

α -E-Catenin (CTNNA1) Inhibits Cell Proliferation, Invasion and EMT of Bladder Cancer

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Aim: Bladder cancer (BLCA) is an urogenital system tumor with a high morbidity. We aimed to explore the function and potential mechanism of α -E-catenin (CTNNA1) in BLCA.

Methods: The CTNNA1 expression in BLCA tissues was detected using qRT-PCR and immunohistochemistry. QRT-PCR and Western blot were performed to measure the CTNNA1 expression in BLCA cell lines. CTNNA1 expression was up-regulated in T24 and UMUC-2 cells by CTNNA1 overexpression plasmid transfection. Cell proliferation, apoptosis, migration and invasion were respectively assessed by CCK-8 assay, flow cytometry, wound healing assay and transwell assay. The expression levels of epithelial-mesenchymal transition (EMT)-related factors were tested by qRT-PCR and Western blot. BLCA nude mice models were constructed to explore the effects of CTNNA1 on BLCA in vivo. Gene set enrichment analysis (GSEA) was proceeded to identify the CTNNA1-related pathways in BLCA.

Results: The expressions of CTNNA1 were down-regulated in BLCA tissues and cell lines, and its low expression indicated poor prognosis of BLCA patients. CTNNA1 inhibited cell proliferation, migration, invasion and EMT and promoted cell apoptosis in BLCA cells. CTNNA1 enhanced E-cadherin expression and suppressed N-cadherin, snail, MMP2 and MMP9 expressions in BLCA cells, which suggested that CTNNA1 repressed EMT in BLCA cells. Moreover, CTNNA1 could inhibit tumor growth in vivo. CTNNA1 was positively associated with P53 and apoptosis pathways in BLCA cells.

Conclusion: CTNNA1 inhibited cell proliferation, migration, invasion and EMT and promoted cell apoptosis in BLCA via activating P53 and apoptosis pathways. CTNNA1 might be a novel target in BLCA therapy.

Keywords: bladder cancer, CTNNA1, cell proliferation, in vivo study, GSEA

Introduction

Bladder cancer (BLCA) is the most common tumor in the urogenital system. Worldwide, BLCA is currently the fourth most common male malignancy and the ninth most common malignancy of all. It affects 429,800 people every year and causes 165,100 deaths.¹ According to the Cancer Statistics 2018, BLCA caused 81,190 new cases and 17,240 deaths in the United States in 2018, leading the list of urinary system cancers.² In China, the morbidity and mortality of BLCA are on the rise year by year due to the change of living environment and lifestyle of Chinese people. In 2015, the total number of BLCA cases exceeded 80,000, and 32,900 people died in China.³ BLCA is one of the most difficult and costly cancers to treat because of its tendency to recur and resistance to chemotherapy. From the epidemiological analysis, the development and occurrence of BLCA are a complex

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process of multi-factors interaction. The detailed pathogenesis of BLCA needs to be studied.⁴

In clinical practice, BLCA is often divided into non-muscle invasive bladder carcinoma (NMIBC) and muscle invasive bladder carcinoma (MIBC).⁵ Among them, NMIBC is manifested as low or high grade. Low-grade NMIBC is easy to recur but not easy to metastasize, while high-grade NMIBC has high malignant degree and may metastasize in addition to recurrence.⁶ Transurethral resection of the bladder tumor (TURBT) is the most common treatment for NMIBC.⁷ Cystectomy, pelvic lymphadenectomy and perioperative platinum-based chemotherapy (PBCT) are the treatment criteria for MIBC.⁸ However, the recurrence rate of NMIBC after surgery is up to 70%.⁹ Tumor recurrence, progression and metastasis are the main causes of death of BLCA, and one-third of patients with MIBC have metastasis at the time of treatment.¹⁰

Although the progression and metastasis of BLCA are an important reason that directly affects the clinical survival of patients, the underlying mechanism of these processes is still unclear. Therefore, further study on the mechanism of progression, recurrence and metastasis of BLCA is the focus of clinical research.¹¹ A large number of relevant studies have shown that the changes of gene expressions can significantly affect the progression of BLCA, such as transcription factor GATA-binding factor 6 (GATA6),¹² stromal antigen 2 (STAG2),¹³ BCL2L2-PABPN1 and CHFR-GOLGA3,¹⁴ growth arrest-specific transcript 5 (GAS5),¹⁵ fibroblast growth factor receptor 3 (FGFR3)¹⁶ and lymph node metastasis associated transcript 1 (LNMAT1).¹⁷ α -E-catenin (CTNNA1) is considered to be an important protein connecting the E-Cadherin/ β -catenin.^{18,19} The down-regulation or absence of CTNNA1 expression may lead to the dysfunction of E-cadherin/catenin complex, which leads to the loss of contact inhibition between cells and the enhancement of tumor cell proliferation and invasion.²⁰ CTNNA1 gene is located on chromosome 5q31, and the protein encoded by CTNNA1 gene is generally expressed in normal tissues, but down-regulated in some tumor tissues, and is closely related to tumor progression and prognosis, such as gastric cancer,²¹ breast cancer²² and colon cancer.²³ However, the function and mechanism of CTNNA1 in BLCA are still unclear.

In the present study, we aimed to explore the functional role of CTNNA1 played in BLCA progression in vivo and in vitro. CTNNA1 may be a potential and novel therapeutic target for the diagnosis and prognosis of BLCA.

Materials and Methods

The Cancer Genome Atlas (TCGA)

Analysis

The expression level of CTNNA1 in 411 cases of BLCA tumor tissues and in 19 cases of adjacent normal tissues was obtained from the TCGA database (<https://portal.gdc.cancer.gov/>). The expression of CTNNA1 in all of these tissues was assessed and compared.

Patients and Tissue Samples

A total of 117 paired of BLCA tumor tissues and adjacent normal tissues were collected in this study. All patients underwent transurethral resection or radical cystectomy of bladder tumors without preoperative radiotherapy or chemotherapy from May 2011 to July 2018 at the Affiliated Hospital of Chengde Medical University. All of these tissues were immediately snap-frozen in liquid nitrogen and then stored at -80°C . The final diagnosis of these patients involved in our study was determined histopathologically. The detailed clinical information of these patients is listed in Table 1. Overall survival (OS) rate, updated on 1 September 2019, was defined as the time from recruitment to death for any reason. The research protocol was reviewed by the Research Ethics Committee of the Affiliated Hospital of Chengde Medical University and adhered to the ethical guidelines of the 1975 Declaration of Helsinki. All patients enrolled in the present study signed the informed consent. SPSS 22.0 (IBM, USA) was performed to analyze the median CTNNA1 expression level in 87 BLCA tissues. The median CTNNA1 expression was taken as truncation value. Those higher than the median were considered as CTNNA1 high expression group, while those lower than the median were considered as CTNNA1 low expression group.

Cell Culture

Human BLCA cell lines, including EJ, 5637, T24 and UMUC-2, and human bladder epithelium immortalized cells SV-HUC-1 were purchased from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, USA), supplemented with 0.1 mg/mL streptomycin, 100 $\mu\text{g/mL}$ penicillin and 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), was used to maintain the cell lines. All of these cells were cultured at 37°C in an incubator containing 5% CO_2 .

Table 1 Correlation Between CTNNA1 expression Level and Clinical Features in Bladder Cancer

Characteristics	Number of Patients (%)	CTNNA1 High Expression ($<$ Median)	CTNNA1 Low Expression (\geq Median)	P value
Number	117	57	60	
Ages(years)				0.536
<65	58 (49.6)	28	30	
≥ 65	59 (50.4)	29	30	
Gender				0.432
Female	43 (36.8)	20	23	
Male	74 (63.2)	37	37	
Pathological stage				0.011
pTa-pT1	50 (42.7)	31	19	
pT2-pT4	67 (57.3)	26	41	
Tumor grade				0.017
Low	51 (43.6)	31	20	
High	66 (56.4)	26	40	
Tumor size				0.041
<3 cm	53 (45.3)	31	22	
≥ 3 cm	64 (54.7)	26	38	
Lymph node metastasis				0.540
Yes	57 (48.7)	28	29	
No	60 (51.3)	29	31	

Cell Transfection

The CTNNA1 overexpression plasmid pcDNA3.1-CTNNA1 (CTNNA1 group) and control plasmid pcDNA3.1-NC (NC group), synthesized and obtained from GenePharma (Shanghai, China), were employed in our study. Human BLCA cell lines T24 and UMUC-2 were both transfected with pcDNA3.1-CTNNA1 or pcDNA3.1-NC by Lipofectamine 2000TM reagent (Invitrogen) according to the manufacturer's instruction. The cell lines were harvested 48 h after transfection for further experiments.

In vivo Xenograft Tumor Assays

BALB/c nude mice (20 ± 2 g, 6–8 weeks) were provided by Shanghai Laboratory Animal Center (Shanghai, China). The mice were housed in specific pathogen-free barrier facilities. Twelve BALB/c nude mice were randomly divided into two groups (NC group and CTNNA1 group). T24 cell lines (5×10^6 cells/mouse) transfected with pcDNA3.1-CTNNA1 were injected into the right axilla of the mice of the CTNNA1 group while T24 cell lines (5×10^6 cells/mouse) transfected with pcDNA3.1-NC were injected into NC group mice. The tumor volume was detected weekly for 4 weeks using the

formula: Tumor volume (mm^3) = $0.5 \times \text{length} \times \text{width}$.² After 4 weeks, the experimental mice were sacrificed by air embolization and the tumors were removed, photographed, weighted and fixed in 4% paraformaldehyde for further experiment. All animal studies were in accordance with the UK Animal (Scientific Procedures) Act, 1986 and associated guidelines and approved by the Institutional Animal Care and Use Committee of Affiliated Hospital of Chengde Medical University.

Immunohistochemistry (IHC) Staining

All of the tissues in our study were paraffin embedded and sliced into thick paraffin sections ($4 \mu\text{m}$). IHC staining was performed to handle these tissue sections as previously described.²⁴ Briefly, the target retrieval solution was applied to immerse the tumor sections in water bath for 30 min. H_2O_2 (3%) and goat serum were respectively used to block the endogenous peroxidase (15 min) and nonspecific bindings (50 min). Then, the slides stained with CTNNA1 or Ki-67 primary antibodies (Solaibao Biotechnology Co., Ltd., Beijing, China) and secondary antibody polymer HRP (Solaibao) successively. Then, the

slices stained with DAB were used to stain the slices and methyl green was used to counterstain them. Images were taken with a microscope (IX71, Olympus Corporation, Tokyo, Japan).

Cell Counting Kit-8 (CCK-8) Assay

CCK8 detection kit (Dojindo, Kumamoto, Japan) was applied to monitor the cell proliferation activity at 24, 48, and 72 h after inoculation according to the manufacturer's protocol. In brief, experimental BLCA cell lines were inoculated into the 96-well plate at a density of 5000 cells/well. After 48 h of transfection, CCK8 reagent (10 μ L) was supplemented into each well and cultured at room temperature. Then, DMSO (150 μ L) was added and the absorbance at 450 nm was tested via a microplate spectrophotometer (Thermo Labsystems, Vantaa, Finland). CCK-8 assay was repeated 3 times.

Cell Apoptosis

After 48 h of transfection, cold phosphate-buffered saline (PBS) was used to collect and wash BLCA cell lines T24 and UMUC-2. The cells were then resuspended in 1 \times binding buffer (400 μ L, BD Bioscience, NJ, USA). Next, the cell solution (100 μ L) was transferred into a culture tube (5 mL), and PE Annexin V (5 μ L, BD Bioscience) and 7-AAD (5 μ L, BD Bioscience) were added into the culture tube. Cells were then incubated for 15 min at 37°C in the dark, and 1 \times binding buffer (400 μ L) was added to each culture tube. The cell apoptosis was measured in triplicate by flow cytometry (BD Bioscience) in accordance with the manufacturer's protocol.

Wound Healing Assay

The cells were seeded into 6-well plates (5×10^4 cells/well). Then, a pipette tip was used to create a scratch on the cell surface at 95%-100% confluence. The cells were subsequently washed with PBS (Invitrogen) to remove cell debris. At 0 h and 24 h after scratch creation, cells were observed and imaged under microscope (Olympus Corporation). Three fields of vision were randomly adopted for counting, and each treatment was performed in triplicate.

Transwell Assay

A Matrigel pre-coated 8 μ m insert was used to fill the cells with serum-free medium into upper chamber. DMEM, containing FBS, was added into lower chamber. After 48 h of incubation, cells that did not migrate to the lower

chamber were removed, while invasive cells were fixed with 70% ethanol, stained with 0.1% crystal violet and then counted under a microscope (Olympus Corporation). The experiment was repeated three times.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from BLCA tissues or cells using TRIzol reagent (Invitrogen) following the manufacturer's instruction. A microspectrophotometer (Thermo Scientific, Waltham, USA) was applied to measure the RNA concentration. Then, the PrimerScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) was employed to reverse transcribe the total RNA (1 μ g). QRT-PCR assay was proceeded using the SYBR Premix Ex TaqTM II kit (TaKaRa) with the RT-PCR system (Heraeus, Hong Kong, China). The realtime PCR was performed with the following conditions: 95°C for 3 min; 41 cycles of 95°C for 25 s, 60°C for 30 s and 72°C for 30 s. β -actin was used as the internal control. The sequence of primers used in qRT-PCR was designed as follows: CTNNA1 forward primers 5'-TCCTGCTGTGTCATGGAA-3' and reverse primers 5'-GCTTTGAACTCGCTGAGG-3', β -actin forward primers 5'-GCTCGTCGTCGACACGGCTC-3' and reverse primers 5'-CAAACATGATCTGGGTCATCTTCTC-3', E-cadherin (E-cad) forward primers 5'-GGTTGATCCTGGCTTTGTT-3' and reverse primers 5'-GCCCTGTTGTCCTTCTTTT-3', N-cadherin (N-cad) forward primers 5'-AGCGCAGTCTTACCGAAGG-3' and reverse primers 5'-TCGCTGCTTTTCATCTGAACCTT-3', snail forward primers 5'-ACATCCGAAGCCACACG-3' and reverse primers 5'-TGGGGACAGGAGAAGGG-3', MMP2 forward primers 5'-CGCCTTTAACTGGAGCAAA-3' and reverse primers 5'-AGGTTATCGGGGATGGC-3', MMP9 forward primers 5'-ACGCAGACATCGTCATCC-3' and reverse primers 5'-CCAGGGACCACAACCTCG-3'. The expression level was calculated using the $2^{-\Delta\Delta C_t}$ method.

Western Blot

BLCA cells used in our study were harvested and lysed in Radio Immunoprecipitation Assay (RIPA) lysis buffer (Sigma, St. Louis, MO, USA). The protein concentration was examined using a BCA detection kit (TaKaRa). Each protein sample (20 μ g) was separated via SDS-polyacrylamide gel (10%) and then electro-transferred to polyvinylidene difluoride membranes. Then, the membranes

were blocked using milk (5%) at 37°C for 1 h, incubated using primary antibodies at 4°C for 16 h, treated using secondary antibodies at 37°C for 1 h, and finally visualized via an Amersham prime ECL Plus detection system (GE Healthcare Life Sciences), and β -actin was used as the internal reference protein. Primary antibodies were all provided by Cell Signaling Technology (Danvers, MA, USA) and the dilutions of primary antibodies were as follows: anti-CTNNA1 (1:1000, CST# 3240), anti-E-cad (1:1000, CST# 14472), anti-N-cad (1:1000, CST# 13116), anti-snail (1:1000, CST# 3879), anti-MMP2 (1:1000, CST# 40994), anti-MMP9 (1:1000, CST# 13667), anti-cleaved-caspase3 (anti-cleaved-CASP3, 1:1000, CST# 9664), anti-caspase3 (anti-CASP3, 1:1000, CST# 9662), anti-apoptotic protease activating factor 1 (anti-APAF1, 1:1000, CST# 5088), anti-cleaved-caspase8 (anti-cleaved-CASP8, 1:1000, CST# 9748), anti-caspase8 (anti-CASP8, 1:1000, CST# 4790), anti-PI 3 kinase p85 alpha antibody (anti-PIK3R1, 1:1000, CST# 4257), anti- β -actin (1:1000, CST# 4970).

Gene Set Enrichment Analysis (GSEA)

GSEA of pathways and genes was carried out based on TCGA BLCA dataset using the GSEA version 2.0 from the Broad Institute at MIT. The gene sets of fewer than 10 genes were excluded in our study. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was used to compute the t-statistic mean of the genes. Using a permutation test 1000 times, the cutoff for the significance level of P values was chosen as 0.01 for the most significant pathways related to CTNNA1 expression.

Statistical Analysis

SPSS 22.0 (IBM, USA) was performed to analyze quantitative data in our paper. And, data were all expressed by mean \pm standard deviation (SD) of at least three independent experiments. Student's *t*-test or ANOVA were used to compare the differences among groups. Pearson's χ^2 tests were performed to assess the relationship between CTNNA1 expression and BLCA clinical pathology contained patients' ages, gender, pathological stages, tumor grades, tumor size and lymph node metastasis. Kaplan–Meier survival analysis was performed to measure the overall survival rate. P-value was analyzed via the Log rank test and $P < 0.05$ presented statistically significant.

Results

CTNNA1 Down-Expressed in BLCA Tissues and Cell Lines

Data from TCGA database showed that the expression level of CTNNA1 in 411 cases of BLCA tissues was significantly lower than that in 19 cases of adjacent non-tumor tissues ($P = 0.0075$, Figure 1A). The expression levels of CTNNA1 in 117 paired of clinical BLCA patients tumor tissues and adjacent normal tissues were detected using qRT-PCR. As shown in Figure 1B, the expression of CTNNA1 in normal tissues was obviously higher than that in BLCA tumor tissues ($P < 0.01$). Then, 117 cases of BLCA tumor tissues were divided into NMIBC group ($n = 62$) and MIBC group ($n = 55$) based on their clinical characteristics, and the CTNNA1 expressions were reanalyzed. Data from qRT-PCR showed that the expression of CTNNA1 in the NMIBC group was also remarkably higher than that in the MIBC group ($P < 0.05$, Figure 1C). A total of 5 paired BLCA tissues and normal tissues were randomly selected and then their CTNNA1 expressions were monitored by IHC. The results from IHC exhibited that the expression levels of CTNNA1 in BLCA tissues were lower than that in normal tissues (Figure 1D). In addition, the 117 cases of BLCA tumor tissues were divided into CTNNA1 high expression group ($n = 57$) and CTNNA1 low expression group ($n = 60$) according to the CTNNA1 expression level, and the overall survival rate was calculated using Kaplan–Meier survival analysis. Figure 1E displays that the overall survival rate of BLCA patients in CTNNA1 high expression group was markedly more than that in CTNNA1 low expression level ($P = 0.0339$). Besides, the relationship between CTNNA1 expression level and BLCA clinical pathology including contained patients' ages, gender, pathological stages, tumor grades, tumor size and lymph node metastasis was also analyzed. Table 1 elucidates that the expression of CTNNA1 was associated with pathological stages, tumor grades and tumor size, but not with patients' ages, gender or lymph node metastasis.

The expression of CTNNA1 in human BLCA cell lines containing EJ, 5637, T24 and UMUC-2 and human bladder epithelium immortalized cells SV-HUC-1 was determined using qRT-PCR and Western blot. Data from qRT-PCR and Western blot showed that CTNNA1 was markedly downregulated in BLCA cell lines compared to that in SV-HUC-1 cell lines ($P < 0.01$, Figure 1F and G). These results confirmed that CTNNA1 was lowly expressed in BLCA tissues and cell lines and its low

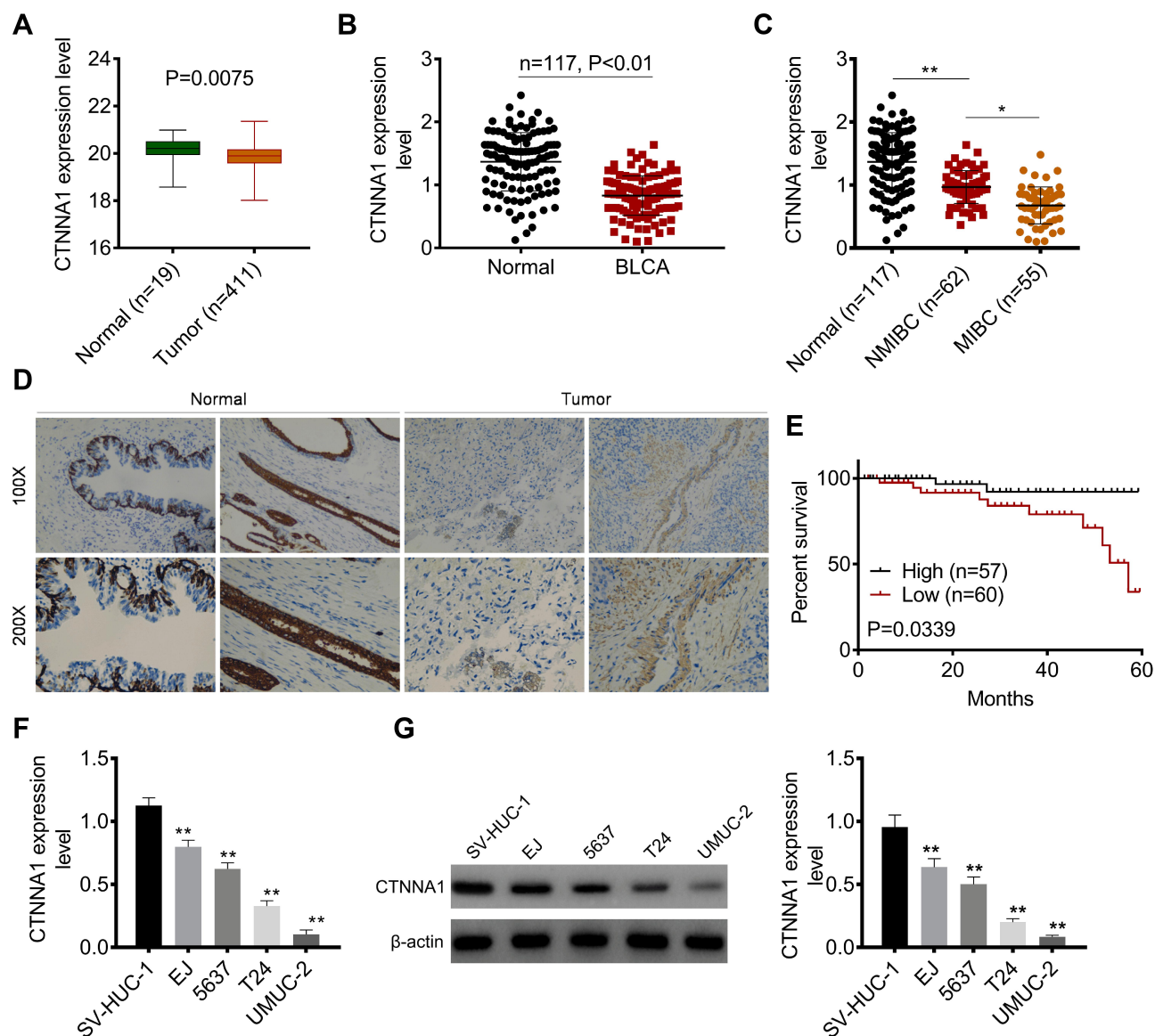


Figure 1 CTNNA1 was down-regulated in BLCA tissues and cell lines. **(A)** The expression levels of CTNNA1 in 411 cases of BLCA tumor tissues and 19 cases of adjacent normal tissues were downloaded from TCGA database. **(B)** The CTNNA1 mRNA expressions in 117 paired of BLCA tumor tissues and matched adjacent normal tissues were detected by qRT-PCR. **(C)** The expression levels of CTNNA1 in 117 cases of normal tissues, 62 cases of NMIBC tumor tissues and 55 cases of MIBC tumor tissues were detected using qRT-PCR. **(D)** The CTNNA1 expressions in 5 paired of BLCA tumor tissues and adjacent normal tissues were determined by IHC. **(E)** Kaplan-Meier survival analysis was performed to measure the overall survival rate. **(F)** The CTNNA1 mRNA expressions in human bladder epithelium immortalized cells SV-HUC-1 and human BLCA cell lines (EJ, 5637, T24 and UMUC-2) were measured by qRT-PCR. **(G)** The CTNNA1 mRNA expressions in human bladder epithelium immortalized cells SV-HUC-1 and human BLCA cell lines (EJ, 5637, T24 and UMUC-2) were assessed by Western blot. Data were shown as mean \pm SD for three-independent experiments. * $P < 0.05$, ** $P < 0.01$ vs normal tissues or SV-HUC-1.

expression was related to poor prognosis of BLCA patients.

CTNNA1 Inhibited BLCA Proliferation in vivo and in vitro

BLCA cell lines T24 and UMUC-2 were chosen to be transfected with pcDNA3.1-NC or pcDNA3.1-CTNNA1. The transfection efficiency was detected using qRT-PCR and Western blot. As shown in Figure 2A and B, CTNNA1

expressions were significantly up-regulated in CTNNA1 groups compared with that in NC groups ($P < 0.01$). Then, CCK-8 assay and flow cytometry were respectively performed to measure cell proliferation and apoptosis. The cell proliferation was remarkably inhibited while the cell apoptosis was notably promoted in CTNNA1 groups compared to that in NC groups ($P < 0.01$, Figure 2C and D).

For in vivo experiment, BLCA nude mice models were successfully constructed. As shown in Figure 2E-G,

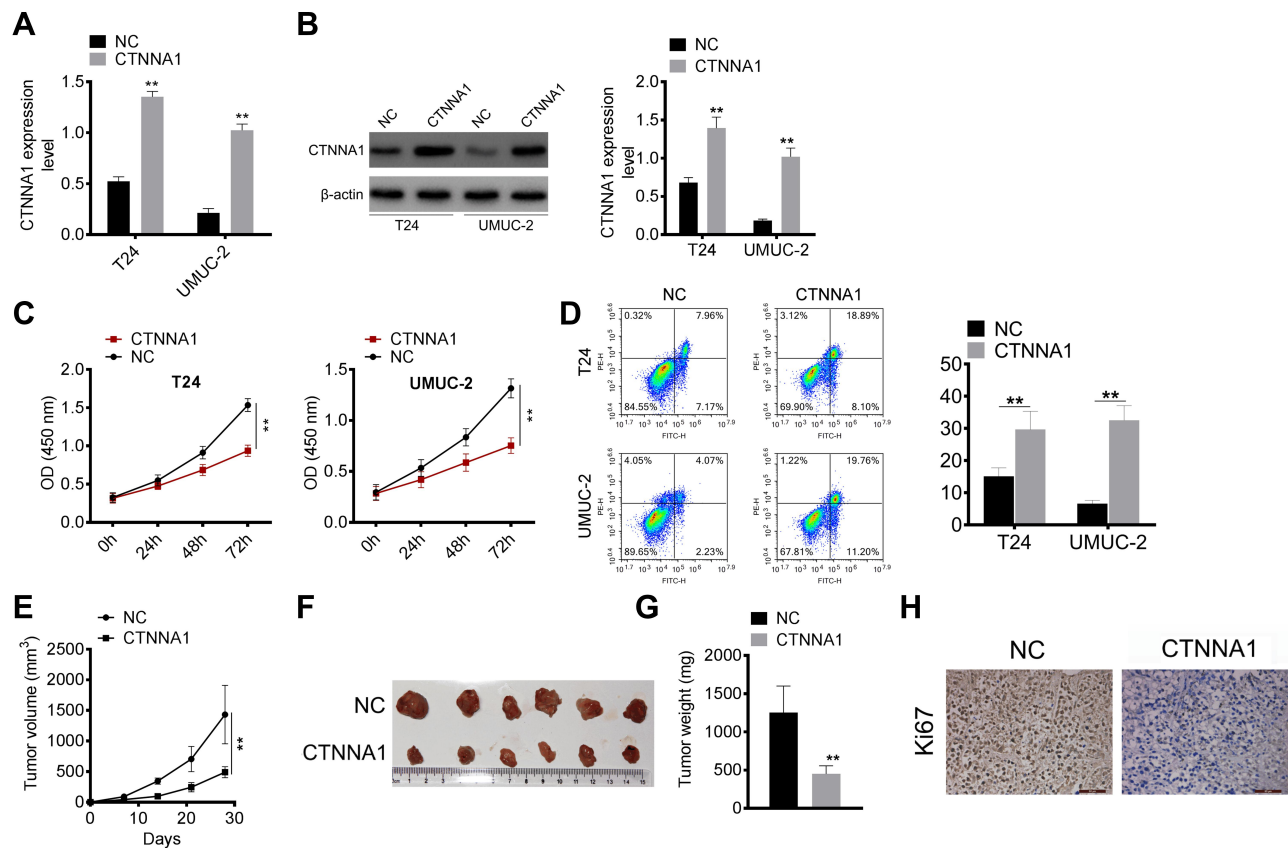


Figure 2 CTNNA1 inhibited tumor proliferation in vivo and in vitro. (A) The transfection efficiency of T24 and UMUC-2 was detected by qRT-PCR. (B) The transfection efficiency of T24 and UMUC-2 was determined by Western blot. (C) Cell proliferation activity was measured by CCK-8 assay. (D) Cell apoptosis was monitored by flow cytometry. (E) BLCA nude mice models were constructed, the tumor volume was detected weekly. (F) The tumor sizes were determined after the mice were killed. (G) The tumor weights were measured after the mice were killed. (H) The tumor proliferation was assessed by Ki-67 assay. Data were shown as mean \pm SD for three-independent experiments. ** $P < 0.01$ vs NC group.

CTNNA1 overexpression obviously inhibited BLCA tumor volume, size and weight compared to the NC group ($P < 0.01$). Furthermore, the results from IHC suggested that the expression of Ki-67 in the CTNNA1 group was significantly lower than that in the NC group (Figure 2H). These results illustrated that CTNNA1 could inhibit BLCA proliferation and facilitate apoptosis in vivo and in vitro.

CTNNA1 Inhibited Cell Migration, Invasion and Epithelial–Mesenchymal Transition (EMT) in BLCA Cell Lines

To evaluate the effect of CTNNA1 on cell migration and invasion of BLCA, wound healing assay and transwell assay were performed, respectively. After 24 h of scratch formation, the results showed that the relative wound widths of CTNNA1 groups were significantly more than that of NC groups ($P < 0.01$, Figure 3A). Next, as shown in

Figure 3B, the invasion cell number of CTNNA1 groups was obviously fewer than that of NC groups ($P < 0.01$). In addition, the expression levels of EMT-related factors including E-cad, N-cad, snail, MMP2 and MMP9 were determined using qRT-PCR and Western blot. The results exhibited that E-cad expressions were markedly increased while N-cad, snail, MMP2 or MMP9 expressions were memorably decreased in CTNNA1 groups when compared to that in NC groups ($P < 0.01$, Figure 3C and D). These data elucidated that CTNNA1 suppressed cell migration, invasion and EMT in BLCA.

CTNNA1 Was Associated with P53 and Apoptosis Pathways in BLCA

To further explore the molecular mechanism of CTNNA1 in BLCA, we performed GSEA in BLCA samples with higher CTNNA1 expression (\geq median of CTNNA1 expression) vs lower CTNNA1 expression ($<$ median of FGF5 expression) based on TCGA dataset.

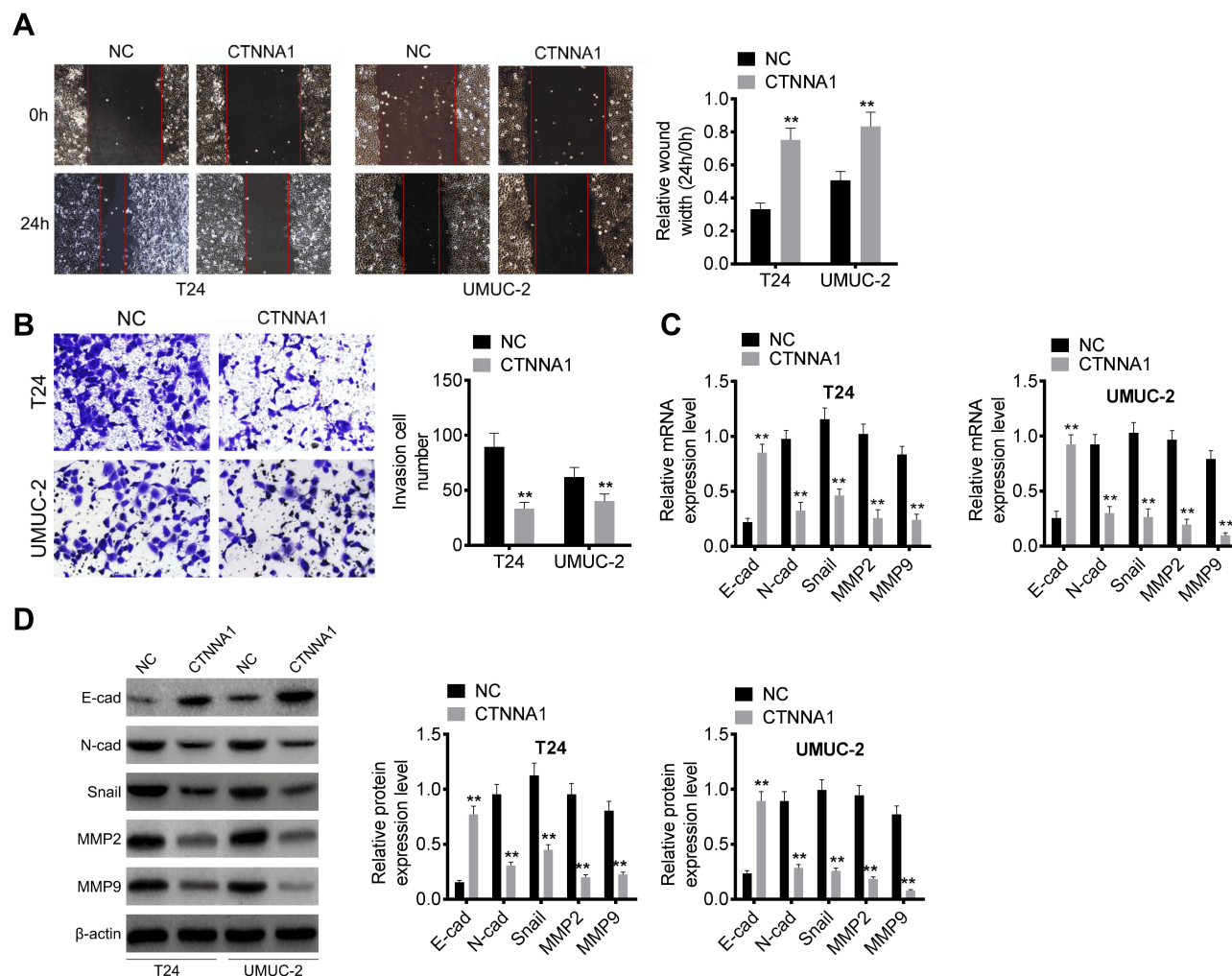


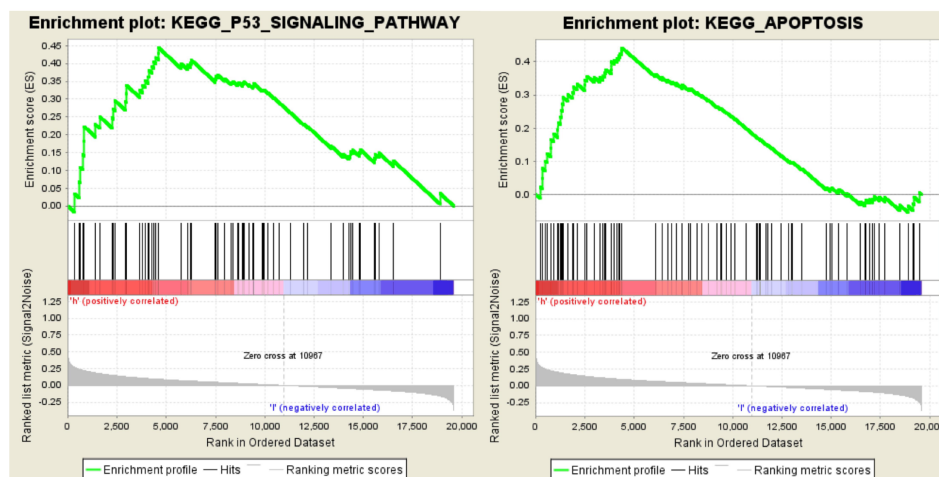
Figure 3 CTNNA1 inhibited cell migration, invasion and EMT in BLCA cell lines T24 and UMUC-2. **(A)** Cell migration activity was detected by wound healing assay. **(B)** Cell invasion activity was determined by transwell assay. **(C)** The mRNA expression levels of EMT-related factors including E-cad, N-cad, snail, MMP2 and MMP9 were measured by qRT-PCR. **(D)** The protein expression levels of EMT-related factors including E-cad, N-cad, snail, MMP2 and MMP9 were measured by Western blot. Data were shown as mean \pm SD for three-independent experiments. $**P < 0.01$ vs NC group.

Figure 4A shows that higher CTNNA1 expression was positively related to P53 and apoptosis pathways in BLCA samples. To further verify the GSEA data, the protein expression of P53-related factors (cleaved-CASP3, CASP3 and APAF1) and apoptosis pathway-related proteins (cleaved-CASP8, CASP8 and PIK3R1) in T24 and UMUC-2 cells that transfected with CTNNA1 overexpression plasmid were measured via Western blot. The protein expression levels of detected factors were significantly enhanced in both T24 and UMUC-2 cells ($P < 0.01$, Figure 4B) after the upregulation of CTNNA1. These results revealed that CTNNA1 expression was positively correlated to P53 and apoptosis signaling pathways in BLCA.

Discussion

BLCA is one of the most common urinary malignancies, and its incidence ranks high worldwide.²⁵ Although the treatment of BLCA has made great progress in recent years, the 5-year survival rate is still unsatisfied.²⁶ Previous studies have found a series of functional biomarkers related to BLCA. Goldstein et al²⁷ reported that PPARG was activated in MIBC through RXRA hotspot mutations in its heterodimer partner and focal PPARG amplification, and revealed the potential resistance mechanisms for ERBB2 and FGFR inhibitors. Chen et al²⁸ found that differentiation antagonizing non-protein coding RNA (DANCR) accelerated proliferation and metastasis in BLCA cell lines through up-regulating CCND1 expression and

A



B

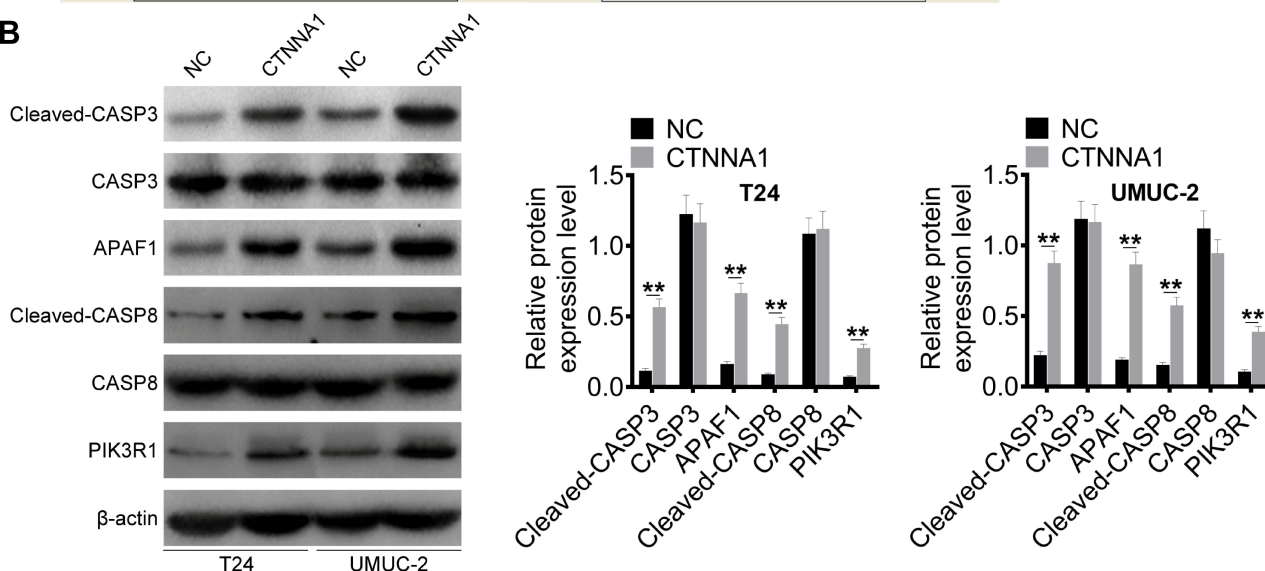


Figure 4 Mechanisms related to CTNNA1 function in BLCA. **(A)** GSEA analysis in BLCA patients with higher CTNNA1 expression vs lower CTNNA1 expression based on TCGA datasets. P53 and apoptosis pathways have the strongest relationship with CTNNA1-higher expression. **(B)** Expression levels of key proteins in the P53 (CASP3 and APAF1) and apoptosis (CASP8 and PIK3R1) pathways were detected by Western blot. Data were shown as mean \pm SD for three-independent experiments. ** $P < 0.01$ vs NC group.

activating IL-11-STAT3 signaling pathway. Chen et al²⁹ confirmed that low expressed in BLCA stem cells (LBCS), a novel long noncoding RNA (lncRNA), suppressed chemoresistance and self-renewal of BLCA stem cells via epigenetic silencing of SOX2. Liu et al³⁰ proved that hsa_circ_0001361, a circular RNA (circRNA), facilitated BLCA invasion and metastasis by inhibiting miR-491-5p expression to enhance MMP9 expression. However, the mechanism of biomarker regulation in the development of BLCA is still not fully understood. CTNNA1, which was considered as a tumor suppressor, played a role in several pathological processes.³¹ Vermeulen et al²³ confirmed that CTNNA1 played a tumor-inhibitor role and suppressed cell invasion in human colon cancer. Li et al³² reported that in

acute myeloid leukemia, CTNNA1 hypermethylation is a frequent event, and it is independently related to an adverse outcome. However, the function and mechanism of CTNNA1 in BLCA are still unclear.

In the present study, we found that CTNNA1 was down-regulated in BLCA tissues based on TCGA database. However, we failed to find out the dissimilarity of CTNNA1 expressions in BLCA in GEO dataset. It is a limitation of our study. Then, we firstly confirmed that CTNNA1 was lowly expressed in BLCA tissues and cell lines in our experiments. The low expression of CTNNA1 indicated poor prognosis of BLCA patients. CTNNA1 expressions were successfully up-regulated in human BLCA cell lines T24 and UMUC-2 through CTNNA1

overexpression plasmid transfection. CCK-8 assay³³ and Ki-67 assay³⁴ were commonly used to detect proliferation in cell lines and tissues, as well as flow cytometry³⁵ was often used to measure cell apoptosis. Nude mice models were used in a variety of tumor researches, such as colon cancer,³⁶ pancreatic cancer³⁷ and BLCA.³⁸ In the present study, nude mice BLCA models were constructed. Next, our data revealed that CTNNA1 inhibited cell proliferation and promoted cell apoptosis in vitro experiment, and suppressed tumor growth in vivo experiment.

In addition, CTNNA1 was reported to be related to cell invasion and migration in tumors.²³ In our study, wound healing assay and transwell assay were respectively performed to assess the cell migration and invasion, and the results exhibited that CTNNA1 could repress cell migration and invasion activities in BLCA cell lines. Previous studies demonstrated that EMT was an important progress in tumor development and metastasis.³⁹ Guan et al⁴⁰ found that microRNA-218 suppressed EMT and migration in prostate cancer. Chockley et al⁴¹ reported that in lung cancer, EMT could cause metastasis-specific immunosurveillance that mediated by natural killer cells. Xu et al⁴² revealed that microRNA-22 inhibited EMT in BLCA by suppressing MAPK1/Slug/vimentin and snail and MAPK1/Slug/vimentin feedback pathway. We also investigated whether CTNNA1 has an effect on EMT in BLCA. E-cad⁴³ was known as an inhibitor in EMT while N-cad,⁴⁴ snail,⁴⁵ MMP2 and MMP9⁴⁶ were defined as promoters in EMT. In our study, we confirmed that CTNNA1 could enhance E-cad expression and repress N-cad, snail, MMP or MMP9 expressions. These results proved that CTNNA1 inhibited cell migration, invasion and EMT in BLCA.

To clarify the molecular mechanism of CTNNA1 in BLCA, GSEA was performed. The results of KEGG analysis indicated that higher CTNNA1 expression level was positively related to the P53 and apoptosis pathways in BLCA samples. CASP3 and APAF1 were key proteins in P53 pathway⁴⁷ while PIK3R1 and CASP8 were key proteins in apoptosis pathway.⁴⁸ The expression of P53 pathway-related factors (CASP3 and APAF1) and apoptosis pathway-related factors (PIK3R1 and CASP8) were significantly increased by CTNNA1 overexpression. Above data suggested CTNNA1 regulated BLCA via P53 and apoptosis signaling pathways.

In conclusion, our paper investigated that CTNNA1 inhibited cell proliferation, migration, invasion and EMT and promoted cell apoptosis of BLCA in vitro by positively regulated P53 and apoptosis signaling pathways, and

suppressed BLCA tumor growth in vivo. The present study provided a new insight into the mechanisms for the relationship between CTNNA1 expression and BLCA cell activities and elucidated that CTNNA1 played as an inhibitor in adoptive immunotherapy of BLCA.

Data Sharing Statement

All data generated and/or analyzed during this study are included in this published article.

Ethics Approval

The project protocol was authorized by the Ethic Committee of Affiliated Hospital of Chengde Medical University and carried out in accordance with the Guidelines of Affiliated Hospital of Chengde Medical University and adhered to the ethical guidelines of the 1975 Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests.

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