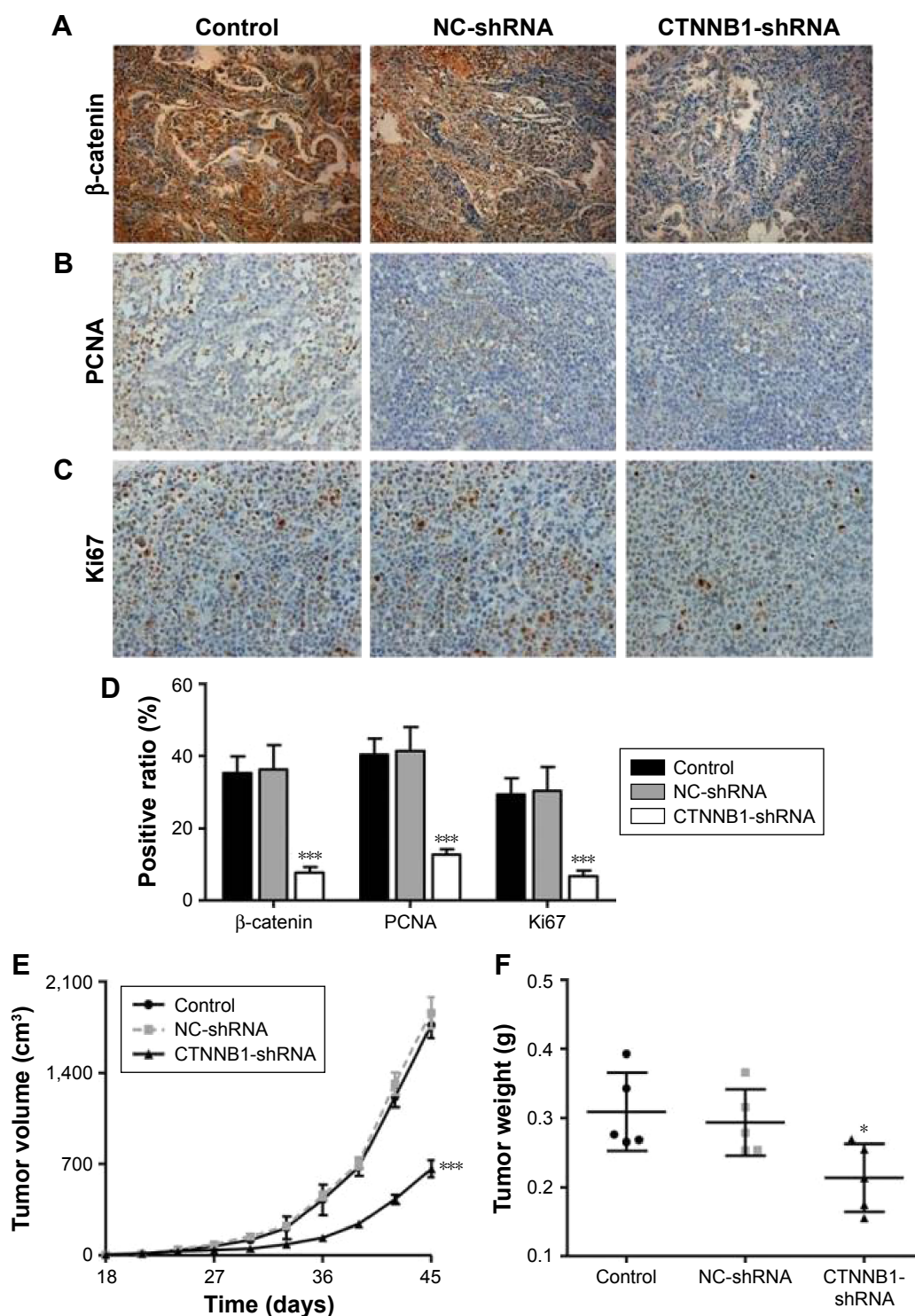


Figure 6 *CTNNB1* regulated tumor growth in vivo. Xenograft tumor from nude mice was injected with A498 cells stably expressing *CTNNB1*-shRNA or negative control (NC-shRNA) as described in "Materials and methods." Expressions of β -catenin (**A** and **D**), PCNA (**B** and **D**), and Ki67 (**C** and **D**) were determined by immunohistochemistry staining. Magnification, $\times 200$. (**E**) Growth curve of tumor volumes. (**F**) Tumor weights. Data are presented as mean \pm SD. * $P < 0.05$, *** $P < 0.001$ compared with control groups.

Abbreviations: SD, standard deviation; NC, negative control.

some additional mechanisms are possible, including direct target genes and regulation of other signaling pathways.

Cell mobility is an important characteristic of many physiologic and pathologic phenomena, such as development, wound repair, angiogenesis, and metastasis.¹ Downregulation

of *CTNNB1* significantly inhibited cell adhesion in invasive breast cancer, and metastatic disease is always accompanied with a higher activity of motility.²⁹ It is arguable that activation of *CTNNB1* is observed in migratory melanoma cells³⁰ and associated with transendothelial migration of

melanoma cells.³¹ Because the metastasis of cancer cells is dependent on the characteristics of migration and invasion, the relationship between the expression of *CTNNB1* and the invasion or migration ability of RCC cell lines was studied. Indeed, it is found that *CTNNB1* is essential for the migration and invasion of RCC cell lines. Knockdown of *CTNNB1* expression in RCC cell lines reduced cell migration and invasion significantly, whereas overexpression of *CTNNB1* increased cell migration and invasion. In order to determine the molecular mechanism of *CTNNB1* action in the migration and invasion, the expression of various migration- and invasion-related genes was evaluated. CXCR4, CCL18, ICAM-1, and VCAM-1 expressions were significantly downregulated in the *CTNNB1* knockdown cells. However, *CTNNB1* overexpression increased the expression of these genes at both mRNA and protein levels. These cytokine factors, including CXCR4, CCL18, ICAM-1, and VCAM-1, have been reported to play critical role in RCC cell invasion and migration.^{32–34} It has also been reported that CXCR4 signaling activates *CTNNB1* transcriptional activity in embryonic rat spinal cord neural progenitors, suggesting that CXCR4 signaling may interact with β -catenin to regulate the development of the central nervous system.³⁵ A previous study found that loss of *CTNNB1* affects VCAM-1 levels in multiple cell types.³⁶ However, ICAM-1 was not downregulated by *CTNNB1* expression in prostate cancer cells.³⁷ Moreover, wnt-signaling inhibitor ethacrynic acid (100 μ M) significantly decreased the expression of these proteins induced by *CTNNB1* overexpression in 786-O (Figure S3). The precise molecular mechanisms of *CTNNB1* regulating these gene expressions call for more research studies. However, our findings may be important in the studies of invasion and migration of RCC.

A limitation of the study is that *CTNNB1* expression in papillary RCC and other histologic subtypes are not being addressed, which should be investigated in the future. Understanding the mechanisms involved in oncogenesis has wide-ranging implications for targeting the treatment of cancer. This seems to be the first report showing *CTNNB1* involved in cell growth, apoptosis, migration, and invasion of RCC cell lines. Moreover, *CTNNB1* involved in tumor cell motility, and invasion could represent a molecular target for RCC and could eventually provide new leads for improved therapeutic approaches against RCC.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

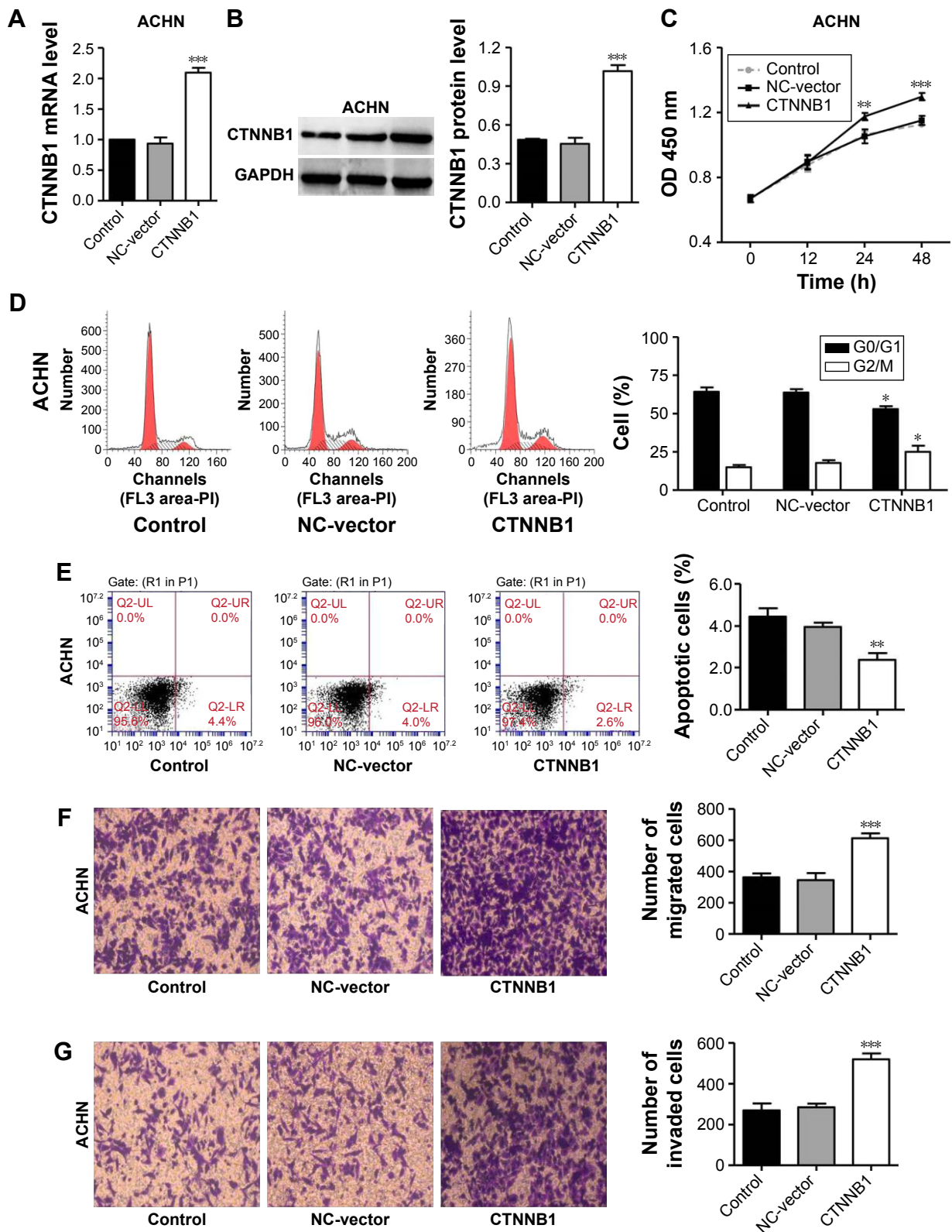


Figure S1 Effect of *CTNNB1* on proliferation, apoptosis, migration, and invasion of ACHN cells. After treatment of ACHN cells (**A** and **B**) with pLKO.1-EGFP-*CTNNB1* vector (*CTNNB1*), expression of *CTNNB1* assay was analyzed by real-time PCR and Western blot, and cell proliferation (**C**), cell cycle (**D**), apoptosis (**E**), migration (**F**), and invasion (**G**) were performed as described in "Materials and methods." Representative pictures of three independent studies are shown. Magnification, $\times 200$. Data are presented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control groups.

Abbreviations: PCR, polymerase chain reaction; SD, standard deviation; NC, negative control.

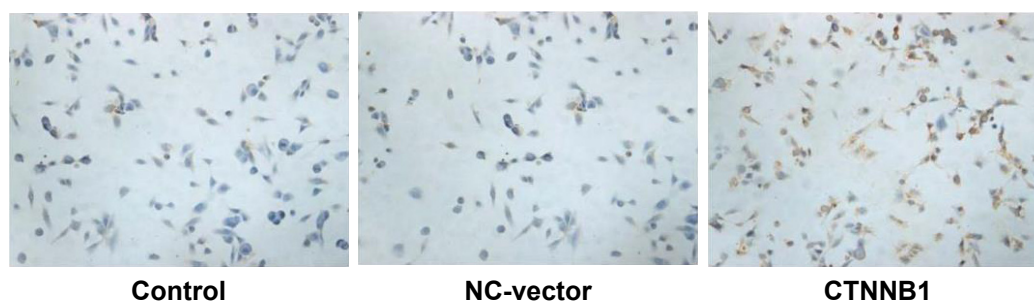


Figure S2 Intracellular distribution of β -catenin in ACHN cells after *CTNNB1* overexpression. Immunohistochemistry staining was performed at 48 h after treatment with pLKO.1-EGFP-*CTNNB1* vector (*CTNNB1*) or the negative control vector. Magnification, $\times 200$.

Abbreviation: NC, negative control.

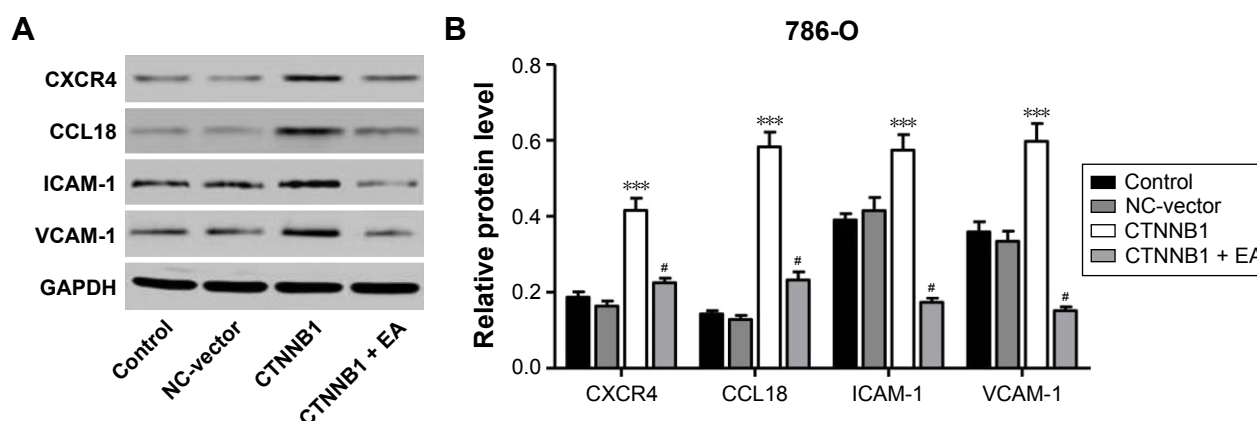


Figure S3 Ethacrynic acid (EA) inhibited *CTNNB1* overexpression-induced upregulation of CXCR4, CCL18, ICAM-1 and VCAM-1 in 786-O cells. Pretreatment of 786-O cells with wnt-signaling inhibitor EA (100 μ M) prior to pLKO.1-EGFP-*CTNNB1* vector (*CTNNB1*) infection, and the expressions of CXCR4, CCL18, ICAM-1, and VCAM-1 were measured by Western blot (A) and (B), as described in "Materials and methods." Representative pictures of three independent studies are shown. Data are presented as mean \pm SD. *** $P < 0.001$ compared with control groups. # $P < 0.001$ compared with *CTNNB1* overexpression groups.

Abbreviations: SD, standard deviation; NC, negative control.

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