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Original Paper

RUNX2 Plays An Oncogenic Role in Esophageal Carcinoma by Activating the PI3K/AKT and ERK Signaling Pathways

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Key Words
Esophageal carcinoma • Runt Related Transcription Factor 2 • PI3K/AKT and ERK signaling pathways • Proliferation • Apoptosis • Migration • Invasion

Abstract
Background/Aims: Esophageal carcinoma is a frequently occurring cancer at upper gastrointestinal tract. We aimed to evaluate the roles and possible mechanism of Runt Related Transcription Factor 2 (RUNX2) in the development of esophageal cancer. ***Methods:*** The expression of RUNX2 in esophageal carcinoma tissues and cells was investigated by qRT-PCR. Effects of RUNX2 on cell viability, apoptosis, migration and invasion were assessed using MTT assay, flow cytometry assay/western blot analysis, and Transwell assays, respectively. Afterwards, effects of RUNX2 on of the activation of the PI3K/AKT and ERK pathways were explored by Western blot analysis. In addition, a PI3K/AKT pathway inhibitor LY294002 and an ERK inhibitor U0126 were applied to further verify the regulatory relationship between RUNX2 and the PI3K/AKT and ERK signaling pathways. Besides, the RUNX2 function on tumor formation *in vivo* was investigated by tumor xenograft experiment. ***Results:*** The result showed that RUNX2 was highly expressed in esophageal carcinoma tissues and cells. Knockdown of RUNX2 significantly inhibited TE-1 and EC-109 cell viability, repressed TE-1 cell migration and invasion, and increased TE-1 cell apoptosis. RUNX2 overexpression showed the opposite effects on HET-1A cells. Moreover, RUNX2-mediated TE-1 cell viability, migration and invasion were associated with the activation of the PI3K/AKT and ERK pathways. Besides, knockdown of RUNX2 markedly suppressed tumor formation *in vivo*. ***Conclusion:*** Our results indicate that RUNX2 may play an oncogenic role in esophageal carcinoma by activating the PI3K/AKT and ERK pathways. RUNX2 may serve as a potent target for the treatment of esophageal carcinoma.

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Introduction

Esophageal carcinoma is a frequently occurring cancer at upper gastrointestinal tract [1, 2], in which the predominant type is esophageal squamous cell carcinoma (ESCC) [3]. It ranks the sixth among leading causes of death and is one of the most common malignant tumors globally [4, 5]. The overall five-year survival rate of esophageal cancer is reported to be only 20% [6]. Despite great advances in surgical techniques and therapies, the overall patient's prognosis remains poor [7, 8]. Therefore, elucidation of key mechanism underlying esophageal cancer will be helpful in designing promising biomarkers or therapeutic targets for diagnosis and treatment.

Runt Related Transcription Factor 2 (RUNX2) is a bone-specific transcriptional regulator that are essential for bone formation [9-11]. Recently, accumulated evidences have highlighted the key roles of RUNX2 in a variety of prevalent cancers. For instance, RUNX2 is abnormally expressed in highly metastatic prostate cancer cells and may function as a key regulator in prostate cancer metastatic bone disease [12, 13]. Targeting of RUNX2 is found to impair the progression of breast cancer to metastatic bone disease [14] and repress cell growth and metastasis of lung cancer cells [15]. In addition, RUNX2 can promote the metastasis of human gastric cancer through up-regulation of the chemokine receptor CXCR4 [16]. Furthermore, the critical role of miR-30a in inhibiting the progression of human osteosarcoma is mediated by targeting RUNX2 [17]. RUNX2 is also confirmed as a target of miR-338-3p to mediate the tumor-suppressive function of miR-338-3p in ovarian epithelial carcinoma [18]. Although the roles of RUNX2 in various prevalent cancers are increasingly disclosed, its molecular pathogenesis underlying esophageal carcinoma development remains largely unknown.

In the present study, the expression of RUNX2 in esophageal carcinoma tissues and cells was investigated. Meanwhile, RUNX2 was knocked down in esophageal carcinoma TE-1 and EC109 cells and was overexpressed in normal esophageal HET-1A cells. The effects of RUNX2 on the cell malignant behaviors were explored. Moreover, the regulatory relationship between RUNX2 and the PI3K/AKT and ERK signaling pathways was detected. Besides, the influence of RUNX2 on tumor formation *in vivo* was testified in mice. The objective of our study was to evaluate the potential role of RUNX2 in the development of esophageal carcinoma, as well as its underlying mechanism. All efforts of this study will provide a theoretical basis or new insight for the treatment of this disease.

Materials and Methods

Patient samples

From Oct, 2014 to Jan, 2016, 20 patients (10 females and 10 males, average age 57.20 ± 6.11 years) who were diagnosed with esophageal carcinoma in our hospital were included in this study. The diagnosis of esophageal carcinoma was pathologically confirmed. Esophageal cancer tissues and corresponding adjacent non-tumorous esophageal tissues were collected from clinically ongoing surgical samples. All samples were snap frozen in liquid nitrogen and stored at -80°C. This study was approved by the ethics committee of our hospital and all patients have signed informed consents before using their tissues for research.

Cell culture, transfection and treatment

The human esophageal carcinoma cell lines Eca109, TE-1 and EC109 and normal esophageal cell line HET-1A were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI-1640 medium that were prepared with 10% fetal bovine serum (FBS, Gibco, Victoria, Australia) and then kept in incubators with humidified atmospheres of 5% CO_2 at 37°C.

An RUNX2 overexpression vector (pc-RUNX2) was constructed by cloning the full-length wild-type RUNX2 coding sequence into pcDNA3.1 plasmid, and confirmed by sequencing. The small interfering RNAs (siRNAs) specifically targeting RUNX2 (si-RUNX2) and negative control of si-RUNX2 (si-NC) were purchased from GenePharma (Shanghai, China). Transfection was carried out using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Furthermore, to explore the possible regulatory mechanism of RUNX2 in esophageal carcinoma, a PI3K/AKT pathway inhibitor LY294002 and an ERK inhibitor U0126 were obtained from Selleck Chemicals (Houston, TX, USA) and used for treatment of TE-1 cells.

MTT assay

The viability of cells was evaluated with the MTT assay. Briefly, TE-1, EC109 and HET-1A cells were seeded in 96-well plates at the density of 5×10^3 per well. Cell viability was measured with a Cell Viability Kit (MTT, Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The optical density (OD) value at wavelength of 570 nm was read with a microtiter plate reader (Promega, Fitchburg, WI, USA). Each experiment was repeated at least three times.

Flow cytometer

Cell apoptosis was detected using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) following the protocols recommended by manufacturer. Briefly, cells (1×10^5 cells/well) were seeded in a 6 well-plate. Cells were double stained by Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instruction of the Annexin V-FITC/PI apoptosis detection kit, and then stained cells were measured with a flow cytometer (Beckman Coulter, Miami, FL, USA). Apoptotic cells (Annexin-V positive and PI-negative) were analyzed with CELL Quest 3.0 software (BD Biosciences, San Jose, CA, USA). All experiments were repeated in triplicate.

Transwell assay

Cell migration and invasion assays were carried out using Transwell culture chambers (8-μm pore size; Costar, Corning, NY, USA) coated with (invasion assay)or without (migration assay) Matrigel (BD Biosciences) as described previously [19]. Briefly, total 2.5×10^4 cells that were suspended in 200 μL RPMI-1640 medium with 1% FBS were plated into the upper Transwell chamber and 600 μL RPMI-1640 medium with 10% FBS was filled into the lower chamber. After 24 h of incubation, the migrated/invaded cells on the lower side of the chamber were fixed and stained with 1% crystal violet solution. Under a microscope (Leica, Heidelberg, Germany), the migrated/invaded cells were counted. The experiments were carried out at least three times.

Quantitative real time PCR (qRT-PCR) analysis

Total RNA was extracted from esophageal cancer tissues and cells using TRIzol reagent (Invitrogen), and was then reverse-transcribed into cDNA with the PrimeScript RT reagent kit (TaKaRa, Dalian, China). Real-time PCR analysis for detecting the expression of RUNX2 mRNA was conducted with SYBR Advantage qPCR Premix (Takara). GAPDH was used as internal control, and the relative expression of RUNX2 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method [20].

Western blot analysis

The protein was extracted from cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) that were prepared with protease inhibitors (Roche, San Francisco, CA, USA). The concentration of protein extracts were quantified by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) according to the manufacturer's instructions. The western blot assay was performed based on a Bio-Rad Bis-Tris Gel system. After blotted onto polyvinylidene difluoride (PVDF) membrane, primary antibodies to cleaved PARP, cleaved caspase-3, RUNX2, p-AKT, AKT, p-GSK3β, GSK3β, p-ERK, ERK and GAPDH (Abcam, Cambridge, UK) prepared in 5% blocking buffer at a dilution of 1:1000 were used for incubating the membranes overnight at 4°C. After incubation with horseradish peroxidase-marked secondary antibody for 1 h at room temperature, the PVDF membranes were transferred into the Bio-Rad ChemiDoc™ XRS system, and visualized by the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

In vivo mouse model used for detecting RUNX2 function

The *in vivo* function of RUNX2 was investigated by tumor xenograft experiment. Approximately 2×10^6 TE-1 cells that were transfected with si-RUNX2 or si-NC were injected subcutaneously into the right hind legs of 4-week-old nude mice (10 mice for each group). On week 5, tumor weight and volume were

measured. Tumor volume (V) was calculated as follows: $V = 0.5 \times L \times W^2$ [21, 22]. All animal experiments were performed in compliance with the care and use of research animals of our hospital.

Statistical analysis

Data collected from multiple experiments are presented as the mean ± SD. Statistical differences were analyzed and calculated by a one-way analysis of variance (ANOVA) or unpaired two-tailed t-test. Statistical analyses were carried out using SPSS 19.0 statistical software and statistical significance was obtained when $P < 0.05$.

Results

RUNX2 was highly expressed in esophageal carcinoma tissues and cells

As shown in Fig. 1A, the mRNA expression of RUNX2 in esophageal carcinoma tissues was significantly higher than that in adjacent non-tumorous tissues ($P < 0.001$). Consistent results were obtained that RUNX2 was highly expressed in esophageal carcinoma cells (TE-1 and EC109) relative to that in normal esophageal HET-1A cells ($P < 0.01$ or $P < 0.001$, Fig. 1B).

Knockdown of RUNX2 inhibited TE-1 cell viability, induced apoptosis and suppressed cell migration and invasion

To investigate the role of RUNX2 in esophageal carcinoma, TE-1 and EC109 cells were transfected with si-RUNX2 to knock down RUNX2 expression. The knockdown efficiency was confirmed by qRT-PCR ($P < 0.001$, Fig. 2A). MTT assay showed that knockdown of RUNX2 significantly inhibited TE-1 and EC-109 cell viability at 3 and 4 days after transfection ($P < 0.05$ or $P < 0.01$, Fig. 2B and 2C). Because RUNX2 mRNA expression in TE-1 cells was higher than that in EC109 cells and the inhibitory effects of RUNX2 knockdown on TE-1 cell

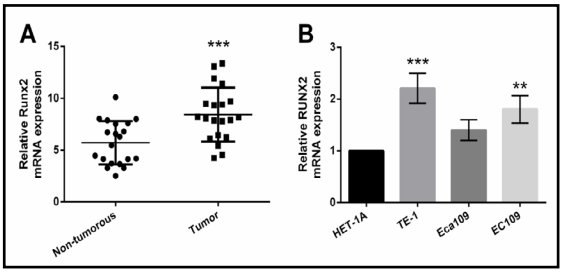

Fig. 1. RUNX2 was highly expressed in esophageal carcinoma tissues and cells. RUNX2 mRNA expression in tissues (A) and cell lines (B) was determined by qRT-PCR. Data are presented as the mean ± SD. ** P<0.01 and *** P<0.001 compared with the non-tumorous group or the HET-1A group.

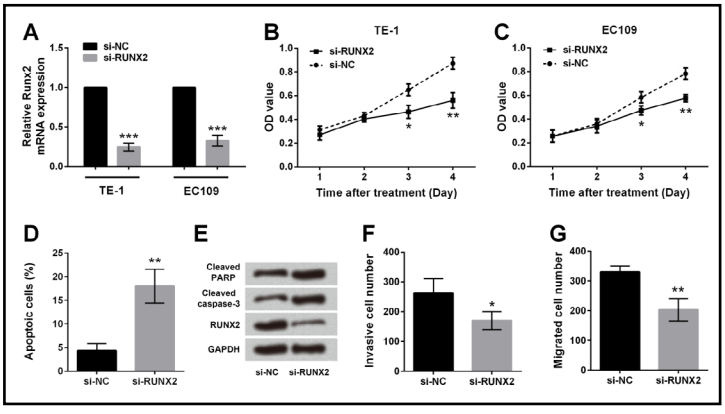

Fig. 2. Knockdown of RUNX2 inhibited TE-1 and EC109 cell viability, induced TE-1 cell apoptosis and suppressed TE-1 cell migration and invasion. TE-1 and EC109 cells were transfected with si-RUNX2 or si-NC. (A) The mRNA expression of RUNX2 after transfection by qRT-PCR assay. (B-C) Cell viability after transfection by MTT assay. (D) Percentage of apoptotic cells after cell transfection by flow cytometry assay. (E) Expression of apoptosis-related proteins by western blot analysis. Migration (F) and invasion (G) were measured by Transwell assay. Data are presented as the mean ± SD. * P<0.05, ** P<0.01 and *** P<0.001 compared with the si-NC group.

viability was obvious, TE-1 cells were selected for subsequent analysis. TE-1 cell apoptosis in si-RUNX2 group was significantly increased compared to the si-NC group ($P < 0.01$, Fig. 2D). Also, the expression of apoptosis-related proteins exhibited consistent changes: cleaved PARP and cleaved caspase-3 expression levels were obviously increased in the si-RUNX2 group relative to the si-NC group (Fig. 2E). Furthermore, the number of migrated and invasive TE-1 cells was significantly reduced by RUNX2 knockdown in comparison with the si-NC group ($P < 0.05$ or $P < 0.01$, Fig. 2F and 2G).

RUNX2 overexpression enhanced HET-1A cell viability, repressed apoptosis and promoted cell migration and invasion

RUNX2 was overexpressed in HET-1A cells to explore the effects of RUNX2 on normal esophageal cells. In Fig. 3A, RUNX2 mRNA expression in cells transfected with pc-RUNX2 was remarkably up-regulated relative to the pcDNA3.1 group ($P < 0.001$). MTT assay showed that RUNX2 overexpression significantly elevated HET-1A cell viability at 3 and 4 days after transfection ($P < 0.05$ or $P < 0.01$, Fig. 3B). Flow cytometry results in Fig. 3C showed HET-1A cell apoptosis was significantly reduced by RUNX2 overexpression compared to the pc-DNA3.1 group ($P < 0.05$). Also, the expression of cleaved PARP and cleaved caspase-3 was obviously down-regulated by RUNX2 overexpression relative to the pcDNA3.1 group (Fig. 3D). Furthermore, HET-1A cell migration and invasion were significantly increased by RUNX2 overexpression in comparison with the pcDNA3.1 group (both $P < 0.01$, Fig. 3E and 3F).

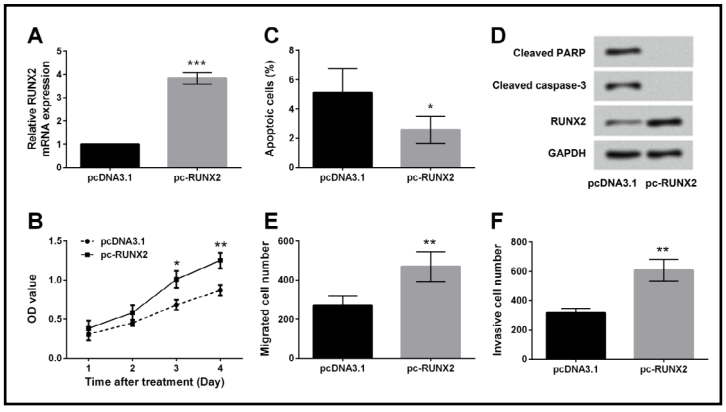

Fig. 3. Overexpression of RUNX2 elevated cell viability, reduced cell apoptosis and promoted migration and invasion in HET-1A cells. HET-1A cells were transfected with pc-RUNX2 or pcDNA3.1. (A) The mRNA expression of RUNX2 after transfection by qRT-PCR assay. (B) Cell viability after transfection by MTT assay. (C) Percentage of apoptotic cells after cell transfection by flow cytometry assay. (D) Expression of apoptosis-related proteins by western blot analysis. Migration (E) and invasion (F) were measured by Transwell assay. Data are presented as the mean ± SD. * P<0.05, ** P<0.01 and *** P<0.001 compared with the pcDNA3.1 group.

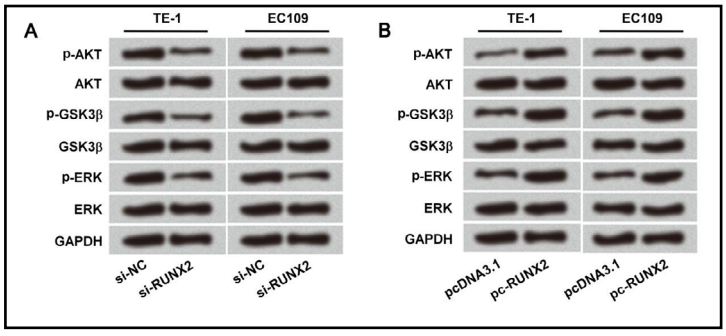

Fig. 4. RUNX2 overexpression activated the PI3K/AKT and ERK signaling pathways in esophageal carcinoma. TE-1 and EC109 cells were transfected with si-NC, si-RUNX2, pcDNA3.1 or pc-RUNX2. Expression of key kinases in the PI3K/AKT and ERK signaling pathways was measured by western blot analysis. (A) RUNX2 knockdown inhibited the PI3K/AKT and ERK signaling pathways. (B) RUNX2 overexpression activated the PI3K/AKT and ERK signaling pathways.

RUNX2 overexpression activated the PI3K/AKT and ERK signaling pathways

To explore the regulatory mechanism of RUNX2 in esophageal carcinoma, the expression levels of key kinases in the PI3K/AKT and ERK signaling pathways were determined after RUNX2 knockdown and overexpression. The results showed that the expression levels of p-AKT, p-GSK3β and p-EKT in TE-1 and EC-109 cells were decreased by RUNX2 knockdown compared with the si-NC group (Fig. 4A), while were increased by RUNX2 overexpression relative to the pcDNA3.1 group (Fig. 4B). These data indicated that RUNX2 overexpression activated the PI3K/AKT and ERK signaling pathways, whereas knockdown of RUNX2 had the opposite effect.

RUNX2-mediated TE-1 cell viability, migration and invasion depended on PI3K/AKT and ERK signaling pathways

To further verify the regulatory relationship between RUNX2 and the PI3K/AKT and ERK signaling pathways in esophageal carcinoma, a PI3K/AKT pathway inhibitor LY294002 and an ERK inhibitor U0126 were used to treat TE-1 cells transfected with pcDNA3.1 or pc-RUNX2. The results showed that transfection with pc-RUNX2 resulted in a significant increase of TE-1 cell viability compared with transfected with pcDNA3.1 ($P < 0.001$), which was significantly reversed by LY294002 and/or U0126 treatment ($P < 0.001$, Fig. 5A). Moreover, TE-1 cell migration (Fig. 5B) and invasion (Fig. 5C) were significantly promoted after RUNX2 overexpression relative to the pcDNA3.1 group ($P < 0.001$), which was also markedly reversed by LY294002 and/or U0126 treatment ($P < 0.001$). These data confirmed that RUNX2-mediated TE-1 cell viability, migration and invasion depended on the PI3K/AKT and ERK signaling pathways.

Knockdown of RUNX2 suppressed tumor formation in vivo

The effects of RUNX2 knockdown on tumor formation *in vivo* were finally detected in mice. The results showed that tumor weights (Fig. 6A) and volume (Fig. 6B) in mice injected with TE-1 cells silencing RUNX2 were significantly lower than those mice injected with si-

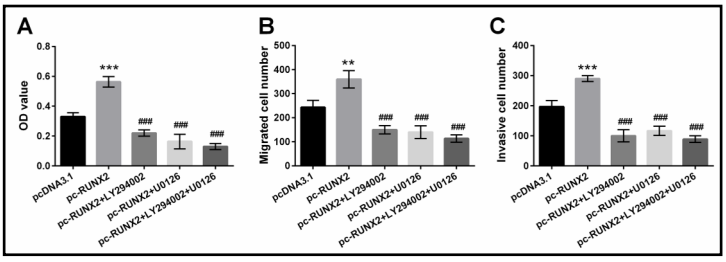

Fig. 5. RUNX2-mediated TE-1 cell viability, migration and invasion depended on the PI3K/AKT and ERK signaling pathways. A PI3K/AKT pathway inhibitor LY294002 and an ERK inhibitor U0126 were used to treat TE-1 cells transfected with pcDNA3.1 or pc-RUNX2. (A) Cell viability by MTT assay. Migration (B) and invasion (C) were measured by Transwell assay. Data are presented as the mean ± SD. *** P<0.001 compared with the pcDNA3.1 group. ### P<0.001 compared with the pc-RUNX2 group.

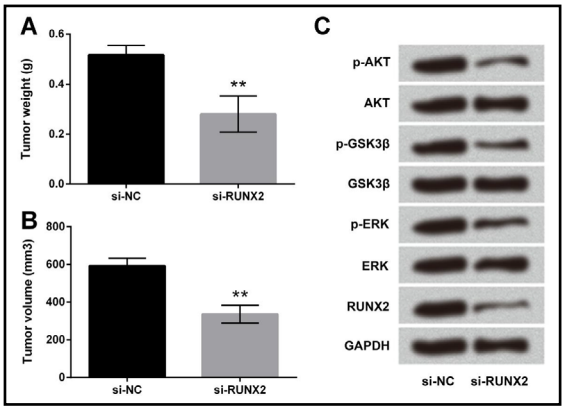

Fig. 6. Knockdown of RUNX2 suppressed tumor formation in vivo. TE-1 cells transfected with si-RUNX2 or si-NC were injected subcutaneously into the right hind legs of 4-week-old nude mice. (A) Tumor weights. (B) Tumor volume. (C) The expression of key kinases in the PI3K/AKT and ERK signaling pathways. Data are presented as the mean ± SD. ** P< 0.01 compared with the si-NC group.

NC-transfected cells (both $P < 0.01$). Moreover, the expression levels of p-AKT, p-GSK3β, p-EKT and RUNX2 in mice injected with si-RUNX2-trasnfected TE-1 cells were significantly decreased compared with mice injected with si-NC-transfected cells (Fig. 6C). These data indicated that knockdown of RUNX2 suppressed tumor formation *in vivo* via regulating the PI3K/AKT and ERK signaling pathways.

Discussion

The present study investigated the function of RUNX2 in esophageal cancer. The results showed that RUNX2 was highly expressed in esophageal carcinoma tissues and cells. Knockdown of RUNX2 significantly inhibited TE-1 and EC109 cell viability, repressed TE-1 cell migration and invasion, and induced TE-1 cell apoptosis. Overexpression of RUNX2 showed the opposite effects on HET-1A cells. Moreover, RUNX2-mediated cell viability, migration and invasion of TE-1 cells were associated with the activation of the PI3K/AKT and ERK signaling pathways. Besides, by tumor xenograft experiment, we found that knockdown of RUNX2 suppressed tumor formation *in vivo*. These findings confirmed the key role of RUNX2 in esophageal cancer development, which merits further discussion.

Accumulating evidences have confirmed the oncogenic roles of RUNX2 in several cancers. Lucero et al. demonstrated that aberrant expression of RUNX2 modulated cell proliferation in human osteosarcoma cell line [23]. Targeting RUNX2 by miR-218 can suppress cell proliferation and invasion in ovarian cancer [24]. Silencing of the RUNX2 is also confirmed to inhibit SGC7901 cell proliferation and promote apoptosis in gastric cancer [25]. Wang et al. revealed that RUNX2 suppression of RUNX2 could inhibit epithelial ovarian carcinoma cell proliferation, migration and invasion [26]. Consistent with these findings, our results showed that knockdown of RUNX2 knockdown inhibited TE-1 and EC109 cell viability, repressed TE-1 cell migration and invasion, and induced TE-1 cell apoptosis *in vitro* and suppressed tumor formation *in vivo*. Meanwhile, RUNX2 overexpression showed the opposite effects on normal esophageal cells. Therefore, we speculated that RUNX2 might play an oncogenic role in regulating the viability, migration, invasion and apoptosis of esophageal carcinoma cells.

Furthermore, the PI3K/AKT pathway has been found widely involved in the regulation of numerous cellular processes, including angiogenesis, cell proliferation and metabolism [27-29]. In addition, activation of the PI3K/AKT signaling pathway is shown to be a key mechanism to mediate the roles of Id-1 (inhibitor of differentiation or DNA binding) in promoting tumorigenicity and metastasis of human esophageal cancer cells [30]. Baicalein can induce cell apoptosis in ESCC via modulation of the PI3K/AKT pathway [31]. Importantly, it has reported that RUNX2 enhances the differentiation and migration of osteoblast and chondrocyte through coupling with the PI3K/AKT signaling [32]. The interaction of RUNX2 with the PI3K/AKT signaling pathway is confirmed to act a driving force in controlling tumor progression and aggressiveness [33]. A previous study has confirmed that RUNX2, acting as an important downstream mediator of the PI3K/AKT pathway, can regulate metastatic properties of breast cancer cells [34]. In this study, overexpression of RUNX2 promoted TE-1 cell viability, migration and invasion, which was significantly reversed by LY294002 and/or U0126 treatment. Therefore, it can be speculated that RUNX2 may regulate TE-1 cell proliferation, migration and invasion through mediating the PI3K/AKT and ERK signaling pathways.

Our results indicate that RUNX2 is highly expressed in esophageal carcinoma tissues and cells and may play an oncogenic role in esophageal carcinoma by activating the PI3K/AKT and ERK signaling pathways. RUNX2 may serve as a potent target for the treatment of esophageal carcinoma.

Disclosure Statement

The authors declare to have no competing interests.

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