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Knockdown of Upregulated Gene 11 (URG11) Inhibits Proliferation, Invasion, and β-Catenin Expression in Non-Small Cell Lung Cancer Cells

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Upregulated gene 11 (URG11), a new gene upregulated by hepatitis B virus X protein, was found to be involved in the development and progression of several tumors. However, the role of URG11 in human non-small cell lung cancer (NSCLC) has not yet been determined. Therefore, the aim of the present study was to explore the role of URG11 in human NSCLC. Our results found that URG11 was highly expressed in human NSCLC tissues compared with matched normal lung tissues, and higher levels were found in NSCLC cell lines in comparison to the normal lung cell line. Moreover, we also found that knockdown of URG11 significantly inhibited proliferation, migration/invasion of NSCLC cells, as well as suppressed tumor growth in vivo. Furthermore, knockdown of URG11 suppressed the expression of β-catenin, c-Myc, and cyclin D1 in NSCLC cells. Taken together, the study reported here provided evidence that URG11 downregulation suppresses proliferation, invasion, and β-catenin expression in NSCLC cells. Thus, URG11 may be a novel potential therapeutic target for NSCLC.

Key words: Non-small cell lung cancer (NSCLC); Upregulated gene 11 (URG11); Proliferation; Invasion; Wnt/β-catenin signaling pathway

INTRODUCTION

Lung cancer is the most common reason for cancer-related deaths, with approximately 2 million new cases diagnosed in the world annually (1). Non-small cell lung cancer (NSCLC) accounts for 80% of primary lung cancer (2). Despite great improvements in chemotherapy and molecular-targeted treatment, the 5-year survival rate of NSCLC patients is still around 15% (3–5). Thus, it is urgent to reveal the molecular mechanisms underlying NSCLC carcinogenesis and progression.

Upregulated gene 11 (URG11), a new gene upregulated by hepatitis B virus X protein, encodes a 70-kDa protein that consists of five chordin-like cysteine-rich repeats and a single C-type lectin domain (6). These domains have been implicated in cell adhesion, migration, and cell/matrix interaction (7–9). Previous studies demonstrated that URG11 plays an important role in tumor development and progression. For example, Peng et al. reported that URG11 is highly expressed in pancreatic cancer specimens, and knockdown of URG11 obviously regulated the expression pattern of epithelial–mesenchymal transition (EMT) markers and reduced the invasion of pancreatic cancer cells (10). Another study showed that URG11 was highly expressed in gastric cancer tissues compared with adjacent nontumorous tissues, and knockdown of URG11 expression by small interfering RNA (siRNA) significantly inhibited the proliferation, anchorage-independent growth, invasiveness, and metastatic potential of gastric cancer cells (11). However, the role of URG11 in human NSCLC has not yet been determined. Therefore, the aim of the present study was to explore the role of URG11 in human NSCLC. Our results demonstrated that URG11 might play an important role in human NSCLC progression.

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MATERIALS AND METHODS

Sample Collection

NSCLC samples and paired adjacent nontumor samples were collected from Hunan Cancer Hospital, The Affiliated Cancer Hospital of Xiangya School of Medicine (China). No local or systemic treatment had been conducted on these patients before the operation. The samples were immediately stored in liquid nitrogen in preparation for use. The informed consents from all of the patients involved were obtained prior to the initiation of this study. The study was approved by the Research Ethics Committee of Hunan Cancer Hospital, The Affiliated Cancer Hospital of Xiangya School of Medicine.

Cell Culture

Three human NSCLC cell lines (A549, 95-D, and H1299) and a normal human bronchial epithelial cell line (HBE) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM (Gibco BRL, Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100 IU/ml penicillin (Sigma-Aldrich) at 37°C with 5% CO_2 and 95% humidity.

Cell Transfection

The short hairpin RNA (shRNA) used against URG11 (shURG11: 5′-GCACCTACACAGGCAGAATCTCTCG AGAGATTCTGCCTGTGTAGGTGC-3′) was designed and chemically synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). The A549 and H1299 cells were transfected with shURG11 using Lipofectamine 2000 (Invitrogen).

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from the tissues or cells using TRIzol reagent (Invitrogen). Up to 3 μg of the total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). RT-qPCR was carried out on an ABI7500 cycler with the following protocol: initial denaturation at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 5 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s. The following PCR primers were used: URG11: 5′-TGAATCAAGGAGTCGCTGGAC-3′ (sense) and 5′-GCATCTCACTGGAACACAAG-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-AATGAAGGGGTCATTGATGG-3′ (sense) and 5′-AAGGTGAAGGTCGGAGTCAA-3′ (antisense). GAPDH was used as a quantitative and qualitative control to normalize the gene expression. Data were analyzed using the formula: $R = 2^{-[\Delta CT\ sample - \Delta CT\ control]}$.

Western Blotting

Proteins were extracted using RIPA lysis buffer [100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EDTA, 10 mM β-glycerophosphate, 2 mM sodium vanadate, and protease inhibitor]. The protein concentration was measured using a BCA Protein Assay Kit (BioTeke, Beijing, China). The proteins were isolated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). The membrane was incubated with primary antibodies against URG11, β-catenin, cyclin D1, and c-Myc, and GAPDH (1:2,000; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Next, peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted in 1:3,000 was added and incubated for 1 h at room temperature. Finally, the protein binding was detected by an enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK).

MTT Assay

For MTT assay, cells transfected with shURG11 or scramble were seeded into 96-well plates at a density of 1×10^5 cells/well. After 1, 2, 3, or 4 days of culture, 20 μl of MTT was added to each well and incubated for 4 h at 37°C with 5% CO_2. Next, 100 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals, and the absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Transwell Migration and Invasion Assays

Cell migration and invasion were performed in a 24-well plate using Transwell chambers (BD Biosciences, San Jose, CA, USA). For the invasion assay, the chamber was precoated with 30 ml of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C for 2 h to form a reconstituted basement membrane, whereas the migration assay was not. In brief, 1×10^5 cells resuspended in 100 μl of serum-free medium were added to the upper chamber, and 500 μl of DMEM containing 10% FBS was added into the lower compartment. The noninvading cells were removed by scraping 12 h (for migration assay) or 24 h (for invasion assay) after incubation at 37°C in 5% CO_2. The cells that invaded through the membrane were fixed in 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 30 min. Then cells invading cells across the membrane were counted under a light microscope (Olympus, Tokyo, Japan).

Tumor Xenograft Growth Assay In Vivo

Female Balb/C nude mice (5–6 weeks old) were from SLAC Laboratory Animal Corp. (Shanghai, China). Cells were digested and resuspended with cold

phosphate-buffered saline (PBS) at a density of 1×10^6 cells/ml. The cell suspension (100 µl) was subcutaneously injected into the flank region of nude mice. Tumors were harvested 35 days after injection. Tumor growth was monitored by tumor volume, which was measured with calipers and calculated as described, V (mm^3) = width^2 (mm^2) × length (mm)/2. Finally, tumors were harvested, and tumor volume and weight were measured. Animal experiments were carried out with the approval of the ethics committee of Hunan Cancer Hospital, The Affiliated Cancer Hospital of Xiangya School of Medicine.

Statistical Analysis

Results are expressed as mean ± SD. Differences between the two groups were analyzed by Student's *t*-test. Differences between multiple groups were analyzed by analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

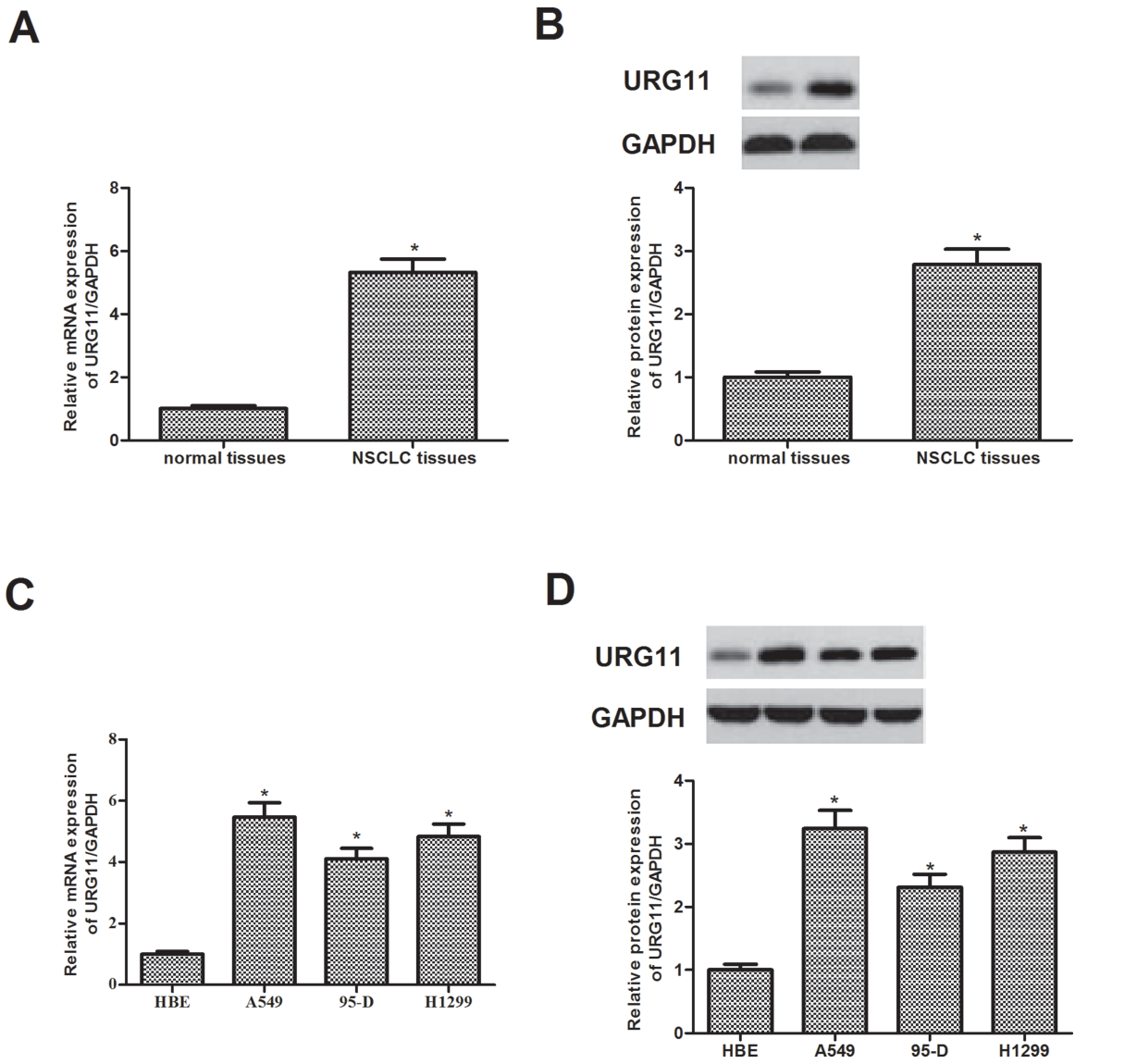

Figure 1. URG11 is highly expressed in NSCLC tissues and cell lines. (A) The expression levels of URG11 mRNA in human NSCLC tissues were analyzed by real-time quantitative polymerase chain reaction (RT-qPCR). (B) URG11 protein was also significantly upregulated in human NSCLC tissues. (C) Expression levels of URG11 mRNA in human NSCLC cell lines. (D) Representative Western image of URG11 protein in human NSCLC cell lines. Data are mean ± SD. All experiments were repeated at least three times. *$p < 0.05$ compared with the scramble group.

RESULTS

URG11 Is Overexpressed in NSCLC Tissues and Cell Lines

First, we measured the expression of URG11 in human NSCLC and pair matched adjacent lung normal tissues by RT-qPCR. As shown in Figure 1A, URG11 expression in mRNA level was 5.32 times in NSCLC tissues of that in adjacent normal tissue. Western blot analysis showed that expression of URG11 in NSCLC tissues was much higher than adjacent normal tissues (Fig. 1B). Next, we examined URG11 expression in NSCLC cell lines, and results demonstrated a higher expression of URG11 in

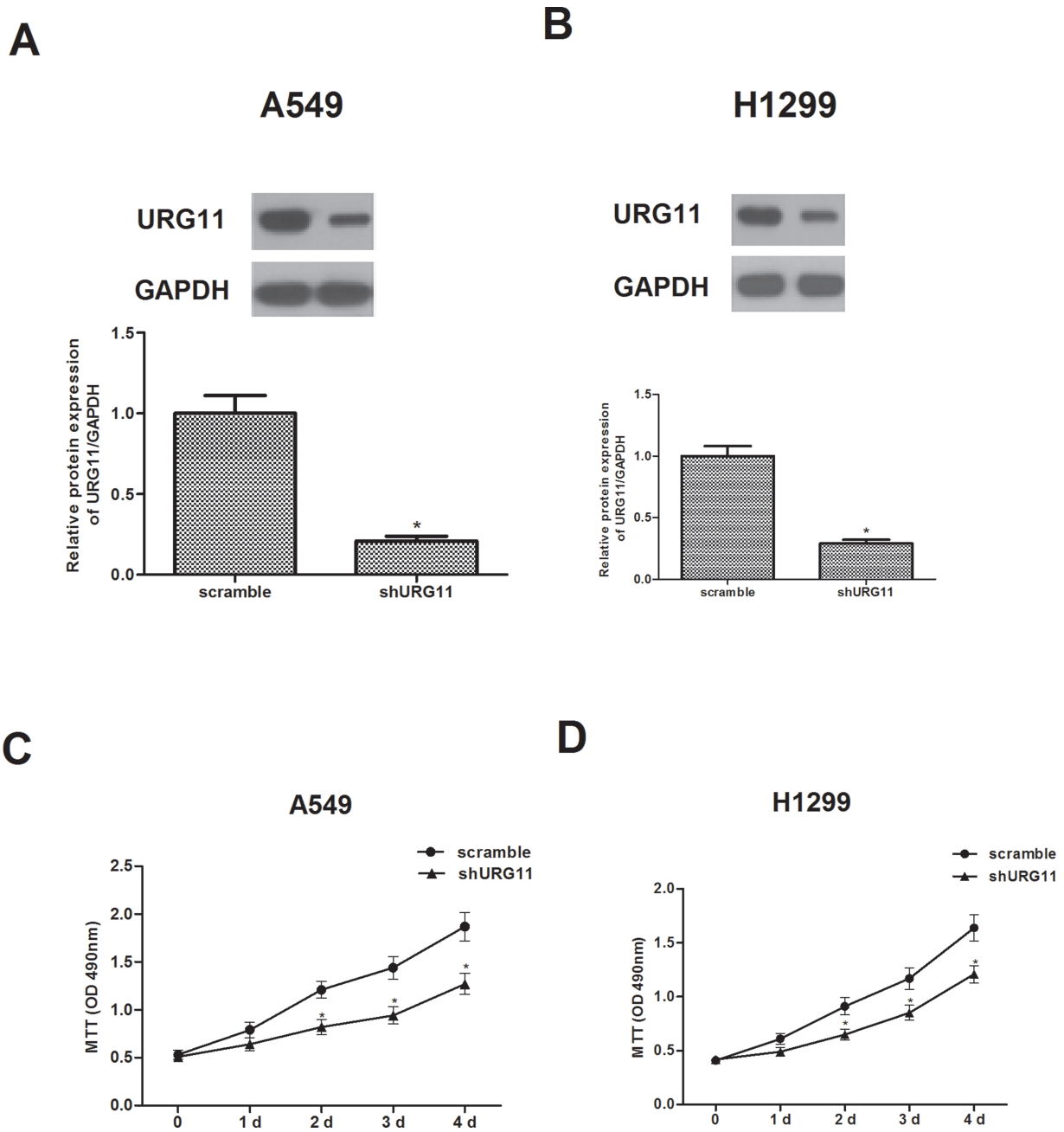

Figure 2. Knockdown of URG11 inhibits NSCLC cell proliferation in vitro. The protein expression of URG11 was confirmed in A549 (A) and H1299 (B) cells after transfection of shURG11, respectively, using Western blot. Knockdown of URG11 significantly inhibited the proliferation in A549 (C) and H1299 (D) cells. Data are mean ± SD. All experiments were repeated at least three times. *$p < 0.05$ compared with the scramble group.

A549, 95-D, and H1299 cell lines, compared with that in normal human bronchial epithelial cell line (HBE) (Fig. 1C and D).

Knockdown of URG11 Inhibits NSCLC Cell Proliferation in Vitro

To investigate the role of URG11 in NSCLC, we treated A549 and H1299 cells with shURG11 or scramble for 24 h. The efficacy of URG11 knockdown was confirmed by Western blot. Western blot assay revealed that siURG11 significantly decreased the protein level of URG11 in A549 and H1299 cells, respectively (Fig. 2A and B). We then examined the role of URG11 in NSCLC cell (A549 and H1299) proliferation. MTT assay results indicated that knockdown of URG11 significantly inhibited A549 cell proliferation with prolonged culture time, as compared with the scramble control (Fig. 2C). Similar results were observed in H1299 cells (Fig. 2D).

Knockdown of URG11 Reduced Xenografted Tumor Growth In Vivo

To further explore the role of URG11 on colon tumor growth in vivo, the xenografted tumor in nude mouse was employed. As indicated in Figure 3A, knockdown of URG11 significantly suppressed tumor weight of BALB/c nude mice compared with the scramble control. In addition, knockdown of URG11 greatly reduced the volume of the xenografted tumor (Fig. 3B).

Knockdown of URG11 Inhibits NSCLC Cell Migration and Invasion In Vitro

We examined the role of URG11 in A549 and H1299 cell migration and invasion using Transwell migration/invasion assays. Results indicated that knockdown of URG11 markedly inhibited the migration in A549 and H1299 cells, respectively, compared with the scramble control (Fig. 4A and B). In addition, knockdown of URG11 produced a marked inhibition of invasion in A549 (Fig. 4C) and H1299 (Fig. 4D) cells through Matrigel on Transwell assay, with average inhibiting rates of 42.3% and 48.3%, respectively.

Knockdown of URG11 Inhibits the Activation of Wnt/β-Catenin Pathway in NSCLC Cells

There is some evidence showing that the Wnt/β-catenin signaling pathway is involved in the proliferation and invasion of NSCLC cells (12–14). To further investigate a potential mechanism that could be involved in the growth and migration inhibition of NSCLC cells, we investigated the effect of URG11 on the expression of β-catenin, cyclin D1, and c-Myc. The results of Western blot analysis showed that knockdown of URG11 significantly downregulated the expression of β-catenin, c-Myc, and cyclin D1 in A549 cells, compared with the scramble control (Fig. 5A), and the optical density of each protein was quantified by GAPDH optical density (Fig. 5B).

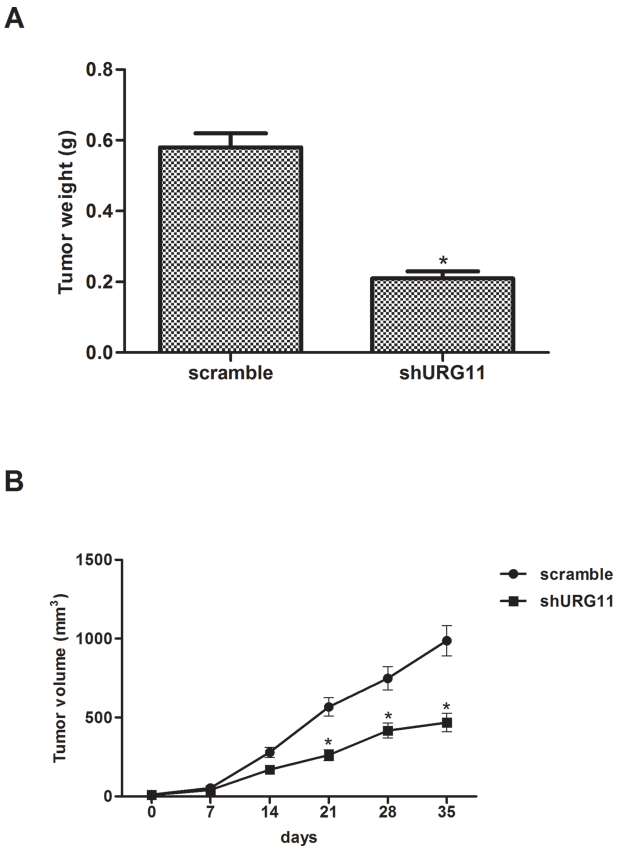

Figure 3. Knockdown of URG11 inhibits tumor growth in an allograft murine model. (A) Stable URG11 knockdown cells or scramble cells were subcutaneously implanted into the flank of nude mice ($n=5$). At day 35, the mice were killed. The tumor volumes were calculated in each group every week. (B) The tumor weights were measured at day 35. Data are mean ± SD. All experiments were repeated at least three times. $*p<0.05$ compared with the scramble group.

DISCUSSION

In the present study, we first found that URG11 was highly expressed in human NSCLC tissues compared with matched normal lung tissues, and higher levels were found in NSCLC cell lines in comparison to the normal lung cell line. Moreover, we also found that knockdown of URG11 significantly inhibited proliferation, migration/invasion of NSCLC cells, as well as suppressed tumor growth in vivo. Furthermore, knockdown of URG11 suppressed the expression of β-catenin, c-Myc, and cyclin D1 in NSCLC cells.

Although URG11 has been shown to be overexpressed in several types of cancer and has been related to cancer progression and development, its roles in human NSCLC remain unclear. In the present study, we found that URG11

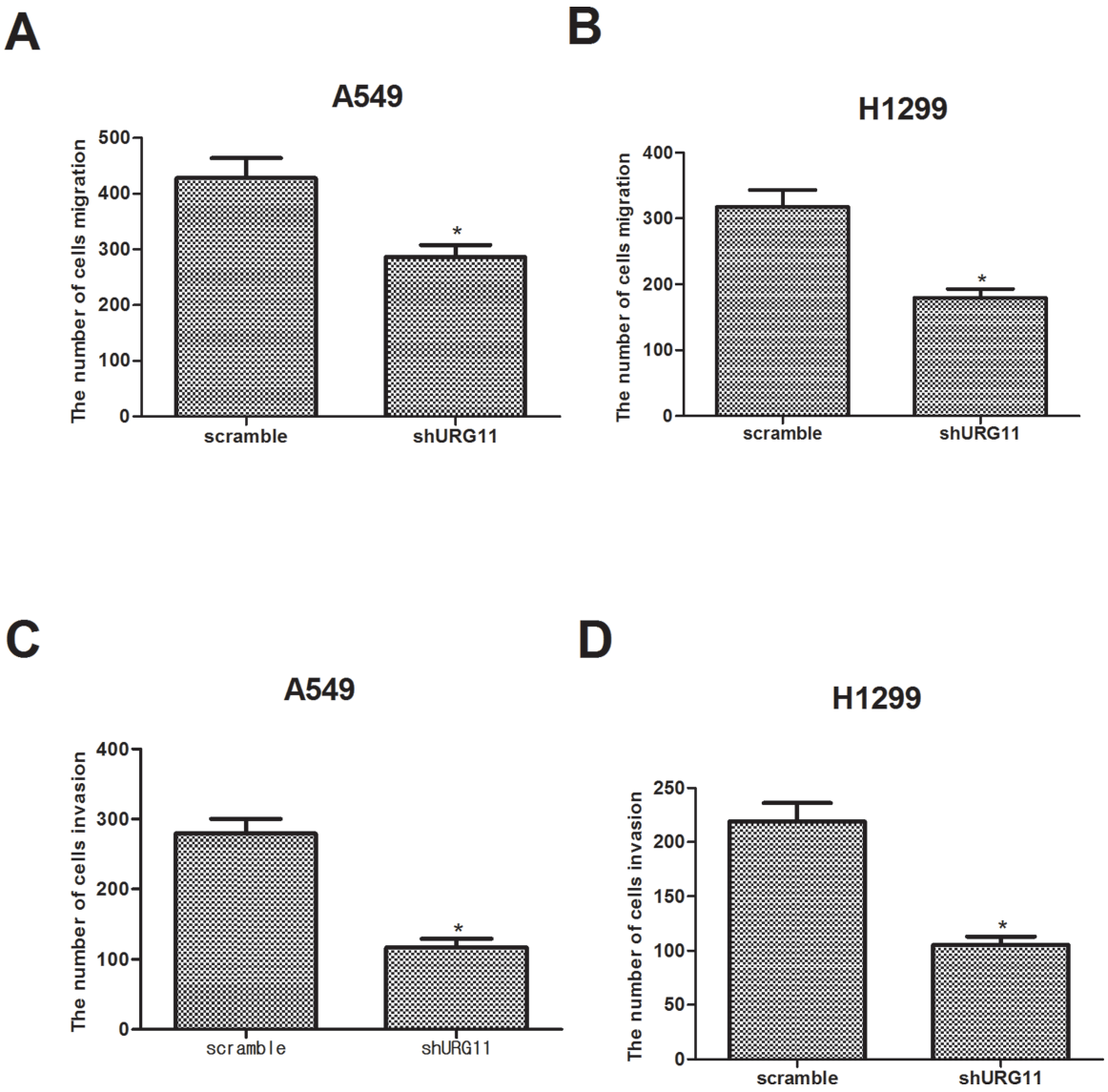

Figure 4. Knockdown of URG11 inhibits NSCLC cell migration and invasion in vitro. The migration of A549 (A) and H1299 (B) cells transfected with shURG11 was evaluated by Transwell assay. The invasion of A549 (C) and H1299 (D) cells transfected with shURG11 was detected by Transwell assay with Matrigel. Data are mean ± SD. All experiments were repeated at least three times. *$p < 0.05$ compared with the scramble group.

was highly expressed in human NSCLC tissues and cell lines, which indicated that URG11 may function as an oncogene involved in the development and progression of NSCLC.

Aberrantly expressed URG11 plays an important role in carcinogenesis. It has been reported that knockdown of URG11 inhibited the proliferation through downregulation of several G_1/S phase-related molecules in hepatocellular carcinoma cells, as well as attenuates hepatocellular carcinoma tumor growth in nude mice (15). Another study showed that knockdown of URG11 significantly inhibited the invasion of pancreatic cancer cells (10). Consistent with previous findings, in this study we found that knockdown of URG11 significantly inhibited proliferation, migration/invasion of NSCLC cells, as well as suppressed tumor growth in vivo. All of these data suggest that URG11 may play an important role in the development and progression of NSCLC.

Wnt/β-catenin signaling pathway plays an important role in promoting tumor progression, dysregulation of cell proliferation, and invasion (16–18). β-Catenin is a critical end component of the Wnt signaling pathway that regulates the expression of cyclinD1, c-Myc, and matrix metalloprotease-7, leading to uncontrolled cell proliferation

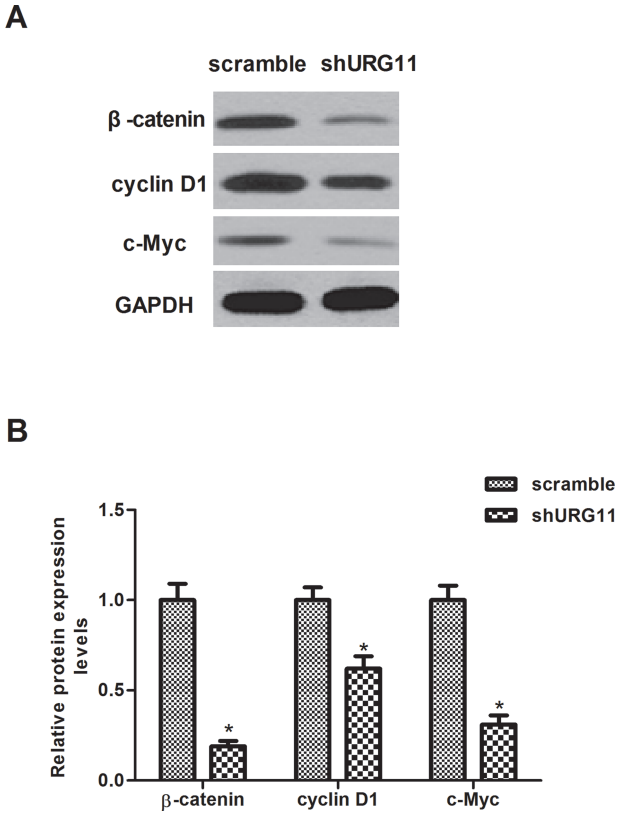

Figure 5. Knockdown of URG11 inhibits the activation of Wnt/β-catenin signaling pathway in NSCLC cells. (A) Western blot assays for the cell lysates showed the downregulation of β-catenin, c-Myc, and cyclin D1 in A549 cells transfected with shURG11. GAPDH served as the loading control. (B) The optical density of each protein was quantified by GAPDH optical density. Data are mean ± SD. All experiments were repeated at least three times. *$p < 0.05$ compared with the scramble group.

and invasion (19–21). Emerging studies have indicated that the Wnt/β-catenin signaling is implicated in NSCLC tumorigenesis (22–24). Hypermethylation of β-catenin promoters was seen in two primary NSCLC cell lines, and loss of β-catenin was correlated with lymph node metastasis and high TNM stages in multiple cancers and associated with poor prognosis in NSCLC (25–27). Akiri et al. confirmed that β-catenin is frequently overexpressed in NSCLC cell lines and in primary NSCLC, and downregulation of activated Wnt signaling inhibited NSCLC proliferation and induced a more differentiated phenotype (28). Most interestingly, one study showed that suppression of endogenous URG11 expression decreased the activation of β-catenin/TCF and its downstream effector genes, cyclin D1, and membrane type 1 matrix metallopeptidase (MT1-MMP) in gastric cancer cells (11). In agreement with the previous studies, herein we found that knockdown of URG11 suppressed the expression of β-catenin, c-Myc, and cyclin D1 in NSCLC cells.

Taken together, the study reported here provided evidence that URG11 downregulation suppresses proliferation, invasion, and β-catenin expression in NSCLC cells. Our study uncovered a novel role for URG11 in NSCLC progression; thus, URG11 may be a novel potential therapeutic target for NSCLC.

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