Am J Transl Res 2017;9(12):5422-5431
www.ajtr.org /ISSN:1943-8141/AJTR0059654

Original Article

Depletion of astrocyte elevated gene-1 suppresses tumorigenesis through inhibition of Akt activity in bladder cancer cells

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Received June 17, 2017; Accepted October 2, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Astrocyte elevated gene-1 (AEG-1) has been reported to promote tumorigenesis, however the molecular mechanisms by which AEG-1-induced bladder cancer progression has remained elusive. Here, we identified that depletion of AEG-1 in bladder cancer cells suppressed cell growth. Moreover, we observed that down-regulation of AEG-1 induced apoptosis and inhibited cell migration and invasion. Furthermore, depletion of AEG-1 inhibited Akt activity and suppressed Bcl-2 expression, but upregulated the levels of p21 and p27. Our findings reveal that AEG-1 carries out its oncogenic function via activation of the Akt pathway. Therefore, inhibition of AEG-1 could be a novel treatment approach for bladder cancer.

Keywords: AEG-1, bladder cancer, proliferation, invasion, pAkt

Introduction

Bladder cancer is one of the most common malignancies worldwide with an estimated 429,800 new cases and 165,100 deaths occurring in 2012 [1]. An approximate annual incidence of 76,960 and mortality of 16,390 are expected in the United States in 2016 [2]. Interestingly, the majority of bladder cancer occurs in men and smoking is the most well-established risk factor for this deadly disease [1]. Incidence rates of bladder cancer have declined in most Western countries due to reduction in smoking prevalence. In addition, increasing the consumption of fruits and vegetables reduces bladder cancer development [1]. However, there is currently no adequate screening method to diagnose bladder cancer. Although multiple therapeutic approaches, including surgery, cystoscopy, laparoscopy micro invasion operation, immunotherapy, and chemotherapy, have been utilized, bladder cancer still has high mortality rate [3-5]. Thus, it is necessaryto identify and develop new therapeutic strategies to treat this disease.

AEG-1, also known as MTDH or LYRIC, was initially identified as a HIV-1 (human immunodeficiency virus type 1)-inducible gene in fetal astrocytes [12]. Multiple studies have revealed that AEG-1 plays a key role in tumorigenesis [6-11] and over-expression of AEG-1 has beenobserved in a wide range of human cancers [11]. Moreover, multiple studies have identified mechanisms by which AEG-1 exerts its oncogenic function [13]. AEG-1 is a target gene of oncogenic Ha-ras and c-Myc [14]. Furthermore, AEG-1 activates the NF-κB (nuclear factor kappa B) and PI3K (phosphatidylinositol 3-kinase)-Akt signaling pathways, leading to tumor progression and metastasis [15-17]. Accordingly, depletion of AEG-1 inhibited prostate cancer progression via upregulation of FOXO3a (forkhead box 3a) activity [18]. Remarkably, AEG-1 was identified as a novel prognostic marker for breast cancer progression and overall patient survival [19]. These studies have highlighted an important role of AEG-1 in tumor progression.

Despite the fact that AEG-1 has been extensively investigated in a variety of human cancers, the precise mechanism of AEG-1-mediated tumorigenesis in bladder cancer cells is less well understood. Here, we investigated the role of AEG-1 in cell growth, apoptosis, migration,

and invasion in bladder cancer cells. Moreover, we explored the molecular mechanism of AEG-1-mediated bladder tumorigenesiss. Our findings demonstrate that down-regulation of AEG-1 inhibited cell growth, enhanced apoptosis, and retarded cell migration and invasion. Intriguingly, we identified that depletion of AEG-1 exhibited anti-tumor activity partly through down-regulation of the Akt pathway in bladder cancer cells. Our data indicates that inhibition of AEG-1 could be a potential therapeutic approach for the treatment of bladder cancer.

Materials and methods

Reagents

Primary antibody for AEG-1 was purchased from Proteintech Group Company. Antibodies against p21, p27, Akt, pAkt (S473), AEG1, Bcl-2 were purchased from Cell Signaling Technology (Danvers, MA). All secondary antibodies were obtained from Thermo Scientific. Lipofectamine 2000 was purchased from Invitrogen. Monoclonal β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). CCK-8 cell viability assay kit was purchased from Beyotime Biotechnology (Shanghai, China), and Transwell inserts and Matrigel were obtained from BD Biosciences.

Cell culture

The bladder cancer cell line T24 was maintained in DMEM medium (Thermo Fisher Scientific, MA, USA). RT4 cells were cultured in McCoy's 5A medium. Both DMEM and McCoy's media were supplemented with 10% FBS (HyClone, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, USA) and cultured in 5% CO_2 at 37°C.

Transfection

Bladder cancer cells (3×10^5 cells/well) were cultured in 6-well plates, and transfected with AEG-1 shRNA or empty vector using lipofectamine 2000 following the manufacturer's instructions. The plasmids for pSuper-AEG-1-shRNA1, pSuper-AEG-2-shRNA2, and pSuper-control-shRNA were provided by Dr. Jun Li (Sun Yat-Sen University Medical School, China). The shRNA sequences for AEG-1 were as follows: shRNA1 GGAGGAGGCTGGAATGAAA; shRNA2 GGTCTCAGATGATGATAAA; Control shRNA CUCCGAACGUGUCACGAAA.

Western blot analysis

Cells were harvested and lysed in RIPA lysis buffer and equal amounts of denatured proteins were subjected to SDS-PAGE electrophoresis and transferred onto PVDF membranes. Membranes were blocked with 5% milk for 1 hour, and subsequently incubated with specific primary antibody at 4°C overnight. Membranes were then washed three times with TBST and incubated with horse dadish peroxidase-conjugated second antibody for 1 hour. ECL reagents (Pierce, Rockford, IL, USA) were used to visualize the protein bands. The membranes were stripped and reprobed with β-actin antibody as the loading control. ImageJ software was utilized for densitometric quantification of the western blot bands.

Immunofluorescence staining

Transfected cells were seeded on cover slips in each well of an 8-well chamber for 48 hours. Cells were then fixed with paraformaldehyde for 15 minutes. After washing cells with PBS, 5% goat serum was added for 30 minutes. Subsequently, the cells were incubated with anti-AEG-1 antibody for 2 hours. After washing with PBS, the cells were incubated with FITC-conjugated secondary antibody for 45 minutes. Cell images were observed with fluorescent microscopy.

Cell viability assay

Transfected bladder cancer cells (4×10^3) were seeded into each well of a 96-well plate. After 48 hours in culture, cells were incubated with CCK-8 at 37°C for 1 hour. Absorbance was measured at 450 nm following the manufacture's instruction.

Cell apoptosis analysis

Transfected bladder cancer cells were seeded in 6-well plates and allowed to incubate at 37°C for 48 hours. Cells were then trypsinized, collected by centrifugation, and washed in PBS. Cells were resuspended in 500 μl binding buffer containing 5 μl Propidium iodide (PI) and 5 μl annexin V-FITC for 15 min. Cells were then subjected to FACS analysis using a FACS calibur

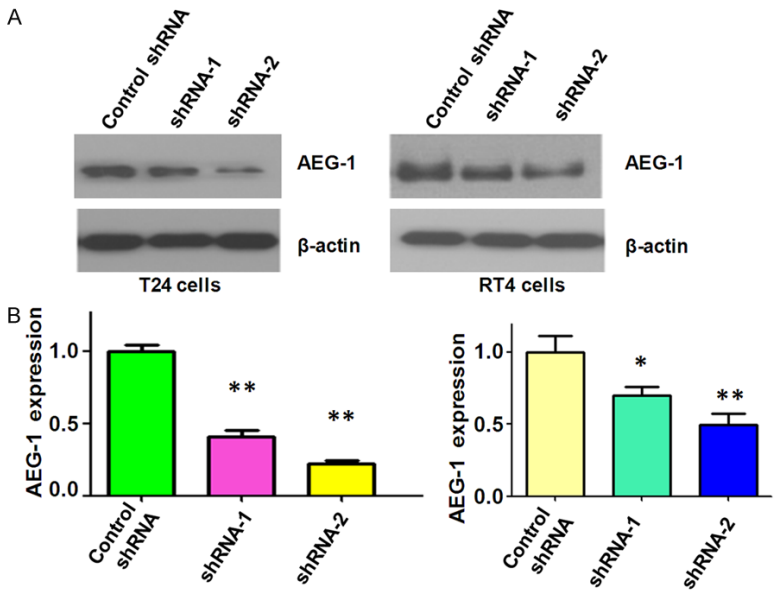

Figure 1. Expression of astrocyte elevated gene-1 (AEG-1) was down-regulated by its shRNA. (A) Western blot analysis of T24 and RT4 cells expressing control shRNA and two AEG-1 shRNAs was used to assess the efficiency of AEG-1 knockdown. Control shRNA: Cells infected with the negative shRNA recombinant virus as the negative control; shRNA-1: Cells infected with AEG-1 shRNA1 recombinant virus; shRNA-2: Cells infected with AEG-1 shRNA2 recombinant virus. (B) Quantitation of results was shown for (A).

flow cytometer, and percent apoptotic cells determined.

Wound healing assay

The transfected bladder cancer cells were incubated in 6-well plates at 37°C overnight. Cells were grown until the cells reached more than 90% confluency, at which time a wound was generated by scratching the surface of the plates with small pipette tip. Detached cells from the plates were removed with PBS and fresh culture medium was added to the remaining cells for 20 hours incubation. The scratched areas were photographed under phase-contrast microscopy. The widths of the wounds of the scratch area were determined by Adobe Photoshop software.

Cell invasion assay

Cell invasion of transfected bladder cancer cells were analyzed by a Transwell insert (8 μm pore size, Corning) with Matrigel according to the manufacturer's instructions. Briefly, bladder cancer cells were added to the upper chamber in 200 μL of serum-free medium. The lower chambers were filled with 500 μL of complete medium (containing 10% FBS as chemo-attractant). After 20 hours incubation in standard culture conditions, cells in the upper surface of the membranes were removed with a cotton swab. The invasive cells on the underside of the membrane were stained with Wright's-Giemsa. At least five randomly-selected images were counted under a light microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 (Graph Pad Software, La Jolla, CA). Differences between transfected group and its control group were evaluated by the *Student's t* test. Independent experiments were repeated in triplicate. All data analyses were presented as mean ± SD. $P<0.05$ were considered significant.

Results

Down-regulation of AEG-1 expression inhibited cell growth

Emerging evidence has shown that AEG-1 plays an important role in the development and progression of human cancers including bladder cancer [7, 20]. To explore whether AEG-1 was critically involved in bladder tumorigenesis, we depleted AEG-1 using shRNA in both T24 and RT4 bladder cancer cell lines. We found that depletion of AEG-1 significantly down-regulated the expression of AEG-1 in T24 and RT4 cells compared to control shRNA expressing cells (**Figure 1A** and **1B**). To investigate whether depletion of AEG-1 in bladder cancer cells influenced cell viability, control cells and cell expressing AEG-1 shRNAs were incubated with CCK-8. We observed that down-regulation of AEG-1 expression led to cell growth inhibition in both T24 and RT4 cells (**Figure 2A** and **2B**). These results suggest that inhibition of AEG-1 may suppress growth of bladder cancer cells.

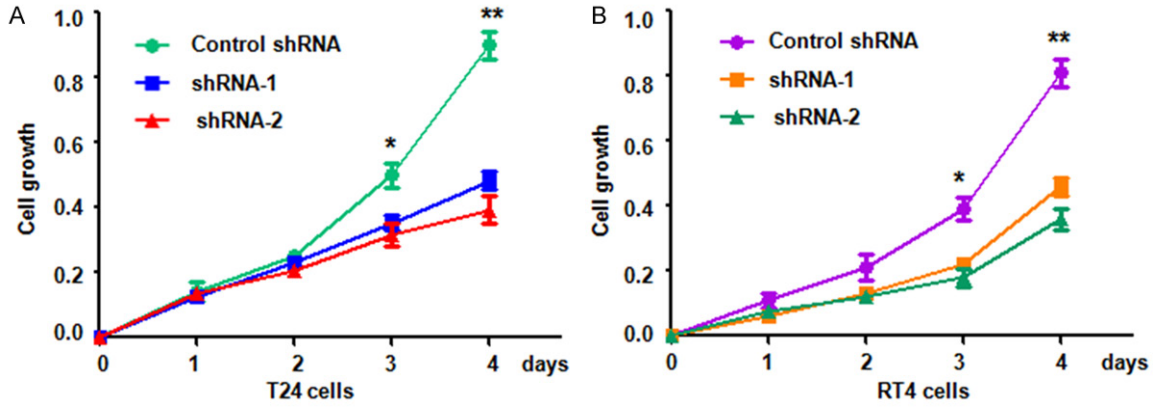

Figure 2. Depletion of AEG-1 inhibited cell proliferation in bladder cancer cells. (A, B) Cellular proliferation was measured with CCK-8 in T24 cells (A) and RT-4 cells (B) expressing control shRNA, AEG-1 shRNA1, and shRNA2.

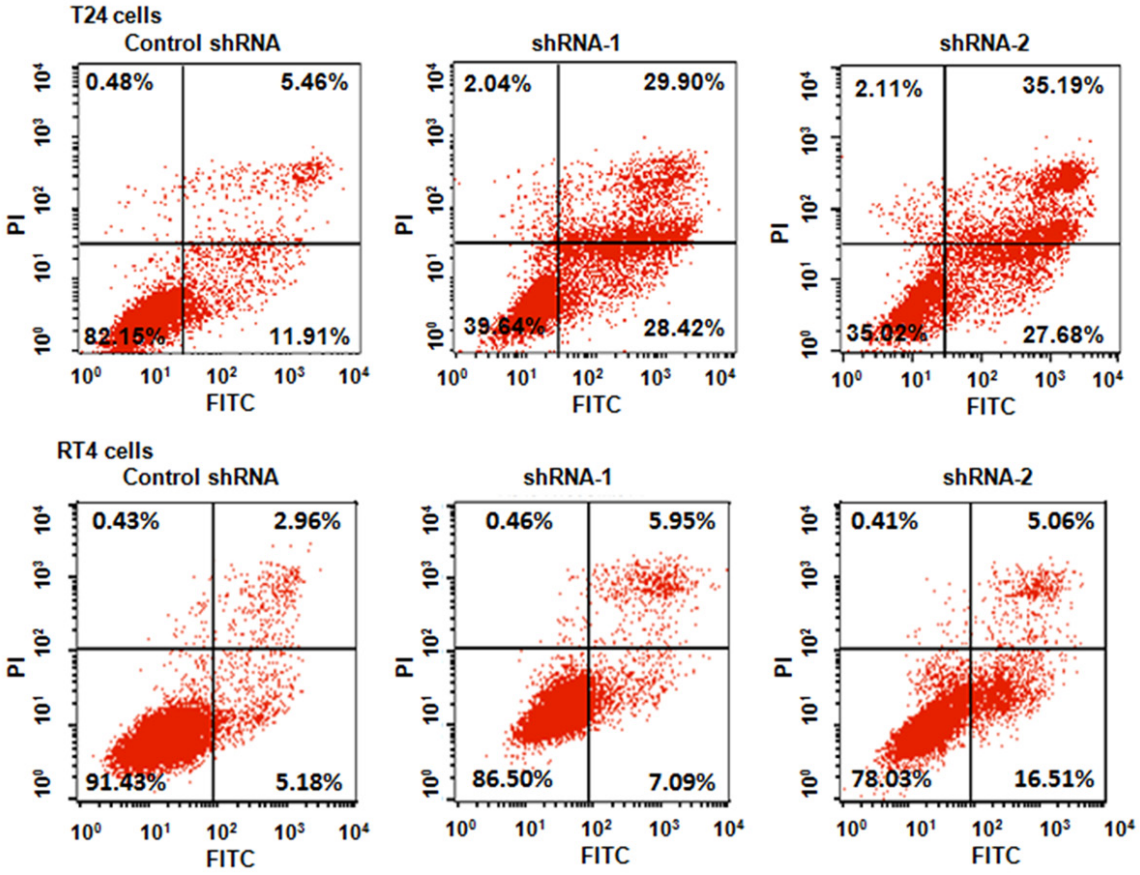

Figure 3. Down-regulation of AEG-1 promotes cellular apoptosis. Cellular apoptosis was measured by assessing PI uptake and Annexin-V staining of T24 and RT4 bladder cancer cells expressing control and AEG-1 shRNAs by Flow cytometry. Depletion of AEG-1 enhanced cell apoptosis in both bladder cancer cell lines.

Down-regulation of AEG-1 expression promoted apoptosis

Several studies have revealed that AEG-1 could regulate cell apoptosis in human cancers [21, 22]. To further determine whether the cell growth inhibition induced by depletion of AEG-1 was in part due to the induction of apoptosis in T24 and RT4 cells, cell apoptotic death was measured by PI-FITC-annexin-V assay in both bladder cancer cell lines expressing control and AEG-1 shRNAs. We observed that depletion of

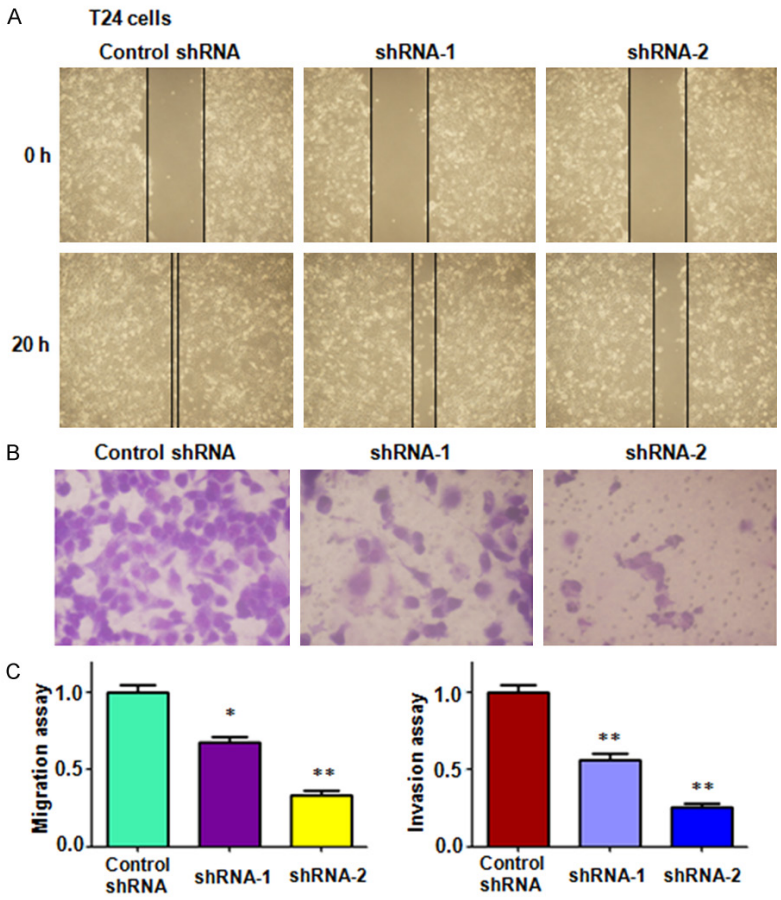

Figure 4. Depletion of AEG-1 inhibited cell migration and invasion in T24 cells. (A) Wound healing assay in T24 cells expressing control shRNA, AEG-1 shRNA1, and AEG-1 shRNA2. Wounds were initiated by scratching cellular monolayer with a pipet tip. 20 hours post scratch, cells were imaged again and width of wound was determined. (B) Cellular invasion was assessed in T24 cells expressing control shRNA, AEG-1 shRNA1, and AEG-1 shRNA2 using a Transwell assay. Cells were placed in upper chamber in serum-free media, and lower chamber contained media supplemented with 10% serum. Following 20 hours, the membrane was stained with Wright's-Giemsa to visualize invasive cells. (C) Quantitation of results was shown for (A and B). *P < 0.05, **P < 0.01, vs control shRNA group.

AEG-1 increased the percentage of apoptotic cells from 17% to 60% in T24 cells (**Figure 3**). Depletion of AEG-1 similarly induced cell apoptosis in RT4 cells (**Figure 3**). In line with previous reports [20, 23], down-regulation of AEG-1 can trigger apoptosis in bladder cancer cells. These data indicated that the growth inhibition of AEG-1 depletion is partly due to an increase in cellular apoptosis.

Depletion of AEG-1 inhibited cell migration and invasion

To investigate whether AEG-1 governs cell migration, we utilized a wound healing assay with T24 and RT4 cell lines expressing both control and AEG-1 shRNA. We observed that down-regulation of AEG-1 remarkably inhibited cell migration in T24 cells (**Figure 4A** and **4C**). To further assess whether AEG-1 controls cell invasion, we assessed the ability of T24 bladder cancer cells to penetrate through a matrigel-coated membrane. We found that down-regulation of AEG-1 reduced the number of cells that migrated through matrigel (**Figure 4B** and **4C**). Furthermore, depletion of AEG-1 similarly retarded cell migration and invasion in RT4 cells (**Figure 5**). These results suggestthat inactivating AEG-1 could suppress cell invasion in bladder cancer cells.

Down-regulation of AEG-1 inhibited Akt activity

It has been well established that the Akt pathway is a key oncogenic pathway regulating tumorigenesis [24]. Increased Akt activity, as observed by Akt phosphorylation status, has been observed in various types of human cancers [25]. Therefore, we explored whether AEG-1 exerts its oncogenic function through regulating the Akt pathway in bladder cancer cells. We observed that depletion of AEG-1 significantly inhibited Akt phosphorylation in both T24 and RT4 cells (**Figure 6**). We also observed that depletion of AEG-1 suppressed Bcl-2 expression, but increased the expression of p21 and p27 in T24 cells (**Figure 6**). Thus, our results indicate that inhibition of AEG-1 expression decreased Akt activation and Bcl-2 expression, but increased p21 and p27 protein levels, which could lead to anti-tumor activity in bladder cancer cells. These findings suggest that inhibiting AEG-1 could be a novel strategy for the treatment of bladder cancer.

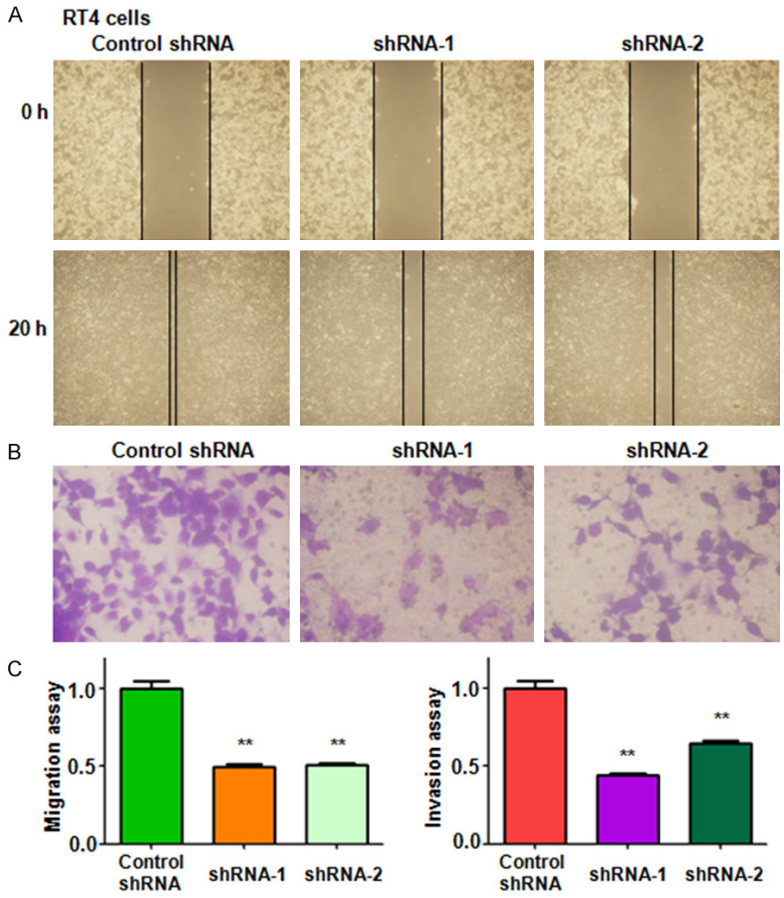

Figure 5. AEG-1 shRNA inhibited cell migration and invasion in RT4 cells. (A) Wound healing assay in RT4 cells expressing control shRNA, AEG-1 shRNA1, and AEG-1 shRNA2. Wounds were initiated by scratching cellular monolayer with pipet tip. 20 hours post scratch, cells were imaged again and width of wound was determined. (B) Cellular invasion was assessed in RT4 cells expressing control shRNA, AEG-1 shRNA1, and AEG-1 shRNA2 using a Transwell assay. Cells were placed in upper chamber in serum-free media, and lower chamber contained media supplemented with 10% serum. Following 20 hours, the membrane was stained with Wright's-Giemsa to visualize invasive cells. (C) Quantitation of results was shown for (A and B). *P < 0.05, **P < 0.01, vs control shRNA group.

Discussion

Several lines of evidence have defined that AEG-1 plays an essential role in the development and progression of many human malignancies. Over-expression of AEG-1 is associated with tumor progression and pathogenesis in esophageal squamous cell carcinoma [26], non-small cell lung cancer [27], and gastric cancer [28]. Our previous results revealed that AEG-1 was associated with tumor grade and progression as well as progression-free survival in bladder cancer [29], suggesting that AEG-1 could represent a novel biomarker to predict bladder cancer progression. Consistent with this notion, over-expression of AEG-1 correlated with TNM stage and recurrence of bladder urothelial carcinoma [30]. Intriguingly, down-regulation of AEG-1 reduced cell viability via apoptosis and inhibited migration in RT112 and 647V bladder cancer cells [20]. Similarly, our current study validated that down-regulation of AEG-1 significantly suppressed cell growth, triggered apoptosis, and retarded cell migration and invasion in T24 and RT4 bladder cancer cells. These findings along with other reports implicate AEG-1 as a key oncoprotein in human tumorigenesis.

The Akt pathway is a key regulator of tumorigenesis in various types of human cancers [24]. Consistently, increased Akt activation has been reported in human tumors [25]. A growing body of evidence suggests that AEG-1 regulates Aktactivity. For example, Lee et al. found that AEG-1 activated cell survival pathways via PI3K/Akt signaling [16, 31]. AEG-1 also promoted invasion and angiogenesis partly through activation of PI3K/Akt [32]. Moreover, AEG-1 over-expression was required for maintenance of the malignant state of human AML cells via activation of Akt1 [33]. Furthermore, AEG-1 interacted with Akt isoform 2 to govern glioma growth and survival [34]. Similarly, AEG-1 inhibited apoptosis via activation of PI3K/Akt pathway and Bcl-2 in non-small cell lung cancer [35]. In line with these results, we found that depletion of AEG-1 down-regulated the activation of Akt (as observed by Akt phosphorylation) and Bcl-2, which may lead to cell growth inhibition and apoptosis in bladder cancer cells.

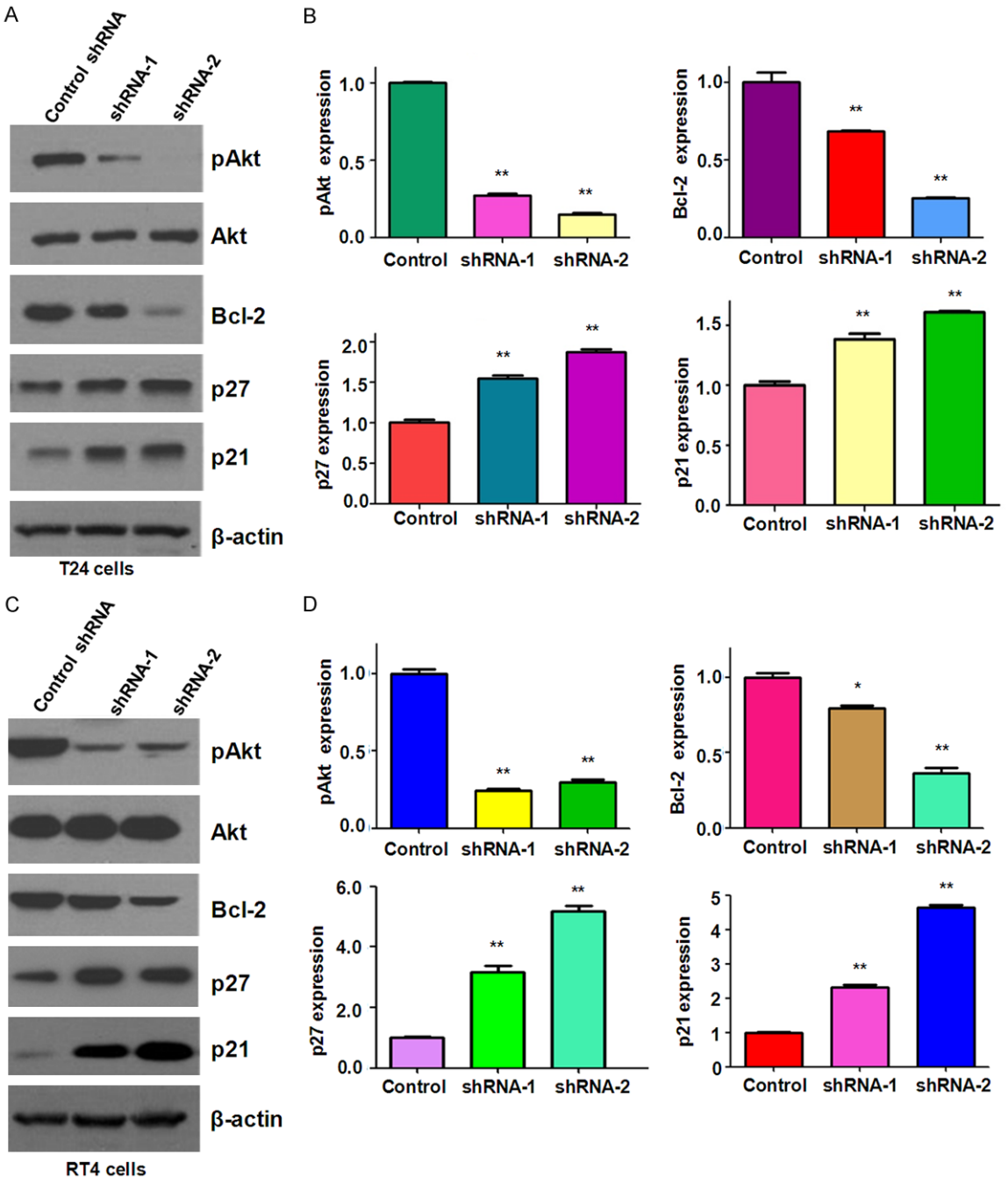

Figure 6. Depletion of AEG-1 suppresses the Akt pathway in bladder cancer cells. (A, C) Western blot analysis of lysates from T24 (A) and RT4 (C) bladder cancer cells expressing control shRNA, AEG-1 shRNA1, and AEG-1 shRNA2. Membranes were probed with pAkt-S473, total Akt, Bcl-2, p27, p21, and β-actin. (B, D) Quantitation of results was shown for (A and C). *P < 0.05; **P < 0.01, vs control shRNA group.

Since AEG-1 is an oncoprotein, it is essential to identify and develop inhibitors of AEG-1. It has been reported that the PI3K inhibitor, LY294002, down-regulated AEG-1 expression in hepatocellular carcinoma cells [36]. Perifosine, an Akt inhibitor, inhibited AEG-1 expression in gastric cancer cells [37]. Ursolic acid isolated from Oldenlandia diffusa has been observed to suppress the expression of AEG-1 in ovarian cancer cells [38]. Interestingly, an AEG-1-based DNA vaccine suppressed lung metastasis and enhanced chemosensitivity in

breast cancer [39] and prostate cancer [40]. Recently, multiple miRNAs, including miR-124, miR-136, miR-137, miR-497, and miR-506, were reported to target AEG-1 expression in various types of human cancers [41-48]. Therefore, upregulation of these miRNAs could be an alternative approach to inhibit AEG-1 expression. However, it remains necessary to discover novel specific inhibitors of AEG-1 for the treatment of human cancers.

Conclusion

Down-regulation of AEG-1 suppressed cell growth, induced apoptosis, and inhibited cell migration and invasion. Mechanically, depletion of AEG-1 may exhibit anti-tumor activity partly through inhibition of the Akt pathway in bladder cancer cells. Thus, inhibition of AEG-1 could be a promising method for the treatment of bladder cancer.

Acknowledgements

This work is supported by grant from Science and Technology Committee of Shanghai Municipal (Grant No. 12ZR1417700), and Shanghai Municipal Health Bureau (Grant No. 2013-4387), and New Leading Technology Project for Municipal Hospitals supported by Shanghai Shen Kang Hospital Development Center (Grant No. SHDC12015125).

Disclosure of conflict of interest

None.

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