

# Long non-coding RNA Z38 promotes cell proliferation and metastasis in human renal cell carcinoma

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**Abstract.** Long non-coding RNAs (LncRNAs) have been reported to serve roles in various types of malignancy, including human renal cell carcinoma (RCC), which is among the most common types of kidney cancer worldwide. The present study aimed to investigate the effects of a newly-discovered LncRNA, Z38, on cell proliferation and metastasis in RCC cells. Reverse transcription-quantitative polymerase chain reaction analysis was used to detect the transcription levels of Z38 in clinical RCC tissues and cultured RCC cells. The expression of Z38 was notably increased in patients with stage III and IV RCC compared with patients with stage I and II. Knockdown of Z38 with specific short hairpin RNAs notably decreased the proliferation rate of A498 and ACHIN cells. In addition, a colony formation assay was included to investigate the role of Z38 in cell proliferation. Transwell assays demonstrated that Z38 deprivation inhibited the migratory and invasive capability of RCC cells. The association between Z38 and the epithelial-mesenchymal transition process was investigated using western blot analysis. The results of the present study demonstrated that Z38 may serve as an important biomarker in the diagnosis and treatment of RCC in the clinic.

## Introduction

Renal cell carcinoma (RCC) is among the most common kidney cancers worldwide, accounting for 2-3% of all human cancer cases, and the occurrence continues to increase (1,2). The morbidity of RCC is >209,000/year worldwide, of which ~102,000 mortalities are recorded annually (3,4). The conventional therapy for RCC is radical surgery, which is only effective for early-stage RCC. However, 20-30% of patients with RCC are diagnosed with metastases at presentation, with

the most frequent sites including the lungs, liver, brain and lymph nodes (5). The metastatic potential of RCC makes it one of the most refractory types of tumor, with a 5-year survival rate of 5-10% (6). Therefore, further studies are required in order to elucidate novel molecular markers to identify RCC in the early stages.

Only 2% of the mammalian genome is able to be translated into protein; however, >85% of the genome exhibits the potential to be transcribed into RNA, following which most RNAs serve roles in epigenetic regulation (7). Notably, long non-coding RNAs (LncRNAs) are a large family of regulatory RNAs, characterized by a length of >200 nucleotides. LncRNAs have been reported to interact with DNAs, RNAs and proteins, and to be involved in the processes of DNA transcription, RNA translation, the cell cycle and apoptosis (8,9). Various LncRNAs have been demonstrated to participate in the tumorigenesis of RCC. LncRNA Lnc-ZNF180-2 was observed to be overexpressed in RCC tissues and acted as a prognostic biomarker of the progression of RCC (10). LncRNA MALAT1 was demonstrated to promote RCC aggression through the histone-lysine N-methyl transferase Ezh2, and to interact with microRNA-205 in RCC cells, leading the identification of MALAT1 to be a novel theranostic marker in the treatment of RCC in the clinic (11).

Z38 was a newly-discovered LncRNA by Deng *et al* (12). Z38 was identified to be a protein coding isoform of claudin domain containing 1 mRNA, one of which is additionally termed claudin-25 and belongs to the claudin family, which contains >26 members and is characterized by a common motif (GLWCC) in the paracellular loop (13,14). Z38 was demonstrated to be an LncRNA by *in vitro* translation experiments and was notably upregulated in human breast cancer. Knockdown of Z38 in breast cancer cells inhibited cell proliferation and metastasis (12). However, the detailed mechanism of the inhibitory roles of Z38 in breast cancer remain unknown.

In the present study, the expression of Z38 was examined in clinical patients with RCC and cultured RCC cells. The role of Z38 in cell proliferation and metastasis was examined using bromodeoxyuridine (BrdU), colony formation and Transwell assays. A preliminary study focusing on the effects of Z38 on the epithelial-mesenchymal transition (EMT) process was also included. The results of the present study indicated that Z38 may act as a potential biomarker in the diagnosis and treatment of RCC in the clinic.

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## Patients and methods

**Patient samples.** The present study was approved by the Ethics Committee of Linyi People's Hospital (Linyi, China). A total of 40 patients with RCC (26 male, 14 female; 35–65 years old; mean age, 48 years), who were admitted to the Department of Urology of Linyi People's Hospital between January 2012 and December 2014 were randomly collected. RCC tissues and adjacent non-cancerous tissues were dissected immediately following surgery and frozen in liquid nitrogen, prior to total mRNA extraction. All patients expressed their intentions to participate in the present study and written informed consent was obtained from each patient.

**Cell culture and lentiviral infection.** The human RCC cell lines 786-O, A498, ACHIN and SN12PM6 were purchased from the American Type Culture Collection (Manassas, VA, USA) and the Caki-2 RCC cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The epithelial cell line HKC from the human proximal renal tubules was purchased from ATCC (Manassas, VA, USA) and included as a control. All of the cell lines were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The specific short hairpin (sh)RNA against human Z38 (GCA TCT GGG ATG AAT TCAT) was designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The lentivirus containing specific shRNAs against Z38 was packaged and assessed by Shanghai GenePharma Co., Ltd. (Shanghai, China).

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA from human tissues and cultured cells was extracted using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The total RNA was reverse transcribed to obtain the first-strand cDNAs using PrimeScript™ RT Master mix (Perfect Real Time; Takara Biotechnology Co., Ltd.). qPCR was performed using the SYBR Premiers Ex Taq kit (Takara Biotechnology Co., Ltd.) in an ABI PRISM 7900 real time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The protocol was as follows: Denaturation, 95°C for 2 min; annealing, 40 repetitions of 95°C for 30 sec and 60°C for 60 sec; and final extension, 72°C for 10 min. The primers used in the present study were synthesized by Shenggong Co., Ltd. (Shanghai, China). Z38 forward, 5'-AGTGGGATTGTG GAGACGGTGT-3' and reverse, 5'-AGGTAAAAGGAAGT GCAACGC-3'; GAPDH forward, 5'-GTGGACATCCGCAAA GAC-3' and reverse, 5'-AAAGGGTGTAAACGCAACTA-3'. GAPDH was included as an internal control. Each experiment was repeated ≥3 times in triplicate and the 2<sup>-ΔΔC<sub>q</sub></sup> method was used for quantification (15).

**Cell proliferation assay.** Cell proliferation was assessed by cell counting and BrdU incorporation using a BrdU Cell Proliferation kit (EMD Millipore, Billerica, MA, USA). Cells were infected with a lentivirus [containing specific or scramble (shNC) shRNA] and allowed to grow for 48 h. The culture medium was replaced with serum-free medium for 24 h. Cells

were trypsinized and collected using low-speed centrifugation (850 × g) at 37°C for 5 min. An equal number (5,000 cells) of A498 and ACHIN cells from each group (control, shNC, shZ38-1 and shZ38-2) were seeded into 96-well plates and incubated in culture medium (10% FBS) supplemented with 10 μM BrdU. BrdU incorporation was examined with additional peroxidase substrates from the kit. Spectrophotometric detection was performed at a wave length of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.) Each assay was repeated ≥3 times in triplicate.

**Colony formation assay.** For the colony formation assay, A498 and ACHIN cells (500 cells/well) were seeded into 6-well culture plates and infected with specific shRNA against Z38. Following incubation for 14 days, the colonies were fixed with 4% paraformaldehyde solution at room temperature for 10 min, stained with 1% crystal violet for 5 min at room temperature, imaged using an inverted microscope (Nikon Corporation, Tokyo, Japan; magnification, x200) and counted in 5 randomly-selected fields. Any colony containing >50 cells was considered to be a successfully-formed colony and was counted.

**Transwell assay.** Cell abilities of migration and invasion were examined using Transwell chambers (pore size, 8 μm; Corning Incorporated, Corning, NY, USA). A498 and ACHIN cells were infected with specific shRNAs or scramble shRNA and allowed to grow for 48 h. The cells were washed 3 times with PBS, trypsinized for ~30 sec and collected with low-speed centrifugation (850 × g) at 4°C for 5 min. The cells were re-suspended in serum-free medium. A total of 5 × 10<sup>4</sup> cells were seeded into the upper chamber of 24-well plates, and 600 μl DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS was added to the lower chamber. Following incubation for 24 h, cells were fixed with ice-cold methanol for 5 min and stained with crystal violet for an additional 5 min. The membrane was washed in water and the cells on the upper surface of the membrane were wiped off using cotton swabs. The cells on the bottom surface were imaged at a magnification of x100 and counted with a light microscope (Nikon Corporation) in 5 random fields. For the invasion assay, the membranes were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C for 6 h.

**Western blot analysis.** The total protein from A498 cells was extracted using lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and the protein quality and quantity were determined using the Bradford dye-binding protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 40 μg protein from each sample was loaded onto a 10% gel and subjected to SDS-PAGE, following which the protein was transferred to a polyvinylidene fluoride membrane. The primary antibodies against cyclin B1 (ab72; 1:1,000), M phase inducer phosphatase 3 (CDC25C; ab32444; 1:1,000), N-cadherin (ab18203; 1:1,000) and E-cadherin (ab1416; 1:1,000) were purchased from Abcam (Cambridge, MA, USA) and the membranes were incubated with primary antibodies overnight at 4°C. Primary antibody against GAPDH (sc47724; 1:2,000) and secondary antibodies [goat anti-rabbit IgG-HRP (sc-2004; 1:2,000) and goat anti-mouse IgG-HRP (sc-2005;

1:2,000)] were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The secondary antibodies were incubated for 1 h at room temperature. The immunoreactivity of proteins was determined using enhanced chemiluminescent autoradiography (Thermo Fisher Scientific, Inc.) and FluorChem Q (ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation unless otherwise stated. The Student's t-test was used to investigate the statistical significance between variables. All statistical analyses were performed using SPSS PASW software version 18.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**LncRNA Z38 is overexpressed in clinical RCC tissues and cultured RCC cells.** In order to investigate the role of Z38 in human RCC, the expression of Z38 was examined in 40 clinical RCC tissues and adjacent non-cancerous counterparts. As presented in Fig. 1A, the average transcription level of Z38 in tumor tissue was increased 4-fold compared with adjacent control tissue. According to their medical records, the 40 patients were classified into two categories: Stage I/II ( $n=14$ ) and stage III/IV ( $n=26$ ). The expression of Z38 in stage III/IV patients was increased  $\sim 2.5$ -fold compared with the stage I/II counterparts (Fig. 1B), implying that the transcription level of Z38 may be an auxiliary indicator of late stage of RCC in the clinic. A total of 5 RCC cell lines and a normal renal epithelial cell line, HKC, were obtained and subjected to RT-qPCR analysis. As presented in Fig. 1C, the transcription levels of Z38 in all of the 5 RCC cell lines were notably enhanced compared with the control cells, of which A498 and ACHIN cells demonstrated the greatest increase in Z38 expression at 5.8-fold and 4.7-fold that in HKC cells, respectively. Therefore, these 2 cell lines were selected for subsequent analysis. The results of the present study indicated that the expression of Z38 was upregulated in RCC, and associated with the tumor node and metastasis staging of RCC in the clinic.

**Knockdown of Z38 in RCC cells inhibits cell proliferation.** In the present study, 2 specific shRNAs against human Z38 were designed and transfected into RCC cell lines A498 and ACHIN. RT-qPCR analysis demonstrated that, in the 2 cell lines, transfection of specific shRNAs markedly decreased the expression of Z38 to  $<50\%$  of that in control cells (Fig. 2A). The second shRNA (shZ38-2) inhibited the transcriptional levels by 58% in A498 cells and 61% in ACHIN cells, making it the more effective shRNA. The effects of Z38 on cell proliferation were investigated. On the 4th day, the cell proliferation rate was decreased to 70% by shZ38-1 and to 71% by shZ38-2 in A498 cells (Fig. 2B). Similarly, cell proliferation was suppressed by 24 and 25% when ACHIN cells were transfected with shZ38-1 and shZ38-2, respectively (Fig. 2C). The inhibitory effects were more apparent on the 5th day for the 2 cell lines (Fig. 2B and C).

Colony formation assays were conducted to further investigate the role of Z38 in cell proliferation. A total of  $\sim 150$  colonies were formed in control A498 cells following 14 days of

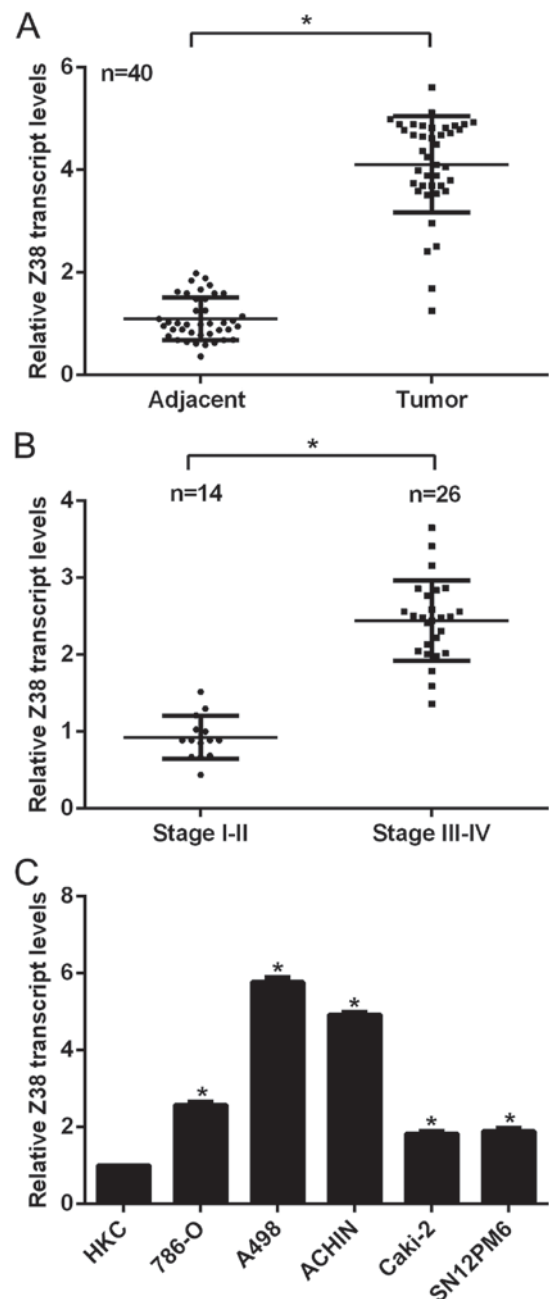


Figure 1. Long non-coding RNA Z38 is overexpressed in clinical RCC tissues and cultured RCC cells. (A) RT-qPCR analysis demonstrated that the expression of Z38 in tumor tissues was increased compared with adjacent non-cancerous tissue.  $^*P < 0.05$ . (B) RT-qPCR assays demonstrated that the transcription level of Z38 in stage III-IV patients was increased compared with stage I-II patients.  $^*P < 0.05$ . (C) The expression of Z38 in RCC cell lines was increased compared with HKC normal renal cells.  $^*P < 0.05$  vs. HKC cells. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RCC, renal cell carcinoma.

incubation; however, 74 colonies in the shZ38-1-treated group, and 60 colonies in the shZ38-2-treated group, were observed following staining with crystal violet (Fig. 3A). Similarly, transfection of shZ38-1 or shZ38-2 inhibited the ability of ACHIN cells to form colonies (Fig. 3B). All of the present observations suggested that the knockdown of Z38 in RCC cell lines A498 and ACHIN suppressed cell proliferation.

**Knockdown of Z38 inhibits metastasis in A498 and ACHIN RCC cells.** Cell proliferation and metastasis are two primary

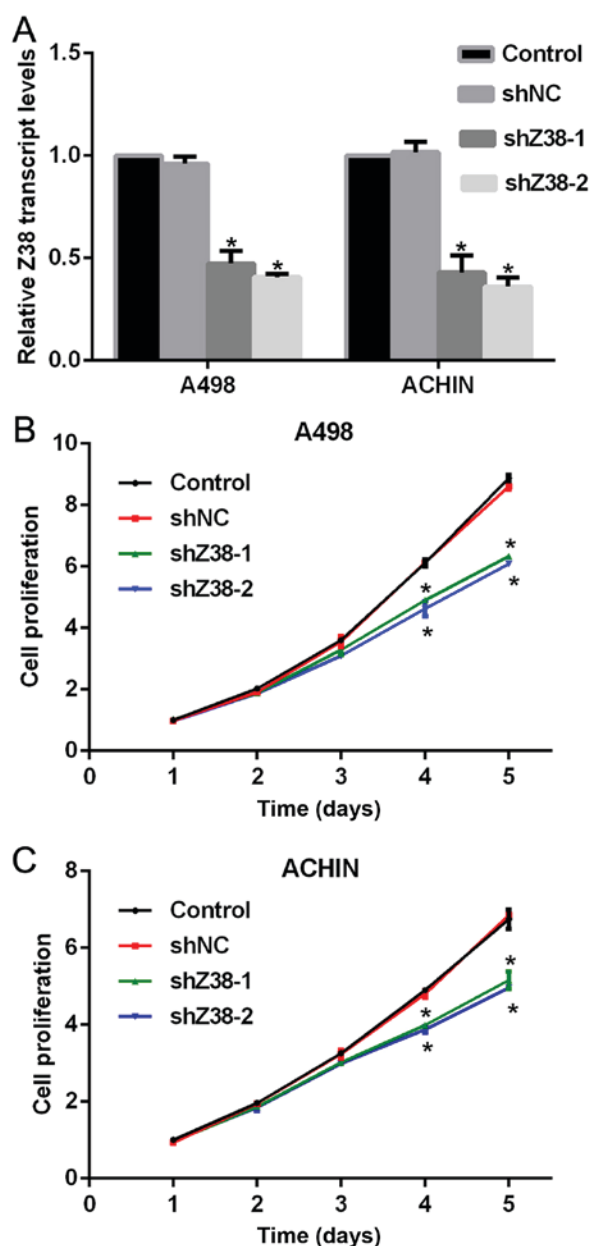


Figure 2. Knockdown of Z38 in renal cell carcinoma cells inhibits cell proliferation. (A) A total of 2 specific shRNAs against human Z38 were transfected into A498 and ACHIN cells, and the transcription level of Z38 in each group was assessed by reverse transcription-quantitative polymerase chain reaction analysis. (B) The cell proliferation rate was decreased when A498 cells were infected with shZ38-1 or shZ38-2. (C) The cell proliferation rate was decreased when ACHIN cells were treated with shRNAs against Z38. \* $P < 0.05$  vs. respective control. shRNA, short hairpin RNA; shNC, scramble shRNA.

manifestations of malignancy; therefore, the effects of Z38 knockdown on cell metastasis were investigated in A498 and ACHIN cells (Fig. 4). Transwell assays were performed and the cells stained with crystal violet were imaged in 5 random fields in each experimental group. Representative images of cell migration (Fig. 4A) and cell invasion (Fig. 4C) are presented, and transmigrated cells were counted. In cell migration assays, >200 A498 cells were inhibited from migrating through the membrane by shZ38-1, and ~60% of the cells were inhibited by shZ38-2 following 24 h of incubation. Similar results were observed in ACHIN cells (Fig. 4B). In invasion assays, infection of shZ38-1 or shZ38-2 suppressed ~60% of A498 cell

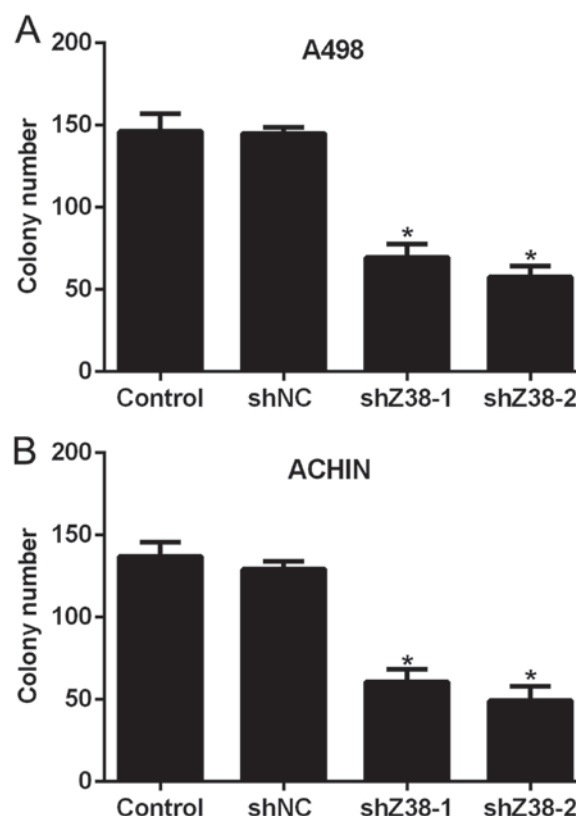


Figure 3. Knockdown of Z38 in A498 and ACHIN cells inhibits colony formation. (A) A total of >50% colonies were inhibited when A498 cells were transfected with specific shRNAs against Z38 following 14 days of incubation. (B) A total of >50% colonies were suppressed when ACHIN cells were treated with specific shRNAs against Z38. \* $P < 0.05$  vs. Control. shRNA, short hairpin RNA; shNC, scramble shRNA.

invasion through the membrane; similarly, >61% of ACHIN cells were inhibited from invading to the lower surface of the membrane (Fig. 4D). The results of the present study suggested that the knockdown of Z38 in A498 and ACHIN cells inhibited cell metastasis.

*Knockdown of Z38 in A498 cells decreases the expression of cell cycle markers and interrupts EMT.* EMT is associated with tumorigenesis. Therefore, cell cycle regulators cyclin B1 and CDC25C, and EMT markers including mesenchymal N-cadherin and epithelial E-cadherin, were detected in A498 cells with shZ38 transfection using western blot analysis. As presented in Fig. 5, when Z38 was knocked down by specific shRNAs in A498 RCC cells, the protein levels of cyclin B1, CDC25C and N-cadherin were decreased, while the expression of E-cadherin was increased. The results of the present study were consistent with the above functional observations, demonstrating that knockdown of Z38 inhibited cell proliferation and metastasis.

## Discussion

RCC accounts for ~3% of all cases of malignancy in adults, with 61,560 new cases and 14,080 mortalities in 2015 in the United States (16). Although progress has been made in RCC diagnosis and treatment, >33% of patients present with metastatic disease when first diagnosed (17). Therefore, further

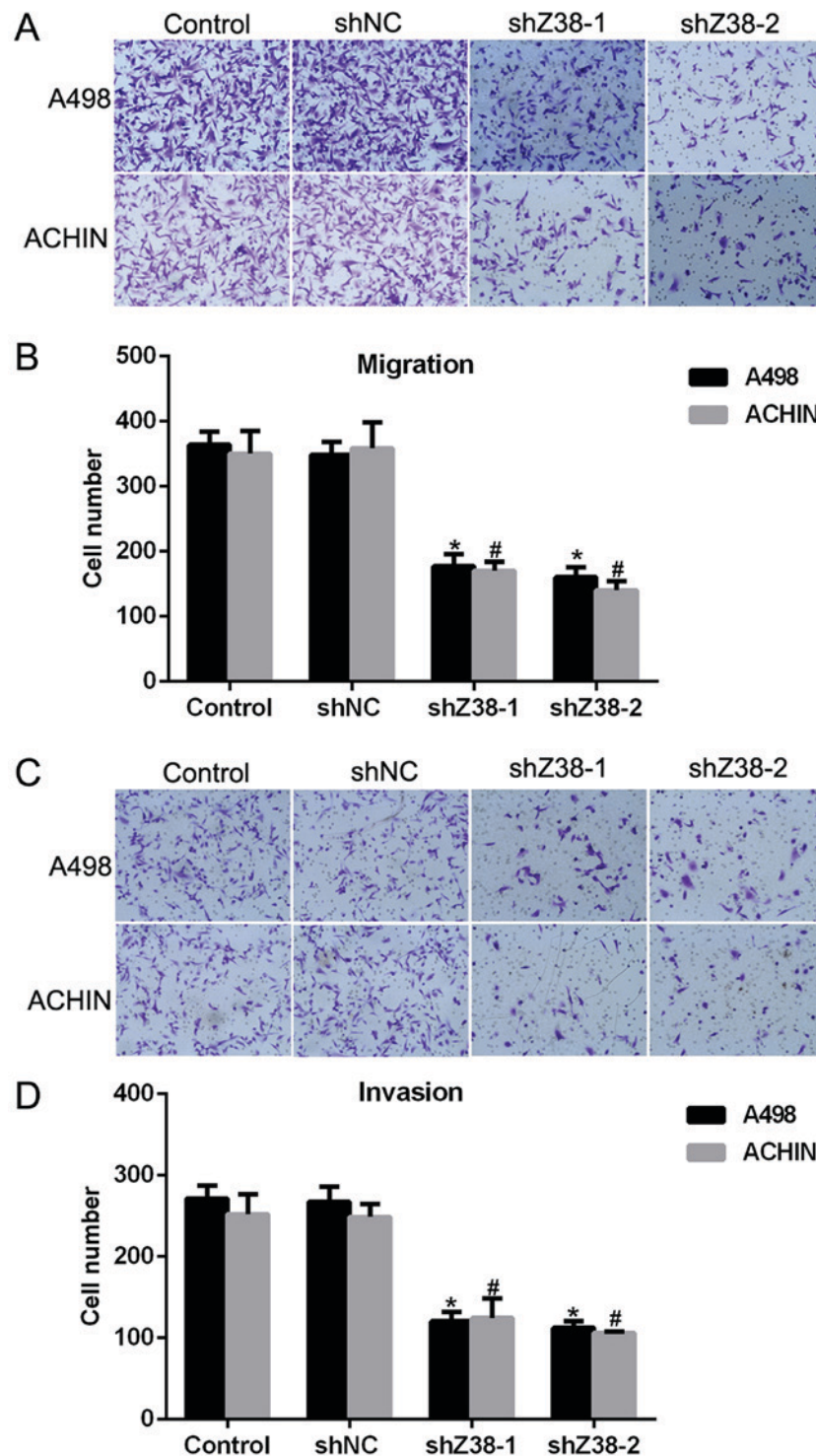


Figure 4. Knockdown of Z38 suppresses cell metastasis in A498 and ACHIN renal cell carcinoma cells. (A) Representative images of cell migration in A498 and ACHIN cells. The images were captured under a light microscope at a magnification of x100, with 5 random fields in each treatment group. (B) Quantification of cell migration assays when A498 and ACHIN cells were infected with shRNAs. (C) Representative images of cell invasion in A498 and ACHIN cells. (D) Quantification of cell invasion assays when A498 and ACHIN cells were infected with shRNAs. \*P<0.05 vs. A498 control; #P<0.05 vs. ACHIN control. shRNA, short hairpin RNA; shNC, scramble shRNA.

studies are required in order to identify novel biomarkers to diagnose RCC in the early stages.

In the present study, the role of LncRNA Z38 in RCC cell proliferation and metastasis was investigated *in vitro*. Cancer cells are cells which no longer respond to the signaling pathways that determine cell growth or death. Instead, cancer cells divide aggressively and obtain a high proliferative rate. This

immortality property underlies the rapid growth of tumors and makes cell proliferation a common research target in the study of tumorigenesis (18,19). Metastasis is an additional feature of malignant cancer cells (20). Despite the proliferative and metastatic properties of RCC cells, the results of the present study demonstrated that the knockdown of Z38 significantly inhibited cell proliferation and metastasis in RCC cell lines,

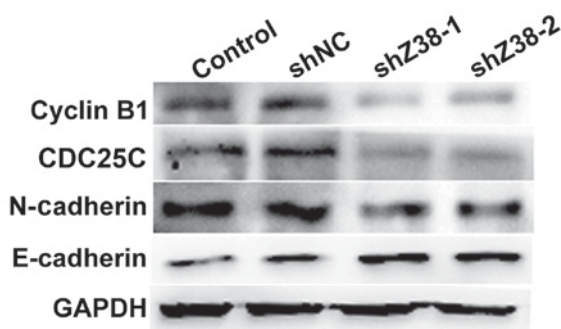


Figure 5. Knockdown of Z38 in A498 cells decreases the expression of cell cycle markers and interrupts epithelial-mesenchymal transition. Western blot analysis demonstrated that the knockdown of Z38 in A498 cells inhibited the expression of cyclin B1, CDC25C and N-cadherin; whereas it increased the protein level of E-cadherin. GAPDH was included as an internal control. CDC25C, M phase inducer phosphatase 3; sh, short hairpin; shNC, scramble shRNA.

which demonstrated the role of Z38 in cell proliferation and metastasis in RCC. The inhibitory effects of Z38 knockdown on cell proliferation and metastasis suggest that it may be a potential therapeutic target in the treatment of RCC.

EMT is a process, during which epithelial cells lose their cell-cell adhesion and polarity, and gain the migratory and invasive potential for transition to mesenchymal cells. This process is considered to serve a role in the initiation of cell metastasis for cancer progression (21,22). Mesenchymal N-cadherin and epithelial E-cadherin are two biomarkers of EMT (23,24). As EMT induction has been associated with metastasis in clinical observations and in the literature, biomarkers of EMT were investigated in the present study. The results of the present study demonstrated that when the expression of Z38 was knocked down by shZ38-1 or shZ38-2, the protein level of N-cadherin was decreased, while the expression of E-cadherin was notably increased. The results of the present study suggested that Z38 deletion suppressed the EMT process. As EMT induction is a hallmark of cell metastasis, the results of the present study demonstrated that Z38 promoted metastasis in RCC cells.

In conclusion, the present study demonstrated that Z38 was overexpressed in clinical patients with RCC and was associated with RCC stage. Knockdown of Z38 in A498 and ACHIN cells inhibited cell proliferation and metastasis. Z38 knockdown in A498 cells suppressed the expression of cyclin B1, CDC25C and N-cadherin, whereas it upregulated the E-cadherin protein level. The results of the present study additionally demonstrated that Z38 deprivation in RCC cells inhibited the EMT process. The present study may provide novel clues for RCC diagnosis in the clinic and Z38 may serve as a novel therapeutic target for the treatment of RCC.

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