Targeting Overexpressed Activating Transcription Factor 1 (ATF1) Inhibits Proliferation and Migration and Enhances Sensitivity to Paclitaxel in Esophageal Cancer Cells

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Background: Previous reports showed that Activating Transcription Factor 1 (ATF1) plays an important role in tumor progression in a tumor-specific manner. However, little is known about the expression and role of ATF1 in esophageal cancer.

Material/Methods: The expression of ATF1 was examined by immunohistochemistry and Western blotting. The correlation between the expression of ATF1 and clinical characteristics of esophageal squamous cell carcinomas (ESCC) patients was analyzed by Fisher’s exact test. The role of cell proliferation, clonogenic survival, migration, and invasion in vitro, as well as the sensitization to paclitaxel, were determined after knockdown of ATF1 by siRNA.

Results: ATF1 was overexpressed in ESCC tissues, which was positively correlated with lymph node metastasis, poor differentiation, and early tumor invasion of esophageal cancer patients. Knockdown of ATF1 effectively reduced cell proliferation, induced S phase cell cycle arrest, and inhibited cell migration and invasion. Moreover, silencing of ATF1 significantly enhanced the sensitivity of esophageal cancer cells to paclitaxel.

Conclusions: These findings suggest that ATF1 is a promising drug target for esophageal cancer.

MeSH Keywords: Activating Transcription Factor 1 • Cell Migration Assays • Cell Proliferation • Esophageal Neoplasms

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Background

Esophageal squamous cell carcinoma (ESCC), the major histological subtype of esophageal cancer, occurs at a high frequency in southern and eastern Africa, Japan, and especially in certain areas of China [1,2]. Because of a lack of early screening tools and effective biomarkers for the general population, the majority of patients with ESCC are diagnosed with wide lymph node metastasis [3,4]. Despite primary surgical and systematic administration of chemotherapy regimens, the 5-year survival rate for patients with advanced disease remains below 20% [3,4]. Therefore, it is important to find effective biomarkers for esophageal cancer that can predict the progression of advanced esophageal cancer and provide an effective anticancer target.

Activating transcription factor 1 (ATF1) belongs to the ATF/CREB family of transcription factors, which specifically binds to the consensus ATF/CRE site ‘TGACGTCA’ and regulates the transcription of target genes to participate in various cellular processes [5]. Previous reports showed that ATF1 was overexpressed in several cancers, including lymphomas [6], nasopharyngeal carcinoma [7,8], and melanoma [9], and involved in cell growth and differentiation. On the other hand, several reports indicated that ATF1 might act as a tumor suppressor in breast cancer [10], and higher ATF1 expression was a predictor of a favorable outcome for the overall survival in colorectal cancer [11]. These results suggest that ATF1 plays an important role in tumor progression in a tumor-specific manner. However, the role of ATF1 in ESCC is still unclear.

Here, we investigated the expression and role of ATF1 in ESCC. Results showed that ATF1 was overexpressed in ESCC tissues, which was positively correlated with lymph node metastasis, poor differentiation, and early tumor invasion of esophageal cancer patients. To further confirm the role of ATF1, we downregulated the expression of ATF1 using siRNA, and found that knockdown of ATF1 effectively reduced cell proliferation, induced S phase cell cycle arrest, and inhibited cell migration and invasion. Moreover, silencing of ATF1 significantly sensitized ESCC cells to paclitaxel. These findings suggest that ATF1 is an attractive drug target for esophageal cancer.

Material and Methods

Cell lines

ESCC cell lines (EC1, EC9706, EC109, TE1, TE13, Kyse140, and Kyse450) were cultured in Dulbecco’s modified Eagle’s medium (Hyclone) containing 10% FBS (Biological Industries, Inc.) at 37°C with 5% CO₂.

Immunohistochemistry (IHC) staining of human esophageal cancer tissue array

Human esophageal cancer tissue array was purchased from Xi’an Alenabio Biotech Co. Ltd. The detailed clinicopathological characteristics of esophageal squamous cell carcinoma patients are listed in Table 1 for statistical analysis. IHC staining was done with specific ATF1 antibody (Abcam Trading Company Ltd, Shanghai, China) according to our previous reports [12]. Briefly, the tissue array sections (5 μm) were dehydrated and subjected to peroxidase blocking. Primary antibodies were added and incubated at 4°C overnight, followed by staining with a Histostain-SAP kit (ZSGB-Bio, Beijing, China). The slides were counterstained with hematoxylin. The stained slides were observed by microscopy and images were acquired. Based on staining intensity, samples were classified into 5 groups, from the lowest density (–) to the highest (++++) [13].

Collection of esophageal cancer tissues

The esophageal cancer tissues and adjacent esophageal tissues used in Western blotting assay were collected from 16 esophageal squamous cell carcinoma patients undergoing resection at the Linzhou Cancer Hospital (Linzhou, Henan, China) from July 2012 to September 2014. Histological diagnosis and tumor-node-metastasis (TNM) stages of cancers were determined in accordance with the American Joint Committee on Cancer (AJCC) manual criteria for esophageal cancer.

Gene silencing using small interfering RNA (siRNA)

EC1 and Kyse450 cells were transfected with siRNA oligonucleotides, synthesized by GenePharma (Shanghai, China) using Lipofectamine 2000. The sequences of the siRNA are as follows: siATF1-1: CGAACUACACCUUCAGCUA; siATF1-2: GAAUCUUUGCACGUA; siControl: UUCUCGGAACUGGUCACGU.

Cell viability and clonogenic assay

EC1 and Kyse450 cells were transfected with siControl or siATF1 for 24 h and then seeded in 96-well plates (2.5×10³ cells per well) for 96 h. Cell viability was determined by MTT according to the manufacturer’s protocol.

For the clonogenic assay, cells transfected with siControl or siATF1 were seeded into 6-well plates with 500 cells per well in triplicate and incubated for 9 days. The colonies formed were fixed, stained, and counted. The colonies with more than 50 cells were counted.

Cell cycle analysis

EC1 and Kyse450 cells were transfected with siControl or siATF1 and then cells were harvested, fixed in 70% ethanol at –20°C,
stained with propidium iodide (PI, 50 μg/ml, Sigma) containing RNase A (30 μg/ml, Sigma) at 37°C for 30 min, and analyzed for cell cycle profile by flow cytometry (Becton Dickinson FACScan). Data were analyzed with ModFit LT software (Verity).

**Soft agar assay**

We prepared 0.4% and 0.8% agar dissolved in DMEM, and 2 ml 0.8% agar was added in the bottom of the 6-well plate. EC1 cells were transfected with ATF1 siRNA or negative control siRNA. Twenty-four hours later, about $5 \times 10^4$ cells were mixed with 0.4% agar and added on top of 0.8% agar. After routine culture for 2 weeks, colonies were captured under a microscope.

** Invasion assay**

The invasion assay was carried out in matrigel (Becton Dickinson)-coated transwell inserts with a pore size of 8 μm, as described previously [13]. Briefly, the inserts were pre-coated with matrigel. EC1 cells ($3 \times 10^4$) were transfected with siRNA and seeded in serum-free medium in the upper chamber, and medium with 10% FBS was added to the lower well. After incubating for 24 h, the cells in the upper chamber were carefully removed with a cotton swab. The inserts were then fixed in methanol and stained with 0.4% crystal violet.

### Table 1. Correlation between the expression of ATF1 and clinical characteristics of ESCC patients.

| Characteristics                   | Total n | Negative n (%) | Positive n (%) | P   |
|-----------------------------------|---------|----------------|----------------|-----|
| Overall                           | 114     | 56 (49)        | 58 (51)        | –   |
| Sex                               |         |                |                | 0.745|
| Male                              | 85      | 41 (48)        | 44 (52)        |     |
| Female                            | 29      | 15 (52)        | 14 (48)        |     |
| Age (y)                           |         |                |                | 0.703|
| <60                               | 55      | 26 (47)        | 29 (53)        |     |
| ≥60                               | 59      | 30 (51)        | 29 (49)        |     |
| Lymph node metastasis (N)         |         |                |                | 0.034|
| N0                                | 38      | 24 (63)        | 14 (37)        |     |
| N1–2                              | 76      | 32 (42)        | 44 (58)        |     |
| TNM stage                         |         |                |                | 0.006|
| I                                 | 14      | 11 (79)        | 3 (21)         |     |
| II                                | 62      | 22 (35)        | 40 (65)        |     |
| III                               | 35      | 22 (63)        | 13 (37)        |     |
| IV                                | 3       | 1 (33)         | 2 (67)         |     |
| Tumor invasion                    |         |                |                | 0.034|
| T1                                | 14      | 7 (50)         | 7 (50)         |     |
| T2                                | 50      | 18 (36)        | 32 (64)        |     |
| T3                                | 50      | 31 (62)        | 19 (38)        |     |
| Differentiation                   |         |                |                | 0.021|
| Well                              | 21      | 16 (76)        | 5 (24)         |     |
| Moderate                          | 49      | 20 (41)        | 29 (59)        |     |
| Poor                              | 44      | 20 (45)        | 24 (55)        |     |

* *P* < 0.05 was defined as significant, Fisher’s exact test.
Wound-healing assay

For wound-healing assay, cells were seeded on 6-well plates in culture medium. The confluent monolayer was scratched by a plastic pipette and then washed 3 times with PBS. Images were taken at 0 or 36 h after wounding.

Statistical analysis

The statistical significance of differences between groups was assessed using GraphPad Prism5 software. The t test was used for the comparison of parameters between groups.

Results

**ATF1 was overexpressed in human esophageal squamous cell carcinoma tissues**

To investigate the clinical significance of ATF1 in esophageal cancer, we firstly examined the expression levels of ATF1 by IHC staining of the human ESCC tissue arrays. According to the staining intensity, the samples were classified into 5 groups with increasing staining intensity from the weakest (−) to the strongest (+++). Western blotting analysis was used to determine the expression of ATF1 in ESCC tissues and adjacent esophageal tissues. The results of Western blotting are shown in the upper panel and analysis of Western blotting is shown in the lower panel. Furthermore, the overexpression of ATF1 protein was positively correlated with lymph node metastasis.
metastasis, poor differentiation, and early tumor invasion (T1–2) ($P=0.034$, $P=0.006$, and $P=0.015$, respectively; Table 1). ATF1 was expressed in all ESCC cell lines (EC1, EC9706, EC109, TE1, TE13, Kyse140, and Kyse450) tested (Figure 1C).

Downregulating the expression of ATF1 inhibited the proliferation of esophageal cancer cells

To further assess the role of ATF1 on cell proliferation of esophageal cancer, the expression of ATF1 was downregulated by siRNA. Results showed that knockdown of ATF1 by specific siRNA, siATF1-1, significantly inhibited cell proliferation (Figure 2A–2C) and colony formation (Figure 2D, 2E) in both EC1 and Kyse450 cells. However, there were no similar effect using siControl or siATF1-2, which could not inhibit the expression of ATF1 (Figure 2A); therefore, we tested the function of ATF1 in the following experiment using siATF1-1.

Knockdown of ATF1 induced S cell cycle arrest in esophageal cancer cells

To elucidate the mechanism of the growth suppression by ATF1 knockdown, we examined the cell cycle profile of the ATF1-silencing cells. As shown in Figure 3, knockdown of ATF1 triggered S cell cycle arrest in both EC1 and Kyse450 cells.

Silencing of ATF1 inhibited cell migration and invasion

Previous reports showed that ATF1 plays an important role in the process of carcinogenesis, including cell transformation and tumor invasion [14–18]. To test this hypothesis, we examined the effect of ATF1 silencing on esophageal cancer cell growth in soft agar and invasion. Results showed that knockdown of ATF1 inhibited soft agar colony capacity of EC1 cell (Figure 4A), and downregulating the expression of ATF1 inhibited EC1 cell...
Knockdown of ATF1 enhanced the cytotoxicity of paclitaxel to ESCC cells

To investigate ATF1 as a novel chemosensitizer, ESCC cells EC1 and Kyse450 were treated with paclitaxel for 72 h after transfection with siControl or siATF1-1, then cell viability was examined by ATPLite assay. Results showed that knockdown of ATF1 significantly enhanced the cytotoxicity of paclitaxel and inhibited cell growth in a dose-dependent manner (Figure 5).

Discussion

Despite the improvement of screening and therapy, the prognosis of ESCC remains poor. It is still important to identify novel tumor markers and assess their role in tumor progress and tumor response to therapy.

Previous reports showed that ATF1 plays a crucial role in carcinogenesis and participates in various cellular processes, including cell transformation [14,17], cell cycle [19], DNA damage [20], apoptosis [21], and metabolic homeostasis [22]. ATF1 is overexpressed in cancer, including lymphomas [6], nasopharyngeal carcinoma [7], and melanoma [9], and is involved in cell growth and associated with clinical stage. High expression of ATF1 promotes nasopharyngeal carcinoma cell tumorigenesis [8]. However, other reports suggest that ATF1 acts as a tumor suppressor in breast cancer [10] and in colorectal cancer [11]. These results indicate that ATF1 regulates carcinogenesis and progression in a tumor-specific manner. In the present study, we showed that ATF1 was overexpressed in esophageal squamous cell carcinomas tissues, which was positively correlated with lymph node metastasis, poor differentiation, and early tumor invasion of esophageal cancer patients. Moreover, knockdown of ATF1 significantly inhibited cell proliferation of ESCC cells. These results indicate that ATF1 may promote ESCC cell growth.

Invasion and metastasis are important characteristics and major causes of death of malignant tumors, especially ESCC [23]. Previous studies have demonstrated that ATF1 was not detected in normal melanocytes but was easily found in metastatic melanoma cells [6]. Overexpression of ATF1 has been shown

Figure 3. Knockdown of ATF1 induced G0/G1 cell cycle arrest of human esophageal cancer cells. EC1 (A) and Kyse450 (B) cells were transfected with siRNA and then stained by PI staining. Cell cycle profile was analyzed by fluorescence-activated cell sorting (FACS) analysis. Representative images are shown in left panel and statistical results were shown in right panel.
Figure 4. Knockdown of ATF1 inhibited cell migration and invasion in human esophageal cancer cells. (A) Effect of silencing ATF1 on clonogenic survival of EC1 cells. Cells were transfected with siRNA and then mixed with agar. Colonies were captured under a microscope after 2 weeks as described in Materials and Methods. The numbers of colonies were quantified and are shown in the lower panel. (B, C) Knockdown of ATF1 inhibited cell migration and invasion in human esophageal cancer cells. EC1 cells were transfected with siRNA. Cell invasion was determined by transwell assay. Cells invaded were captured (upper panel) and quantified (lower panel) (B). Cell migration was determined by wound-healing assay (C).

to upregulate the expression of matrix metalloproteinase 2 (MMP-2) and contributes to the acquisition of the metastatic phenotype in melanoma cells [16]. Moreover, expression of single-chain antibody fragment anti-ATF-1 in melanoma cells suppressed their tumorigenicity and metastatic potential in nude mice [24]. Jones et al. reported that ATF1 was significantly upregulated and involved in invasive ductal carcinoma (IDC) of the breast-associated blood vessels [25]. ATF1 was reported
to be involved in hepatocyte growth factor-mediated cell invasion in thyroid cancer [18]. The overexpression of ATF1 in nasopharyngeal carcinoma was correlated with its target genes, such as MMP-2 [7]. In accordance with these results, in the present study we found that ATF1 was overexpressed in esophageal squamous cell carcinomas tissues, which is positively correlated with lymph node metastasis and early tumor invasion. Furthermore, knockdown of ATF1 inhibited cell migration and invasion using wound-healing assay and transwell assay. These results suggest that ATF1 is involved in tumor invasion and metastasis and could be an attractive anti-ESCC target.

Paclitaxel is widely used for the treatment of advanced esophageal carcinoma [26,27]. However, the toxicity and the development of chemoresistance usually limited its anticancer efficiency. Thus, the development of novel chemosensitizer or new drug combination therapies against ESCC is imperative. Guo et al. reported that ATF1 was involved in miR-30a-mediated radiosensitivity of NSCLC [28]. Here, we demonstrate that silencing of ATF1 enhanced the cytotoxicity of paclitaxel in ESCC cells and suggests that targeting ATF1 may be an effective means of increasing paclitaxel efficacy. In summary, this is the first report to investigate the expression and clinicopathological significance of ATF1 in ESCC. Results demonstrated that ATF1 protein was hyper-expressed in human ESCC tissues and was positively correlated with lymph node metastasis, poor differentiation, and early tumor invasion in esophageal cancer patients. In addition, knockdown of ATF1 effectively inhibited cell proliferation, migration, and invasion. Furthermore, silencing of ATF1 significantly enhanced the efficacy of paclitaxel. These findings expanded our knowledge of ATF1 in ESCC progression and suggest ATF1 as a novel drug target for esophageal cancer.

Conclusions

These findings suggest that ATF1 is an appealing drug target and a sensitizer to paclitaxel for esophageal cancer.

Conflicts of Interest

None.

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Figure 5. Knockdown of ATF1 sensitized ESCC cells to paclitaxel. EC1 (A) and Kyse450 (B) cells were transfected with siControl or siATF1-1 for 24 h and then treated with different concentrations of paclitaxel for 72 h. Cell viability was measured at the time points indicated using the ATPLite assay. Data were normalized using the mean of siControl in the siControl+Paclitaxel group and siATF1-1 in the siATF1-1+Paclitaxel group.
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