Original Article
AKAP4 mediated tumor malignancy in esophageal cancer

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Abstract: AKAP4 as a new Cancer/Testis (CT) antigen is expressed not only in human germ cells, but also expressed in various tumor cells. AKAP4 is correlated with tumor malignancy; however, the role of AKAP4 in esophageal cancer remains unknown. Here we explored the function of AKAP4 in esophageal cancer. We found that AKAP4 mRNA and protein levels were up-regulated in the esophageal cancer tissues compared to normal control. In KYSE150 cell line, inhibition of AKAP4 suppressed cell growth and invasiveness. Overexpression of AKAP4 promoted cell growth and invasiveness. In addition, expression of epithelial markers (E-cadherin and ZO-1) was up-regulated or down-regulated and expression of mesenchymal markers (vimentin and N-cadherin) was down-regulated or up-regulated after knockdown or overexpression of AKAP4 *in vitro*. *In vivo* in a xenograft model silencing AKAP4 suppressed tumor growth. We also found that NF-κB p65 bound to AKAP4 promoter and regulated expression of AKAP4. In conclusion, overexpression of AKAP4 is associated with esophageal cancer progression. Inhibition of AKAP4 leads to suppressed growth and invasion of esophageal cancer.

Keywords: AKAP4, esophageal cancer, tumor growth

Introduction

Esophageal cancer is one of the most common malignance in the world [1]. In China, esophageal squamous cell carcinoma (ESCC) dominated the most samples of esophageal cancer [2]. Recently, there is much advance in diagnosis and treatment of esophageal cancer; however, the five years overall survival rate especially for metastatic samples is still low [3]. It is becoming imperative to further understand the mechanisms of ESCC progression.

AKAP4 belongs to a member of the A-kinase anchor proteins that function to bind the regulatory subunit of protein kinase A (PKA) [4]. AKAP4 protein is originally localized to the sperm flagellum and involved in the regulation of sperm motility [5, 6]. Cancer/testis (CT) genes are a family of genes that are normally expressed only in human germ cells, but also expressed in various tumor cells [7]. CT antigens are also used to evaluate their role in tumor progression including cell growth, survival, and metastasis [8]. AKAP4 is a new Cancer/ Testis (CT) antigen and observed in a panel of cancer cell lines compared with normal ones [9]. AKAP4 as a tumor antigen is a biomarker and has the potential therapeutical function for glioblastoma [10], myeloma [11, 12], prostate cancers [13], breast cancer [14, 15], cervical cancer [16], ovarian cancer [17], and NSCLCs [18-20]. However, it remains unclear whether the malignant phenotypes would be effective in including esophageal cancer.

In this study, we examined AKAP4 expression in tumor specimens from esophageal patients and assessed the correlation between AKAP4 expression and the clinical features. Additionally, we also investigated whether AKAP4 affected cancer malignancy *in vitro* and *in vivo*. Our findings suggest that AKAP4 may be a novel diagnosis and therapy target for esophageal cancer.
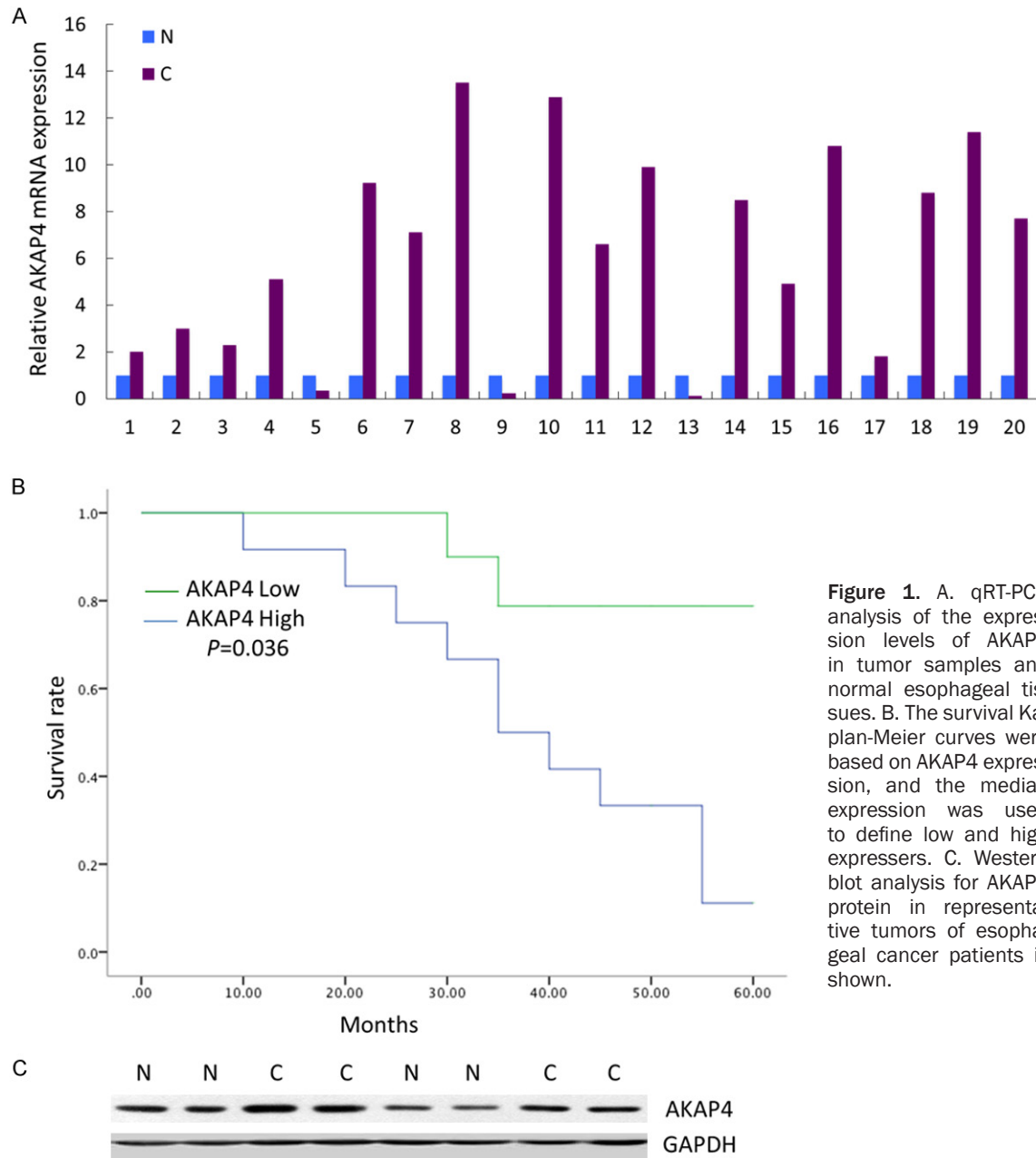
Figure 1. A. qRT-PCR analysis of the expression levels of AKAP4 in tumor samples and normal esophageal tissues. B. The survival Kaplan-Meier curves were based on AKAP4 expression, and the median expression was used to define low and high expressers. C. Western blot analysis for AKAP4 protein in representative tumors of esophageal cancer patients is shown.

Results

AKAP4 high expression in esophageal cancer with poor prognosis

To determine the role of AKAP4 in esophageal cancer, we firstly detected the expression of AKAP4 mRNA in tumor samples and normal esophageal tissues by qRT-PCR. We found the high expression of AKAP4 in esophageal cancer tissues; however, it is less detectable in normal esophageal tissues (**Figure 1A**). To fur-

ther understand the clinical significance of a high AKAP4 expression level in esophageal cancer, we analyzed the correlation between the expression of AKAP4 in esophageal cancer and survival in esophageal cancer patients. The Kaplan-Meier analysis revealed that high expression levels of AKAP4 were associated with poor survival (**Figure 1B**; P = 0.036). Statistical analysis indicated that AKAP4 high expression was also associated with lymph node metastasis (**Table 1**). The western blot analysis revealed that AKAP4 protein was strongly

Table 1. Clinical characteristics of the participating patients in this study

Features	Total	AKAP4		P
		Low	High	
Median Age (Range)	63.5 (42-77)	64.5 (43-77)	61.5 (42-74)	0.338
Gender				1
Male/Female	22/8	11/4	11/4	
Stage				0.696
I+II	14	8	6	
III+IV	16	8	8	
Lymph node status				0.028
Negative	16	11	5	
Positive	14	4	10	
AKAP4 mRNA expression				0.000
Low	15	15	0	
High	15	0	15	

expressed in esophageal cancer tissues and low expressed in normal ones (**Figure 1C**). These findings suggest a possible link between AKAP4 expression and esophageal cancer progression.

Knockdown of AKAP4 suppressed the migration and invasion of esophageal cancer cells

To explore the role of AKAP4 in esophageal cancer, we knocked down AKAP4 expression and firstly study the proliferation of esophageal cancer cells. Western blot analysis confirmed that the knockdown efficiency of AKAP4 in KYSE150 cells transfected with siRNA especial target for AKAP4 (siAKAP4) compared to the negative control (siCon) (**Figure 2A**). Down-regulation of AKAP4 expression was found to decrease cell proliferation in **Figure 2B**. We also found that AKAP4 silencing inhibited the cell growth by colony formation assay (**Figure 2C**). Clinical data analysis showed that AKAP4 was also associated with lymph node metastasis. Next, to determine whether AKAP4 can affect metastasis of esophageal cancer cells, wound healing migration and transwell invasion was analyzed. We found that the down-regulation of AKAP4 suppressed the migration of the KYSE150 cells (**Figure 2D**). Down-regulation of AKAP4 expression also resulted in fewer cells infiltrating the membranes in the transwell assay compared to the control (**Figure 2E**). Change of cell migration and invasion may accompany the EMT (Epithelial-Mesenchymal Transition, EMT). Finally, we investigate wheth-

er AKAP4 induces EMT. We found that the mesenchymal markers N-cadherin and vimentin were significantly down-regulated in KYSE150 cells of knocking down AKAP4 compared with the control group (**Figure 2F**). At the same time, AKAP4 down-regulation also resulted in an up-regulation of the epithelial markers E-cadherin and ZO-1 (**Figure 2F**).

Overexpression of AKAP4 enhanced the migration and invasion of esophageal cancer cells

Since the knockdown of AKAP4 suppressed the migration and invasion of esophageal cancer cells, we wonder if overexpression of AKAP4 affects the cell migration and invasion. By transfecting with pCDNA3.1-AKAP4, we found the increased expression of AKAP4 compared with esophageal cancer cells transfected with the pCDNA3.1-Control vector (**Figure 3A**). **Figure 3B** showed that the up-regulation of AKAP4 expression increased cell proliferation. Our results also demonstrated that AKAP4 promoted KYSE150 cell migration and invasion (**Figure 3C** and **3D**). Moreover, expression of the epithelial-mesenchymal transition markers was detected. We found that up-regulation of AKAP4 increased the expression of mesenchymal markers N-cadherin and vimentin and dereased the epithelial markers E-cadherin and ZO-1 expression (**Figure 3E**).

Downregulation of AKAP4 attenuated tumorigenicity in vivo

To further understand the role of AKAP4 in tumorigenesis and development in vivo, we subcutaneously inoculated esophageal cancer KYSE150-luc cells into the nude mice. The esophageal cancer KYSE150-luc cells were transfected with the stably constructed AKAP4 shRNA-expressing or control clones. We used the bioluminescence imaging system to detect the primary tumor burden in vivo. The stable knockdown efficiency is firstly confirmed (**Figure 4A**). The tumors of AKAP4-knockdown group produced decreased bioluminescent signals com-
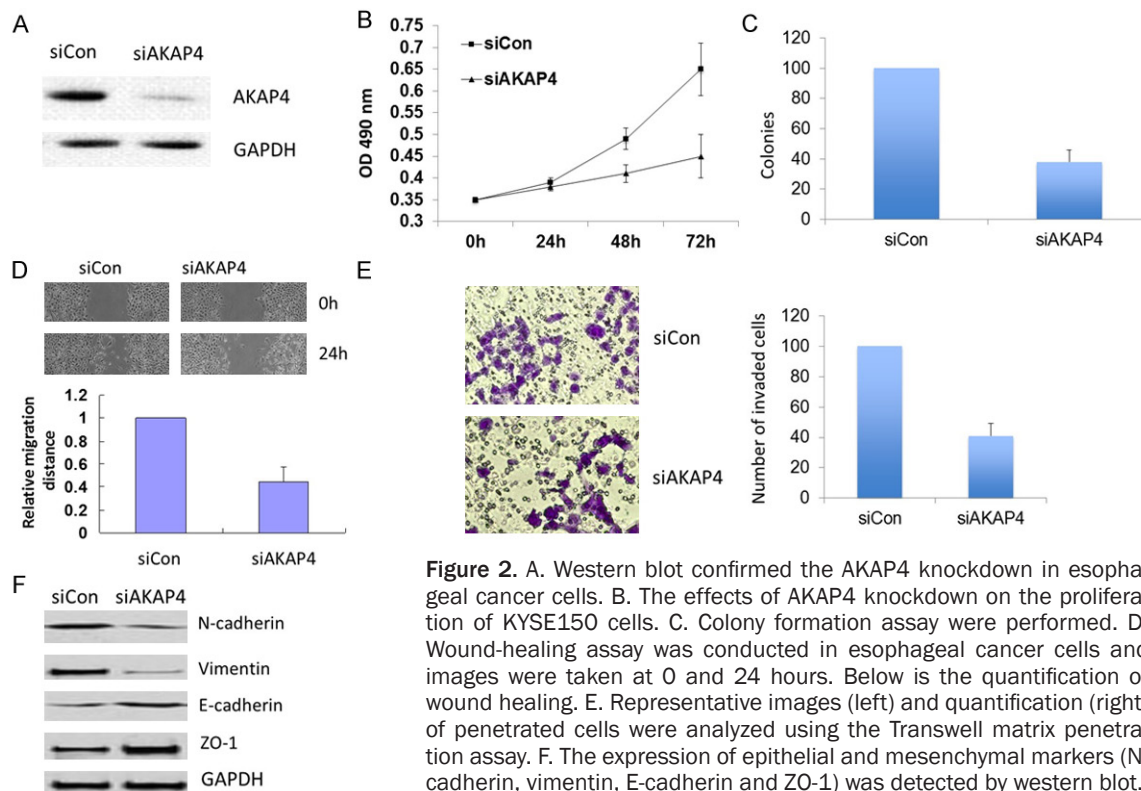
Figure 2. A. Western blot confirmed the AKAP4 knockdown in esophageal cancer cells. B. The effects of AKAP4 knockdown on the proliferation of KYSE150 cells. C. Colony formation assay were performed. D. Wound-healing assay was conducted in esophageal cancer cells and images were taken at 0 and 24 hours. Below is the quantification of wound healing. E. Representative images (left) and quantification (right) of penetrated cells were analyzed using the Transwell matrix penetration assay. F. The expression of epithelial and mesenchymal markers (N-cadherin, vimentin, E-cadherin and ZO-1) was detected by western blot.

pared with control (**Figure 4B**). The tumors formed by silencing AKAP4 were smaller than the control tumors. We also found that the tumor growth of KYSE150-luc-shAKAP4 cells was slow than that of KYSE150-luc-shConrol cells by bioluminescence imaging (**Figure 4C**). The survival rates of these mice were also investigated, and we found that KYSE150-luc-shAKAP4-bearing mice survived longer than control mice (**Figure 4D**). The western blot analysis of tumors in the AKAP4 knockdown and control groups also showed that the protein levels of E-cadherin and ZO-1increased significantly, while the mesenchymal phenotype marker N-cadherin and vimentin decreased compared with control groups (**Figure 4E**).

Binding of transcription factor NF-κB p65 to human AKAP4 promoter

P65 as an important transcription factor has been reported to affect the esophageal cancer progression [21, 22]. To determine whether p65 is involved in AKAP4 expression, we firstly analyzed whether p65 bond to human AKAP4 promoter in esophageal cancer cells by CHIP. CHIP results showed that p65 specially bond to AKAP4 promoter (**Figure 5A**). Furthermore we

performed knockdown of p65 experiment to check the expression of AKAP4. We found that the knockdown of p65 reduced the expression of AKAP4 (**Figure 5B**). To further confirm the involvement of p65 in AKAP4 expression in esophageal cancer, we used p65 inhibitor PDTC. Treatment of KYSE150 cells with the inhibitor resulted in the decrease of AKAP4 (**Figure 5C**). Theses results indicated that p65 was involved in the expression of AKAP4 in esophageal cells.

Discussion

In the present study, we showed that the AKAP4 gene was overexpressed in esophageal cancer and is significantly correlated with lymph node metastasis as well as poor survival in esophageal cancer patients. AKAP4 as a new CT antigen has been reported to be overexpressed and new biomarker in a series of tumors [17]. Our results are consistent with these data. These results indicate that AKAP4 is a potential biomarker and might be applied to cancer prevention and treatment.

Furthermore, AKAP4 silencing by RNA interference suppressed esophageal cancer cell grow-
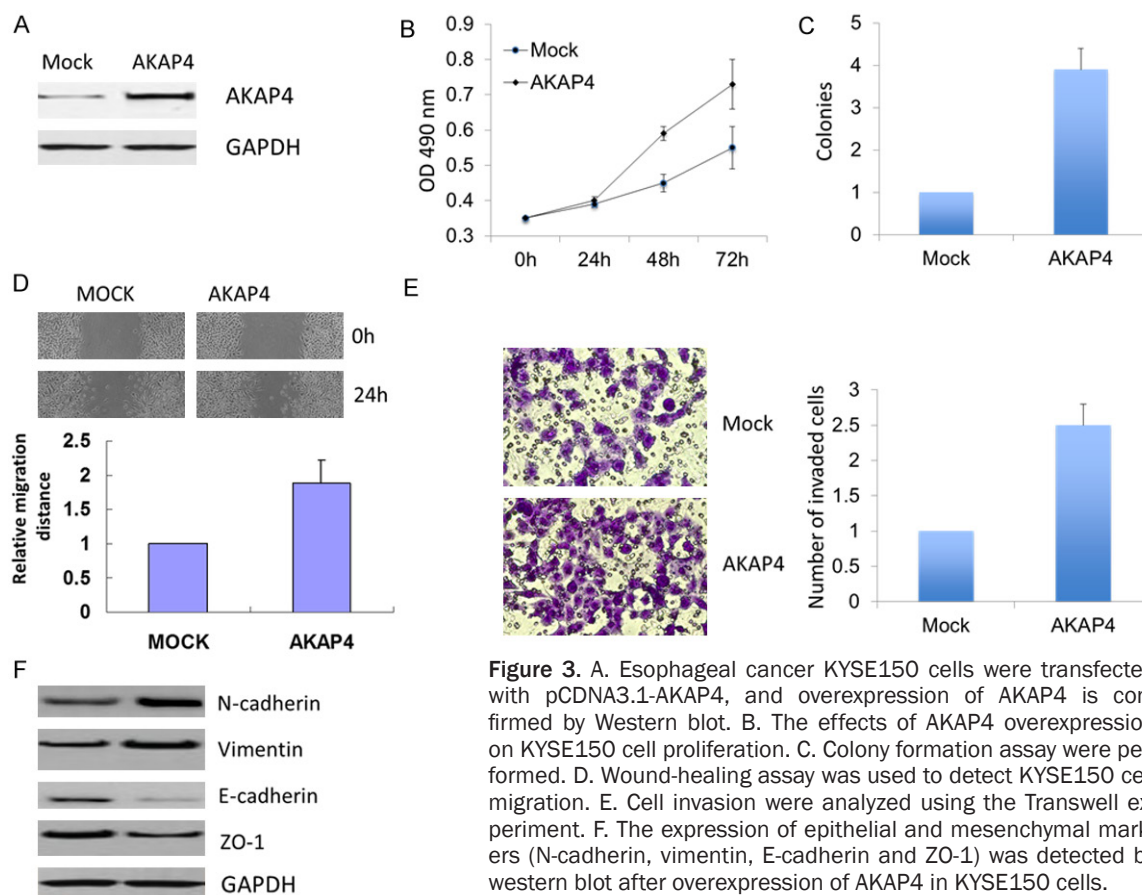
Figure 3. A. Esophageal cancer KYSE150 cells were transfected with pCDNA3.1-AKAP4, and overexpression of AKAP4 is confirmed by Western blot. B. The effects of AKAP4 overexpression on KYSE150 cell proliferation. C. Colony formation assay were performed. D. Wound-healing assay was used to detect KYSE150 cell migration. E. Cell invasion were analyzed using the Transwell experiment. F. The expression of epithelial and mesenchymal markers (N-cadherin, vimentin, E-cadherin and ZO-1) was detected by western blot after overexpression of AKAP4 in KYSE150 cells.

th and invasiveness *in vitro*. However, overexpression of AKAP4 promoted cell growth and invasiveness *in vitro*. Notably, we also found that AKAP4 silencing by shRNAs suppressed esophageal tumor growth and resulted in a long survival by *in vivo* experiment. These data suggested that AKAP4 promoted esophageal cancer cell malignancy and is a promising novel target for RNAi-based therapeutics against esophageal cancer. It is reported that the ablation of AKAP4 protein caused significant inhibition in cellular growth, migration and invasion ability of cervical cancer cells [16].

Epithelial-mesenchymal transition (EMT) is an important process during development by which epithelial cells acquire mesenchymal, fibroblast-like properties and show decreased intercellular adhesion and increased motility [23]. During tumorigenesis and development, EMT plays a crucial role and may induce the motility and invasiveness of cancer cells [24, 25]. AKAP4 is an important tumor biomarker, but the mechanism in tumor pathogenesis is still not unknown. In this study, silencing AKAP4 led to increased expression of the epithelial markers E-cadherin and ZO-1 and decreased expression of the mesenchymal markers N-cadherin and vimentin in KYSE150 cells; however, overexpression of AKAP4 was reverse. These results indicated that EMT was involved in the process of AKAP4 inducing esophageal cancer development. This data provide new concept on understanding the function of AKAP4 in tumor progression.

NF-κB p65 is a ubiquitously expressed and essential transcription factor involved in all types of cellular processes, including cell proliferation, differentiation, apoptosis, metastasis and tumorigenesis [26, 27]. p65 activation is correlated with cancer development and the activation of p65 can suppress apoptosis, thus promoting chemoresistance and tumorigenesis [28, 29]. Here we found that p65 bond to the promoter and promoted the expression of AKAP4 in esophageal cancer. This further explained the mechanism of AKAP4 function.
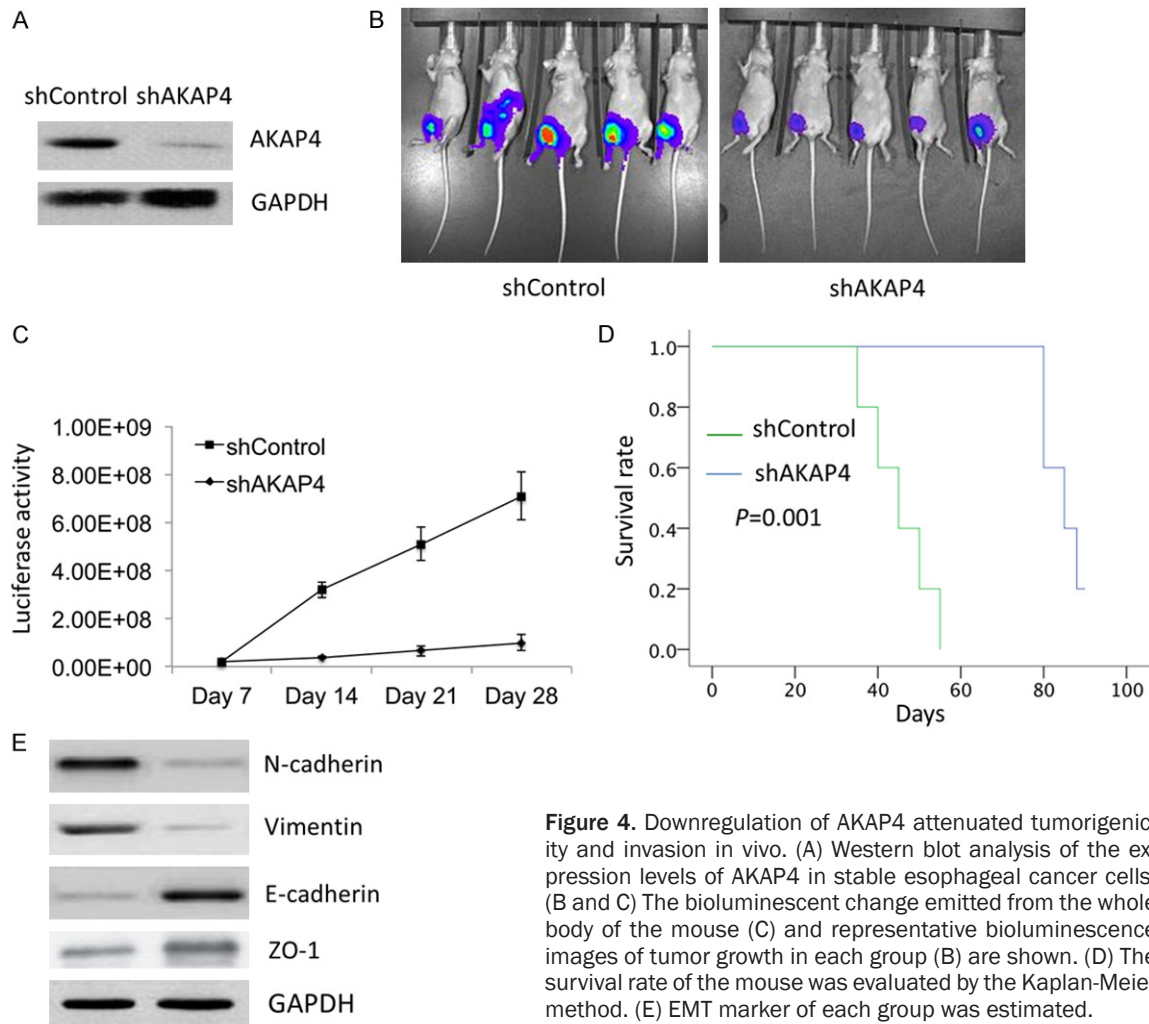
Figure 4. Downregulation of AKAP4 attenuated tumorigenicity and invasion in vivo. (A) Western blot analysis of the expression levels of AKAP4 in stable esophageal cancer cells. (B and C) The bioluminescent change emitted from the whole body of the mouse (C) and representative bioluminescence images of tumor growth in each group (B) are shown. (D) The survival rate of the mouse was evaluated by the Kaplan-Meier method. (E) EMT marker of each group was estimated.

In conclusion, this study suggests that AKAP4 is a biomarker of esophageal cancer and change of AKAP4 expression contributes to the regulation of esophageal cancer phenotypes. We further put forward the mechanisms of AKAP4 in esophageal cancer progression. These might provide new ways to prevent and treatment of esophageal cancer and improve the clinical outcome of the patients.

Materials and methods

Samples

The protocol for this study was approved by the ethics committee of Hebei Medical University. The patients from the Second Hospital of Hebei Medical University diagnosed with esophageal aquamous cell caicinoma were selected for the study. All patients in the study provided the written informed consent. The pathological specimens were reviewed, and histological classifications of esophageal aquamous cell caicinoma were made based on the World Health Organization (WHO) Classification.

Cell culture and transfection

The human esophageal carcinoma cell lines KYSE-150 was kindly provided by Dr. Zhang Xun (Tianjin Chest Hospital). The cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) medium containing 10% fetal bovine serum (FBS, GIBCO), penicillin and streptomycin at 37°C in humidified air containing 5% CO_2. For transfection, cells were cultured to 80% confluence and transfected with recombinant plasmids or siRNA using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's recommendation.
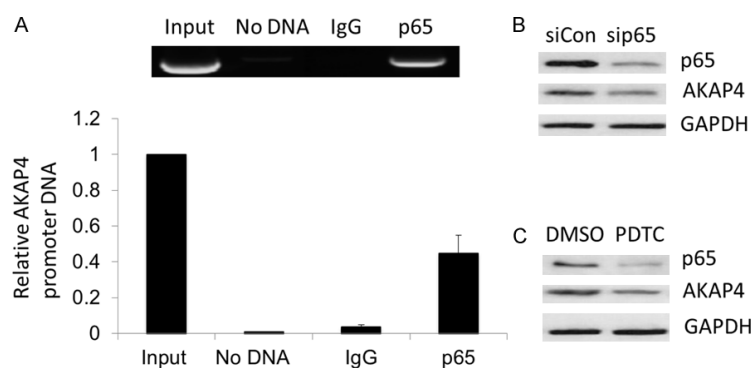
Figure 5. Binding of transcription factor NF-κB p65 to human AKAP4 promoter. A. CHIP was used to analyze binding of p65 to AKAP4 promoter. Transcription factor NF-κB p65 subunit directly interacted with human AKAP4 promoter in esophageal cancer cells. B. Knockdown of endogenous p65 and expression of AKAP4 were analyzed by Western blotting. GAPDH was used as a loading control. C. Inhibition of P65 by P65-specific inhibitor PDTC prevented AKAP4 expression in KYSE150 cell lines.

Quantitative real-time PCR

mRNA expression level was identified by quantitative real-time PCR. The detail was described as previously [30].

Western blotting

Total cell or tissues lysates were extracted using RIPA buffer and run on 10% gradient SDS-polacrylamide gel that was then transferred to NC membrane. The membrane was blocked for 1 hour at room temperature (RT) in 5% milk and then incubated with primary antibody overnight at 4°C. Finally, the membrane is incubated with the secondary antibody.

Plasmid constructions

For overexpression of AKAP4, the open reading frame (ORF) of AKAP4 was amplified from human mRNA and was cloned into the pcDNA3.1 vector (Invitrogen). The recombined vector was named as AKAP4. Empty pcDNA3.1 vector was used as control and named as MOCK. The construction was confirmed by sequencing.

MTT assay

MTT assay was used to analyze cell proliferation. Cells were seeded into 96-well plate at 5.0×10^3 cells/ml and cultured for 24, 48, and 72 h, respectively. At each time point, 10 μl MTT reagent (5 mg/ml, Sigma) was added to each well, and then incubated for 4 h at 37°C. DMSO (Invitrogen) was added to the wells to dissolve the formazan crystals. The wavelength of 490 nm was measured on microplate reader (Spectra Max M5, MD, USA). Each sample was tested in triplicate and all experiments were performed three times.

Wound-healing assay

The wound-healing assay was used to detect cell migration ability. Cells were plated into 12-well plates. 24 h after transfection cells were wounded with a 100 μl micropipette tip. Width of the wound was measured at different time points. Three different locations were visualized and photographed under a microscope.

Cell invasion assay

Boyden chamber assay was used to examine cell invasion ability. 1.0×10^4 cells in 200 μl RPMI-1640 medium with 1% FBS were placed into the upper chambers. The lower chambers were filled with 600 μl complete medium with 10% FBS. After incubation for 12 h at 37°C, the invasion cells on the lower surface of the inserts were fixed and stained with 0.1% crystal violet, and five random fields for each insert were counted.

Lentiviral shRNA transduction

Cell lines stably expressing AKAP4 lentiviral shRNA or negative control of non-target shRNA were established. The shRNA target for AKAP4 or control fragment was subcloned into lentivirus vector. KYSE-150-luciferase cells were infected with recombinant lentiviruses expressing shRNA for AKAP4 (shAKAP4) or shRNA control (shControl). After that, stable cell lines were generated by selection with puromycin.

In vivo assay

For the in vivo assays, the stable cell line were collected and suspended in 0.2 ml PBS for each sample and the cells were injected into left side of the posterior flank of nude mouse.

The luciferase substrate was added to each mouse at a dose of 150 mg/kg and lives images of the mouse were obtained using an IVIS200 (Xenogene, USA). Luciferase activity was measured every 7 days. Tumor growth was also measured.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to manufacture manufacturer's instructions. Briefly, cells were crosslinked in 1% formaldehyde for 15 min at room temperature. Chromatin was captured at 4°C with the following 2 µg primary antibody for each sample: p65 (sc-8008) and rabbit IgG (sc-2027) (Santa Cruz, CA). After capture, the chromatin was collected, purified and then decrosslinked at 65°C. The enrichment of DNA was detected by qPCR.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All experiments were repeated three times, and the results are expressed as the mean ± SD. The difference among groups was conducted by using Student's t-test or one-way ANOVA. The Kaplan-Meier method was used to estimate survival and survival differences were analyzed by the log-rank test. Differences were considered significant when P value was < 0.05.

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