IGFBP7 remodels the tumor microenvironment of esophageal squamous cell carcinoma by activating the TGFβ1/SMAD signaling pathway

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Abstract. Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer, and its development, growth, and invasiveness are regulated by the tumor microenvironment (TME). Insulin-like growth factor-binding protein-7 (IGFBP7), which is closely related to various tumors, transforming growth factor-β1 (TGFβ1), which is a key signal mediator in oncogenesis, α-smooth muscle actin (α-SMA), and collagen I are important components of the TME. IGFBP7 can upregulate the expression of TGFβ1 and activate the TGFβ1/SMAD signaling pathway, which leads to an increase in collagen I in hepatic stellate cells (HSCs). However, the contribution of IGFBP7 to TGFβ1 and the TME in the progression of ESCC remains unknown. In the present study, we investigated IGFBP7 expression and its effects on TGFβ1 and the TME in ESCC. A total of 45 patients were divided into three groups: early-tumor group (n=15), advanced-tumor group (n=15), and paracancer control group (n=15). The EC109 cell line was cultured and treated with AdIGFBP7 and LvshTGFβ1, and the expression levels of IGFBP7, TGFβ1, α-SMA, collagen I, and p-SMAD2/3 were determined by immunohistochemical staining and western blotting analysis. IGFBP7, TGFβ1, α-SMA, and collagen I were upregulated in the ESCC samples compared with the control samples (P<0.05), and the values peaked in the advanced-tumor group (P<0.05). Compared with the control group, the TGFβ1, α-SMA, p-SMAD2/3, and collagen I proteins were gradually increased from 24 to 72 h in the EC109 cells treated with AdIGFBP7 (P<0.05). Inhibition of TGFβ1 expression in the EC109 cells treated with AdIGFBP7 gradually reduced the expression of α-SMA, collagen I, and p-SMAD2/3 from 24 to 72 h (P<0.05). These findings suggest that increased IGFBP7 may accelerate the progression of ESCC by upregulating TGFβ1, α-SMA, and collagen I via activating the TGFβ1/SMAD signaling pathway, which could remodel the TME.

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Introduction

Esophageal cancer (EC) is one of the most serious types of cancer worldwide, and it presents a high mortality rate and poor prognosis (1). Esophageal squamous cell carcinoma (ESCC), which is the most common type of esophageal cancer, is often diagnosed at an advanced stage (2), and ESCC patients have a high recurrence rate and low 5-year survival rate (3). Various factors are involved in the development, growth, and invasion of ESCC. For example, insulin-like growth factor-binding protein-7 (IGFBP7), which is also known as insulin-like growth factor binding protein related protein 1 (IGFBPrP1), is a secretory protein with a molecular mass of approximately 30 kDa (4), and it has distinct characteristics and participates in cell proliferation, senescence, and apoptosis in numerous cancers (5). IGFBP7 expression has been reported in gastrointestinal cancers, including esophageal adenocarcinoma (EAC) (6).

Cancer invasion and metastasis are not only determined by cancer cells but also by the tumor microenvironment (TME), with cancer-associated fibroblasts (CAFs) and the extracellular matrix (ECM) playing important roles during the progression of cancer (7). The TME also plays a critical role in ESCC (8). α-smooth muscle actin (α-SMA) is a marker of CAF activation, and the collagen I content in the stroma cells of the TME is correlated with the aggressiveness and outcome of tumors (9).

Transforming growth factor-β1 (TGFβ1) has been recognized as a key signal mediator involved in oncogenesis (10,11), and it plays an important role in the TME by accelerating invasion, metastasis, angiogenesis, and immunosuppression. TGFβ1 upregulates α-SMA and collagen I, thus creating an important proinvasion and proangiogenesis niche for cancer development (12,13). The TGFβ signaling pathway is a critical pathway for generating a fibrotic TME, and the TGFβ1/SMAD signaling pathway is a classical pathway associated with cancer. Recent studies have shown that TGFβ1 cannot activate p-SMAD2/3-deficient hepatic stellate cells (HSCs) to synthesize ECM (14,15). Therefore, the TGFβ1/SMAD signaling pathway plays an important role in ECM deposition.

IGFBP7 can upregulate the expression of TGFβ1 and α-SMA and act as an upstream factor for TGFβ1 in the activation of the SMAD signaling pathway, thus leading to increases in collagen I in HSCs (16). However, whether IGFBP7 expression activates the SMAD signaling pathway to upregulate the expression of TGFβ1, α-SMA, and ECM to remodel the TME during esophageal squamous cell carcinoma (ESCC) progression remains unknown. The present study investigated the expression of IGFBP7 and its effect on TGFβ1 and the TME in ESCC and analyzed the associated changes in the expression of TGFβ1, α-SMA, collagen I, p-SMAD2/3, and SMAD2/3.

Materials and methods

Tissue samples. A total of 45 patients aged 18 to 80 years who were diagnosed with ESCC via biopsy at the Affiliated Lianyungang Oriental Hospital of Xuzhou Medical University (Lianyungang, China) between April 2017 and June 2019 were eligible for inclusion and enrolled in this study. Early esophageal cancer refers to the cancer that only invades the esophageal mucosa or submucosa without lymph node metastasis. When the tumor has involved the muscularis or adventitia or outside the adventitia, and has local or distant lymph node metastasis, the cancer has developed to the advanced stage. The samples were divided into three groups: early tumor group (n=15), advanced tumor group (n=15), and paracancer control group (n=15). Normal paracancer tissues adjacent to the tumor were sampled. The present study was formally approved by the Medical Ethics Committee of the Affiliated Lianyungang Oriental Hospital of Xuzhou Medical University (2017-006-01), and written informed consent was obtained from each patient.

Cell culture and treatment with AdIGFBP7 and LvshTGFβ1. The EC109 cell line was purchased from the Aiyan Biological Co. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell lines were grown in a humidified 37˚C incubator with 5% CO_2. AdIGFBP7 was purchased from GenePharma Co. Adenoviral vectors carrying no cDNA (CAd) were used as the negative control. LvshTGFβ1 was designed and synthesized by Sangon Biotech Co. the Lentiviral vectors carrying negative shRNA (LvshNC) served as negative control. The TGFβ1 shRNA sequence was as follows: 5'-GCAGCTGTACATTGACTTTAG-3'. The negative shRNA sequence was 5'-TTCTCCGAACGTGTCACGT-3'. The shRNA and LV10 vector were constructed by Sangon Biotech Co. at the final concentration of 200 nM and 50 ng/μl, respectively. The 293T cell line was purchased from the Cell Bank of the Chinese Academy of Sciences and used to generate the virions. RNAi-Mate (A615555; bbi-biotech GmbH) was used as the transfection reagent according to the manufacturer's instructions. Transfection was performed at 37˚C for 6 h and then the transfection medium was replaced with full culture medium for 72 h. Viral supernatant was collected and centrifuged at 1,800 x g at 4˚C for 4 min, and then at 26,000 x g at 4˚C for 2 h. The transfection efficiency was assessed by the percentage of the number of RFP-positive cells to the total cells. Further experiments with EC109 cells were followed. In the treatment group, EC109 cells were transfected with AdIGFBP7 and LvshTGFβ1 at a multiplicity of infection (MOI) of 40 and 100, respectively. Cells were harvested at 24, 48 and 72 h.

Immunohistochemistry. Immunohistochemistry was performed to examine the expression of IGFBP7. The patient samples were washed with PBS, fixed in formalin, embedded in paraffin, and then sliced. The tissue slices were then deparaffinized, hydrated, and incubated with hydrogen peroxide (3%) for 20 min to block endogenous peroxidase activities. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH=6.0) and a microwave histoprocessor for 10 min. Primary antibodies against IGFBP7 (1:100; cat. no. ab171085; Abcam) were then added and incubated overnight at 4˚C, followed by biotinylated secondary antibodies (1:500; cat. no. ab207995; Abcam) at 37˚C for 20 min. In this experiment, PBS was used as the negative control instead of the primary antibody. Slices were then incubated with 3,3-diaminobenzidine (DAB) solution for 5 min. Integrated optical density (IOD) values were measured using the Image Pro Plus software (version 6.0; Media Cybernetics, Inc.).

Western blot analysis. The total protein from the tissue specimens or cells were extracted using a kit following the manufacturer's protocol (cat. no. 3100; KeyGEN BioTECH Co., Ltd.). The protein concentration was determined using a BCA protein concentration assay kit following the manufacturer's instructions (cat. no. 23227; Thermo Fisher Scientific, Inc.). Proteins (40 μg) from the tissue samples and cell homogenates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) and subjected to western blotting. The separated proteins were subsequently transferred onto PVDF membranes and then incubated in a blocking solution with 3% skimmed milk for 2 h at room temperature and with primary antibodies against IGFBP7 (1:1,000; cat. no. ab171085; Abcam), TGFβ1 (1:1,000; cat. no. ab92486; Abcam), α-SMA (1:1,000; cat. no. ab5694; Abcam), collagen I (1:1,000; cat. no. ab34710; Abcam), p-SMAD2/3 (1:1,000; cat. no. ab272332; Abcam), SMAD2/3 (1:1,000; cat. no. ab217553; Abcam), and β-actin (1:1,000; cat. no. ab8227; Abcam) overnight at 4˚C. After washing, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. D110066; Sangon Biotech, Co., Ltd.) was added and incubated for 2 h at room temperature. An enhanced chemiluminescence system (Bio-Rad Laboratories, Inc.) was used to visualize

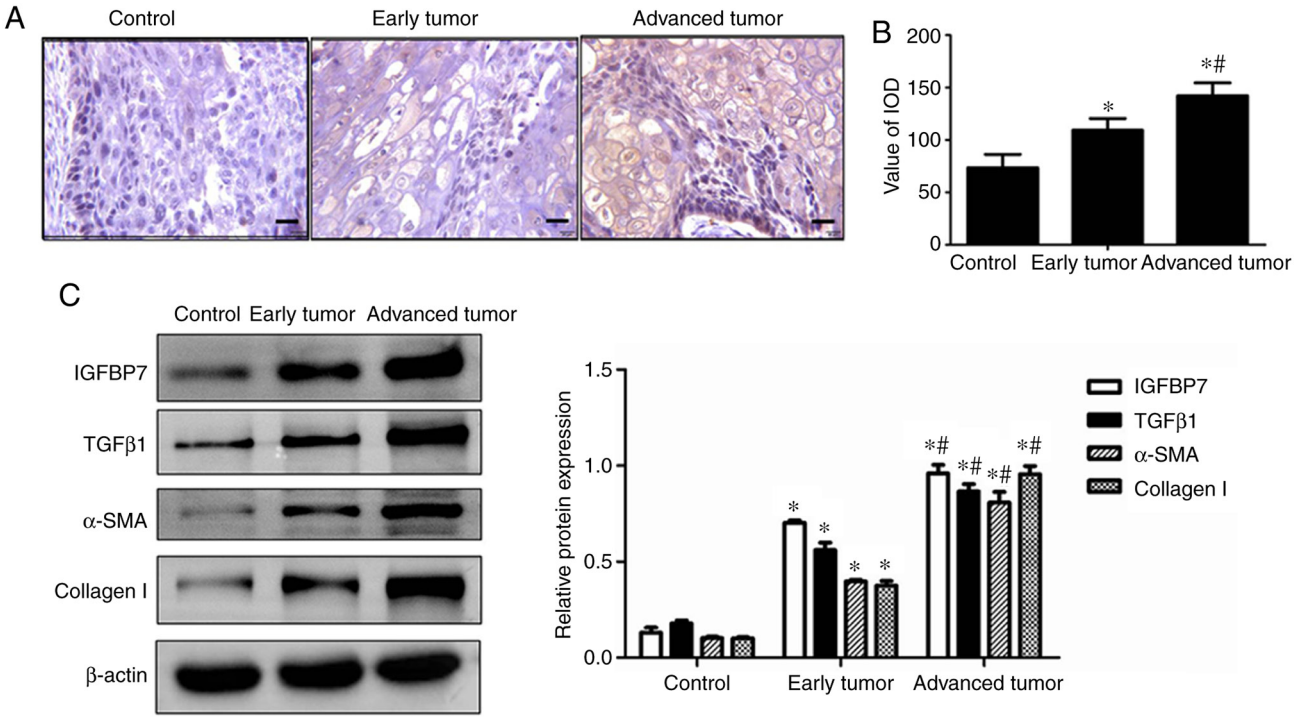

Figure 1. Expression of IGFBP7, TGFβ1, α-SMA, and collagen I in esophageal squamous cell carcinoma. (A) IGFBP7 expression was examined by immunohistochemistry staining (scale bar, 50 μm). (B) IOD value of the positive-brown particles was calculated. (C) Expression of IGFBP7, TGFβ1, α-SMA, and collagen I was examined by western blotting. β-actin served as an internal control (n=15). *P<0.05 vs. the control group; #P<0.05 vs. the early tumor group. IGFBP7, insulin-like growth factor-binding protein-7; TGFβ1, transforming growth factor-β1; α-SMA, α-smooth muscle actin.

the protein bands, and the relative protein expression was normalized to that of β-actin. Protein expression was quantified using Bio-Rad Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc.) Data are presented as mean ± SD. An analysis of variance (ANOVA) and SNK-*q* test were performed to determine whether the differences among the groups were significant, with P≤0.05 considered to indicate a statistically significant difference.

Results

Expression of IGFBP7, TGFβ1, α-SMA, and collagen I are increased in esophageal squamous cell carcinoma. Tissue samples from the patients in each experimental group were examined to identify changes in IGFBP7, TGFβ1, α-SMA, and collagen I expression. Immunohistochemistry staining showed that the IGFBP7 protein was upregulated in both the early tumor group and advanced tumor group compared with the control group, with IGFBP7 expression markedly upregulated in the advanced tumor group compared with the early tumor group (Fig. 1A). A semi-quantitative evaluation of IGFBP7, as indicated by the integrated optical density (IOD), showed that the expression of IGFBP7 was gradually increased in the early tumor group and advanced tumor group relative to the control group (P<0.05; Fig. 1B), with the highest IOD observed in the advanced tumor group. As shown in Fig. 1C, the western blot analysis showed that IGFBP7, TGFβ1, α-SMA, and collagen I were significantly upregulated in both the early tumor group and advanced tumor group compared with the control group. In the advanced tumor group, the expression of IGFBP7, TGFβ1, α-SMA, and collagen I was markedly upregulated compared with that of the early tumor group (P<0.05). These results suggest that IGFBP7 is positively correlated with TGFβ1 and participates in the process of esophageal squamous cell carcinoma.

Effect of IGFBP7 on the expression of TGFβ1 and α-SMA in the EC109 cell line. After the EC109 cells were treated with AdIGFBP7, western blotting was performed to assess the expression of IGFBP7, TGFβ1, and α-SMA in each experimental group. As shown in Fig. 2A, the IGFBP7 protein level gradually increased from 24 to 72 h, thus demonstrating that AdIGFBP7 transfection was effective. The western blot analysis showed that the TGFβ1 and α-SMA proteins were upregulated at 24, 48, and 72 h in the treatment groups compared with the control group (P<0.05); moreover, the levels gradually increased in a time-dependent manner and peaked at 72 h. There were no changes in the normal and CAd groups (P<0.05; Fig. 2B and C). These results suggest that IGFBP7 upregulates the expression of TGFβ1 and α-SMA in esophageal squamous cell carcinoma.

Effect of IGFBP7 on the expression of p-SMAD2/3, SMAD2/3, and collagen I in the EC109 cell line. Following treatment with AdIGFBP7, the expression of p-SMAD2/3, SMAD2/3, and collagen I was examined via western blotting, and the results showed that the p-SMAD2/3 protein was upregulated at 24, 48, and 72 h and peaked at 72 h (P<0.05; Fig. 3A). No changes in SMAD2/3 protein expression were observed in the

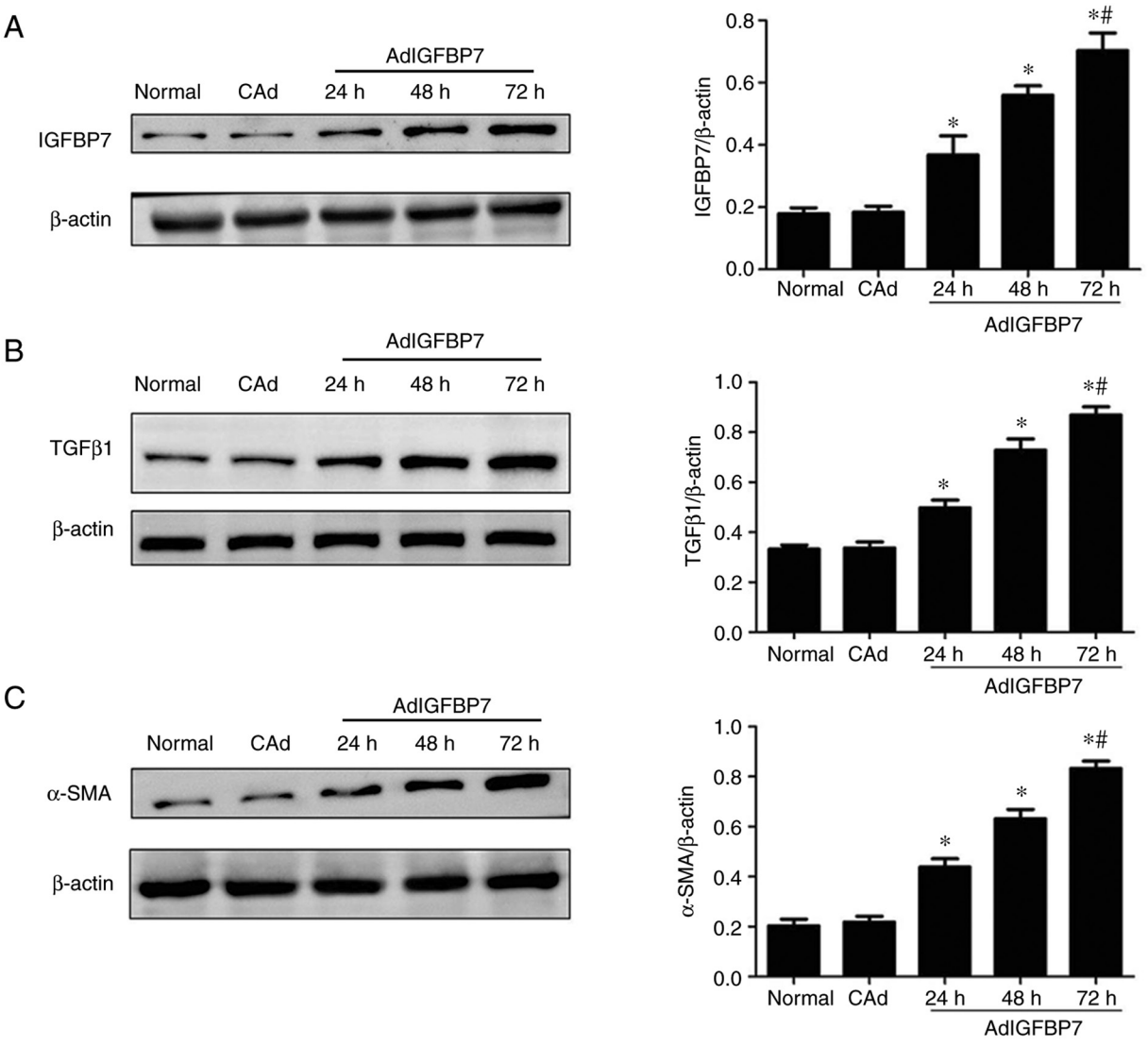

Figure 2. Effect of IGFBP7 on the expression of TGFβ1 and α-SMA in the EC109 cell line. The protein expression of IGFBP7 (A), TGFβ1 (B) and α-SMA (C) was examined by western blotting. β-actin served as an internal control (n=4). *P<0.05 vs. the control group; #P<0.05 vs. the 48-h group. IGFBP7, insulin-like growth factor-binding protein-7; TGFβ1, transforming growth factor-β1; α-SMA, α-smooth muscle actin.

experimental groups (Fig. 3A). The ratio of p-SMAD2/3 to SMAD2/3 was also significantly upregulated from 24 to 72 h in a time-dependent manner (P<0.05; Fig. 3B). As shown in Fig. 3C, collagen I expression gradually increased from 24 to 72 h, with a peak observed at 72 h (P<0.05). These results suggest that IGFBP7 may promote collagen I expression by activating the TGFβ1/SMAD signaling pathway in esophageal squamous cell carcinoma.

Inhibition of TGFβ1 expression reduces the expression of α-SMA, collagen I, and p-SMAD2/3 in IGFBP7-treated EC109 cell line. Following treatment of the EC109 cell line with LvshTGFβ1, the TGFβ1 protein level was gradually and significantly decreased from 24 to 72 h, which showed that LvshTGFβ1 transfection was effective (Fig. 4A). After the EC109 cell line was treated with both AdIGFBP7 and LvshTGFβ1, the western blotting analysis showed that the expression of α-SMA, collagen I, and p-SMAD2/3 and the ratio of p-SMAD2/3 to SMAD2/3 were gradually decreased from 24 to 72 h compared with that of the control group. There was no changes in the CAd plus LvshNC compared with the normal group. Changes in the expression of SMAD2/3 protein were not observed among the groups (Fig. 4B-D). These results suggest that TGFβ1 possibly plays an important role in IGFBP7-induced α-SMA and ECM production in esophageal squamous cell carcinoma.

Discussion

Esophageal carcinoma can be divided into two different histological subtypes, i.e., squamous cell carcinoma and adenocarcinoma (EAC), of which squamous cell carcinoma accounts (ESCC) for the majority of EC cases (3). Many factors are involved in the occurrence and development of ESCC. For example, insulin-like growth factor-binding protein-7 (IGFBP7) participates in the progression of many tumors (17), and previous research has demonstrated that the protein level of IGFBP7 is significantly increased in EAC (18). In this study, we observed that IGFBP7 was significantly upregulated in ESCC; thus, IGFBP7 is increased

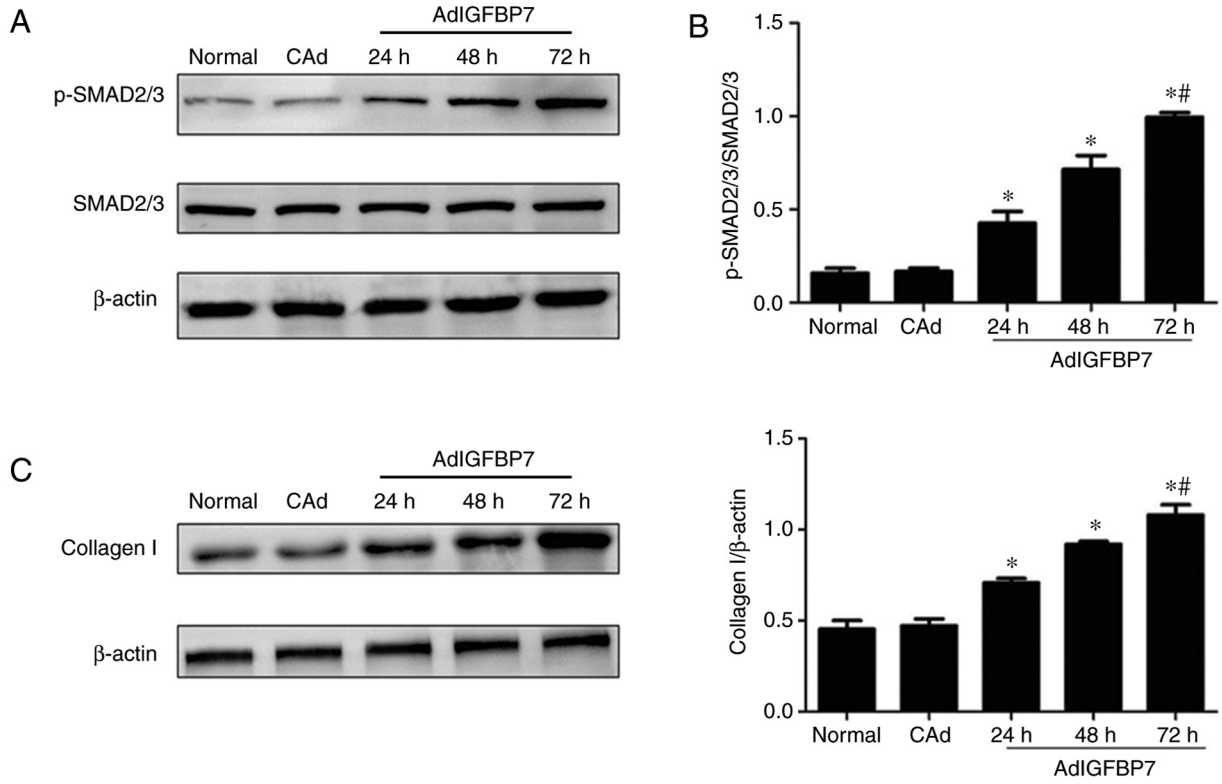

Figure 3. Effect of IGFBP7 on the expression of phosphorylated (p-)SMAD2/3, SMAD2/3, and collagen I in the EC109 cell line. (A) The protein expression of p-SMAD2/3 and SMAD2/3 was examined by western blotting. (B) p-SMAD2/3 and SMAD2/3 ratio. (C) Western blotting for collagen I. β-actin served as an internal control (n=4). *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the 48-h group. IGFBP7, insulin-like growth factor-binding protein-7.

not only in EAC but also in ESCC. Furthermore, we found that the expression of IGFBP7 was markedly increased in the advanced tumor group compared with the early tumor group. These data suggest that IGFBP7 possibly performs an important function in the process of esophageal squamous cell carcinoma.

Transforming growth factor-β1 (TGFβ1) is an important cytokine that regulates the tumor microenvironment (TME) (19) and affects the formation and development of tumors in many ways (20). A previous study showed that TGF-β-induced cell proliferation and apoptosis play important roles in the progression of ESCC (21). In addition, TGFβ1 expression in cancer-associated fibroblasts (CAFs) was significantly associated with the overall survival of patients with ESCC (22). In the present study, we found that the TGFβ1 protein was significantly upregulated in ESCC. α-smooth muscle actin (α-SMA) and collagen I represent the most important cell components of the TME, and they are upregulated in ESCC. In addition, TGFβ1, α-SMA, and collagen I were markedly upregulated in the advanced tumor group compared with the early tumor group. Furthermore, the changes in TGFβ1, α-SMA, and collagen I were consistent with those of IGFBP7. These data suggest that IGFBP7 is positively correlated with the expression of TGFβ1, α-SMA, and collagen I; thus, IGFBP7 may promote the development of esophageal cancer.

TGFβ1 expression was induced by IGFBP7 in the liver of rats *in vivo* (23) and hepatic stellate cells (HSCs) *in vitro* (24). However, whether IGFBP7 directly induces TGFβ1 expression in ESCC has not been clarified. Therefore, we transfected the EC109 cell line with AdIGFBP7, and subsequent changes in TGFβ1 expression were observed. Our experiments showed that the expression of TGFβ1 was gradually increased in a time-dependent manner, which indicates that IGFBP7 can upregulate TGFβ1 expression in other cell lines (as indicated above) as well as the EC109 cell line. Previous studies have demonstrated that the expression of α-SMA is induced by IGFBP7 in fibroblasts (25) and HSCs (26). In this study, we also found that the expression of α-SMA was gradually increased in the EC109 cell line treated with AdIGFBP7. Considering the above results, we suggest that IGFBP7 is likely to participate in ESCC through upregulating the expression of TGFβ1 and α-SMA, which may result in TME remodeling.

The TGFβ1/SMAD signaling pathway performs an important function in the regulation of ECM production. Previous studies revealed that IGFBP7 can activate and induce ECM production in HSCs via the TGFβ1/SMAD signaling pathway (27,28). Thus, we tested the effects of IGFBP7 on p-SMAD2/3 and collagen I in the EC109 cell line and found that the protein expression levels of p-SMAD2/3 and collagen I were significantly increased following treatment with AdIGFBP7, which was consistent with previous reports. Endogenous TGFβ1 in fibroblasts might be involved in the induction of α-SMA by IGFBP7. We observed that the expression of α-SMA was significantly decreased by LvshTGFβ1 in the AdIGFBP7-treated EC109 cell line, which suggests that α-SMA expression was induced by IGFBP7 in a TGFβ1-dependent manner. Our data also showed that the expression of p-SMAD2/3 and collagen I were significantly

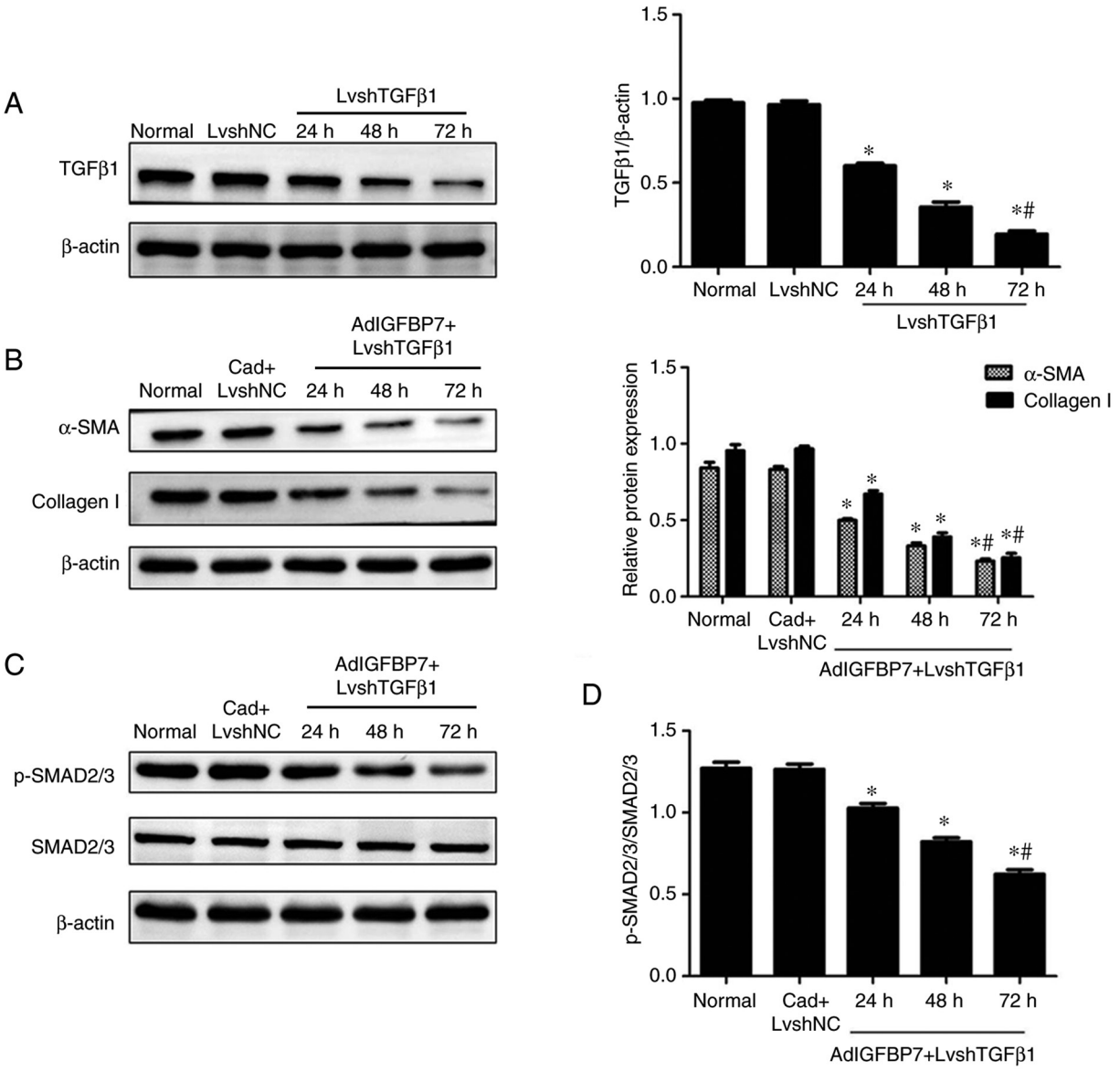

Figure 4. Effect of LvshTGFβ1 on the expression of α-SMA, p-SMAD2/3, SMAD2/3, and collagen I in AdIGFBP7-treated EC109 cell line. Western blotting for (A) TGFβ1 and (B) α-SMA and collagen I and (C) p-SMAD2/3 and SMAD2/3. (D) p-SMAD2/3 and SMAD2/3 ratio. β-actin served as an internal control (n=4). *P<0.05 vs. the control group; #P<0.05 vs. the 48-h group. TGFβ1, transforming growth factor-β1; α-SMA, α-smooth muscle actin.

decreased by LvshTGFβ1 in the AdIGFBP7-treated EC109 cell line. These results demonstrated that the inhibition of TGFβ1 expression possibly affected the IGFBP7-induced SMAD pathway and collagen I expression in the EC109 cell line. Taken together, our results indicate that IGFBP7 has an important effect on the TGFβ1/SMAD signaling pathway and induces α-SMA upregulation and ECM production, which may remodel the TME in ESCC.

In conclusion, the present study suggests that increased IGFBP7 may accelerate ESCC progression by promoting the expression of TGFβ1, α-SMA, and collagen I by activating the TGFβ1/SMAD signaling pathway, which could remodel the TME. However, the effects of IGFBP7 knockdown on TGFβ1/SMAD signaling and IGFBP7 on TME cell components in ESCC were not analyzed in this study, which will be used as a future research perspective. Further investigations are required to elucidate additional mechanisms underlying the effect of IGFBP7 on ESCC.

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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

XL designed the experiments and prepared the manuscript. JZ, YW, CM, DW, and LP performed the experiments. LC analyzed the data. All authors read and approved the final

manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved. XL and LC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study strictly conformed to the ethical rules of the Medical Ethics Committee of the Affiliated Lianyungang Oriental Hospital of Xuzhou Medical University (2017-006-01), and written informed consent was obtained from each patient (Jiangsu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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