TGIF1 plays a carcinogenic role in esophageal squamous cell carcinoma through the Wnt/β-catenin and Akt/mTOR signaling pathways

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Abstract. TGFB induced factor homeobox 1 (TGIF1), a transcriptional corepressor, has been reported to be involved in tumorigenesis and cancer development. However, the role of TGIF1 in the growth and metastasis of esophageal cancer is poorly studied. In the present study, it was found that TGIF1 was highly expressed in esophageal cancer tissues and cell lines. The silencing of TGIF1 by siRNA interference significantly inhibited the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) process of KYSE-150 esophageal cancer cells, and promoted cell apoptosis. Correspondingly, the upregulation of TGIF1 significantly promoted the proliferation and metastatic potential of Eca-109 cells, and reduced apoptosis. Furthermore, the data indicated that the Wnt/β-catenin and Akt/mammalian target of rapamycin (mTOR) signaling pathways were inhibited by TGIF1 knockdown, and were promoted by the overexpression of TGIF1. It was also confirmed that TGIF1 knockdown reduced tumor growth, inhibited Wnt/β-catenin and Akt/mTOR pathway activation, and reversed the TGF-β1-mediated EMT process in a tumor xenograft model. Taken together, the data of the present study suggest that TGIF1 plays an oncogenic role in the progression of esophageal cancer. It may carry out this role by regulating the Wnt/β-catenin and Akt/mTOR signaling pathways.

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Introduction

Esophageal cancer is the seventh most common malignant tumor, with >483,000 new cases and >439,000-related deaths in 2015 worldwide (1,2). It has been reported that the global incidence of esophageal cancer has sharply increased by >6-fold (3,4). With the development and improvement of esophageal cancer treatment strategies, the 5-year survival rate of patients with early-stage esophageal cancer has improved significantly; however, the prognosis of patients with advanced disease remains dismal (5). However, due to the lack of early typical clinical symptoms, the majority of cases are already in the late stages of the disease at the time of diagnosis. Moreover, local recurrence or distant metastasis results in the poor prognosis of patients with esophageal cancer (6). Thus, a novel therapeutic target for esophageal cancer needs to be identified, and the further understanding of the pathogenesis of esophageal cancer is urgently required.

It has been widely accepted that TGFB induced factor homeobox 1 (TGIF1), a member of the TGIF family, functions as a transcriptional repressor of TGF-β signaling and retinoid X receptor (RXR) signaling (7,8). Recent studies have demonstrated that TGIF is also involved in Wnt/β-catenin signaling (9,10) and regulates basic energy metabolism in cells (11). Consistent with the critical roles of TGIF1 in the regulation of important signaling pathways, TGIF1 has also been shown to be involved in tumorigenesis and tumor development, such as in lung (12), breast (13) and colorectal (14) cancer. Wang *et al* reported that the knockdown of TGIF inhibited the proliferation of EC-109 cells (15). However, the roles of TGIF in the carcinogenesis and metastasis of esophageal cancer and the underlying mechanisms are not yet fully understood.

Thus, the present study aimed to investigate the roles of TGIF1 in the growth and metastasis of esophageal cancer. The data presented herein indicate that TGIF1 is significantly upregulated in esophageal cancer tissues and cells, and that TGIF1 knockdown reduces the proliferation and tumorigenicity of esophageal cancer *in vivo* and *in vitro*. Moreover, it is demonstrated that TGIF1 functions as a potential tumor promoter in esophageal cancer by regulating the Wnt/β-catenin and Akt/mammaliain target of rapamycin (mTOR) signaling pathways.

Materials and methods

Cells, cell culture and transfection. Human esophageal cancer cell lines (TE-10, KYSE-150, TE-1, kyse410 and Eca-109) and the human esophageal epithelial cell line, Het1A, were obtained from Cell Bank of Chinese Academy of Sciences. Cells were maintained in RPMI-1640 medium supplementing with 10% fetal bovine serum (FBS) at 37˚C. A total of 3 siRNAs targeting TGIF1 (si-TGIF1-1#/-2#/-3#) were synthesized from Guangzhou RiboBio Co., Ltd. and transfected (100 nM) into the cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h, and scramble siRNA sequence was used as the negative control (si-NC). The siRNA sequences were as follows: TGIF1-siNC, 5'-UUCUCCGAA CGUGUCACGUTT-3'; TGIF1-siRNA1, 5'-GAAAGAUGU CCCUUUCUCUCU-3'; TGIF1-siRNA2, 5'-CCAAAUCAG UUCACAAUUUCC-3'; TGIF1-siRNA3, 5'-GUGGAUUUC AGCUUCUAGUGG-3'. The lentiviral vector overexpressing TGIF1 were established from Shanghai GeneChem Co., Ltd. (3 μl/6-wells plates), which was transfected for 48 h. The blank plasmid was used as the negative control (OE-NC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells using TRIzol reagent and reverse transcribed into cDNA using the HiFiScript cDNA Synthesis kit (CW2569M; Beijing CWBio). qPCR was performed using the Ultra SYBR Mixture kit (CW0956;Beijing CWBio). The PCR thermocycling conditions were as follows: 95˚C for 5 min and 40 cycles of 95˚C for 12 sec and 55˚C for 40 sec. Primers used in the present study were as follows: TGIF1 forward, 5'-GACATTCCCTTGGACCTTTCT-3' and reverse, 5'-TACAGCCAATCCCGAAGAATC-3'; β-actin forward, 5'-CCCGAGCCGTGTTTCCT-3' and reverse, 5'-GTC CCAGTTGGTGACGATGC-3'. Relative gene expression was calculated by the $2^{-\Delta\Delta Cq}$ method (16).

Analysis of TGIF1 expression in esophageal cancer. The expression data of TGIF1 was obtained from (Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/index.html) (17). A total of 182 cases of esophageal cancer and 286 normal samples were included in the GEPIA dataset. |log2FC| The cut-off was set to 1. The log2(TPM + 1) was used for log-scale.

Western blot analysis. Proteins were extracted from the cells with 48 h following transfection by using RIPA buffer (Beyotime Institute of Biotechnology) and then quantified using a BCA Protein Assay kit (Beijing CWBio). Proteins (30 μg) were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. Following blocking with skimmed milk for 1 h, the membranes were incubated with the following primary antibodies (1:1,000) overnight at 4˚C: Anti-TGIF1 (cat. no. ab220965), anti-N-cadherin (cat. no. ab18203), anti-E-cadherin (cat. no. ab15148) and anti-Snail (cat. no. ab53519) antibodies were obtained from Abcam. Anti-Bcl-2 (cat. no. 12789-1-AP), anti-Bax (cat. no. 50599-2-Ig), anti-cyclin 1 (cat. no. 60186-1-Ig) and anti-GAPDH (cat. no. 60004-1-lg) antibodies were obtained from Proteintech Group, Inc. Anti-cleaved caspase-3 (cat. no. 9661), anti-Wnt3a (cat. no. 2391), anti-β-catenin (cat. no. 9562), anti-Akt (cat. no. 9272), anti-p-Akt (cat. no. 9271), anti-mTOR (cat. no. 2972) and anti-p-mTOR (cat. no. 2971) antibodies were obtained from Cell Signaling Technology, Inc. The membranes were further incubated with HRP-conjugated secondary antibodies (1:5,000; cat. no. S A00004-3, Proteintech Group, Inc.) at room temperature for 1 h. Signals were developed using an ECL kit (BioVision, Inc.). The data were analyzed using ImageJ software (version 1.8.0; National Institutes of Health) and GraphPad Prism software (version 7.04; GraphPad Software, Inc.).

Cell counting kit (CCK-8) assay. Following transfection for 24 h, the cells were plated in a 96-well plate at a density of $1x10^3$ cells/well. Cell viability was measured using a CCK-8 assay (Beijing Solarbio Science & Technology Co., Ltd.) at 0, 24, 48 and 72 h, respectively. Prior to detection, the cells were incubated with 10 μl CCK8 regent at 37˚C for 1.5 h, and the OD_{450nm} value was measured.

Colony formation assay. Cells were seeded in 60-mm plates at 500 cells/plate and cultured in RPMI-1640 medium supplementing with 10% fetal bovine serum (FBS) for 10 days at 37˚C. Following washing with PBS, cells were stained with 0.1% crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) 30 min at room temperature and colonies were then counted and imaged under a light microscope (Leica Microsystems GmbH) at x100 magnification.

Transwell assay. Transwell chambers with Matrigel gel (BD Biosciences) or not were used to measure cell invasion and migration, respectively. Cells were suspended in a serum-free medium and plated into the upper chamber ($1x10^4$ cells/well). The lower chambers were filled with 500 μl medium containing 10% FBS. Following incubation for 24 h at 37˚C, the migrated or invaded cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min at room temperature, which were then counted and imaged under a microscope (Leica Microsystems GmbH; magnification, x100).

Flow cytometric assay. Cells were incubated at 37˚C in a serum-free medium for 24 h and suspended in 1X binding buffer. Approximately $1\text{-}5x10^5$ cells were incubated with Annexin V/FITC-PI (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. The cell samples were then analyzed using a flow cytometer (BD FACSCanto™ II; BD Biosciences), and the rate of apoptosis was calculated using BD FACSDiva™ software (BD Biosciences).

Tumor xenograft assay. A total of 30 female nude mice (18-22 g; 4-6 weeks old) were purchased from Huafukang Biotechnology Co., Ltd. The study protocols were approved by the Institutional Animal Experimentation Committee of

Shandong Cancer Hospital (Shandong, China). All animals were housed at the Animal Care Facility of Shandong Cancer Hospital at 25˚C with a 12/12-h light/dark cycle in a vivarium with humidified airflow, and were allowed free access to normal chow and water during the study period. Five different cells ($5x10^6$) in 150 μl of PBS were subcutaneously injected into the right hind legs of each mouse. To maintain the transfected characteristics *in vivo*, cholesterol-modified TGIF1 siRNA for *in vivo* RNA delivery was designed and synthesized by Guangzhou RiboBio Co., Ltd. For the delivery of cholesterol-conjugated RNA, 10 nmol RNA in 0.1 ml saline buffer were locally injected into the tumor mass once every 3 days for 3 weeks, as previously described (18). Five different tumor models were established: i) si-TGIF1-1#; ii) si-TGIF1-2#; iii) si-NC; iv) OE-NC; v) TGIF1-OE (n=5 per group).Tumor volumes were calculated every day using a Vernier caliper (volume=length x width x width x 0.5), as previously described (19). When the volumes of the tumors reached approximately 100-150 mm^3, the tumor growth delay experiment was performed. As described in a previous study by the authors (20), the relative tumor volume (RTV; RTV = Vt/V0, where Vt is the volume of the tumor at any given time and V0 is the initial volume before treatment) was calculated and the growth curve was analyzed. The mice were euthanized by pentobarbital (100 mg/kg) followed by cervical dislocation at 20 days post-injection or when the tumor volume had reached 2,000 mm^3. Tumors were collected immediately following the euthanasia of the mice.

Immunohistochemistry of the mouse tumor tissue. Immunohistochemistry was performed as previously described (21). The tumor tissues were sectioned (4-μm-thick) and dewaxed. After antigen retrieval was performed using 10 mmol/l citrate buffer, the sections were incubated with 3% H_2O_2 and blocked with 5% BSA at 37˚C for 30 min. The related detection protein included the primary antibodies (1:1,000) described above and anti-Ki67 (1:500, cat. no. ab15580) obtained from Abcam, and anti-TGIF1 (1:1,000, cat. no. orb47063) obtained from Biorbyt. The sections were then incubated with primary antibodies at 4˚C overnight. After rewarming at room temperature the following day, the sections were incubated with secondary antibody (1:200; cat. no. A-21442; Thermo Fisher Scientific, Inc.) using the two-step polymer HRP (cat. no. PV-9005; OriGene Technologies, Inc.) detection system at room temperature for 1 h. The samples were visualized using 3,3-diaminobenzidine. Images were acquired using a light microscope (Leica Microsystems GmbH; magnification, x100).

Statistical analysis. In the present study, data are presented as the means ± SD from 3 independent experiments and statistically analyzed using GraphPad Prism7 software. Statistically significant differences between groups were analyzed using a Student's t-test or one-way ANOVA followed by Bonferroni's post hoc test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

TGIF1 increases the proliferation of esophageal cancer cells. The present study first investigated TGIF1 mRNA expression in esophageal cancer by Gene Expression Profiling Interactive Analysis. As shown in Fig. 1A, the mRNA expression of TGIF1 in esophageal cancer tissues (n=182) was higher than that in normal tissues (n=286). Moreover, RT-qPCR was performed to examine the mRNA expression of TGIF1 in 5 esophageal cancer cell lines (TE-10, KYSE-150, TE-1, kyse410 and Eca-109) and the human esophageal epithelial cell line, Het1A. The results revealed that compared with the Het1A cells, the mRNA expression of TGIF1 was significantly upregulated in all esophageal cancer cell lines (Fig. 1B). Thus, these data indicated that TGIF1 was upregulated in esophageal cancer tissues and cells.

To investigate TGIF1 function in the progression of esophageal cancer, loss-of-function and gain-of-function experiments were performed. As shown in Fig. 1C, TGIF1 mRNA expression was significantly knocked down by 3 different siRNA-TGIF1 sequences (si-TGIF1-1#, -2# and -3#) in the KYSE-150 cells, which exhibited the highest TGIF1 expression; si-TGIF1-1#, and si-TGIF1-2# were used in the following experiments due to their greater knockdown efficiency. Accordingly, the TGIF1 protein level was also significantly suppressed by si-TGIF1-1# or si-TGIF1-2# (Fig. 1D). As the Eca-109 cells exhibited the lowest expression of TGIF1, they were transfected with pcDNA3.1-TGF1 to upregulate its expression at both the mRNA and protein level (Fig. 1E and F). Subsequently, CCK-8 and colony formation assays were performed to examine the proliferative ability of the 2 esophageal cancer cells following transfection with si-TGIF1 or pcDNA3.1-TGF1 (Fig. 1G-J). As shown in Fig. 1G, the silencing of TGIF1 markedly decreased the viability of the KYSE-150 cells when compared with the NC group. Notably, TGIF1 overexpression significantly enhanced the viability of the Eca-109 cells (Fig. 1H). Moreover, the silencing of TGIF1 significantly decreased the colony-forming ability of the KYSE-150 cells, which was increased by TGIF1 overexpression in the Eca-109 cells (Fig. 1I and J).

TGIF1 enhances the migratory and invasive abilities of esophageal cancer cells. Transwell assay was conducted to detect the preliminarily effect of TGIF1 on the metastatic capacity of esophageal cancer cells. The data demonstrated that the silencing of TGIF1 significantly decreased the migratory ability of the KYSE-150 cells (Fig. 2A), while the upregulation of TGIF1 enhanced the migratory ability of the Eca-109 cells (Fig. 2B). Correspondingly, the invasive ability of the KYSE-150 cells was also suppressed by TGIF1 knockdown (Fig. 2C), while in the Eca-109 cells in which TGIF1 was overexpressed, the opposite results were observed (Fig. 2D). Furthermore, western blot analysis was used to examine the expression of epithelial-mesenchymal transition (EMT)-related proteins in esophageal cancer cells. The results indicated that the expression of N-cadherin and Snail was significantly decreased in the KYSE-150 cells transfected with si-TGIF1, while E-cadherin expression was upregulated (Fig. 2E); however, the expression of the above-mentioned proteins exhibited an opposite trend in the TGIF1-overexpressing cells (Fig. 2E). Therefore, it was demonstrated that TGIF1 can enhance the metastatic potential of esophageal cancer cells by regulating the EMT process.

TGIF1 impairs the apoptosis of esophageal cancer cells. Flow cytometric assay indicated that compared with the control group, the percentage of apoptotic KYSE-150 cells

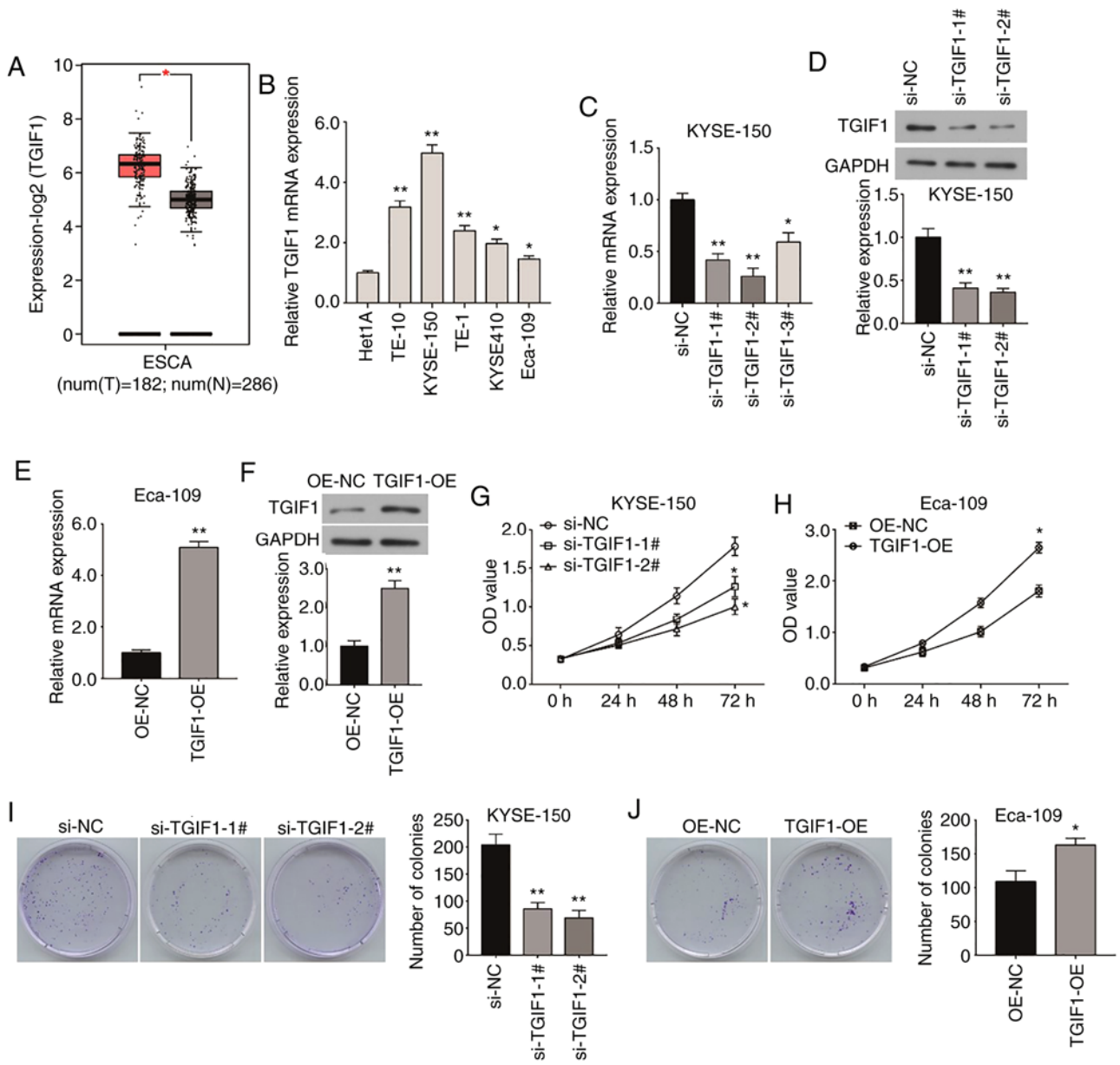

Figure 1. TGIF1 is upregulated in esophageal cancer and promotes cell proliferation. (A) mRNA expression of TGIF1 in esophageal cancer tissues (red box) and normal tissues (black box) analyzed using the GEPIA database. (B) Expression of TGIF1 mRNA in esophageal cancer cell lines (TE-10, KYSE-150, TE-1, kyse410 and Eca-109) and the human esophageal epithelial cell line, Het1A. (C) mRNA expression of TGIF1 in KYSE-150 cells transfected with siRNA-TGIF1-1#/2#/3# or siRNA control. (D) Western blot analysis was used to detect TGIF1 protein expression in KYSE-150 cells transfected with siRNAs. (E) mRNA expression of TGIF1 in Eca-109 cells transfected with pcDNA3.1-TGIF1 or pcDNA3.1 vector. (F) Western blot analysis was used to detect TGIF1 protein expression in Eca-109 cells. (G and H) CCK-8 assay was used to measure the viability of (G) KYSE-150 and (H) Eca-109 cells transfected with siRNAs or pcDNA3.1-TGIF1. (I and J) Colony formation assay was performed to examine colony-forming abilities of the (I) KYSE-150 and (J) Eca-109 cells. *P<0.05, **P<0.01 vs. respective control. TGIF1, TGFB induced factor homeobox 1.

was significantly increased by si-TGIF1 (Fig. 3A), while the percentage of apoptotic Eca-109 cells was significantly decreased by TGIF1 overexpression (Fig. 3B). Furthermore, western blot analysis revealed that the expression of the anti-apoptotic protein, Bcl-2, was significantly downregulated by si-TGIF1, whereas it was upregulated by TGIF1 overexpression. However, the expression of the pro-apoptotic proteins, Bax and cleaved caspase-3, was upregulated in the TGIF1-silenced KYSE-150 cells, and decreased in TGIF1-overexpressing Eca-109 cells (Fig. 3C). Taken together, these results revealed that the silencing of TGIF1 induced the apoptosis of esophageal cancer cells by regulating the Bcl-2/Bax axis and caspase-3 activation.

TGIF1 promotes the activation of Wnt/β-catenin and Akt/mTOR signaling pathways in esophageal cancer cells. It has been reported that TGIF1 can promote Wnt signaling in breast cancer cells (10). Herein, as shown in Fig. 4, it was found that the silencing TGIF1 inhibited the expression of Wnt3a and β-catenin, which was upregulated in TGIF1-overexpressing cells, suggesting that TGIF1 also promoted Wnt/β-catenin signaling in esophageal cancer cells. Additionally, it was observed that Akt/mTOR, another classical signaling pathway involved in cellular functions, was also affected by TGIF1 in esophageal cancer cells (Fig. 4A-C). TGIF1 knockdown suppressed the phosphorylation of Akt, and mTOR and

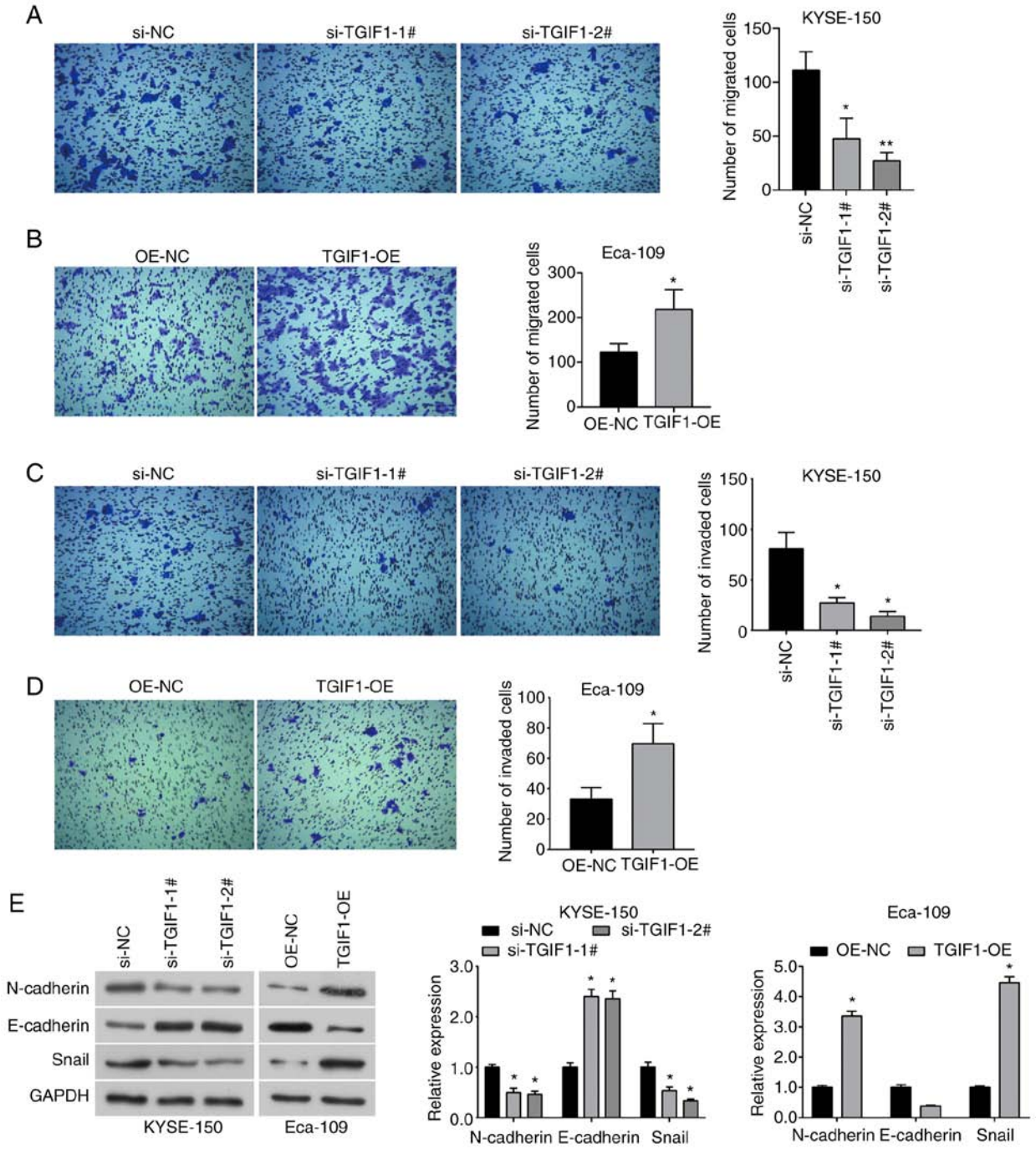

Figure 2. TGIF1 enhances the migration and invasion abilities of esophageal cancer cells. (A and B) Following transfection with siRNAs or pcDNA3.1 vector, (A) KYSE-150 and (B) Eca-109 cell migration was measured by Transwell assay (magnification, x100). (C and D) Transwell assay was performed to examine the invasion of (C) KYSE-150 and (D) Eca-109 cells (magnification, x100). (E) Expression of EMT-related proteins (E-cadherin, N-cadherin and Snail) was detected by western blot analysis in KYSE-150 and Eca-109 cells. *P<0.05, **P<0.01, vs. respective control. TGIF1, TGFB induced factor homeobox 1.

cyclin D1 expression in the KYSE-150 cells, while TGIF1 overexpression increased the expression of these proteins in the Eca-109 cells (Fig. 4), suggesting that TGIF1 cab promote the activation of the Akt/mTOR signaling pathway in esophageal cancer cells and that the knockdown TGIF1 arrested the cell cycle in the G1 phase. The ratio of phosphorylated to total protein is presented in Fig. S1.

Effects of TGIF1 on the growth of esophageal cancer in vivo. To verify the effects of TGIF1 on esophageal cancer *in vivo*, KYSE-150 (si-NC, si-TGIF1-1#, si-TGIF1-2#) and Eca-109 (OE-NC,TGIF1-OE) subcutaneous xenograft tumor models were established using BALB/c nude mice and the relative tumor volume was measured. Tumor growth was found to be markedly attenuated in the mice injected with the KYSE-150 cells and TGIF1 siRNA (si-TGIF1-1#, si-TGIF1-2#) compared with those injected with KYSE-150 cells and the negative control siRNA (si-NC; si-TGIF1-1# vs. si-NC group, P<0.05; si-TGIF1-2# vs. si-NC group, P<0.01; Fig. 5A). Of note, the RTV of the Eca-109-derived tumors overexpressing TGIF1 (TGIF1-OE) was larger than that of the Eca-109-derived tumors injected with the negative control (OE-NC; P<0.05; Fig. 5B).

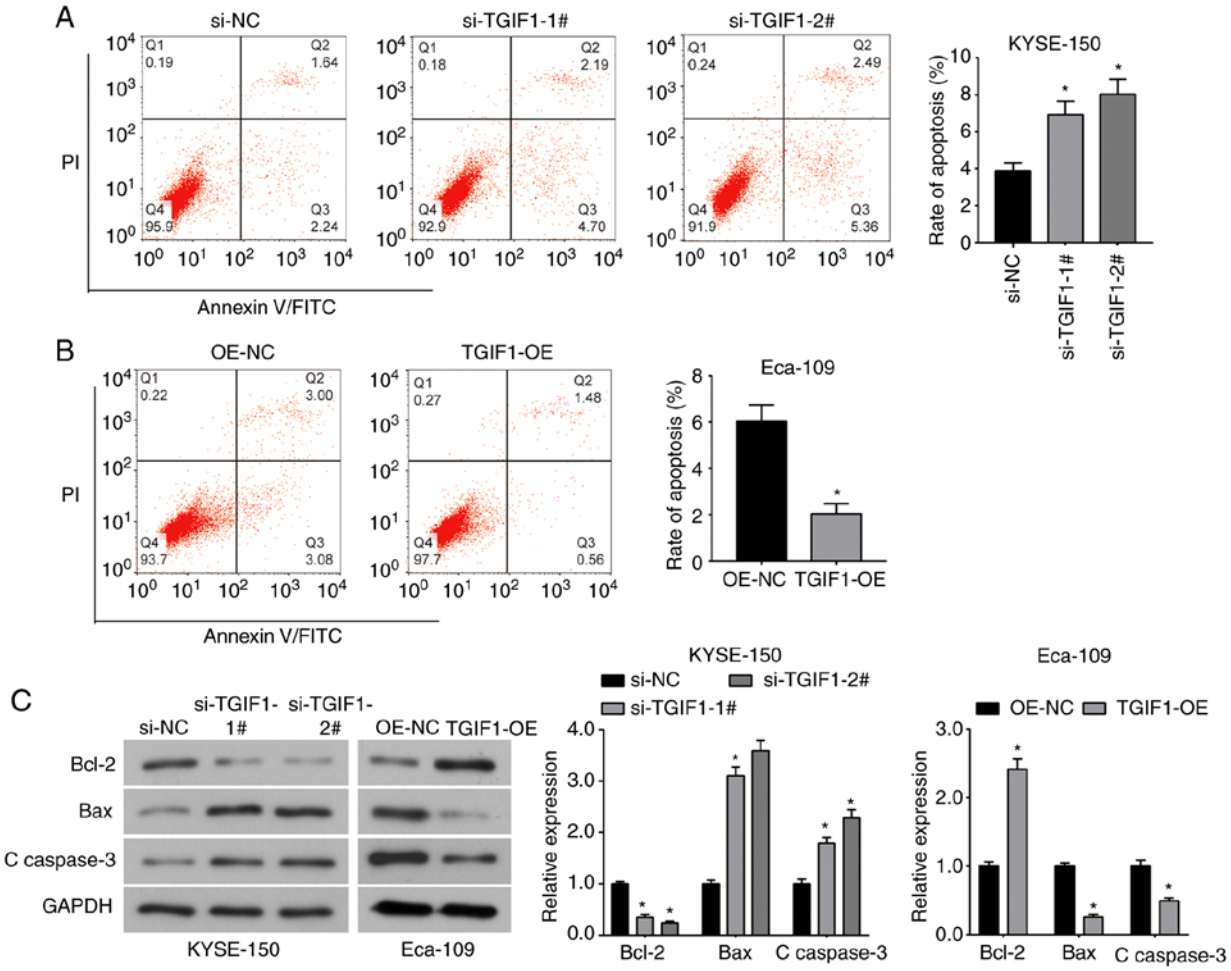

Figure 3. TGIF1 decreases the apoptosis of esophageal cancer cells. (A and B) Flow cytometry was used to determine the rate of apoptosis of (A) KYSE-150 and (B) Eca-109 cells following transfection. (C) Expression of apoptosis-related proteins (Bcl-2, Bax and cleaved caspase-3) was detected by western blot analysis in the KYSE-150 and Eca-109 cells. *P<0.05, vs. respective control. TGIF1, TGFB induced factor homeobox 1.

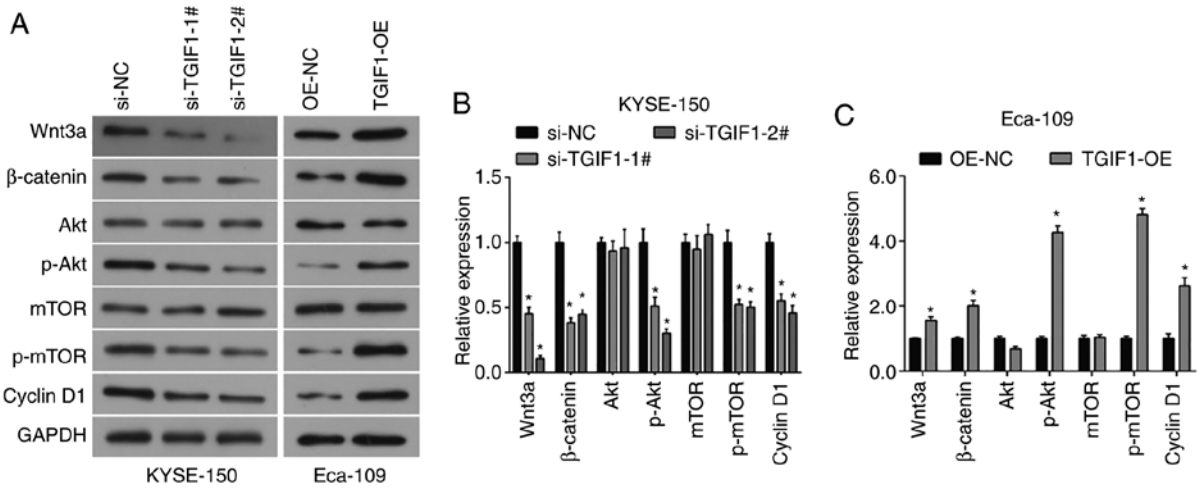

Figure 4. TGIF1 promotes the activation of the Wnt/β-catenin and Akt/mTOR signaling pathways. (A) Expression of Wnt/β-catenin-related and Akt/mTOR-related proteins were detected by western blot analysis in KYSE-150 and Eca-109 cells. (B) Quantitative analysis of the results of western blot analysis in KYSE-150 cells. (C) Quantitative analysis of the results of western blot analysis in Eca-109 cells. *P<0.05, vs. respective control. TGIF1, TGFB induced factor homeobox 1; mTOR, mammalian target of rapamycin.

These data suggested that TGIF1 knockdown suppressed tumor formation and tumor growth *in vivo*, while the upregulation of TGIF1 enhanced tumor growth *in vivo*.

TGIF1 affects proliferation-related genes and pathways in vivo. The expression levels of TGIF1, E-cadherin, N-cadherin, Snail, Wnt/β-catenin, Akt/mTOR and Ki-67 in

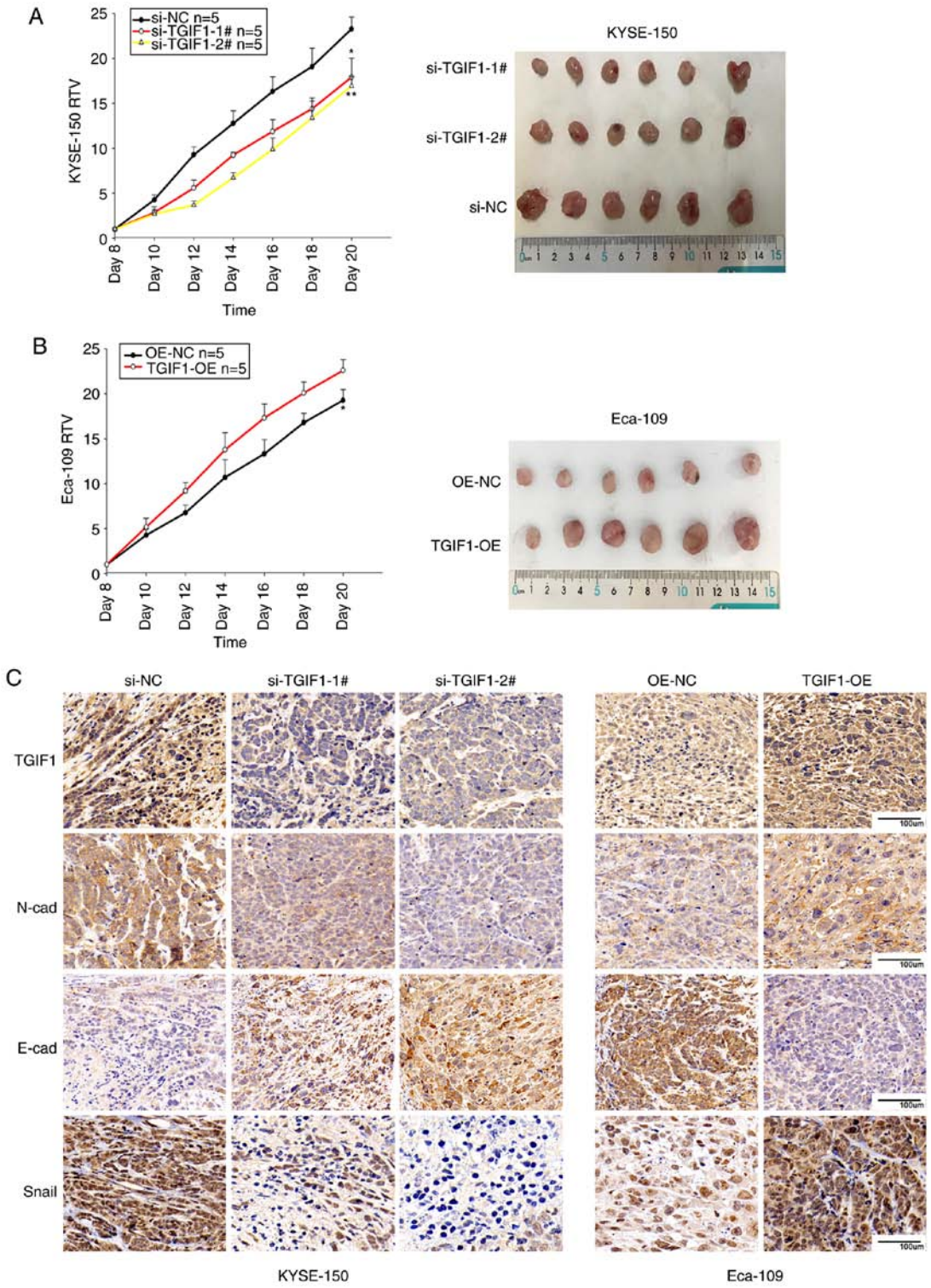

Figure 5. TGIF1 enhances the growth of esophageal cancer and increases the expression of EMT-related proteins *in vivo*. (A) Relative tumor volume growth cure of KYSE-150-derived tumors. (B) Relative tumor volume growth cure of Eca-109-derived tumors. *P<0.05, **P<0.01, vs. respective control. (C) Expression of EMT-related proteins (E-cadherin, N-cadherin and Snail) was detected by immunohistochemistry in the KYSE-150- and Eca-109-derived tumors. TGIF1, TGFB induced factor homeobox 1; RTV, relative tumor volume; N-cad, N-cadherin; E-cad, E-cadherin.

the tumor xenografts were detected by immunohistochemical staining. As shown in Figs. 5C and S2, the TGIF1, Snail and N-cadherin expression levels were downregulated by si-TGIF1 in the KYSE-150-derived tumor xenografts, whereas these were upregulated by TGIF1 overexpression in the Eca-109-derived tumor xenografts. The expression of E-cadherin was upregulated in the TGIF1-silenced KYSE-150-derived tumors, and was decreased in the TGIF1-overexpressing Eca-109-derived tumors. Additionally, as shown in Figs. 6 and S3, the expression levels of Wnt3a and β-catenin were inhibited by the silencing of TGIF1, whereas they were upregulated by TGIF1 overexpression. TGIF1 knockdown suppressed the phosphorylation of Akt/mTOR and Ki-67 expression in the KYSE-150-deriged tumors, while TGIF1 overexpression increased the expression

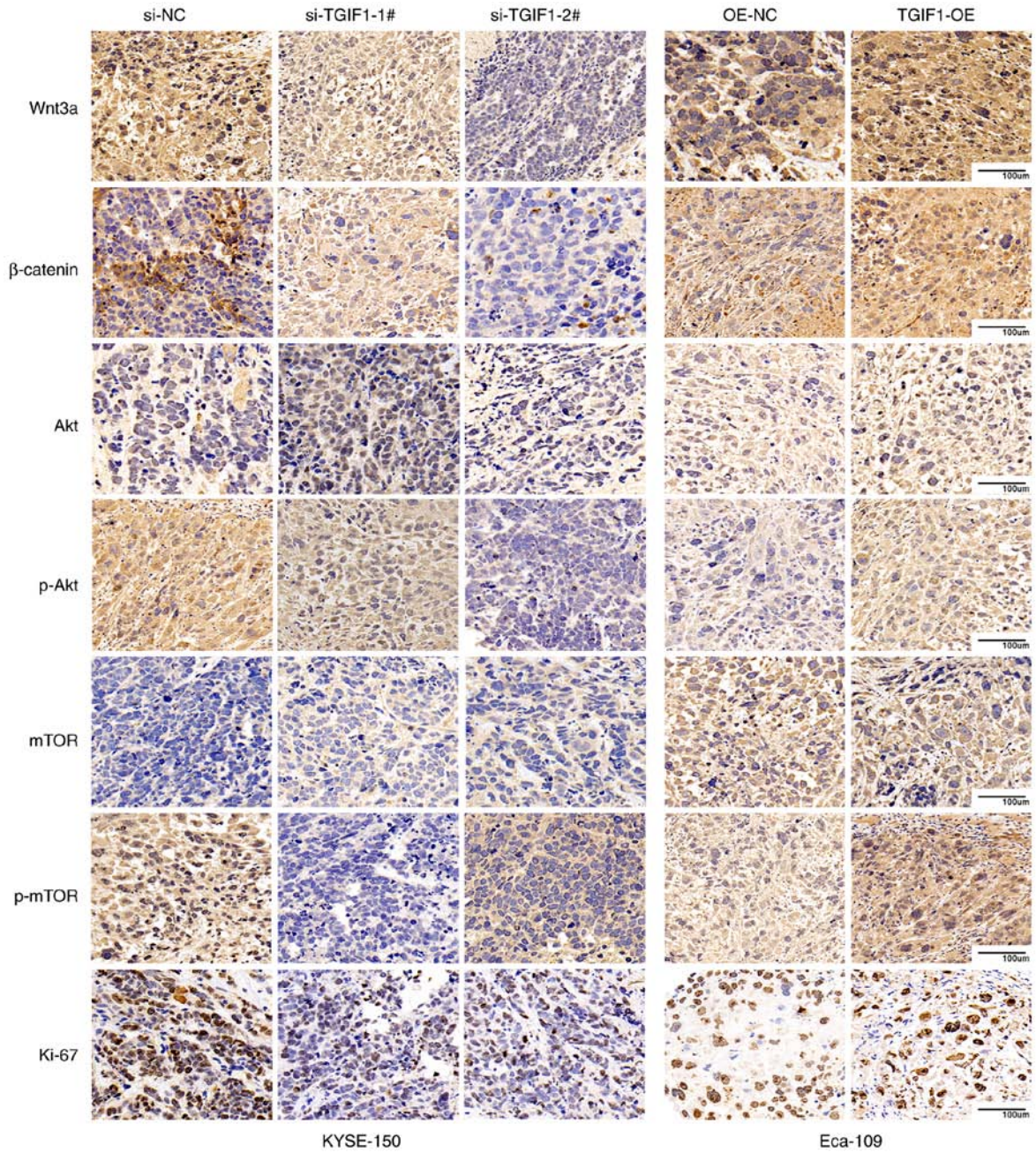

Figure 6. TGIF1 promotes the activation of the Wnt/β-catenin and Akt/mTOR signaling pathways *in vivo*. Expression levels of Wnt/β-catenin-related and Akt/mTOR-related proteins were detected by immunohistochemistry in the KYSE-150 and Eca-109 tumors. TGIF1, TGFB induced factor homeobox 1; mTOR, mammalian target of rapamycin.

of these proteins in the Eca-109-derived tumors. The down-regulation of p-mTOR by the knockdown of TGIF1 may be the key factor of the classical Akt/mTOR signaling pathway. These results were consistent with the results obtained *in vitro* and confirm that TGIF1 increases the proliferation of esophageal cancer cells via the Wnt/β-catenin and Akt/mTOR signaling pathways, and promotes EMT *in vivo*.

Discussion

Previous studies have reported that TGIF1 plays a critical role in tumor initiation and progression, and its overexpression is associated with a poor prognosis of patients with colorectal (10,11), lung (12), gastric (22) and breast (13,23) cancer. The study by Wang *et al* demonstrated that TGIF1 was upregulated in colorectal cancer and functioned as an oncogene, promoting cancer cell proliferation and migration (14). Another study by Wang *et al* reported that TGIF1 knockdown suppressed the migration and invasion of breast cancer cells (13). The study by Haider *et al* further demonstrated that the lack of TGIF1 also restricted the progression of breast cancer bone metastases (23). Inconsistent with the oncogenic role of TGIF1 in tumorigenesis and development reported in previous studies, Parajuli *et al* revealed that TGIF1 funcioned as a tumor suppressor in pancreatic ductal adenocarcinoma (24). Weng *et al* also found that the loss of TGIF1 induced the development of pancreatic cancer (25). The reason for the inconsistency with other cancer types was that TGIF1 is involved in different pathways in

pancreatic cancer. It has been reported that the silencing of TGIF inhibits the proliferation and tumorigenicity of EC109 cells (15). However, the role of TGIF1 in the metastasis of esophageal cancer and the underlying mechanisms remain elusive.

Compared with the study by Wang *et al* (15), the present study used different esophageal cancer cell lines and constructed an TGIF1 overexpression model. Furthermore, the present study verified more functions of TGIF1 as an oncogene and explored the possible mechanisms. Herein, it was found that TGIF1 was upregulated in esophageal cancer tissues and cell lines. Importantly, the data demonstrated that the silencing of TGIF1 significantly inhibited the proliferation and colony-forming capabilities of the KYSE-150 cells, while TGIF1 overexpression resulted in an opposite phenotype in the Eca-109 cells, which was consistent with the findings of a previous study (15). Moreover, it was found that TGIF1 reduced apoptosis of esophageal cancer cells by regulating the Bcl-2/Bax axis and caspase-3 activation. EMT plays a pivotal role in tumor metastasis proven by the upregulated expression of N-cadherin, while the expression level of E-cadherin is downregulated (26-30). Furthermore, the present study demonstrated that TGIF1 increased the migratory and invasive capabilities of esophageal cancer cells and regulated the expression of EMT biomarkers; TGIF1 knockdown downregulated the N-cadherin and Snail expression, and upregulated E-cadherin expression in KYSE-150 cells and tumors, indicating that TGIF1 may increase the metastatic potential of esophageal cancer cells by regulating the EMT process. These findings suggest thatTGIF1 functions as an oncogene in the growth and metastasis of esophageal cancer.

In addition to its well-known ability to suppress TGF-β signaling, TGIF1 has also been confirmed to activate Wnt signaling in breast, colorectal and lung cancer cells (12,14). The Wnt/β-catenin signaling pathway is involved in various biological processes, such as cell proliferation, movement, differentiation and cell death (31-34), which can regulate target gene transcription by β-catenin entering the cell nucleus (35,36). Zhang *et al* demonstrated that TGIF promotes β-catenin abundance by diverting Axin1/2 from the β-catenin destruction complex, and was thus involved in Wnt1-induced mammary tumor formation (10). Wang *et al* reported that TGIF1 promoted the proliferation of colorectal cancer cells by activating Wnt/β-catenin signaling via its DNA binding ability and interaction with β-catenin (14). Based on these studies, it was hypothesized that the Wnt/β-catenin signaling pathway may play a tumor-promoting role with TGIF1 in esophageal cancer. Herein, it was found that Wnt3a and β-catenin expression was significantly downregulated in cells and tumors in which TGIF1 was knocked down, whereas it was upregulated by TGIF1 overexpression, indicating that TGIF1 may promote the progression of esophageal cancer partly by activating the Wnt/β-catenin signaling pathway. Additionally, the activation of the Akt/mTOR signaling pathway was also upregulated by TGIF1, by increasing the p-Akt and p-mTOR levels, suggesting that the Akt/mTOR signaling pathway may be involved in the tumor-promoting effect of TGIF1. This warrants further exploration in future studies.

Some limitations of the present study should be acknowledged. It is known that the incidence of esophageal adenocarcinoma equals or exceeds the incidence of esophageal squamous cell carcinoma in the US and other western countries. Therefore, one limitation of the present study is that esophageal adenocarcinoma cell lines were not been used. Second, the effects of the knockdown of TGIF1 on the cell cycle should be further investigated in future studies. Third, the effects of TGIF overexpression on the related pathways need to be further explored. Moreover, a transgenic animal model could be used to assess the functional role of TGIF in esophageal tumorigenesis.

In conclusion, the present study demonstrated that the silencing of TGIF1 suppressed esophageal cancer cell proliferation, migration and invasion, and promoted apoptosis, suggesting that TGIF1 plays oncogenic role in the progression of esophageal cancer. Moreover, the Wnt/β-catenin and Akt/mTOR signaling pathways may be involved in the tumor-promoting effects of TGIF1. Thus, TGIF1 may be a therapeutic target for the treatment of esophageal cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BL designed the study and revised the draft of the manuscript. YY and LK completed the main experiments and drafted the manuscript. HG created the figures and performed the statistical analyses. XL performed the histological examination of the tumors. WH assisted in the design of the study. FS and LJ analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Ethics Committee at the Shandong Cancer Hospital Affiliated to Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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