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Clinical significance of TBX2 in esophageal squamous cell carcinomas and its role in cell migration and invasion

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Abstract. – OBJECTIVE: To explore the role of T-box 2 (TBX2) in esophageal squamous cell carcinomas (ESCC).

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (PCR) and Western blot (WB) assays were used to detect the expression level of TBX2 in tissues and cells. Transwell assays were conducted for determination of cell invasion and migration.

RESULTS: The results suggested that the TBX2 was upregulated in ESCC tissues. Further, high expression of TBX2 expression was associated with tumor size, differentiation, distant metastasis, and TNM stage. In our *in-vitro* study, we decreased the expression of TBX2 in ESCC cells by transfection using LipofectamineTM 3000. The results from the transwell assay suggested that the downregulation of TBX2 could significantly suppress cell migration and invasion. Besides, WB results indicated that epithelial-mesenchymal transition (EMT)-related protein expressions were also changed after transfection.

CONCLUSIONS: TBX2, as an oncogene, could promote the progress of ESCC by affecting the transfer ability in tumor cells.

Key Words:
Esophageal squamous cell carcinomas (ESCC), T-box 2 (TBX2), Migration, Invasion, Epithelial-mesenchymal transition (EMT).

Introduction

Esophageal cancer (EC) is a common malignant tumor, and its mortality rate ranks 6th in the world[1]. The incidence of EC has notable regional features, and its morbidity and mortality rates in China are both among the top in the world[2]. Esophageal squamous cell carcinomas (ESCC) accounts for more than 90% of EC. Although EC was treated with combination therapy of surgical resection, adjuvant chemotherapy and biotherapy in recent years, its overall prognosis was still poor, and its 5-year survival rate remained low[3], which was associated with the tumor invasion and distant metastasis[4,5]. Currently, it was urgent to find new biomarkers for new therapeutic targets, so as to reduce postoperative tumor invasion and migration, and improve prognosis of EC patients.

During the development of EC, the interaction among multiple pathogenic factors, multiple evolution stages, and multiple mutant genes was involved. EC also had individual differences, which was related to the polymorphism of tumor-related genes[6,7]. The occurrence of EC was similar to that of other malignant tumors, involving the activation of oncogenes and the inactivation of cancer suppressor genes. Thus, studying the molecular pathways related to oncogenes and cancer suppressor genes was extremely important for clarifying the molecular mechanism of EC development, which can not only provide a theoretical basis for the prevention and diagnosis of EC, but also made effective intervention during treatment, so greatly improving the prognosis of EC patients.

As a member of the T-box gene family, T-box 2 (TBX2) widely exists in biological species[8]. Human TBX2 is located on chromosome 17q23, which played an important role in the development of various tissues and organs[9-12]. TBX2 is a transcription factor that can regulate cell proliferation, migration, apoptosis, and could serve as an oncogene to promote the tumor progression. However, the clinical significance and biological function of TBX2 in EC remained unclear yet. Therefore, in the present study, the association between TBX2 expression in ESCC tissues and

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clinicopathological factors was analyzed, and the effects of TBX2 on biological behaviors of EC cells was explored *via in vitro* experiments.

Patients and Methods

Tissue Specimens Collected

A total of 73 ESCC patients undergoing radical surgery in The First Affiliated Hospital of Soochow University from April 2016 to December 2018 were enrolled. At the same time, esophageal tissues from 20 non-cancer patients were included in control group. The tumor histological grading and postoperative tumor node metastasis (TNM) staging were based on the AJCC criteria (Version 7). All patients were pathologically diagnosed with ESCC after surgery, and they received no chemoradiotherapy, targeted therapy and biotherapy before surgery. This investigation was approved by the Hospital Ethics Committee.

Cell Culture

Human ESCC cell lines ECA-109 and human normal esophageal cell lines Het-1A purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were routinely cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C. The cells in the logarithmic growth phase were used for experiments.

Transfection

The cells were inoculated into a 6-well plate (1×10^6 cells/well, 2 mL) and transfected with si-TBX2 or si-NC (negative control) using Lipofectamine™ 3000 and OPTI-MEMI (Invitrogen, Carlsbad, CA, USA) when more than 80% of cells were fused according to the instructions. The total RNA was extracted for later use after 48 h of transfection.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

The total RNA was extracted from tissues or cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the instructions, and the purity and concentration of RNA were detected using a spectrophotometer. Then, the RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions of the reverse transcription kit. Real-time fluorescence quantitative PCR was performed using the qRT-PCR kit (TaKaRa, Komatsu, Japan) and specific primers with cDNA as a template. The relative expression of TBX2 was calculated using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. Primers used were shown below: TBX2: Forward: 5'-AGTGGATGGCTAAGCCTGTG-3'; Reverse: 5'-ACGGGTTGTTGTCGATCTTC-3'; GAPDH: Forward: 5'-GAGA-AGGCTGGGGCTCATTT-3'; Reverse: 5'-AGTGATG-GCATGGACTGTGG-3'

Western-Blot (WB) Assay

After transfection for 48 h, the protein was taken from different group, and the protein concentration was detected using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). After 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA, USA), sealed with 5% skim milk powder overnight, and incubated with primary antibodies of TBX2, E-cadherin, N-cadherin, Vimentin, and GAPDH at 4°C overnight. After the membrane was washed, the protein was incubated with corresponding secondary antibodies at 37°C for 2 h, followed by enhanced chemiluminescence (ECL) color development (Thermo Fisher Scientific, Waltham, MA, USA), exposure, and fixation. Finally, the gray value was detected.

Cell Invasion and Migration Assays

For migration: after transfection for 24 h, the cells were digested, centrifuged and counted, and then diluted to 1.5×10^5 cells/mL with the serum-free medium. The cell suspension was added to the transwell upper chamber (200 μL/well), and 600 μL of complete medium was added to the transwell lower chamber. Then, cells were cultured in the incubator for 24 h, fixed with formaldehyde for 15 min and stained with the crystal violet for 15 min. The cells on the inner membrane were gently wiped with a cotton swab, and the number of cells passing through the filter membrane in the 4 high-power fields (×200) was counted.

For invasion: the transwell chamber membrane was evenly coated with 50 μL Matrigel gel (Corning, Corning, NY, USA), and incubated at 37°C for 15 min, so that the Matrigel gel was coagulated. The treatment after the cells was digested,

centrifuged, and counted was the same as that in transwell migration assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The association between SIRT1 and clinicopathological features was analyzed using χ^2-test. $p<0.05$ suggested the statistically significant difference.

Results

TBX2 Was Upregulated in ESCC

The expression of TBX2 in tissues was detected by qRT-PCR. The results showed that the expression level of TBX2 in 70 ESCC patients was much higher than that in patients from control group (Figure 1A). The results indicated that TBX2 played a role in promoting oncogenes in the development of ESCC.

The Relationship of TBX2 with Clinicopathological Features

The patients were divided into TBX2 high-expression group (n=38) and low-expression group (n=35) according to the mean expression level of TBX2. The association between TBX2 expression and clinicopathological features of patients was further analyzed. The results were shown in Table I and indicated that high expression of TBX2 was associated with tumor size and differentiation as well as distant metastasis and TNM stage. However, there was no correlation between TBX2 expression and patients' gender, age or tumor location.

TBX2 Expression Affects the Metastasis Ability of ESCC Cells

In cell line detection, we also found that the expression of TBX2 in ESCC cell line (ECA-109) was significantly up-regulated by comparing normal esophageal cell line (Het-1A) (Figure 1B). The results of PCR appeared to have determined the role of TBX2 as oncogene gene in ESCC. This was consistent with our clinical results.

After ECA-109 cells were transfected with si-TBX2 or si-NC, the expression of TBX2 in cells was detected again. As we expected, the results from PCR (Figure 2A) and WB (Figure 2B) showed that si-TBX2 could significantly limit the expression of TBX2 in cells at RNA and protein levels.

Invasion and migration are important factors affecting the prognosis of cancer patients, the impacts of TBX2 on the cell migration and invasion were detected by transwell assays. As shown in Figure 3, when we reduce the expression of TBX2 at the cellular level, the migration and invasion ability of ECA-109 cells was significantly changed. The number of migrating and invading cells was significantly reduced under the microscope. We further examined the changes in the expression levels of epithelial-mesenchymal transition (EMT)-related proteins after transfection in our study. The results from Figure 4 indicated that regulating TBX2 expression could affect the procession of EMT. When we attenuated TBX2 expression in ESCC cells, E-cadherin was up-regulated, while that of N-cadherin and Vimentin was downregulated, which means that EMT was weakened after intervention of si-TBX2.

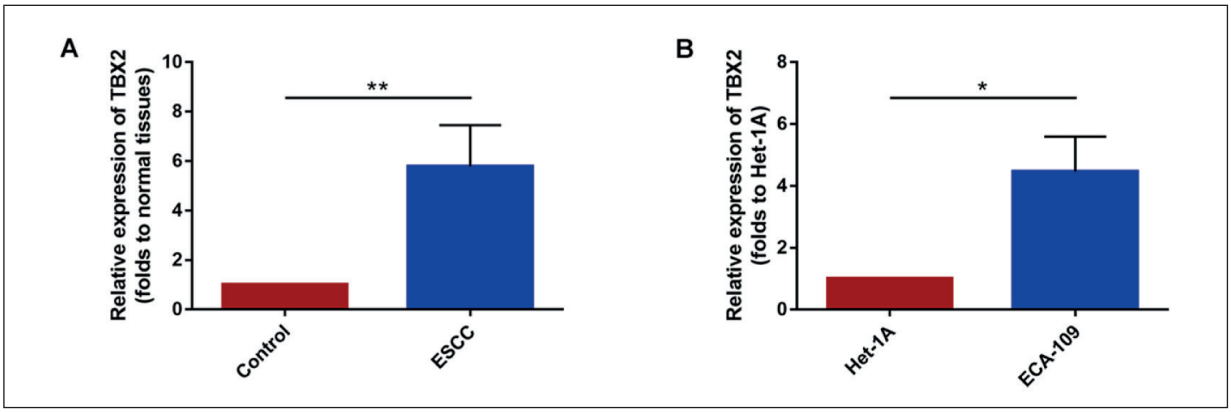

Figure 1. The expression of TBX2 were measured in tissues and cells by qRT-PCR. **A,** In tissues. **B,** In cells. (*$p<0.05$, **$p<0.01$).

Table I. TBX2 expression and clinical features of patients with ESCC.

Features	No.	TBX2 expression		*p*-value
		High	Low	
No.	73	38 (52.05%)	35 (47.95%)	
Gender				0.4594
Female	25	15 (60.00%)	10 (40.00%)	
Male	48	23 (47.92%)	25 (52.08%)	
Age (years)				0.1602
< 60	34	21 (61.76%)	13 (38.24%)	
≥ 60	39	17 (43.59%)	22 (56.41%)	
Tumor size (cm)				0.0001
< 5	39	10 (25.64%)	29 (74.36%)	
≥ 5	34	28 (82.35%)	6 (17.65%)	
Location				0.4376
Upper thoracic	17	11 (64.71%)	6 (35.29%)	
Middle thoracic	47	22 (46.81%)	25 (53.19%)	
Lower thoracic	9	5 (55.56%)	4 (44.44%)	
Differentiation				0.0064
High	20	5 (25.00%)	15 (75.00%)	
Moderate	39	22 (56.41%)	17 (43.59%)	
Poor	14	11 (78.57%)	3 (22.43%)	
Distant metastasis				0.0202
Absence	51	22 (43.13%)	29 (56.87%)	
Presence	22	16 (72.73%)	6 (27.27%)	
TNM stage				0.0104
I-II	43	17 (39.53%)	26 (61.47%)	
III- IV	30	21 (70.00%)	9 (30.00%)	

Discussion

The transcription factors in the T-box family play different roles during embryonic development. T-box family members share a conserved T-box domain, bind to DNA in a sequence-specific manner and act as activators of transcription inhibitors[13]. The dysregulation of T-box family genes was associated with dysplasia of multiple organ systems. The haplotype dysfunction of TBX1 was the major cause of clinical manifestations of many patients with DiGeorge syndrome[14]. Moreover, TBX2 gene was associated with dysplasia of the heart, lung, and kidney tissues[10-12]. The heterozygous mutation of TBX3 would lead to autosomal dominant ulnar mammary syndrome[15]. It had been proved that the heterozygosis, frame shift and missense mutation of TBX4 were the main causes of Ischiocoxopodopatellar syndrome[16]. Besides, the haplotype dysfunction of TBX5 causes Holt-Oram syndrome[17], and the variation of TBX6, TBX15,

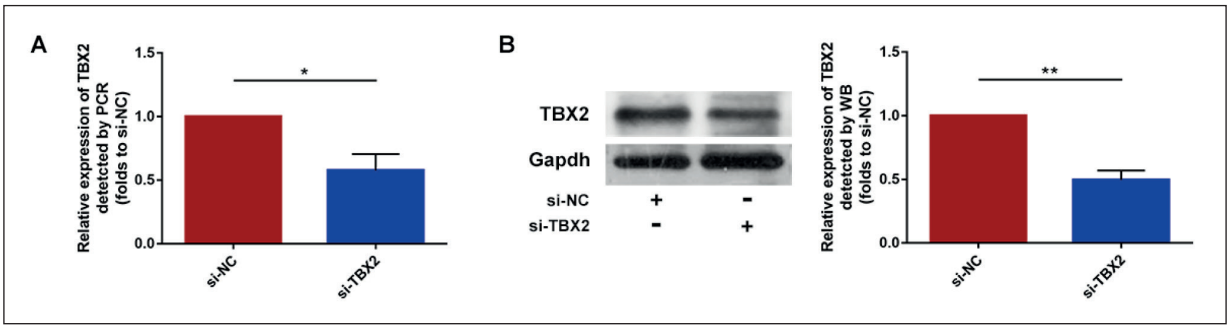

Figure 2. TBX2 was efficiently knocked down by treatment of si-TBX2. The expression of TBX2 (**A**) detected by qRT-PCR; (**B**) detected by Western blot. (*p<0.05, **p<0.01).

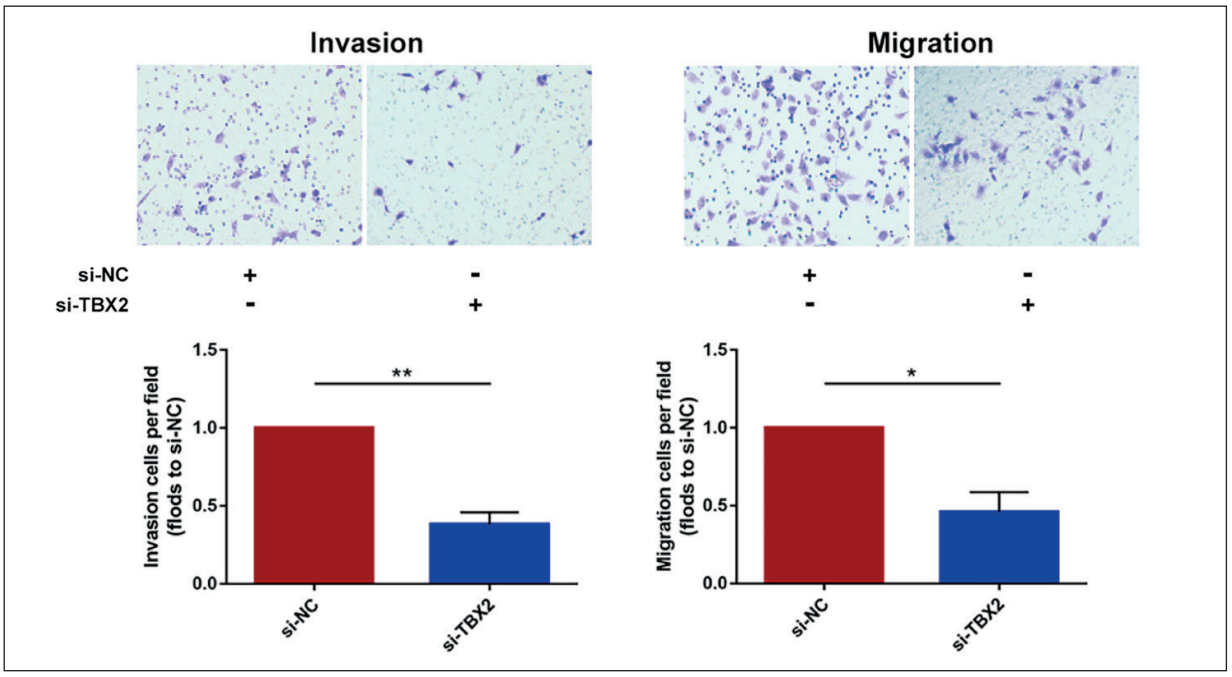

Figure 3. The invasion and migration of ESCC cell post-transfection was analyzed using the transwell assay and detected by microscope (× 200). (*p<0.05, **p<0.01).

and TBX20 was also associated with cardiac and skeletal anomalies. In addition, the dysregulation of T-box factors in different cancers had been reported in increasingly more studies, and the abnormal expression of T-box factors was directly related to tumorigenesis.

In most studies on the role of TBX2 in tumors, TBX2 had been widely studied as a carcinogen; in 2000, Jacobs et al[18] found TBX2 to be amplified in a subset of primary human breast cancers, indicating that it might contribute to breast cancer development. Two years later, in another report on breast cancer by Sinclair et al[19], TBX2 was determined to be preferentially amplified and overexpressed in BRCA1 and BRCA2 mutant tumors. Also, in 2005 Vance et al[20] reported that TBX2 was critically required to maintain proliferation and suppress senescence in melanomas in melanomas. In the past decade, many studies[21-24] on the relationship between TBX2 and cancer have been reported. Unfortunately, there was still a lack of systematic research on the relationship between TBX2 and EC. By detecting the expression of TBX2 in clinical samples, we found that TBX2

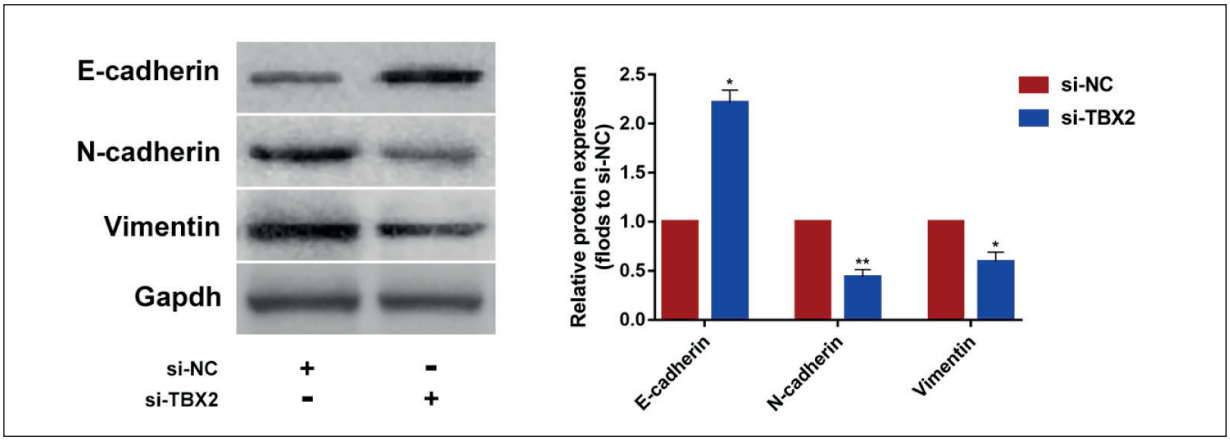

Figure 4. The EMT markers post-transfection were detected by Western blot. (*p<0.05, **p<0.01 *vs.* si-NC group).

was widely expressed in ESCC tissues. Further studies had found that TBX2 expression and tumor size, differentiation, distant metastasis, and TNM stage, confirming the cancer promoting effect of TBX2 in ESCC.

Tumor invasion and migration refer to the process in which tumor cells were detached from the primary lesion, invade adjacent or distant organs and tissues through a variety of ways, and continue to proliferate and grow, forming tumors of the same nature[25,26]. As one of the most essential features of malignant tumors, invasion and migration were an interaction process between tumor cells and host cells, as well as a multi-factor and multi-step biological process, which were not only symbols for exacerbation, but also important causes of poor prognosis[27]. Therefore, the research on mechanism of tumor invasion and migration was of important clinical significance. EMT, a process in which cells with epithelial phenotype were differentiated into those with mesenchymal phenotype under specific pathophysiological conditions, was widely studied. After EMT, the expression of epithelial phenotype such as E-cadherin was downregulated, while that of mesenchymal phenotype such as N-cadherin and Vimentin was up-regulated[28-30]. Such a process enables tumor cells to invade and migrate to the distance, which also indicated the close correlation between EMT and tumor invasion and migration[31]. In our *in vitro* study, the effects of TBX2 on the migration and invasion was performed in ECA-109 cells. When we declined the expression of TBX2 in ECA-109 cells, the migration and invasion ability was suppressed. Further, the reduction in migration and invasion ability was also reflected in the expression level of EMT-related proteins.

Conclusions

In summary, through *in vivo* and *in vitro* experiments, we found TBX2 expression was significantly increased in ESCC. High expression of TBX2 in tissues was associated with tumor size, differentiation, distant metastasis, and TNM stage. Attenuating the expression of TBX2 in cells could significantly reduce the migration and invasion of ESCC cells. The results showed the cancer promoting effect of TBX2 in ESCC, suggesting that TBX2 might play an important role in the development of ESCC. Unfortunately, limitations still existed in this study. Due to the lack of sufficient sample size and further *in vivo* experiments, the specific role and molecular mechanism of TBX2 in ESCC remained to be further studied.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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