

# Knockdown of Ubiquitin-Specific Protease 14 (USP14) Inhibits the Proliferation and Tumorigenesis in Esophageal Squamous Cell Carcinoma Cells

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Ubiquitin-specific protease 14 (USP14), one of three proteasome-associated deubiquitinating enzymes (DUBs), plays an essential role in the development of human carcinoma. However, to the best of our knowledge, the role of USP14 in esophageal squamous cell carcinoma (ESCC) is unknown. In the current study, we investigated the expression and role of USP14 in ESCC. Our results showed that the level of USP14 was significantly increased in ESCC tissues and cell lines. Downregulation of USP14 significantly inhibited ESCC cell proliferation and ESCC tumor growth in nude mice. Downregulation of USP14 also suppressed the migration/invasion in ESCC cells. Mechanically, downregulation of USP14 decreased the protein expression levels of  $\beta$ -catenin, cyclin D1, and c-Myc in ESCC cells. In conclusion, our study shows that USP14 plays an important role in the progression and metastasis of ESCC. Therefore, these data suggest that USP14 may be a potentially useful therapeutic strategy for the treatment of ESCC.

**Key words:** Ubiquitin-specific protease 14 (USP14); Esophageal squamous cell carcinoma (ESCC); Proliferation; Invasion

## INTRODUCTION

Esophageal cancer (EC) is one of the most common malignancies worldwide. Esophageal squamous cell carcinoma (ESCC) is the predominant subtype of cancer of the esophagus. In China, the incidence of EC has tended to increase in the last 5 years<sup>1</sup>. Few significant improvements in the overall survival of ESCC patients have been achieved; the 5-year survival rate of EC patients is less than 30%<sup>2-5</sup>. Furthermore, metastasis is the main cause of mortality in patients with ESCC<sup>6</sup>. Therefore, there is an urgent need to explore the molecular mechanisms underlying EC tumorigenesis and find new potential treatment targets.

Deubiquitinating enzymes (DUBs) are key components of the ubiquitin-dependent protein degradation pathway and regulate a number of metabolic processes including cell growth, differentiation, and apoptosis<sup>7</sup>. Ubiquitin-specific protease 14 (USP14) is a major regulator of the proteasome and one of three proteasome-associated DUBs<sup>8</sup>. Previous studies reported that USP14 can inhibit the proteasome and protein turnover in cells<sup>9,10</sup>. In addition, dysregulation of USP14 has been reported in the development and progression of several

tumors<sup>11-14</sup>. For example, Zhu et al. reported that expression of USP14 was increased in breast cancer tissues, and downregulation of USP14 significantly inhibited breast cancer cell proliferation and metastasis<sup>15</sup>. However, to the best of our knowledge, the role of USP14 in ESCC is unknown. In the current study, we detected the expression of USP14 in ESCC. In addition, we investigated the roles of USP14 in ESCC. Our findings indicate that USP14 plays a pivotal role during ESCC development and progression and may be a novel diagnosis and therapy target for ESCC.

## MATERIALS AND METHODS

### *Patients and Tissue Specimens*

Ten paired ESCC tissues and their matching adjacent noncancerous tissues were obtained from The Second Affiliated Hospital of Xi'an Jiaotong University School of Medicine (P.R. China). All samples were snap frozen in liquid nitrogen and then stored at  $-70^{\circ}\text{C}$ . This study was approved by the ethics committee of The Second Affiliated Hospital of Xi'an Jiaotong University School of Medicine (P.R. China), and all patients involved in the study gave their written informed consent.

### Cell Culture

Human ESCC cell lines (EC109, TE10, and KYSE70) and a human esophageal epithelial cell line (Het-1a) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> humidified incubator.

### Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from 2 µg of total RNA with AMV Reverse Transcriptase Kit (Promega, Madison, WI, USA). qPCR was performed in triplicate using the SYBR Green PCR Master Mix and the Applied Biosystems 7300 RT-PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of the primer pairs were as follows: USP14, 5'-GAGT-TGGACCTTT-CCAGA-3' (forward) and 5'-TGCTTGACAG-ATGTGA-3' (reverse); β-actin, 5'-ATAGCACAGCCTGGATAGCAACGTAC-3' (forward) and 5'-CACCTTCTACAATGAGCTGCGTGTG-3' (reverse). β-Actin was used as an internal control. The expression levels of the relative genes were calculated using the 2<sup>-ΔΔCt</sup> method.

### Western Blot Analysis

Total protein was extracted from ESCC tissues or cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing a mixture of protease inhibitor. The protein concentration was quantified by the Bradford assay. A total of 50 µg of proteins was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). After blocking with 5% fat-free milk in TBS [20 mmol/L Tris, 0.15 mol/L NaCl (pH 7.0), 0.1% Tween 20], the membranes were incubated with primary antibodies (anti-USP14, anti-E-cadherin, anti-N-cadherin, anti-β-catenin, anti-cyclin D1, anti-c-Myc, and anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the blots were visualized by enhanced chemiluminescence (ECL) detection system (Amersham). The gray intensity analysis was performed using the Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

### Short Hairpin RNA and Cell Transfection

The short hairpin RNA (shRNA) expression vector targeting USP14 (sh-USP14) and its negative control (sh-NC) were generated by Shengong Company (Shanghai, P.R. China). The sh-USP14 or sh-NC was transfected into SW480 cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Results were checked by Western blot at 24 h after transfection.

### Cell Proliferation Assay

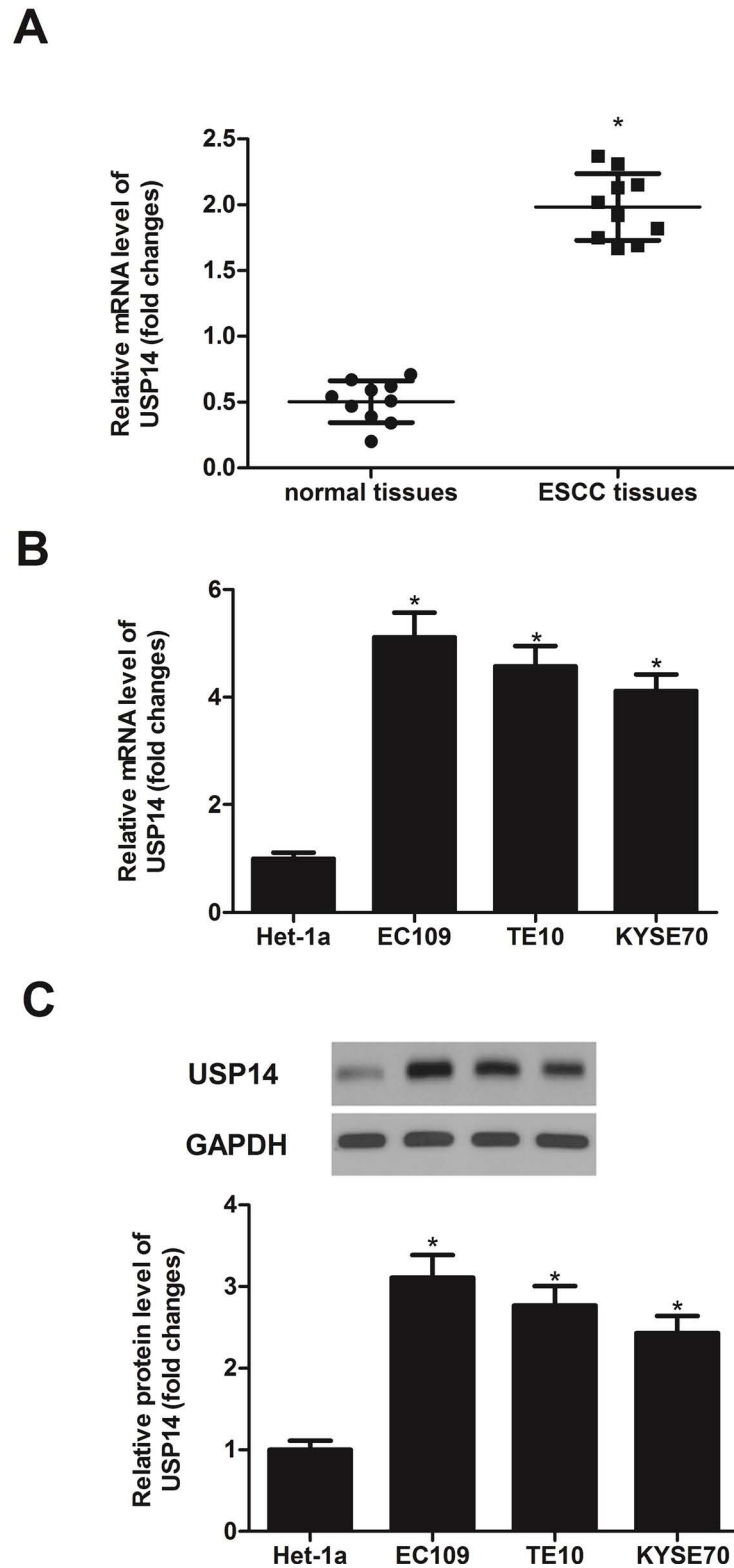
Cell proliferation was evaluated using the MTT assay. Briefly, infected cells at a density of 1 × 10<sup>5</sup> cells/well were seeded in 96-well plates and incubated overnight at 37°C. Cell growth was monitored every day for a period of 4 days. Next, 20 µl of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubation at 37°C for 4 h, the culture medium was removed, and 150 µl of DMSO (Sigma-Aldrich) was then added. Absorbance was measured at 490 nm using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA).

### Cell Migration and Invasion Assays

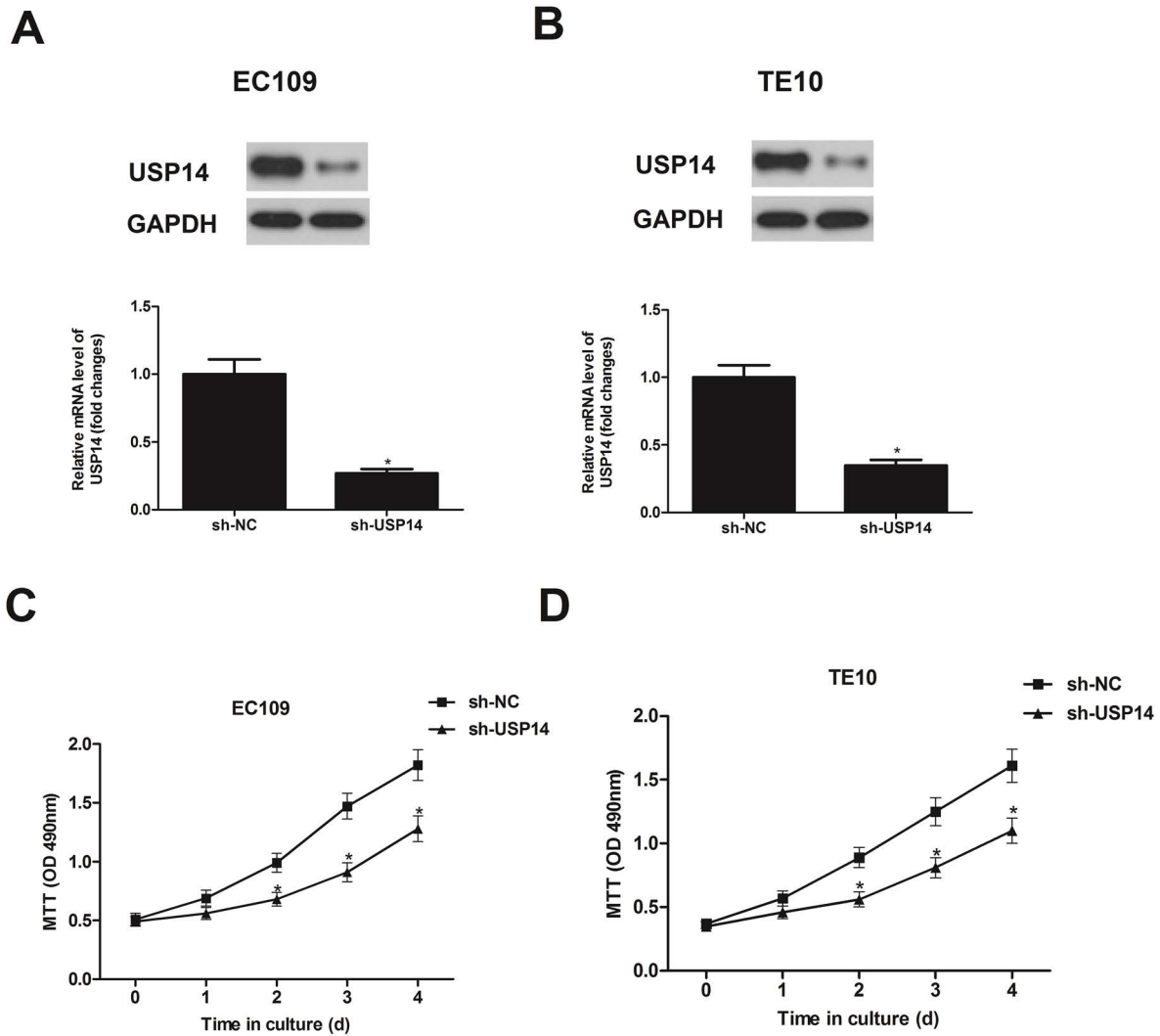
Cell migration and invasion assays were performed as previously described<sup>16</sup>. For the cell invasion assay, infected cells at a density of 1 × 10<sup>5</sup> cells/well were plated onto the top surface of the Transwell insert chambers (Corning, Corning, NY, USA); 600 µl of DMEM containing 10% FBS was added to the lower chamber. After incubation for 24 h, nonmigrated cells were removed, and the membranes containing the migrated cells were fixed in absolute methanol for 10 min, stained with 0.1% crystal violet for 30 min, and counted under a microscope (Olympus, Tokyo, Japan). For the cell invasion assay, the process was analogous to the cell migration assay, except that the membranes were smeared with Matrigel to form a matrix barrier.

### In Vivo Xenograft Tumor Assay

Five-week-old BALB/c female nude mice were purchased from the Experimental Animal Centre of The Second Affiliated Hospital of Xi'an Jiaotong University (P.R. China), and all animal procedures were approved by the Institutional Animal Care and Use Committee at The Second Affiliated Hospital of Xi'an Jiaotong University. For the in vivo study, sh-USP14 EC109 cells (5 × 10<sup>6</sup>) were injected subcutaneously into the dorsal flanks of nude mice. Tumor formation was monitored every 5 days by measuring the tumor size with a caliper. The tumor volume was then calculated using the following formula:  $V = (L \times W^2) / 2$ . Twenty-five days later, the animals were sacrificed by cervical dislocation, and the tumors were excised and weighed.



**Figure 1.** USP14 is highly expressed in ESCC tissues and cell lines. (A) The mRNA expression levels of USP14 were analyzed by qRT-PCR in human ESCC tissues. (B) The mRNA expression levels of USP14 were detected in four ESCC cell lines by qRT-PCR. (C) The protein levels of USP14 were detected in four ESCC cell lines by Western blot. All experiments were repeated at least three times. Data are presented as mean  $\pm$  SD. \* $p$ <0.05 compared to the mock group.



**Figure 2.** Downregulation of USP14 suppresses the proliferation of ESCC cells. Western blot and qRT-PCR analyses were used to detect the knockdown efficiency of USP14 in EC109 (A) and TE10 (B) cells. Cell proliferation was evaluated at indicated time points using CCK-8 assay in EC109 (C) and TE10 (D) cells. All experiments were repeated at least three times. Data are presented as mean  $\pm$  SD. \* $p < 0.05$  compared to the sh-NC group.

### Statistical Analysis

All the statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA). Data are presented as the mean  $\pm$  SD from at least three independent experiments. The significance of differences was analyzed using Student's *t*-test or by one-way ANOVA. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

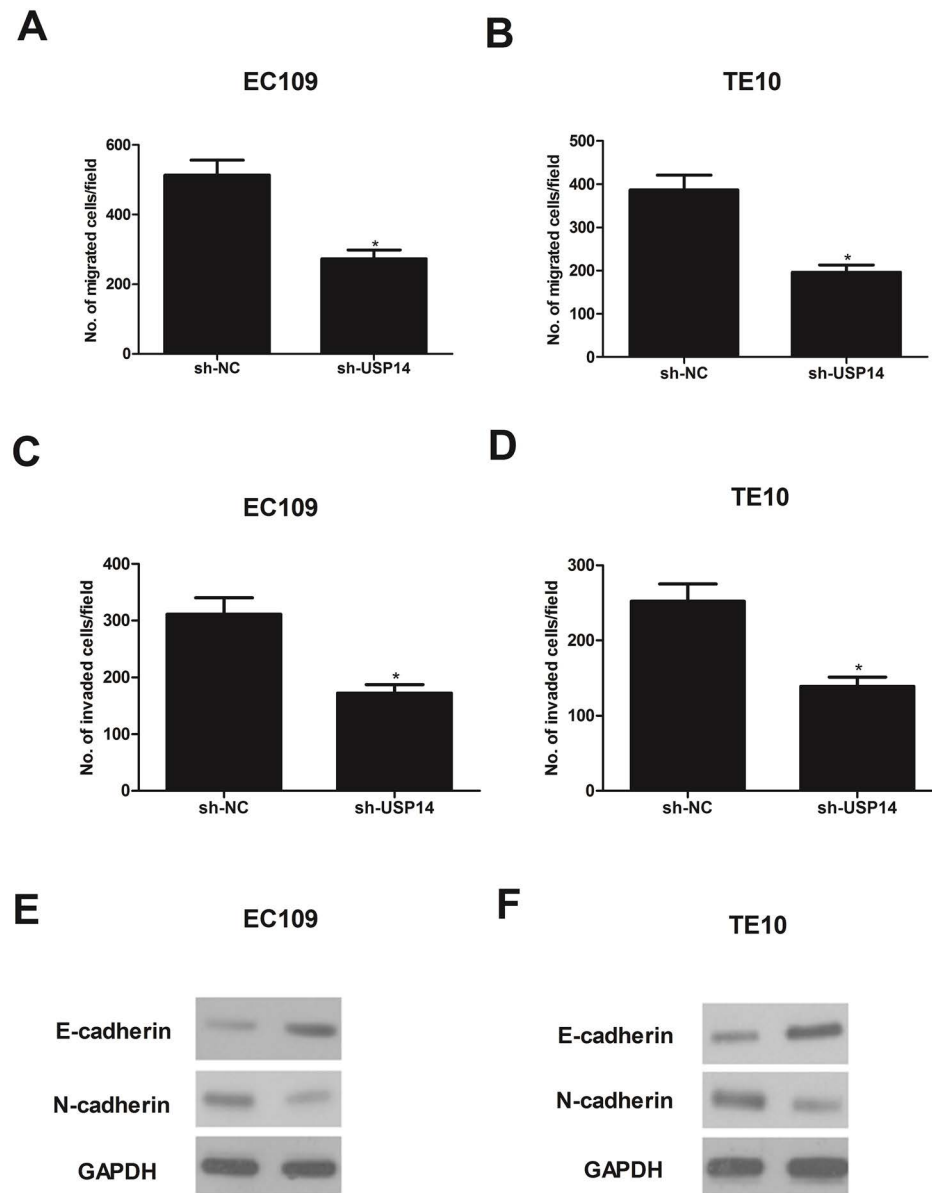
### USP14 Is Highly Expressed in ESCC Tissues and Cell Lines

In order to evaluate the expression of USP14 in ESCC, we examined 10 ESCC tissues via qRT-PCR. We found that the USP14 mRNA levels in ESCC tissues were

higher compared to the corresponding normal control tissue (Fig. 1A). Next, we examined the expression of USP14 in ESCC cell lines. We found that the USP14 expression levels at both mRNA and protein were significantly higher in human ESCC cell lines (EC109, TE10, and KYSE70) than in human esophageal epithelial cell line (Het-1a) (Fig. 1B and C).

### Downregulation of USP14 Suppresses the Proliferation of ESCC Cells

To determine the effect of USP14 on cell proliferation, we depleted endogenous USP14 via a lentivirus vector-based shRNA approach in EC109 and TE10 cells, respectively. After transfection of sh-USP14, both the protein and mRNA levels of USP14 were dramatically decreased in



**Figure 3.** Downregulation of USP14 suppresses the migration and invasion of ESCC cells. EC109 and TE10 cells were transfected with sh-NC or sh-USP14 for 24 h. Transwell assays were performed to evaluate the migration of EC109 (A) and TE10 (B) cells. Matrigel invasion assay was used to detect the invasion of EC109 (C) and TE10 (D) cells. Western blot was used to detect the expression of E-cadherin and N-cadherin in EC109 (E) and TE10 (F) cells. GAPDH was used as a loading control. All experiments were repeated at least three times. Data are presented as mean  $\pm$  SD. \* $p < 0.05$  compared to the sh-NC group.

EC109 (Fig. 2A) and TE10 cells (Fig. 2B), in comparison with control cells. We then examined the effect of USP14 on cell proliferation. The results demonstrated that downregulation of USP14 significantly inhibited the proliferation in EC109 (Fig. 2C) and TE10 cells (Fig. 2D).

#### *Downregulation of USP14 Suppresses the Migration and Invasion of ESCC Cells*

We next evaluated the effect of USP14 on ESCC cell motility. The Transwell assay demonstrated that

downregulation of USP14 significantly inhibited the migration of EC109 (Fig. 3A) and TE10 cells (Fig. 3B). Similarly, the number of EC109 (Fig. 3C) and TE10 cells (Fig. 3D) that invaded the Matrigel membrane was also greatly reduced in sh-USP14-treated groups compared with that in the control groups. In addition, we examined the effect of USP14 on EMT phenotype in ESCC cells. The results of the Western blot indicated that downregulation of USP14 increased the expression of epithelial marker E-cadherin, while it decreased levels

of mesenchymal markers vimentin and N-cadherin in EC109 (Fig. 3E) and TE10 cells (Fig. 3F), respectively.

#### *Downregulation of USP14 Attenuates Tumorigenicity In Vivo*

To further investigate the effect of USP14 on tumor growth,  $1 \times 10^6$  of USP14-depleted cells were subcutaneously inoculated into the left dorsal flanks of female BALB/c-nu mice. The tumor (Fig. 4A) and volume (Fig. 4B) of the tumors were dramatically reduced in the USP14-knockdown group mice compared with the control group ( $p < 0.05$ ).

#### *Downregulation of USP14 Suppresses the Activation of Wnt/ $\beta$ -Catenin Signaling Pathway in ESCC Cells*

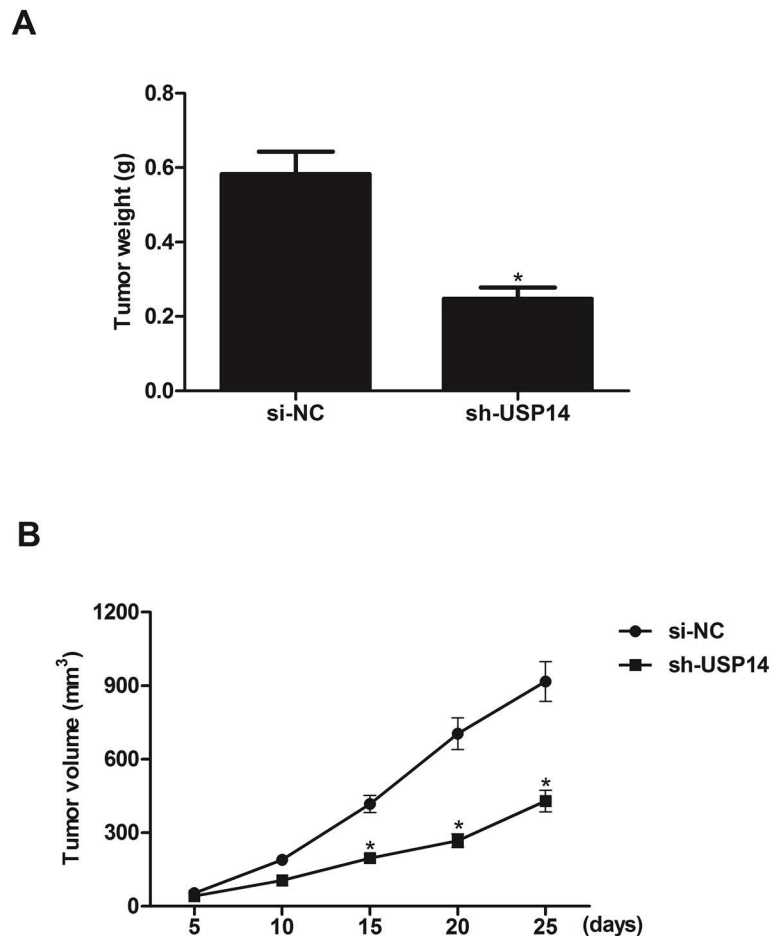
To investigate the molecular mechanism by which USP14 promoted proliferation and invasion in ESCC cells, we examined the effects of USP14 on the activation of the Wnt/ $\beta$ -catenin signaling pathway. The results of the Western blot analysis demonstrated that

the protein expression levels of  $\beta$ -catenin, cyclin D1, and c-Myc were dramatically decreased in EC109 cells transfected with sh-USP14, compared with the sh-NC group (Fig. 5).

## DISCUSSION

To the best of our knowledge, this study is the first to demonstrate the role of USP14 in ESCC. We found that the level of USP14 was significantly increased in ESCC tissues and cell lines. Downregulation of USP14 significantly inhibited ESCC cell proliferation and ESCC tumor growth in nude mice. Downregulation of USP14 also suppressed the migration/invasion in ESCC cells. Mechanically, downregulation of USP14 decreased the protein expression levels of  $\beta$ -catenin, cyclin D1, and c-Myc in ESCC cells.

USP14 has been thought to play an important role in the tumor progression of various cancers. It has been reported that USP14 expression was specifically upregulated in



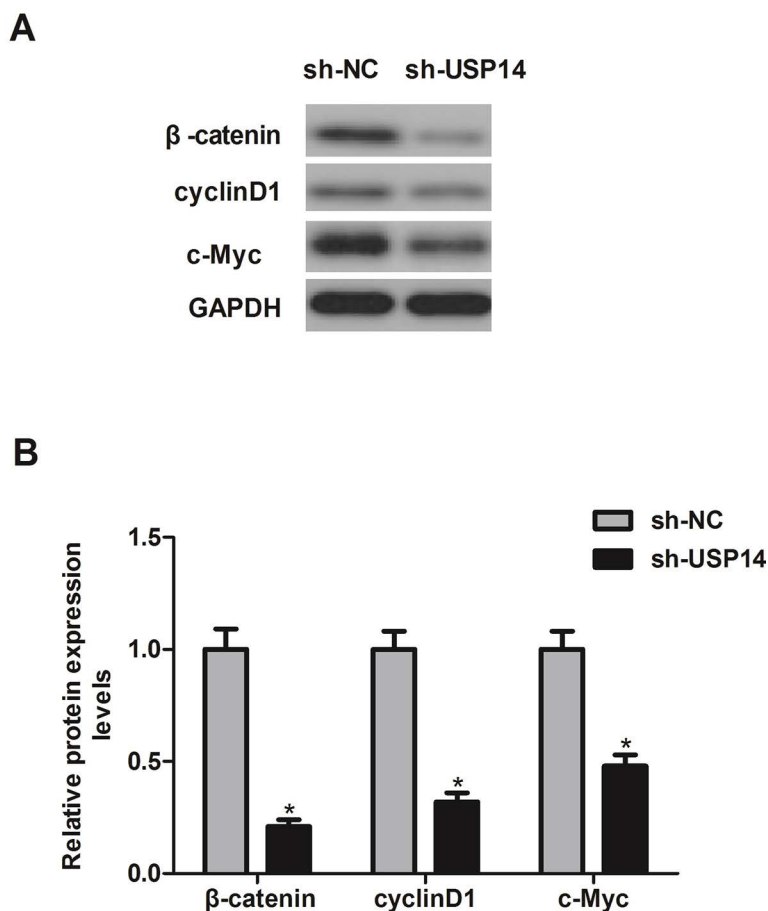
**Figure 4.** Downregulation of USP14 attenuates tumorigenicity in vivo. USP14-depleted cells ( $1 \times 10^6$ ) were subcutaneously inoculated into the left dorsal flanks of female BALB/c-nu mice. (A) Tumor weights of the two groups. (B) Growth curves of tumor size. All experiments were repeated at least three times. Data are presented as mean  $\pm$  SD. \* $p < 0.05$  compared to the sh-NC group.

both lung adenocarcinoma cell lines and tumor tissues, and knockdown of USP14 expression significantly inhibited cell growth and cell cycle arrest in NSCLC cells<sup>12</sup>. Huang et al. found that USP14 is highly expressed in colorectal cancer and human primary hepatocellular carcinoma (HCC) tissues, and knockdown of USP14 with the lentiviral vector delivery of shRNA in human HCC cells suppressed cell proliferation, altered the cell cycle, and induced cell apoptosis<sup>17</sup>. Consistent with these findings, the data obtained in the present study showed that the level of USP14 was significantly increased in ESCC tissues and cell lines. We also found that downregulation of USP14 significantly inhibited ESCC cell proliferation and ESCC tumor growth in nude mice. These findings suggest that USP14 acts as a tumor-promoting gene in the development and progression of ESCC.

EMT is a developmental program associated with cancer progression and metastasis. It initiates the metastatic

cascade and endows cancer cells with invasive and migratory capacities<sup>18</sup>. It has been shown that the repression of E-cadherin acts as one important step driving EMT in various cancers, including ESCC<sup>19,20</sup>. A recent study showed that the positive expression of E-cadherin is significantly increased and N-cadherin<sup>+</sup> and vimentin<sup>+</sup> staining was decreased in the metastatic lymph nodes compared to their paired primary ESCC<sup>21</sup>. In this study, we observed that downregulation of USP14 led to increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal markers N-cadherin and vimentin in ESCC cells, as well as suppressed the migration/invasion of ESCC cells. These results suggest that EMT was involved in the process of USP14-inducing ESCC development.

The Wnt/ $\beta$ -catenin signaling pathway plays an important role in the development and progression of several human malignancies, including ESCC<sup>22-24</sup>.  $\beta$ -Catenin is



**Figure 5.** Downregulation of USP14 suppresses the activation of the Wnt/ $\beta$ -catenin signaling pathway in ESCC cells. (A) EC109 cells were transfected with sh-NC or sh-USP14 for 24 h. Western blot was used to detect the expression of  $\beta$ -catenin, cyclin D1, and c-Myc. GAPDH was used as a loading control. (B) The relative protein expression levels of  $\beta$ -catenin, cyclin D1, and c-Myc were quantified using the Image-Pro Plus 6.0 software. All experiments were repeated at least three times. Data are presented as mean  $\pm$  SD. \* $p < 0.05$  compared to the sh-NC group.

a multifunctional protein that mediates cell–extracellular matrix adhesion and promotes tumor proliferation and metastasis<sup>25</sup>. It was reported that aberrant  $\beta$ -catenin expression was found in 54.3% (144 of 265) of ESCCs, and aberrant  $\beta$ -catenin expression was closely associated with tumor size, tumor location, degree of differentiation, and lymph node status in GSK3 $\beta$ <sup>-</sup> ESCC<sup>26</sup>. He et al. found that inhibition of sal-like protein 4 (SALL4) reduces tumorigenicity via the Wnt/ $\beta$ -catenin signaling pathway in ESCC<sup>27</sup>. Thus, targeting the Wnt/ $\beta$ -catenin pathway potentially represents a promising therapeutic approach for the treatment of ESCC. Our study showed that downregulation of USP14 decreased the protein expression levels of  $\beta$ -catenin, cyclin D1, and c-Myc in ESCC cells. These data suggest that knockdown of USP14 inhibits the proliferation and tumorigenesis in ESCC cells by suppressing and inhibiting the Wnt/ $\beta$ -catenin signaling pathway.

In conclusion, our study shows that USP14 plays an important role in the progression and metastasis of ESCC. Therefore, these data suggest that USP14 may be a potential useful therapeutic strategy for the treatment of ESCC.

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