

Original Article

FBXL20 promotes cell proliferation and metastasis through activating Wnt/ β -catenin signaling pathway in esophageal cancer

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Abstract: Previous studies have demonstrated that FBXL20 can promote tumor progression in human colorectal adenocarcinoma. Additionally, FBXL20, acts as a checkpoint of p53, controls autophagy and receptor degradation. These studies suggest that FBXL20 is involved in several cancer progression. However, the role of FBXL20 in esophageal cancer remains unknown. The present study demonstrated that the expression of FBXL20 in esophageal cancer tissue was significantly higher than its expression in adjacent tissues. In vitro studies, silencing FBXL20 expression by lentiviral vector shRNA induced G0/G1 cell cycle arrest, and inhibited the proliferation, migration and invasion ability of esophageal cancer cells through Wnt/ β -catenin signaling pathway. In addition, FBXL20 silencing could increase the apoptosis of esophageal cancer cells. In summary, this study demonstrated that FBXL20 was an oncogenic gene, which could promote proliferation, migration, invasion, and G0/G1 cell cycle arrest via activating Wnt/ β -catenin signaling pathway and induce apoptosis in esophageal cancer. Therefore, FBXL20 might serve as a potential tumor marker and therapeutic target for esophageal cancer.

Keywords: FBXL20, esophageal cancer, Wnt/ β -catenin, cell cycle arrest, proliferation, migration

Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth most common cause of cancer related deaths [1, 2], and it affects more than 450000 people worldwide with a rapidly increasing incidence [3]. The five year overall survival rates range from 15% to 25% [4]. The mortality rates are highest for both sexes in Eastern and Southern Africa, and Eastern Asia [1]. There are a number of factors which lead to the occurrence of esophageal cancer, including aberrant gene expression and uncontrolled proteolysis. However, the progression of esophageal cancer remains largely unknown and novel biomarkers need to be identified.

FBXL20, a novel member of the F-box protein family, is characterized by an approximately 40-amino acid F-box motif. F-box proteins interact with SKP1 through the F box, and they interact with ubiquitination targets through other

protein interaction domains [5]. SCF complexes, formed by SKP1, cullin and F-box proteins and acting as protein-ubiquitin ligases, play an integral part in of cell proliferation and aberrant cell differentiation. Additionally, SCF complexes deregulation contributes to tumorigenesis [6]. Some F-box proteins have been identified, including SKP2 [7] and FBXW7 [8], which have a variety of structures and functions [9]. FBXL20 acts as a checkpoint of p53, which could control autophagy and receptor degradation by ubiquitination and proteasomal degradation of Vps34 [10]. Recent studies have identified FBXL20 as a proto-oncogenes, and the mechanism on FBXL20-associated oncogenesis has also been extremely explored. Inhibition of FBXL20 results in suppressed proliferation and promoted apoptosis in colorectal carcinoma cells [11]. Moreover, FBXL20 could increase the colorectal adenocarcinoma cells invasion ability by regulation of ubiquitin mediated degradation of E-cadherin [12]. However, the molecular

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and biological functions of FBXL20-associated oncogenesis in esophageal cancer has remained unknown.

In this study, we found that FBXL20 was highly expressed in esophageal cancer tissues and cell lines, and abnormal expression of FBXL20 promoted proliferation, migration and invasion in esophageal cancer. Our results suggests that FBXL20 is a one of the proto-oncogenes, and it could serve as a potential biomarker and treatment target for esophageal cancer.

Materials and methods

Cell lines and tumor samples

Five esophageal cancer cell lines (EC109, EC9706, KYSE150, KYSE510, and TE-10) were studied. These cells were purchased from Chongqing Key Laboratory of Molecular Oncology and Epigenetics (Chongqing, China). Cell lines were grown in RPMI-1640 medium (Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS; PAN Biotech, Aidenbach, Germany) and 100 µg/ml of penicillin and streptomycin, maintained at 37°C in a humidified atmosphere with 5% CO₂. RNA samples were extracted from esophageal tumor tissues and paired surgical-margin tissues. Esophageal cancer tissues and cancer adjacent tissues were obtained from patients who received esophageal cancer surgery. All tissues were obtained from patients who agreed to surgery for the treatment of esophageal cancer at the Department of Surgery, The First Affiliated Hospital of Chongqing Medical University. This study was approved by the Institutional Review Board of Chongqing Medical University.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cell lines and esophageal cancer tissues using TRIzol reagent (Takara, Otsu, Japan) and then reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) according to the manufacturer's protocols. Quantitative RT-PCR (qRT-PCR) was performed by using SYBR Premix Ex Taq™ II (Takara), according to the manufacturer's protocols. Thermal-cycling reaction was performed in the 7500 Real-Time PCR System (Applied Biosystems) and β-actin was used as the endogenous control. Relative expression

levels of FBXL20 in esophageal cancer tissues and cell lines were normalized to the β-actin levels. The primer sequences used were as follows: FBXL20 (Forward: 5'-TAGCCAGGTGAAGC-ATTGAG-3', Reverse: 5'-AGAGGGTGCTTCTTCTTGGA-3'); β-actin (Forward: 5'-CTCCTCCTGAGCGCAAGTACTC, Reverse: 5'-TCCTGCTTGCTGATCCACATC-3').

Immunohistochemical staining

To evaluate FBXL20 expression in esophageal cancer tissues and cancer adjacent tissues, immunohistochemistry was performed. Briefly, sections were dewaxed by soaking in xylene for 10 mins, four times and then the concentration of ethanol was stepwise decreased from 100 to 75% for 5 mins at every concentration. Sections were hydrated and underwent sodium citrate antigen retrieval after deparaffinization. Sections were then incubated with 3% H₂O₂ to block endogenous peroxidase activity. Sections were incubated with primary anti-FBXL20 antibody (1:50 dilution) (Bioss, Beijing, China) overnight at 4°C after blocked with the 5% goat serum. Sections were then incubated in secondary antibody at 37°C for 30 mins. Staining was performed using 3'-Diaminobenzidine (DAB) and hematoxylin. The immunohistochemical staining results were assigned a score based on the depth of staining and the rate of dye uptake by the stained cells. The intensity of staining is reported as: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of positively stained cells was determined as: <5%, 0; 5-25%, 1; >25-50%, 2; >50-75%, 3; and >75%, 4. Nuclear and cytoplasmic staining was included in the statistical analyses. The index of staining was determined by multiplying the score for staining intensity by the score for positivity rate. A score of <4 was considered negative (-), and a score ≥4 was considered positive (+).

Construction and transduction with the lentiviral vector shRNA

As we know the FBXL20 gene sequence, (Gene ID: 84961), we appointed Shanghai Genechem Co. Ltd. to provide lentiviral vectors containing an FBXL20-shRNA. Using a lentiviral vector we were able to stably transduce the FBXL20-shRNA with low cytotoxicity and high transduction efficiency. The sequences used for packaging into the lentivirus vector are as follows:

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Viral, shRNA-LV-FBXL20-RNAi: 5'-ACTCCTGT-TACGGATATTT-3'; Control-VECTOR: 5'-TTCTCC-GAACGTGTCACGT-3'. The target gene and empty lentiviral vector were transduced into esophageal cancer cell lines. After transduction for 8-12 h the cell medium was replaced. The cell fluorescence was observed after transduction for 48-72 h. After stably transduced cells were selected using puromycin, the cells were divided into two groups: LV-FBXL20-RNAi (transduced with shRNA for knockdown of the FBXL20 gene), Vector (transfection with empty viral vectors).

Cell-proliferation assay

CCK-8 assay was used for examining cell proliferation. EC109 and KYSE150 cells were seeded onto 96-well plates at a density of 2×10^3 cells/well. Proliferation assays of the cell lines were recorded at 6, 24, 48 and 72 h using the Cell Counting Kit-8 (CCK-8) (Santa Cruz, CA, USA) according to the company's protocols. Spectrometric absorbance was measured using a microplate reader (Infinite 200 PRO; Tecan) set at 450 nm. Each experiment was replicated three times.

Plate colony-formation assay

Esophageal cancer cells were seeded onto six-well plates at a density of 500 cells/well and maintained at 37°C with 5% CO₂. The medium was replaced every 3-4 days. The colonies were fixed with 4% paraformaldehyde for 30 mins and stained with crystal violet for 30 mins after two weeks. Surviving colonies were counted after staining.

Flow cytometric analysis of cell cycle and apoptosis

Both groups of cells were collected and centrifuged at 93× g/min for 5 mins. The cells were then washed with PBS twice and fixed in ice-cold 70% ethanol overnight at 4°C. Cells were mixed in PBS for apoptosis detection. Cell cycle and apoptosis were assayed at the Chongqing Medical University Life Sciences Institute.

In vitro migration and invasion assay

Migration: two groups of tumor cells (1×10^5 cells) were suspended in RPMI-1640 without serum and added to the upper cell culture inserts with 8- μ m aperture filters (Corning).

RPMI-1640 with fetal bovine serum was added to the lower compartments. Tumor cells migrated from the serum-free media to the high concentration-serum media. Cells that migrate to the lower surface were fixed in paraformaldehyde and stained with hematoxylin after incubation for 24 h.

Invasion: two groups of tumor cells (8×10^4 cells) were seeded into a chamber that was coated with matrigel (Corning). RPMI-1640 with fetal bovine serum was added to the lower compartments. After incubation for 48 h, cells that migrate to the lower surface were fixed in paraformaldehyde and stained with hematoxylin. Both migration and invasion of the cells that migrate to the lower surface were counted under a light microscope in five random and independent visual fields.

Western blot

Cells were washed twice with phosphate buffered saline (PBS). Cells were harvested and lysed on ice in a radio immunoprecipitation assay (RIPA) buffer with 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime Biotechnology, Shanghai, China). The proteins were quantified using the bicinchoninic acid (BCA) assay method. A total of 40 μ g protein lysate was separated by sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skimmed milk powder for 2 h. The primary antibodies used were: FBXL20 (Santa Cruz), β -catenin (Cell Signaling Technology, Danvers, MA, USA), c-myc (Abcam, Cambridge, UK), cyclin D1 (Abcam), MMP-7 (Cell Signaling Technology), E-cadherin (Cell Signaling Technology), cleaved caspase 3 (Cell Signaling Technology), and β -actin (Cell Signaling Technology) used as a control. The membranes were incubated with primary antibodies at 4°C overnight, and then washed with Tris buffered saline and Tween-20 (TBST) three times, and incubated in their respective secondary antibodies for 2 h. Bands were visualized using BeyoECL Plus (Beyotime Biotechnology).

Statistical analysis

The results presented in the current study are expressed as the average \pm standard deviation

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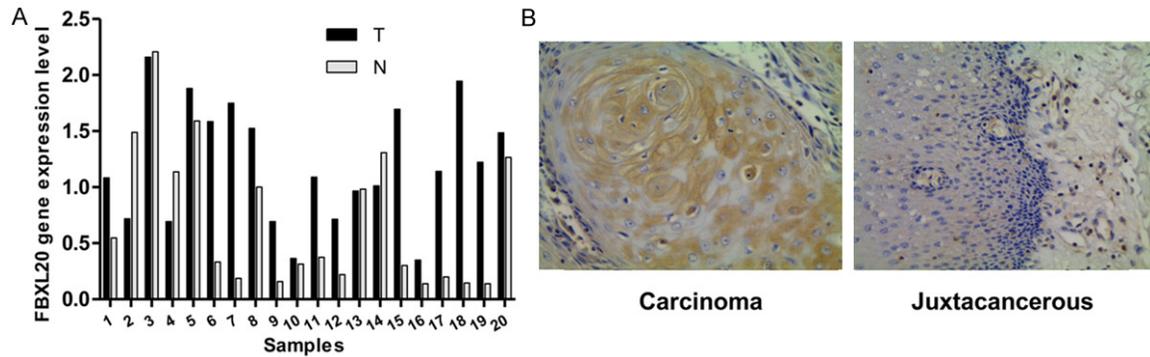


Figure 1. FBXL20 is overexpressed in esophageal cancer tissues. A. qRT-PCR analysis of FBXL20 expression in esophageal cancer tissues (T) and matched cancer adjacent tissues (N). 15 of the esophageal cancer tissues had a higher level of FBXL20 than that in the adjacent cancer tissues. T-test was used for statistical analysis ($P < 0.05$). B. Immunohistochemical staining of FBXL20 in esophageal cancer tissues and cancer adjacent tissues. FBXL20 was upregulated in most cancer tissues compared with that adjacent tissues (photomicrographs are shown at a magnification of $\times 400$). A chi-square test was used for statistics ($P < 0.05$).

Table 1. A chi-square test was used for statistics the FBXL20 expression in esophageal cancer tissues and cancer adjacent tissues ($P < 0.05$)

Type	Expression		p-value
	+	-	
Carcinoma	11	4	0.039
Juxtacancerous	5	10	

Note: Statistical analysis: Chi-square; -: negative (score of 0-4); +: positive (score of 5-12). $P < 0.05$.

(SD). Statistical analysis was performed using the Student's t-test and the Fisher's exact probability test. Student's t-test was used to compare means between two groups. The Chi-square test was used for two constituent ratios in two different groups with SPSS17.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered a statistically significant difference. All graphs were created using GraphPad Prism (GraphPad, La Jolla, CA, USA).

Results

FBXL20 is highly expressed in esophageal cancer tissue and cell lines

Using qRT-PCR, we found that the expression level of FBXL20 in esophageal cancer tissue was significantly upregulated in 75% of the tumor samples (15 out of 20), compared with paired surgical-margin tissues (Figure 1A). In addition, immunohistochemical staining of 15 sample pairs revealed that eleven were positive

in the esophageal cancer group and four samples were positive in the control group (Figure 1B, Table 1). The FBXL20 expression in five esophageal cancer cell lines was also explored by using qRT-PCR, and the results showed that the expression of FBXL20 was relatively high in EC109, KYSE510, and KYSE150 cells (Figure 2A). The above results indicate that the up-regulation of FBXL20 might be associated with the progression of esophageal cancer.

Transduction verification

The shRNA lentivirus and vector were transduced into EC109 and KYSE150 cells. After stable transduction, FBXL20 mRNA expression levels were measured by qRT-PCR (Figure 2B) and the protein expression was measured by western blot (Figure 4C). The results show that both mRNA and protein expression of FBXL20 significantly decrease in LV-FBXL20-RNAi group, while compared with the vector group.

FBXL20 promotes colony formation and proliferation

To investigate the effect of FBXL20 on esophageal cancer cell growth, CCK-8 assays and colony formation were used. The cell proliferation rate in the LV-FBXL20-RNAi group was significantly slower than that in the vector control group when analyzed with a CCK-8 assay (Figure 2C). In addition, Knockdown of FBXL20 reduced colony formation ability in both EC109 and KYSE150 cells (Figure 2D). These results

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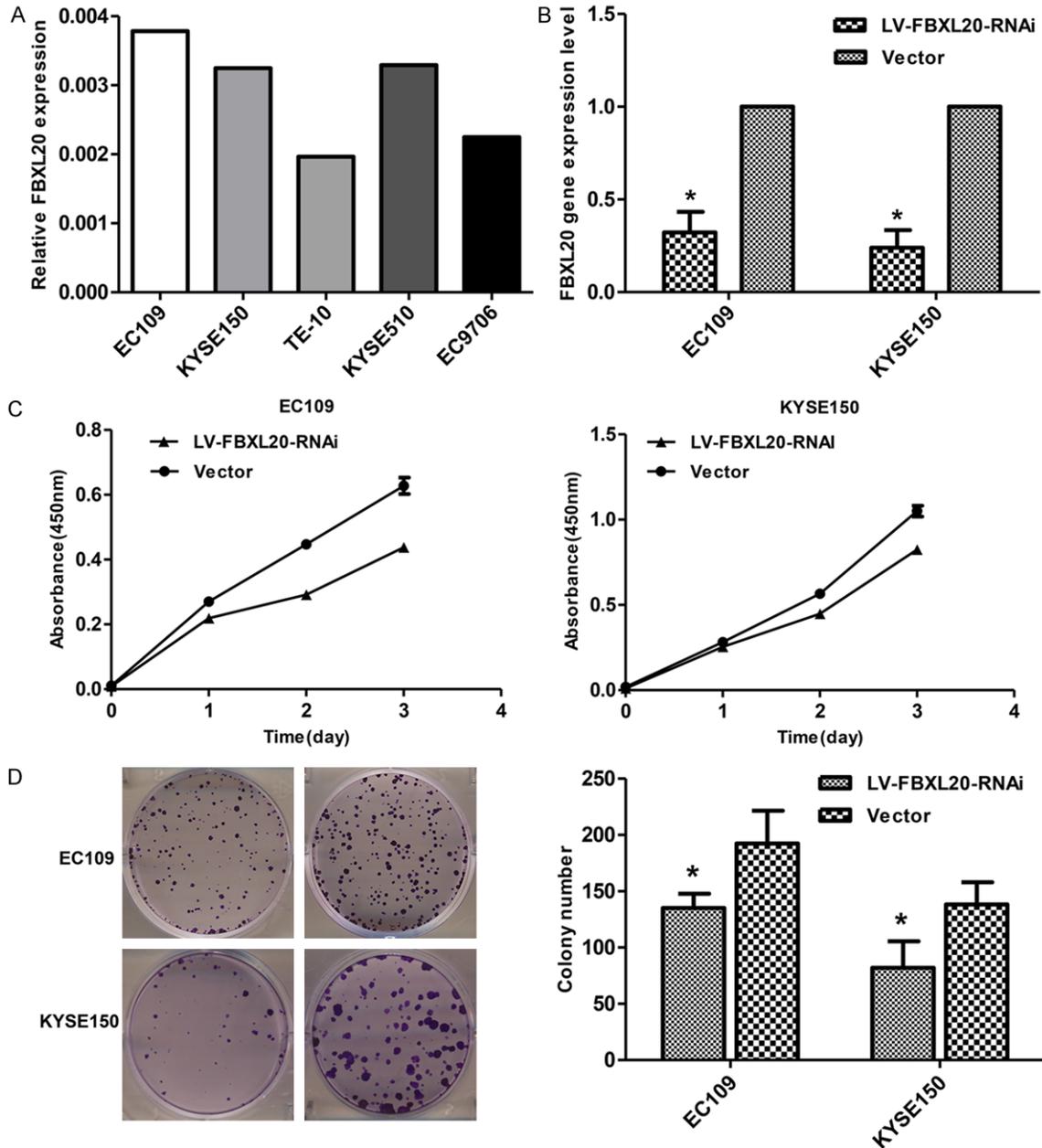


Figure 2. Down-regulation of FBXL20 decreases esophageal cancer cell proliferation and colony formation. A. Expression of FBXL20 in 5 esophageal cancer cell lines. We chose the two relatively high expressing cell lines EC109 and KYSE150. B. FBXL20 is downregulated in EC109 and KYSE150 cells. The mRNA expression of FBXL20 was detected by qRT-PCR in the EC109 and KYSE150 cells ($P < 0.05$). C, D. The results of CCK-8 cell proliferation and colony formation assays showed that the proliferation of EC109 and KYSE150 cells was significantly decreased after the inhibition of FBXL20 ($P < 0.05$).

suggest that FBXL20 promotes esophageal cancer cell proliferation.

Knockdown of FBXL20 induces G0/G1 cell cycle arrest and apoptosis

We further determined the effects of FBXL20 on esophageal cancer cell cycle and apoptosis

using flow cytometry. Cell cycle analysis showed that significantly increased numbers EC109 cells was found in G0/G1 phase in the LV-FBXL20-RNAi group (70.6%), compared to the vector control group (61.3%) (Figure 3A). The numbers of KYSE150 cells in G0/G1 phase (47.1%) were significantly augmented in the

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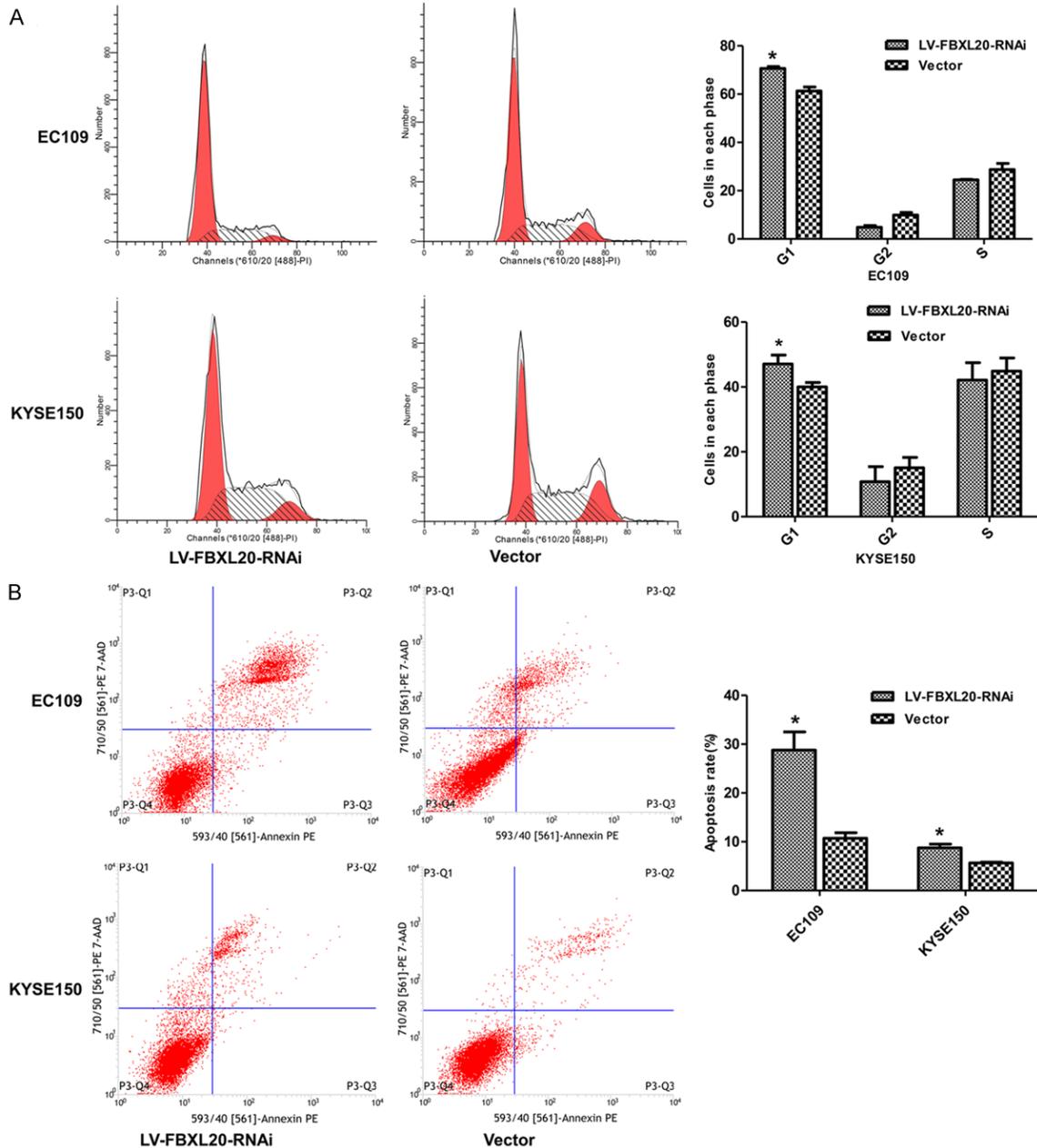


Figure 3. Influence of FBXL20 on the migration and invasion in the EC109 and KYSE150 cells. A, B. The results of transwell migration and invasion assays indicated that the inhibition of FBXL20 could significantly reduce the metastasis of esophageal cancer cells ($P < 0.05$) (photomicrographs are shown at a magnification of $\times 100$).

LV-FBXL20-RNAi group, by comparison of the vector control group (40%) (Figure 3A). Flow cytometric analysis showed apoptosis in the LV-FBXL20-RNAi group (28.8%) was significantly increased when compare to the vector group (10.7%) in EC109 cells (Figure 3B), and apoptosis in LV-FBXL20-RNAi group (8.7%) was also significantly increased when compare to the vector group (5.6%) in KYSE150 cells (Figure 3B). These results indicate that knockdown of

FBXL20 could induce G0/G1 cell cycle arrest and apoptosis in esophageal cancer cells.

FBXL20 promotes metastasis and invasion

In order to validate the effects of FBXL20 on migration and invasion ability in esophageal cancer cell, the transwell assay was performed. The down-regulation of FBXL20 significantly impaired migration and invasion of both EC109

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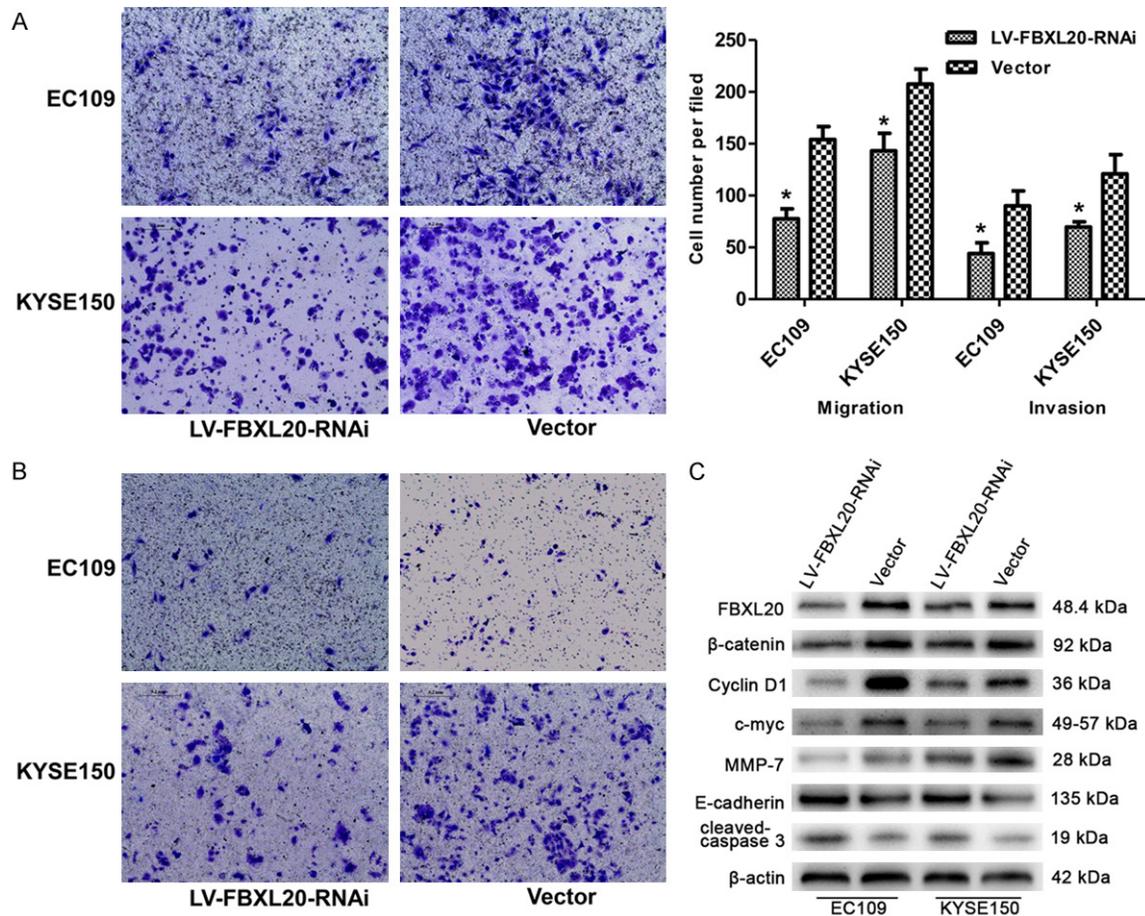


Figure 4. Effect of FBXL20 on the cell cycle distribution and apoptosis and the mechanism underlying FBXL20 regulation of esophageal cancer cell proliferation and metastasis in two types of esophageal cancer cell lines (EC109 and KYSE150). **A.** The cell cycle was analyzed using flow cytometry in EC109 and KYSE150 cells. The LV-FBXL20-RNAi group had a significantly higher proportion of cells arrested in the G₀/G₁ phase ($P < 0.05$). **B.** Flow cytometry demonstrated that the down-regulation of FBXL20 could promote the apoptosis of EC109 and KYSE150 cells ($P < 0.05$). **C.** Western blot assay verified the efficiency of target gene FBXL20. Wnt/ β -catenin and apoptosis related proteins were detected by western blot with β -actin as a control.

and KYSE150 cells (**Figure 4A** and **4B**). These results show that FBXL20 plays as an important promoter in the migration and invasion of esophageal cancer cells.

FBXL20 enhances the activation of caspase 3

To investigate the cleaved effector in the apoptotic pathway, the activation of caspase 3 was measured by western blot. We found that cleaved caspase 3 in the LV-FBXL20-RNAi group was significantly upregulated when compared to the vector control group (**Figure 4C**).

FBXL20 activates Wnt/ β -catenin signaling pathway

To investigate the potential mechanism of FBXL20 in promoting esophageal cancer cell

cycle and metastasis, the relationship between FBXL20 and the Wnt/ β -catenin signaling pathway was explored. The protein expression of β -catenin was significantly downregulated in LV-FBXL20-RNAi group when compared to vector control groups. In both EC109 and KYSE150 cells, the downstream target genes of β -catenin, including cyclin D1, c-myc, and MMP-7, were all downregulated in the LV-FBXL20-RNAi group while compared to the vector control (**Figure 4C**). Meanwhile, the expression of E-cadherin is upregulated in LV-FBXL20-RNAi group.

Discussion

The present study reports, for the first time, the association between FBXL20 expression and esophageal cancer progression. We first detect-

ed the expression level of FBXL20 in 20 matched clinical fresh esophageal cancer tissues and five esophageal cancer cell lines using qRT-PCR, and 15 paired samples of clinical paraffin-embedded esophageal cancer tissues using immunohistochemical staining. We found that the mRNA and protein expression level of FBXL20 was upregulated in esophageal cancer tissues, compared with the adjacent cancer tissues. This result is consistent with the expression levels of FBXL20 in other tumors tissues [11]. Meanwhile, we found that FBXL20 was highly expressed in the five esophageal cancer cell lines. These results support the hypothesis that FBXL20 is an oncogene in human esophageal cancer.

Recent evidence suggests that several F-box proteins play an important role in cancer occurrence and progression. Overexpression of SKP2 was observed in many malignant cancers and was commonly associated with downregulation of p27^{Kip1} levels and loss of tumor differentiation [7]. Abnormal expression of FBXW7 increased the proliferation of colorectal cancer cell, and FBXW7 regulates multiple cellular process, including cell growth and differentiation through regulation of transforming growth factor- β (TGF β) signaling [13, 14]. FBX8 inhibits invasion and metastatic ability of gastric cancer [15]. To assess whether FBXL20 has a similar function in esophageal cancer cell lines, a series of function assays were performed. In the present study, the flow cytometry showed the numbers of cells in G0/G1 phase in LV-FBXL20-RNAi group had significantly increased compared to the vector control group, suggesting that down-regulated of FBXL20 induced G0/G1 cell cycle arrest. The CCK-8 and colony formation results demonstrated that FBXL20 could promote the proliferation rate of esophageal cancer cell. Previous study demonstrated that the Wnt/ β -catenin signaling pathway plays an important role in the growth process of esophageal cancer [16], and a variety of other malignancies, such as breast cancer [17], colorectal cancer [18], and osteosarcoma [19]. The present study shows that the expression of β -catenin in the LV-FBXL20-RNAi group was significantly lower than that in the control vector group. Meanwhile, we found cyclin D1 one of the target genes downstream of the Wnt/ β -catenin signaling pathway was significantly downregulated through the knockdown of

FBXL20. This is of note, as Cyclin D1 is an important regulatory protein for the G1-S cell cycle phase transition [20]. In addition, c-myc, which was a downstream target gene of the Wnt/ β -catenin signaling pathway and correlated with the proliferation capacity of cancer [21] and a key regulator of the cell cycle [22], was also downregulated by the knockdown of FBXL20. These results suggest that the suppression of the cyclin D1 and c-myc in esophageal cancer cells may result from β -catenin downregulation through knock down FBXL20. Our results are in line with previous studies of FBXL20 in other cancer cell lines and tissues [11], indicating that FBXL20 promotes esophageal cancer cell growth and proliferation through the Wnt/ β -catenin pathway.

In the present study, we also found that the migration and invasion ability of the LV-FBXL20-RNAi group was significantly reduced compared to the vector control group. Cancer metastasis and invasion are associated with multiple factors, which involve multistep processes [23]. Previous study demonstrated that E-cadherin/ β -catenin complex was important to epithelial cell-cell contact [24], which is involved in the metastatic and invasion process. Meanwhile, improved metastatic and invasion ability has been associated with the abnormal expression of E-cadherin in breast cancers [25] and esophageal cancer [26]. In the present study, these results show that the expression of E-cadherin was significantly upregulated in the LV-FBXL20-RNAi group compared to the vector group. These results are in line with previous studies of FBXL20 in other cancer cell lines and tissues [12]. Matrix metalloproteinases (MMPs) play an important role in tumor metastasis and invasion [27]. MMP-7, a member of MMPs family, has been shown to be significant correlated with β -catenin activation in tumor invasion [28], and it may exert invasion function in early stage esophageal cancer [29]. Consistently, we also found that the MMP-7 was significantly downregulated in the LV-FBXL20-RNAi group. From the above findings, we could conclude that decreased migration and invasion ability, induced by downregulation of FBXL20, was related to the upregulation of E-cadherin and downregulation of MMP-7 in esophageal cancer cells. Meanwhile, expression of β -catenin in the LV-FBXL20-RNAi group. Therefore, these results suggest that the upregulation of the

E-cadherin and the downregulation of MMP-7 in esophageal cancer cells may have resulted from β -catenin downregulation. Moreover, we also found that FBXL20 also participated in apoptosis in esophageal cancer cells through cleaved caspase 3.

Our results suggest that FBXL20 promotes progression of esophageal cancer through activation of the Wnt/ β -catenin signaling pathway. Moreover, the abnormal expression of FBXL20 can affect various esophageal cancer cell functions, including the cell cycle, apoptosis, proliferation, metastasis and invasion. However, this valuable mechanism requires further investigation.

In summary, our study reveals that FBXL20 is an oncogenic gene in esophageal cancer, indicating that FBXL20 might serve as a potential tumor marker and treatment target for esophageal cancer.

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Disclosure of conflict of interest

None.

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