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Original Article

Tropomyosin3 is associated with invasion, migration, and prognosis in esophageal squamous cell carcinoma

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Abstract: The present study aimed to explore the biological role of tropomyosin3 (TPM3) in esophageal squamous cell carcinoma (ESCC) cell lines via TPM3 knockdown using small interference RNA. Esophageal carcinoma is one of the leading causes of death worldwide. Understanding the mechanism of ESCC and finding effective biomarkers to improve its prognosis is vital. The biological roles of TPM3 have not yet been illustrated in ESCC. This study assessed the *in vitro* migration and invasion potentials via wound-healing and transwell assays. The cell growth was measured by colony formation and Cell Counting Kit-8 assays. No significant differences were found between the knockdown groups and the control group ($P>0.05$). Also, the high expression of TPM3 was associated with T stages, tumor stage, and postoperative chemotherapy, whereas the positive expression of TPM3 had no statistical significance, with regard to gender, age, lymph node involvement, and tumor differentiation. The Kaplan-Meier method was used to analyze the ESCC and adjacent tissues to explore the TPM3 differential expression and the significance of prognosis in the 5-year survival. Silencing of TPM3 inhibited invasion and migration capacities in both cell lines and repressed colony formation. The positive expression of TPM3 was 53.11% in the cancer tissue and 7.34% in the adjacent tissue ($P<0.05$). The high expression of TPM3 was found in the ESCC tissue, which correlated with poor survival (log-rank test, $P = 0.015$). The results of the present study suggested that TPM3 was involved in the migration, invasion, and prognosis in ESCC.

Keywords: Downregulation, esophageal squamous cell carcinoma, invasion, migration, prognosis, tropomyosin3

Introduction

An estimated 455,800 new esophageal cancer cases and 400,200 relative deaths occurred in 2012 worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is predominant worldwide, especially in East Asia, and the overall 5-year survival of patients remains poor [2-4]. However, the mechanism and therapies of esophageal carcinoma remain unclear. As the overall survival is poor, it is necessary to discover effective cancer biomarkers to predict prognosis and target therapy to improve survival.

Tropomyosin3 (TPM3), a member of tropomyosin family, is located in 1q22→1q23 and consists of 13 exons [5, 6]. In the skeletal muscle, TPM3 mediates a myosin-actin response to calcium ions and takes part in the stabilization of cytoskeletal microfilaments [7]. Jang et al. [8] found that TPM3 played an important role in asymmetric cell division and maintenance of cortical integrity in mouse oocytes. Several studies have demonstrated that TPM3 was related to nemaline myopathy [9-12]. Besides, Fan et al. [13] revealed that TPM3 was upregulated in colorectal cancer. However, the function of TPM3 remains obscure in nonmuscular tissues.

Evidences showed that nonmuscular TPM3 was involved in the progression of cancer. Miyado et al. [14] suggested that TPM3 was overexpressed in B16-F10 mouse melanoma cells. Kim et al. [15] found that the high expression of TPM3 significantly increased the risk of hepatocellular carcinoma (HCC). Choi et al. [16] and Tao et al. [17] proved that the silencing of TPM3 reduced invasion and migration capacities in HCC and gliomas through the epithelial-mesenchymal transition (EMT) signaling pathway. Furthermore, TPM3 was associated with some gene fusion rearrangements, such as tyrosine kinase receptor (TRK), platelet-derived growth factor receptor β (PDGFRB), anaplastic lymphoma kinase (ALK), and neurotrophic tyrosine kinase receptor type 1 (NTRK1) fusion formation, which cause the development of tumors [18-22]. Several studies illustrated that the EMT signaling pathway is involved in ESCC, including transforming growth factor-β (TGF-β), wnt/β–catenin, and notch-1 [23-25]. The loss of E-cadherin protein was a sign of prognosis in ESCC [26, 27]. The overexpression of TPM3 induced the development of EMT, and was related to the reduction of E-cadherin protein via upregulation of Snail in HCC and glioma [16, 17]. All evidences suggested that the overexpression of TPM3 might be correlated with the development of cancer, but the role of TPM3 in ESCC was still unknown.

This study explored the biological role of TPM3 in ESCC cell lines via TPM3 knockdown using small interference RNA, and hypothesized that the overexpression of TPM3 in ESCC correlated with poor prognosis.

Methods and materials

Cell lines and cell culture

Human ESCC cell lines, EC109 and EC9706, were chosen for this study. These two cell lines were cultured in Dulbecco's modified Eagle medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in a 5% CO_2 incubator at 37°C [28].

Cell transfection

Three synthetic double-stranded oligonucleotides were purchased from GenePharma, China. Transfection was performed using a siRNA Transfection Reagent (Roche, Germany), according to the manufacturer's instructions. The cells were seeded into six-well plates at 5×10^5 cells/well, and the cellular proteins were extracted after 48 h.

Experimental groups

The study comprised three groups: siTPM3-492, siTPM3-614, and siTPM3-NC. The former two groups were knockdown groups. The siTPM3-NC represented the control group. The sequence that had the maximum inhibition efficiency was screened out for follow-up assays. An independent experiment was performed at least three times.

Western blot detects TPM3 and Snail expression

The cells were collected from each group, and the protein density was detected at 48 h after transfection. The cell proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Primary antibodies used included TPM3 (1:1500; Abcam, USA), Snail (1:2000; Abcam), and β-actin (1:1000; Abcam). The secondary antibody was diluted to 1:2000 (Boster, China). Relative bands were detected using an ImageQuant LAS 4000 mini camera (CE Healthcare, WI, USA) [28]. The ImageJ software (National Institutes of Health, MD, USA) was used to analyze the intensities of band signals.

Wound-healing assay

The transfected cells were grown to 100% confluence in six-well plates. The cell layers were scratched using a 20-μL tip to form wound gaps, washed three times with phosphate-buffered saline (PBS), and photographed at different time points. The cells were counted using a scale label moving away from the original place 48 h after knockdown.

Invasion assay

The cell invasion assay was performed using transwell membranes coated with Matrigel (NY, USA). The transfected cells were plated at a density of 5×10^5 cells/well in the upper chamber with a serum-free medium. FBS (10%) as a chemoattractant was added to the lower chamber. After 48 h of incubation, the cells were stained with crystal violet for 5-10 min. Finally, the invasion cells were counted in five microscopic fields (×200).

Colony formation assay

Stably transfected cells were harvested and seeded in six-well plates at a density of 1×10^3 cells/well. After 2 weeks, the cells were fixed in 3% methanol for 30 min and stained with 1% crystal violet for 10 min. They were then counted from visible colonies using a phase contrast microscope.

Cell proliferation assay

The cells in the logarithmic growth phase were planted in 96-well plates at a density of 4×10^3

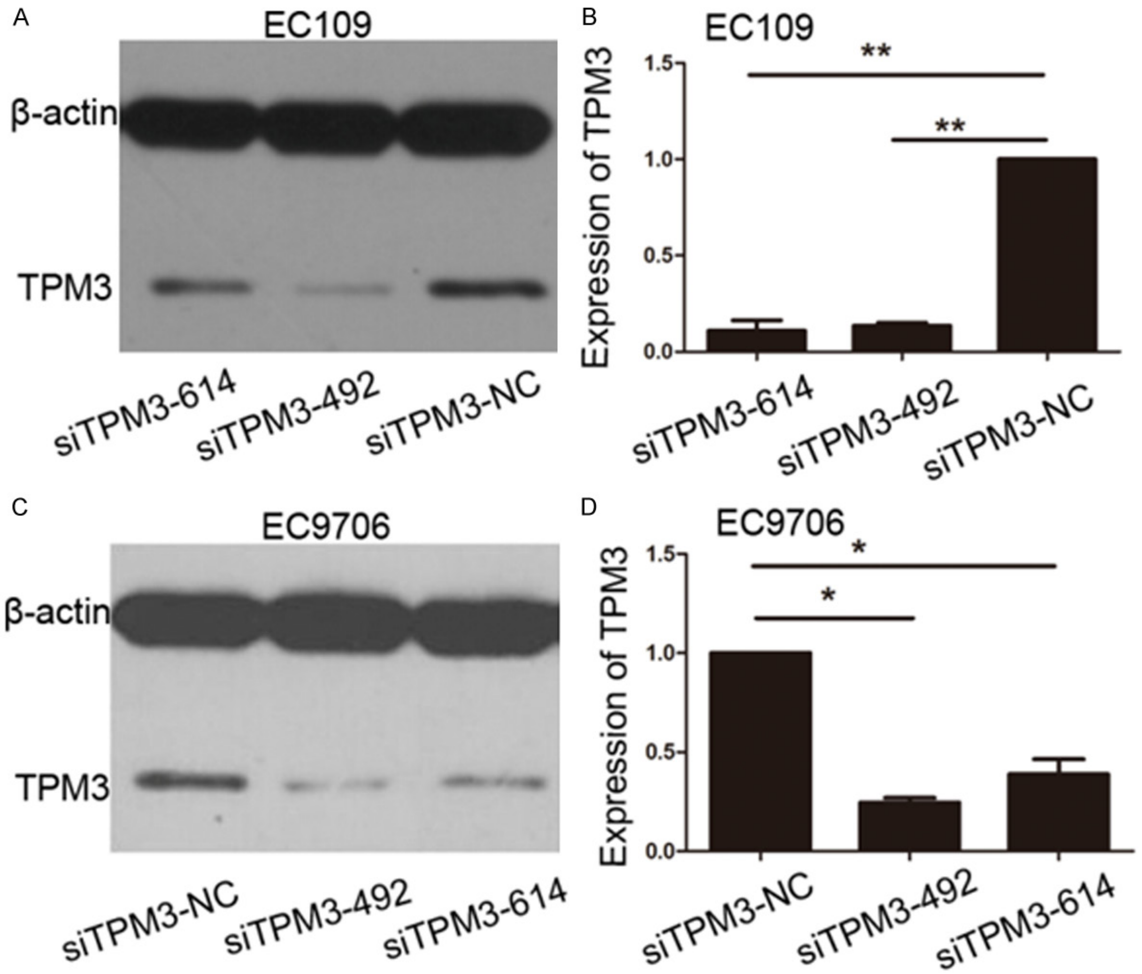

Figure 1. Downregulation of the expression of TPM3 in EC109 and EC9706 measured by Western blot. A, B. Compared with the siTPM3-NC group, the expression of TPM3 decreased obviously in siTPM3-492 and siTPM3-614 groups in EC109 (P<0.001) and EC9706 (P<0.05). β-actin acted as the internal control for Western blot analysis. C, D. Statistical analysis. $^{*}P$<0.05; $^{**}P$<0.001. All results were presented as mean ± standard deviation from three independent experiments.

cells/well on the previous day. A Cell Counting Kit-8 reagent (CCK-8; Donjido, Kumamoto, Japan) was used according to the manufacturer's instructions. The optical density (OD) value was detected using a microplate reader (Bio-Tek, VT, USA) at a regular time per day.

Immunohistochemical staining

Tumor and adjacent tissues were collected from 177 patients with ESCC. These cases ranged from the year 2003 to 2009 for resectable ESCC. This study was approved by the ethics committee of the Union Clinical Medical College of Fujian Medical University, China (NO:2012KY001). All participants signed the informed consent. The patients who underwent neoadjuvant therapy, had incompletely resected tumors (R1 or R2), and survived for less than 3 months were excluded. Staging was performed based on the seventh edition of American Joint Committee on Cancer staging system [29].

Paraffin-embedded and formalin-fixed ESCC tissue was cut into 3-μm sections, deparaffinized in xylene, and then dehydrated in serial ethanol dilutions. These sections were incubated with TPM3 antibody (1:150) for 60 min at room temperature. They were then rinsed in PBS and incubated with secondary antibody (KIT-5910, MaxVision, China) for 15 min at room temperature. After rinsing with PBS again,

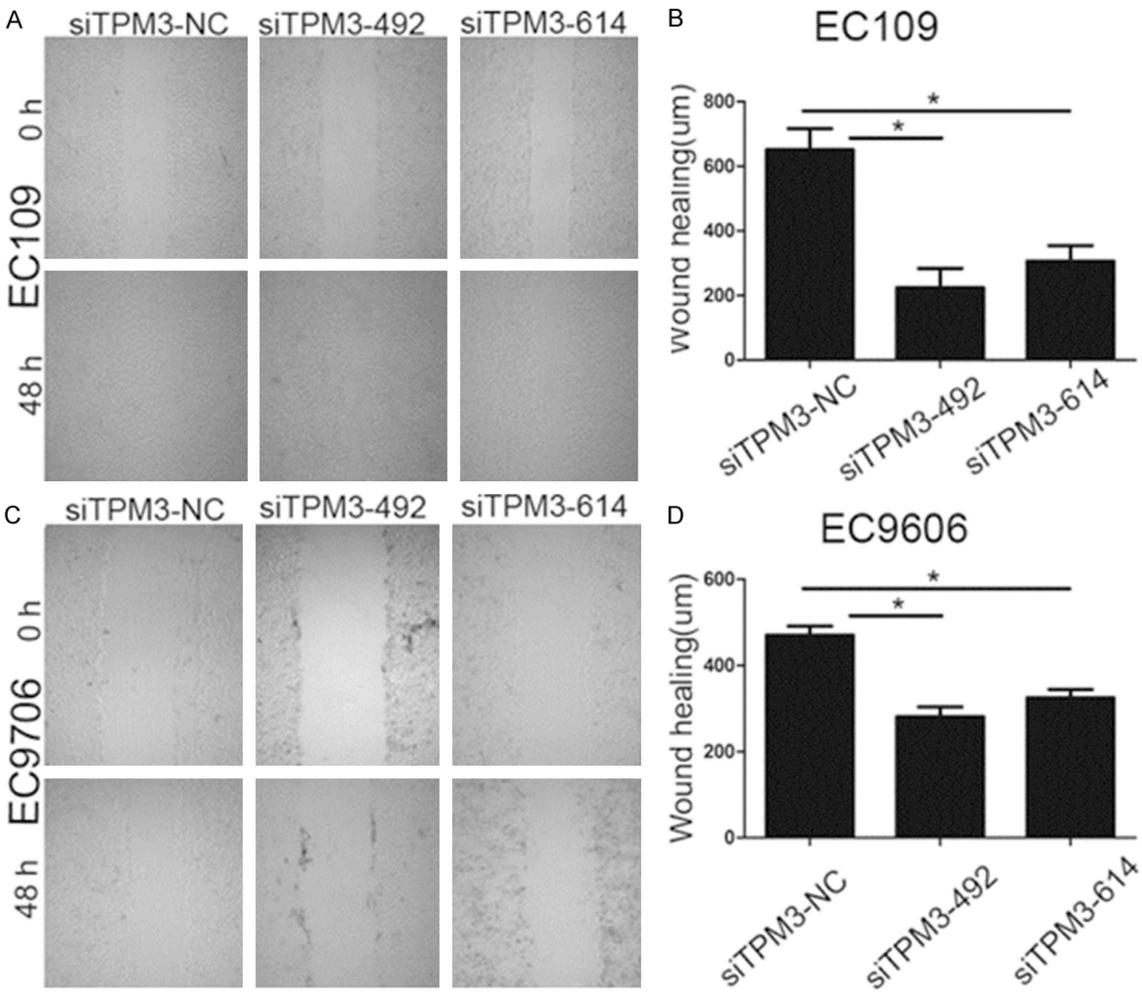

Figure 2. In migration assays, two cell lines were measured *in vitro*. A, B. The effect of siTPM3 on migration evaluated using wound-healing assay 48 h after cell transfer. C, D. Axis of ordinates represents cells relative to surplus space. *P<0.01.

the color was developed using diaminobenzidine (MaxVision, China).

Immunohistochemical scoring

The scoring was based on high-magnification comprehensive staining intensity and the proportion of positive cells in semi-quantitative determination. The dyeing strength criteria were as follows: no signal indicated 0 point, pallideflavens indicated 1 point, yellow indicated 2 points, and brown indicated 3 points. The positive cell criteria were as follows: specimens with <1% positive cells indicated 0 point, 2%-25% indicated 1 point, 26%-50% indicated 2 points, 51%-75% indicated 3 points, and >75% indicated 4 points. Two scores were multiplied and divided into four grades: negative (-, score, 0-1), weak (+, score, 2-4), moderate (++, score, 5-8), and strong (+++, score, 9-12) [30, 31].

Statistical analysis

Each value was obtained from at least three independent experiments and presented as mean ± standard deviation by one-way analysis of variance. The SPSS version 19 was used for statistical analysis. A P value <0.05 was considered statistically significant.

Results

Downregulation of TPM3 expression as detected by Western blot

The expression of TPM3 was detected by Western blot (**Figure 1**). The expression of TPM3 decreased obviously in siTPM3-492 and siTPM3-614 groups compared with the siTPM3-NC group in EC109 (P<0.001) and EC9706 (P<0.05). The success of silencing TPM3 expression was the basis of cell function research.

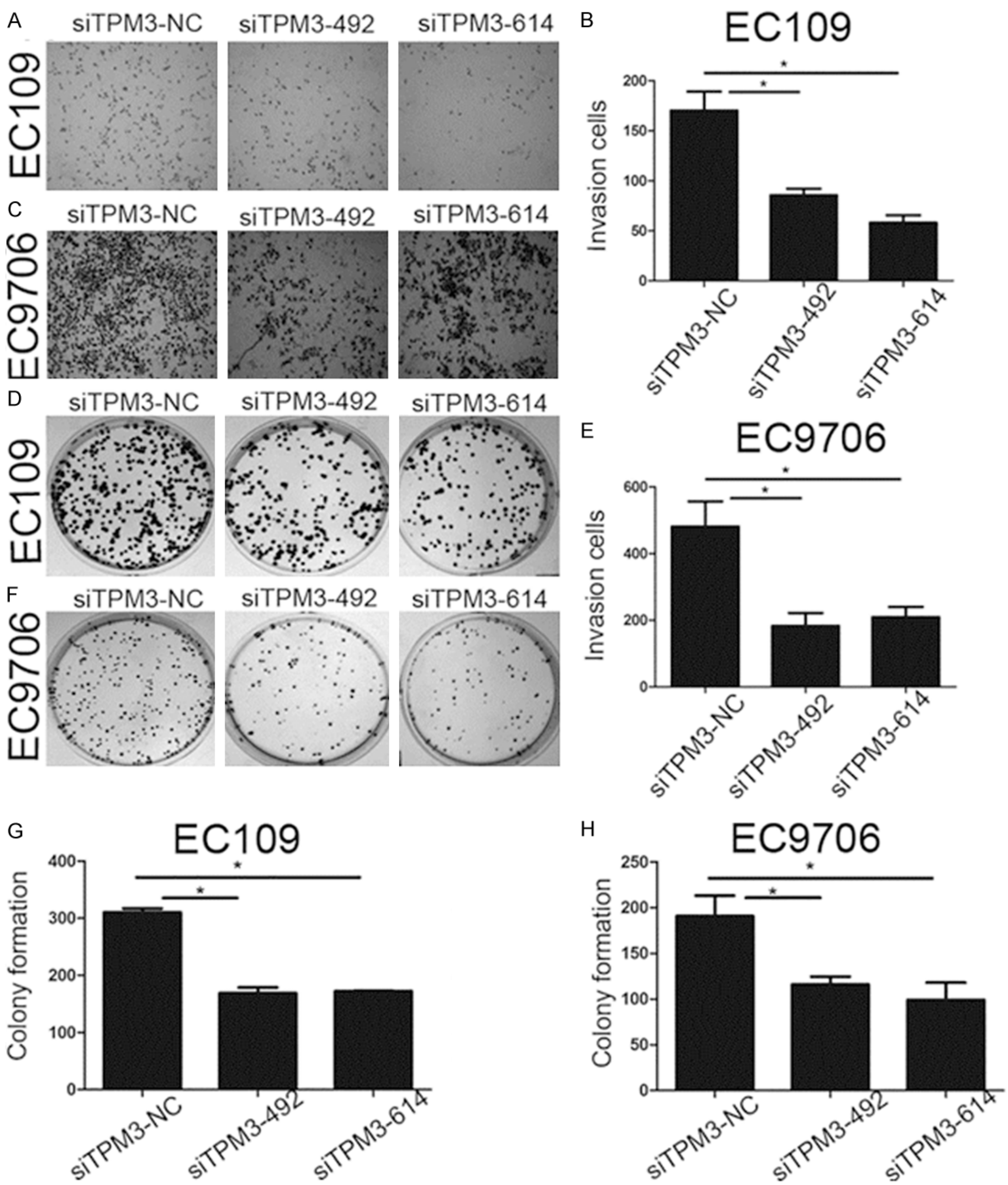

Figure 3. Invasion assay: two cell lines were examined using the Matrigel-coated transwell membrane of transferred cells after 48 h. A, C. After crystal violet staining, the number of colonies in five microscopic fields (×100) was determined. B, E. Error bars represent mean ± standard error of mean. $^{*}P<0.05$. D, F. Colony formation assay results. G, H. Analysis of the results. The number of colonies in the siTPM3-NC group was profoundly repressed compared with the siTPM3-492 and siTPM3-614 groups. $^{*}P<0.05$. Error bars represent mean ± standard error of mean. The results were presented as mean ± standard deviation from three independent experiments.

Effects of TPM3 repression on cellular migration and invasion

To explore the crucial role of TPM3 in the migration of ESCC cells, the migration assay was performed *in vitro* by wound healing (**Figure 2**). Compared with the siTPM3-NC group, the siTPM3-492 and siTPM3-614 groups were relatively less in the blank area in EC109 ($P<0.05$) and EC9706 ($P<0.05$). The migration rate was measured (mean blank areas as to mean initial blank area). The results of this study indicated

Table 1. Clinical and pathological baseline characteristics

Total	177	Positive (n = 94; 53%)	Negative (n = 83; 47%)	P value
Age (year) mean ± SD		58.05 (± 9.19)	56.36 (± 9.87)	0.240*
Age				
<50	43 (24)	18 (10)	25 (14)	0.063†
50-60	67 (38)	42 (24)	25 (14)	
>60	67 (38)	34 (19)	33 (19)	
Gender				
Male	139 (79)	77 (44)	62 (35)	0.107†
Female	38 (21)	17 (10)	20 (11)	
T stage				
T1 + T2	59 (33)	25 (14)	34 (19)	0.017†,‡
T3 + T4	118 (67)	69 (39)	49 (28)	
Tumor stage				
I + II	84 (47)	38 (21)	46 (26)	0.017†,‡
III + IV	93 (53)	56 (32)	37 (21)	
Lymph node involvement				
Yes	99 (56)	56 (32)	43 (24)	0.071†
No	78 (44)	38 (21)	40 (23)	
Tumor differentiation				
High	75 (42)	51 (29)	44 (25)	0.077†
Middle	66 (37)	31 (17)	26 (15)	
Low	33 (19)	12 (7)	13 (7)	
Postoperative chemotherapy				
Yes	103 (58)	33 (19)	41 (23)	0.034†,‡
No	74 (42)	60 (34)	42 (24)	

*Two-sided Student t test. †Two-sided χ^2 test. ‡Bold values are statistically significant (P<0.05).

that TPM3 downregulation inhibited the migration potential significantly.

In the invasion assay (**Figure 3**), Matrigel was used to explore the invasion ability after TPM3 knockdown. siTPM3-492 and siTPM3-614 groups repressed invasion significantly compared with the siTPM3-NC group in ESCC cells. In EC109, siTPM3-NC was 170.3 [95% confidence interval (CI), 88.7-252.1], and siTPM3-492 and siTPM3-614 were 85.5 (95% CI, 2.9-168.1) and 58.1 (95% CI, 25.4-90.6), respectively, P<0.05 (**Figure 3B**). In EC9706, siTPM3-NC was 481.1 (95% CI, 154.3-807.7), and siTPM3-492 and siTPM3-614 were 183.3 (95% CI, 17.4-349.3) and 209.3 (95% CI, 181.5-599.9), respectively, P<0.05 (**Figure 3D**).

Colony formation after TPM3 silencing

As TPM3 repressed migration and invasion capacities, the effect of cell growth assay was also explored (**Figure 3**). First, the colony formation assay was conducted. The results indicated that the numbers of colonies in siTPM3-492- and siTPM3-614-treated cells significantly decreased compared with siTPM3-NC-treated ESCC cells. In EC109, siTPM3-NC was 236.1 (95% CI, 150.5-321.8), and siTPM3-492 and siTPM3-614 were 146.1 (95% CI, 116.8-175.6) and 152.7 (95% CI, 129.9-175.4), respectively, P<0.05 (**Figure 3F**). In EC9706, siTPM3-NC was 190.8 (95% CI, 133.1-248.6), and siTPM3-492 and siTPM3-614 were 116.2 (95% CI, 94.3-137.7) and 99.1 (95% CI, 50.3-147.7), respectively, P<0.05 (**Figure 3H**).

Then, the CCK-8 cell proliferation experiment was conducted (Supplement 1). No significant differences were found between the knockdown and control group (P>0.05).

Clinical and pathological data

Clinical and pathological baseline characteristics are presented in **Table 1**. The expression patterns were initially analyzed in patients with different grades of ESCC between TPM3-positive (n = 94; 53%) and TPM3-negative tissues (n = 83; 47%). The results showed that the high expression of TPM3 was associated with T stages (P = 0.017), tumor stage (P = 0.017), and postoperative chemotherapy (P = 0.034); whereas, the positive expression of TPM3 showed no statistical significance, including gender (P = 0.107), age (P = 0.063), lymph node involvement (P = 0.071), and tumor differentiation (P = 0.077).

Higher expression of TPM3 in esophageal cancer tissues and its association with patient prognosis

Survival was analyzed using the Kaplan-Meier method. The positive expression of TPM3 (n =

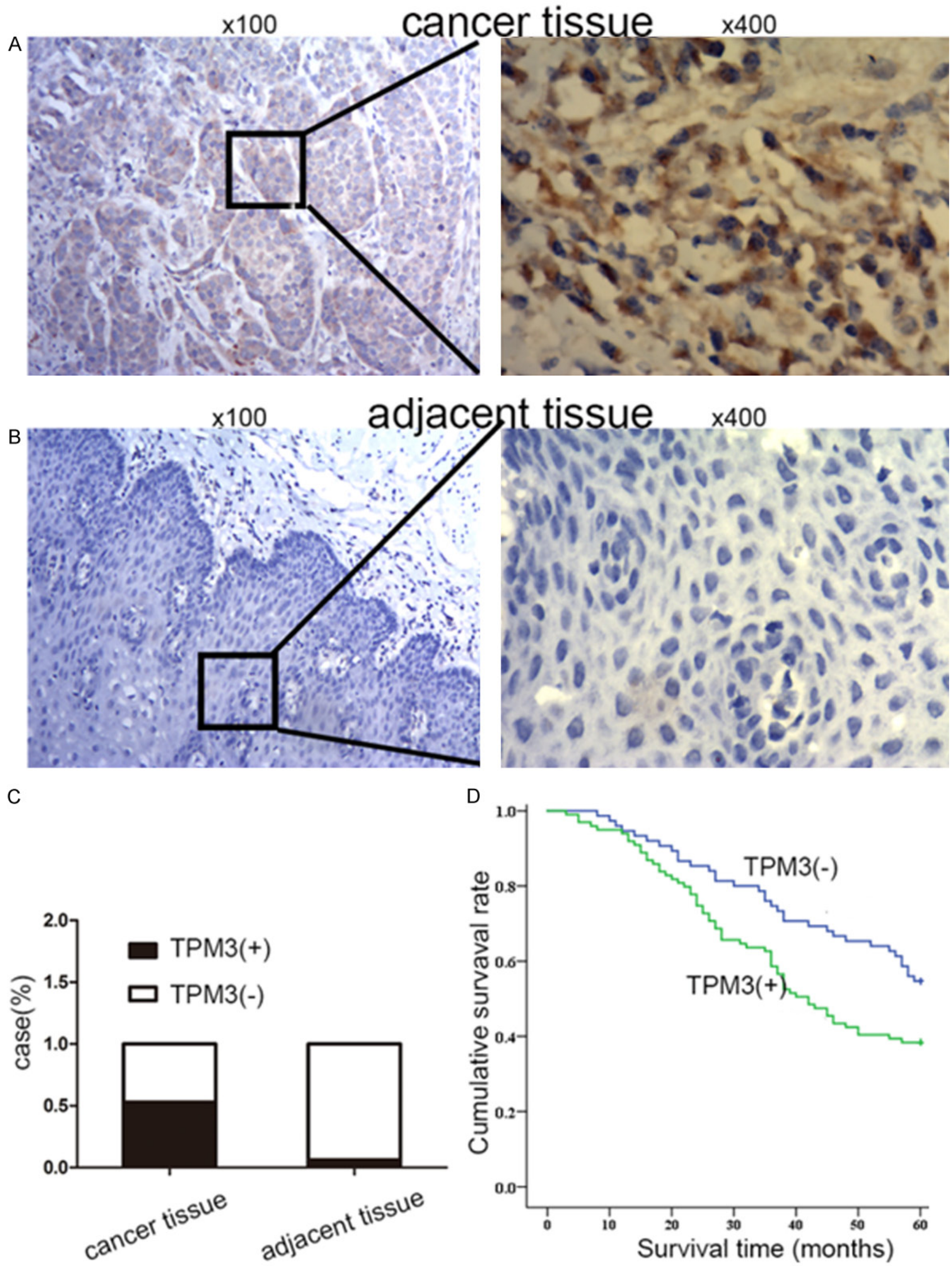

Figure 4. Analysis of TPM3 expression in esophageal cancer and adjacent tissues. A. Higher expression of TPM3 was observed with brown cytoplasm in the cancer tissue. B. Similarly, negative TPM3 was observed in the adjacent tissue. C. Elevated TPM3 expression in cancer tissue. D. Kaplan-Meier survival analysis of overall survival between negative and positive groups. Log-rank $P = 0.015$.

94) was found to be significantly higher in the cancer tissue than in the adjacent tissue (n = 13) (**Figure 4C**). The rate of TPM3-positive expression was 53.11% in the cancer tissue

and 7.34% in the adjacent tissue (χ^2 = 87.88, P<0.001). Among the patients enrolled in this study, patients with the positive expression of TPM3 had a poorer prognosis compared with patients with the negative expression of TPM3 (log-rank test, P = 0.015) (**Figure 4D**).

Discussion

ESCC is a common malignancy in China. Even after an improved surgical technology and trimodality therapy, the overall survival rate is disappointing. Recently, several studies suggested that TPM3 was overexpressed in mouse melanoma cells, HCC, and gliomas [14, 16, 17]. Thus, this study was conducted to explore the biological role of TPM3 in ESCC cells and patients.

This study demonstrated that the downregulation of TPM3 significantly restrained the migration and invasion capacities of ESCC cells compared with the control group. In this study, the positive rate of TPM3 was higher in the cancer tissue than in the adjacent tissue. Furthermore, in the cancer tissue, the Kaplan-Meier analysis of the survival curves showed that the 5-year survival of patients with ESCC having positive TPM3 expression was poorer than that of the patients with negative TPM3 expression. The overexpression of TPM3 was higher in a metastatic mouse melanoma cell line than in a low metastatic one [14], and it was associated with the development of HCC [16, 32]. The effect of TPM3 on tumor's biological behavior had been described in HCC and gliomas, which was involved in the EMT signaling pathway [16, 17]. The *TPM3* gene is located in 1q21.3. Kim et al. found that *TPM3* recurrently amplified copy number alterations in primary HCC [15]. However, the biological effect of TPM3 and its clinical significance in ESCC are yet to be elucidated.

How TPM3 was used as an oncogene alone for the formation of fusion gene remains unclear. TPM3 has been reported to be overregulated and correlated with poor survival in HCC [32]. However, TPM3 has formed through gene fusion. TPM3-ALK activation requires dimerization through the coined-coil structure of TPM3 [33]. TPM3-ALK expression induced changes in cytoskeleton organization and conferred higher metastatic capacities compared with other ALK fusion proteins [34]. A new fusion gene TPM3-ALK was created by a (1,2)(q25,p23) translocation in anaplastic large-cell lymphomas [20, 35], and the TPM3-ALK became an oncogene. The TPM3-ALK fusion gene was also found to have effects on transformation proliferation and invasion properties in NIH3T3 cells [36]. The analysis of gene expression profile of TPM3-ALK and NMP-ALK revealed positive anaplastic large-cell lymphomas [37]. Besides, TPM3 is involved in fusion gene integration with other genes. *TPM3/NTRK1* and *TPM3/PDGFRB* oncogenes were involved in the rearrangements in papillary thyroid carcinoma and chronic eosinophilic leukemia [18, 19, 22]. Furthermore, Giuriato et al. [38] found that *TPM3-ALK* and *NMP-ALK* could inhibit and reverse the early B lymphocyte leukemia in the ALK-positive mice treated with ALK inhibitors.

This study found that TPM3 downregulation in ESCC cell lines profoundly repressed the migration and invasion potentials. Therefore, overexpression of TPM3 was assumed to be linked to the EMT pathway. Of late, several studies on EMT signaling pathway were conducted in ESCC. The EMT signaling pathway mechanism was illustrated to include TGF-β and wnt/β-catenin in ESCC [24, 39]. The loss of E-cadherin protein was an important step in the process of esophageal carcinoma in the EMT signaling pathway [40]. In this study, E-cadherin was not detected because EC109 and EC9706 were poorly differentiated cells. Snail was proved to be important in the EMT signaling pathway, as it mediated esophageal carcinoma [41-44]. Hence, in future, the mechanism underlying the involvement of TPM3 in invasion and metastasis needs to be further explored.

Furthermore, to explore the relationship between TPM3 and proliferation, the colony formation assay was conducted after TPM3 knockdown. The cell communities decreased significantly after TPM3 knockdown. However, no significant difference was found in the level of Snail expression and CCK-8 assay after silencing TPM3.

However, this study had some limitations. First, the EMT signaling pathway and TPM3-ALK fusion gene or other gene formation involved in the mechanism of TPM3 were not formulated. Second, EC109 and EC9706 were poorly differentiated; thus, E-cadherin could not be detected. Finally, although the inhibition of colony formation, migration, and invasion was found in

TPM3 knockdown cell lines, larger-scale screening of TPM3 expression profile and *in vitro* and *in vivo* experiments with higher overexpression of TPM3 are needed to support the findings.

In summary, this study suggested that TPM3 knockdown inhibited migration and invasion potentials in ESCC cell lines. In addition, the overexpression of TPM3 was found in the cancer tissue and was involved in poor prognosis. Given the limitations of biomarkers, the role of TPM3 in migration and invasion, and as a strong prognostic predictor needs to be investigated to find a new targeted therapy for ESCC.

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Disclosure of conflict of interest

None.

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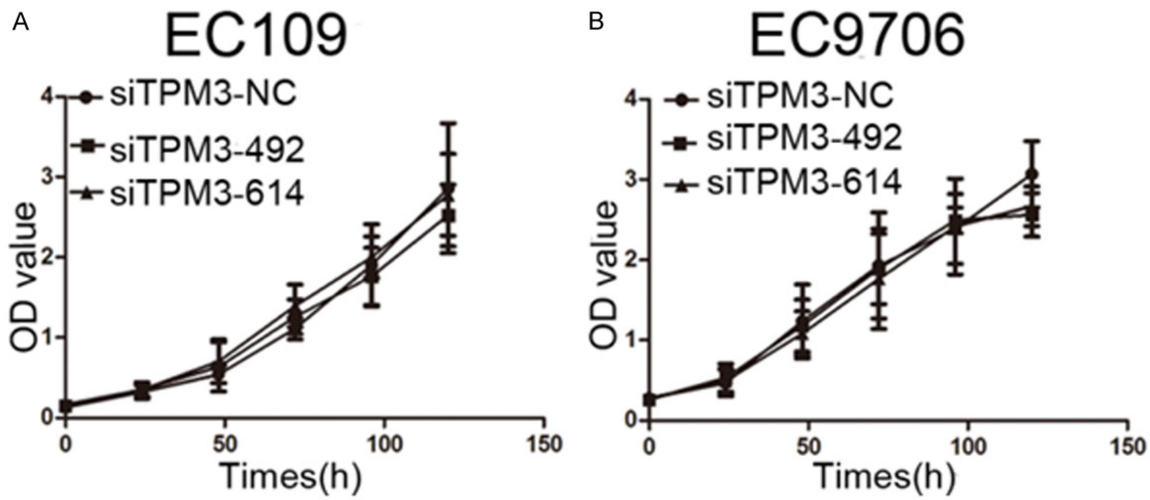

Supplement 1. To explore the cell growth, the proliferation assay was performed after TPM3 knockdown. A, B. The OD value was detected every 24 h. No statistically significant difference in EC109 ($P>0.05$) and EC9706 was found between the three groups ($P>0.05$). Each experiment was performed at least three times.