Introduction

Esophageal squamous cell carcinoma (ESCC) is a common malignant tumor of the digestive tract and an important health-related problem in the world (1). China is one of the countries with the highest incidence and mortality of esophageal cancer, with ESCC as the main subtype (2). Although progress in the diagnosis and treatment of ESCC has been made in recent years, the prognosis is still poor and the 5-year survival rate is less than 30%, mainly because many patients are diagnosed at an advanced stage. ESCC in the early stage has no obvious clinical symptoms and a lack of effective and non-invasive methods for the early diagnosis (3). Therefore, it is urgent to better understand the molecular mechanism and identify biomarkers for the diagnosis, prognosis and treatment of ESCC.
Endothelin-1 (ET-1), as a member of endothelin families including three 21-amino acid peptides, is the most potent vasoconstrictor agent ever identified and participated in the progression of various diseases including pulmonary artery hypertension (4), cardiovascular disease (5), atherosclerosis (6) and osteoarthritis (7). Recently, several studies have proved the aberrant expression of ET-1 in multiple cancers such as ovarian cancer (8), prostate cancer (9), breast cancer (10) and colon cancer (11). Moreover, ET-1 plays an important role in the development and progression of various tumors through mediating cell proliferation, migration and invasion, for example, knockdown of ET-1 significantly inhibited lung cancer cell proliferation and invasion (12). Said et al. showed that silencing of ET-1 decreased tumor metastasis of bladder cancer (13) and osteosarcoma (14). However, the value and mechanism of ET-1 in the progression of ESCC remains poorly understood.

Increasing evidence confirmed that the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway, as a regulatory factor of tumor progression, was activated in various cancers (15,16). For example, inhibition of the PI3K/Akt signaling pathway suppressed cell proliferation, invasion and migration of colorectal cancer (17), lung cancer (18), oral squamous cell carcinoma (19), hepatocellular carcinoma (20) and ESCC (21), etc. Moreover, several tumor-related genes (TRE), as the upstream regulator of the PI3K/Akt signaling pathway, were involved in tumor progression and development via regulating cancer cell malignant biological behavior, for instance, upregulation of gene of phosphate and tension homology deleted on Chromosome 10 (PTEN) inhibited tumor progression and enhanced the sensitivity of cancer cell to chemotherapy through blocking the PI3K/Akt signaling pathway (22,23). Wang et al. showed that human ether a-go-go-related gene 1 (HERG1) promoted ESCC cell proliferation and metastasis through activating the PI3K/Akt signaling pathway (24). Wei et al. found that Yes-associated protein (YAP) suppressed the progression of ovarian cancer by inactivating the PI3K/Akt signaling pathway (25). Interestingly, previous studies have proved that the function of ET-1 in the progression of various diseases by regulating the PI3K/Akt signaling pathway, for example, inhibition of ET-1 alleviated the pathophysiology of amyotrophic lateral sclerosis by blocking the PI3K/Akt signaling pathway (26). Horowitz et al. reported that overexpression of ET-1 promoted myofibroblast resistance to apoptosis through inhibiting the PI3K/Akt signaling pathway (27). However, the effect of ET-1 on tumor growth and metastasis of ESCC via regulating the PI3K/Akt signaling pathway remains elusive.

In the present study, the expression of ET-1 was determined in ESCC tissues and cell lines. Moreover, we further to examine the role of ET-1 in the proliferation, cell cycle, migration, and invasion of ESCC cells. Mechanistically, to explore whether ET-1 regulated the tumor growth and metastasis of ESCC through PI3K/Akt pathway. Collectively, our studies may provide an important indicator of prognosis and guidance for ESCC clinical treatment.

Methods

Clinical samples

The ESCC tissues and their paired normal tissues were collected from the patients (n=45) from 2012 to 2017 at The First Affiliated Hospital of Nanchang University, none of the patients received chemotherapy or radiotherapy prior to surgery. The clinical specimens were immediately frozen at −80 °C refrigerator after resection and prepared for further experiments. Besides, all the patients have signed the informed consent and the approval was also obtained from the Ethics Committee of The First Affiliated Hospital of Nanchang University. All the involved clinical experiments were conducted in accordance with the principle of the “Declaration of Helsinki”.

Cell culture

The human ESCC cell lines (TE-1, ECA109, KYSE150, CaES-17, and EC9706), human normal esophageal epithelial cell line Het-1A and HEK-293 cells were purchased from Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) in the incubator with a humidified atmosphere containing 5% CO₂ at 37 °C.

Transfection

The ET-1 siRNA and scrambled negative control RNA were designed and constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). All the above vectors were transfected into the ECA109 cells using the Lipofectamine
3000 kit purchased from Invitrogen (Thermo Fisher Scientific, USA) according to the manufacturer's instruction.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

The total RNA of tissues and cell lines were extracted using the Trizol reagent purchased from Life Technologies (USA). After that, the TaqMan Reverse Transcription Reagents (Applied Biosystems, USA) were employed to reversely transcribed the total RNA to complementary DNA (cDNA). The SYBR Green PCR Master Mix (Applied Biosystems, USA) was next used to quantify the expression levels of targeted genes at transcriptional levels. The relative mRNA levels were normalized to β-actin. The sequences of the involved primers were listed in Table 1.

**Western blot**

The polyvinylidene fluoride (PVDF) membranes were incubated overnight at 4 ℃ with the primary antibodies ET-1 (1:1,000, #ab117529, Abcam, USA), p-PI3K (1:1,000, #17366, Cell Signaling Technology, USA), PI3K (1:1,000, #4249, Cell Signaling Technology, USA), p-Akt473 (1:2,000, #4060, Cell Signaling Technology, USA), p-Akt308 (1:1,000, #13038, Cell Signaling Technology, USA), Akt (1:1,000, #4685, Cell Signaling Technology, USA) and β-actin (1:1,000, #ab5694, Abcam, USA). The horseradish peroxidase-coupled secondary antibody (anti-rabbit immunoglobulin (Ig)G (H+L); 1:3,000, #14708, Cell Signaling Technology, USA) was incubated with the membranes for 1 h at room temperature. The electrochemiluminescence (ECL) Western blot detection kit (Bio-Rad, USA) was employed to detect the optical density (OD) of the protein bands to evaluate the expression levels of the protein proteins.

**Cell counting Kit-8 (CCK-8) assay**

The ECA109 cells were cultured under the standard conditions and transfected with different vectors until the cell confluency reached about 70–80%. After that, the cells were collected and seeded in the 96-well plates at the density of 5,000 cells per well. After that, the CCK-8 kit (AbMole, USA) was employed to determine cell proliferation according to the manufacturer's protocol. Briefly, the CCK-8 solution (10 μL per well) was added into the wells and incubated with the cells for 2 h. The OD values were then detected in the wavelength of 450 nm to evaluate cell proliferative ability.

**Flow cytometry analysis**

Collected the samples mentioned above, washed with PBS, centrifuged at 800 xg for 6 min, suspended in ice-cold 70% ethanol/phosphate buffered saline (PBS), centrifuged at 800 xg for another 6 min, and suspended with PBS. The cell cycle of ECA109 cells was verified with the Cell Cycle kit (Multiscience Biotech, China) by cytometry. The cytometry was executed with Beckman CytoFLEX FCM.

**Transwell migration and invasion assay**

The ECA109 cells were transfected with different vectors and collected until the cell confluency reached about 70–80%. Paced the 2×10^4 cells on the surface of the Transwell upper chamber. For invasion assay, Matrigel (BD Biosciences, USA) was coated the upper surface of polycarbonate filters. After 24 h, the invaded and migrated cells were fixed with 4% paraformaldehyde and rinsed three times with PBS, then stained with 0.1% crystal violet for 10 min and rinsed three times with PBS. Random selection 5 fields of vision for cell count, observation and photography.

**Animal model**

All animal experiments were performed in accordance with the Laboratory Animal Care guidelines of Animal Ethics Committee of The First Affiliated Hospital of Nanchang University. ECA109 cells were pre-transfected with ET-1 siRNA for 24 h. Afterward, 200 μL cells (1×10^5)
were injected subcutaneously into the flanks of 6-week-old female nude mice (8 mice per group) and allowed to form tumors. Tumors’ length and width were measured every three days. After 4 weeks of incubation, the tumor tissues were collected for further experiments, such as the immunohistochemistry staining was performed to detect the expression of Ki-67 (1:1,500, Abcam, USA) according to the previous studies (28).

Statistical analysis

The data in this study are presented as mean ± standard deviation (SD) of 3 or 8 individual experiments and analyzed by a one-way analysis of variance (ANOVA) test, and the difference between the two groups was determined using the Student t-test, which was considered statistically significantly P<0.05 or P<0.01. Survival data were estimated using the Kaplan-Meier survival curves and analyzed using the log-rank test.

Results

ET-1 was overexpressed in ESCC and associated with poor outcome

Previous studies confirmed that ET-1 plays an important role involved in the progression of multiple tumors (29), we examined the mRNA expression level of ET-1 in ESCC tissues and cell lines. As expected, ET-1 was significantly overexpressed in ESCC tissues relative to the adjacent tissues (P<0.001, Figure 1A). Immunohistochemical staining showed that ET-1 was expressed significantly higher in ESCC tumors than in the adjacent tissues (Figure 1B). Meanwhile, high expression of ET-1 was positively correlated with tumor size (P<0.05), tumor stage (P<0.01), and tumor metastasis (P<0.05) by comparing clinicopathological characteristics (Table 2). In addition, we further to assess the correlation between the expression level of ET-1 and 5-year survival of patients with ESCC using Kaplan-Meier analysis, and the results showed that patients...
with high ET-1 expression in ESCC tumors displayed a worse overall survival (P<0.05, Figure 1C). Furthermore, RT-qPCR analysis results revealed that the protein level of ET-1 in ESCC cells was higher than in normal esophageal epithelial cells (Het-1A; P<0.001, Figure 1D), especially ECA109 cells (P<0.01). Taken together, ET-1 may serve as an oncogene in the progression of ESCC.

Knockdown of ET-1 suppressed the proliferation and metastasis of ESCC cells

To examine whether ET-1 exhibits tumor-promoting activity through regulating the biological behavior of ESCC cells, ECA109 cells were transfected with ET-1 siRNA to decrease the expression of ET-1. As expected, silencing of ET-1 significantly decreased the protein level of ET-1 in ECA109 cells compared with the si-NC group (both P<0.01, Figure 2B). Knockdown of ET-1 significantly decreased the number of ECA109 cells in G2/M phase and increased the number of cells in the G0/G1 phase (P<0.01, Figure 2C,D). Moreover, Transwell assay results showed that ET-1 knockdown markedly reduced the migration and invasion capacity of ECA109 cells (both P<0.001, Figure 2E,F,G,H). Taken together, the above results suggested that the downregulation of ET-1 exerted an anti-proliferation and anti-metastasis effect in ESCC.

Knockdown of ET-1 blocked the PI3K/Akt signaling pathway

Increasing evidence confirmed that the PI3K/Akt signaling pathway plays an important role in the progression of tumors, including ESCC (30), gastric cancer (31), and lung cancer (32), etc. Herein, to further explore whether ET-1 regulated the progression of ESCC via the PI3K/Akt signaling pathway. As shown in Figure 3, compared with the control group, knockdown of ET-1 significantly decreased the levels of p-PI3K, p-Akt\textsubscript{308} and p-Akt\textsubscript{473} in ECA109 cells (both P<0.001), while no change the expression of PI3K and Akt. Of note, ECA109 cells treated with the PI3K/Akt

### Table 2 Relationship between the expression of ET-1 in the tissues and clinicopathological characteristic of esophageal squamous cell carcinoma

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>n</th>
<th>ET-1 expression</th>
<th>(\chi^2)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(^a)Low (%)</td>
<td>High (%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>20</td>
<td>9 (20.00)</td>
<td>11 (24.44)</td>
<td>0.218</td>
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<td>≥60</td>
<td>25</td>
<td>13 (28.89)</td>
<td>12 (26.67)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24</td>
<td>12 (26.67)</td>
<td>12 (26.67)</td>
<td>0.025</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>10 (22.22)</td>
<td>11 (24.44)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>15</td>
<td>11 (24.44)</td>
<td>4 (8.89)</td>
<td>5.380</td>
</tr>
<tr>
<td>≥3</td>
<td>30</td>
<td>11 (24.44)</td>
<td>19 (42.22)</td>
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<tr>
<td>TNM stage</td>
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<tr>
<td>I–II</td>
<td>19</td>
<td>13 (28.89)</td>
<td>6 (13.33)</td>
<td>5.021</td>
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<tr>
<td>III–IV</td>
<td>26</td>
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<td>17 (37.78)</td>
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<td>Lymph node metastases</td>
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</tr>
<tr>
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<td>4 (8.89)</td>
<td>4.132</td>
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<tr>
<td>Yes</td>
<td>31</td>
<td>12 (26.67)</td>
<td>19 (42.22)</td>
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</tr>
</tbody>
</table>

\(^a\), 45 paired of cases of ESCC tissues were divided into groups according to the median expression of ET-1: a high ET-1 expression group (above the median ET-1 expression) and low ET-1 expression group (below the median ET-1 expression).
Knockdown of ET-1 inhibited cell proliferation and metastasis of ESCC cells. (A) The protein level of ET-1 in ECA109 cells transfected with ET-1 siRNA (si-ET-1) and control was determined using western blot; (B) CCK-8 was applied to examine the proliferation ability of ECA109 cells; (C,D) flow cytometry was used to measure the number of ECA109 cells in G0/G1, G2/M and S phase; (E,G) the invasion capacity of ECA109 cells was measured by Transwell assay, Scale bar = 50 μm; (F,H) Transwell assay was used to evaluate the migration ability of ECA109 cells, Scale bar = 50 μm. **, P<0.01, ***, P<0.001 compared with the si-NC group. ESCC, esophageal squamous cell carcinoma.

signaling pathway activator IGF-1 significantly attenuated the inhibitory effect of ET-1-silenced on the expression of p-PI3K, p-Akt$^{308}$ and p-Akt$^{473}$ (both P<0.001). Taken together, knockdown of ET-1 inactivated the PI3K/Akt signaling pathway in ESCC cells.

**Silencing of ET-1 inhibited proliferation and metastasis of ESCC cells through inactivating the PI3K/Akt signaling pathway**

To further examine whether ET-1-silenced serves anti-proliferation and anti-metastasis activities in ESCC through inhibition of the PI3K/Akt signaling pathway. As shown in Figure 4A, treatment with PI3K/Akt signaling pathway activator IGF-1 plus knockdown of ET-1 significantly alleviated the inhibitory effect of ET-1-silenced on the proliferation ability of ECA109 cells (P<0.01). Moreover, compared with the si-ET-1 group, the number of G2/M phase and G0/G1 phase in ECA109 cells transfected with ET-1 siRNA and then treated with IGF-1 were significantly decrease and increased (all P<0.01, Figure 4B,C). Furthermore, we examined whether IGF-1 could
downregulate the effect of ET-1 knockdown on the invasion and migration capacity of ESCC cells. As expected, IGF-1 treatment blocked the knockdown of ET-1 suppressed the invasion and migration of ECA109 cells (both P<0.001, Figure 4D,E,F,G). Of note, no significant difference between the control group and the si-ET-1 + IGF-1 group. The results showed that the activation of the PI3K/Akt signaling pathway could alleviate the inhibitory effect of ET-1 silenced on the proliferation and metastasis effect in ESCC cells.

**ET-1 inhibition suppressed tumor growth of ESCC in vivo**

To further measure the effect of inhibition of ET-1 on tumor growth of ESCC in vivo. We observed that knockdown of ET-1 resulted in a significantly decreased tumor growth (Figure 5A,B) and tumor weight (P<0.001, Figure 5C) when compared with the control group. Immunohistochemistry results showed that inhibition of ET-1 markedly decreased the level of Ki-67 in tumor tissues (Figure 5D). Taken together, knockdown of ET-1 significantly inhibited tumor growth in vivo.

**Discussion**

ESCC was a is a common malignant tumor, which has the characteristics of atypical symptoms, difficult treatment and low survival rate (33). However, current treatment methods and effective diagnosis target of ESCC are limited, leading to the poor overall survival of ESCC. In the present study, we demonstrated that ET-1 plays an important role in the progression of ESCC, inhibition of ET-1 significantly decreased cell proliferation, migration, and invasion of ESCC. Mechanistically, ET-1 exhibits anti-proliferation and anti-metastasis activities via blocking the PI3K/Akt signaling pathway in ESCC.

Increasing evidence confirmed that TRE regulated tumor progression through mediating the malignant biological behavior of cancer cells (34). Importantly, some studies have explored the therapeutic targeting of TRE in multiple cancers, and various pharmaceutical inhibitors targeting TRE have been developed (35,36). For example, ET-1, also called endothelin-1, plays a critical role in the progression and development of cancers, including nasopharyngeal carcinoma (37), gastric cancer (38), and prostate cancer (39), while the role and mechanism of ET-1 in ESCC are unknown. In the present study, our data showed that ET-1 was upregulated in tumor tissues and cell lines of ESCC, silencing of ET-1 inhibited cell proliferation and metastasis in ESCC. Moreover, previous studies have confirmed that overexpression of ET-1 promoted the risk of development and progression of papillary thyroid cancer (40) and breast cancer (41). Furthermore, ET-1 elevated induced the migration and proliferation of tumor cells through regulating the PI3K/ERK/c-FOS/AP1 signaling pathway (42). In this study, we demonstrated that inhibition of ET-1 suppressed ESCC cell proliferation, migration and invasion by blocking the PI3K/Akt signaling pathway.

Accumulated studies have explored the therapeutic targeting of the PI3K signaling pathway in various tumors; however, the clinical effect was not satisfactory (43). For example, inhibition of the PI3K/Akt signaling pathway suppressed tumor progression by decreasing cancer stem-like cell expansion (44). Philip et al. showed that the inactivation of the PI3K/Akt signaling pathway improved the individualized treatment effect of endometrial cancer patients with PTEN mutations (45). Ao et al. reported that inhibition of the PI3K/Akt signaling pathway suppressed
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Figure 4 Silencing of ET-1 suppressed biological behavior of ESCC cells through inactivating of the PI3K/Akt pathway. (A) CCK-8 was used to detect the cell viability; (B,C) flow cytometry was used to measure the number of ECA109 cells in G0/G1, G2/M and S phase; (C,D,F) Transwell assay was used to measure the migration capacity of ECA109 cells, Scale bar=50 μm; (E,G) the invasion capacity of ECA109 cells was examined by Transwell assay, Scale bar=50 μm. **, P<0.01, ***, P<0.001, compared with the control group; ##, P<0.01, ###, P<0.001, compared with the si-ET-1 group. ESCC, esophageal squamous cell carcinoma.

gastric cancer cell proliferation, migration, and invasion and induced cell apoptosis (46). Liu et al. confirmed that the treatment of hepatocellular carcinoma cells with the PI3K/Akt signaling pathway inhibitor leads to the expansion of liver cancer stem cells via the inactivation of SGK3 (47).

In addition, previous studies have proved that the PI3K/Akt signaling pathway serves an important role involved in the process of EMT in various tumors (48). For instance, one of the current studies revealed that inactivation of the PI3K/Akt signaling pathway decreased the epithelial-mesenchymal transition in ovarian cancer (48). In line with the above results, our studies found that activation of the PI3K/Akt signaling pathway induced by IGF-1 addition promoted ESCC cell proliferation, migration, and invasion, while knockdown of ET-1 alleviated this effect.

In conclusion, we have demonstrated that ET-1 plays a
vital role in the progression of ESCC. More importantly, treatment with the PI3K/Akt signaling pathway activator IGF-1 alleviated the inhibitory effect of ET-1 knockdown on tumor growth and metastasis of ESCC cells, which is an attractive therapeutic regimen for the treatment of ESCC.

Acknowledgments

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.04.26). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All patients selected for our study were fully informed about our experimental protocols, which were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval ID: 20180528).

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