EGFL6 Modulates WNT Signaling to Drive Esophageal Cancer Metastasis and Proliferation

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Research

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Abstract

Background

Esophageal cancer (EC) is the sixth deadliest cancer in the world. There has been no breakthrough in the research on EC in the past few decades. Epidermal growth factor-like protein 6 (EGFL6), as a member of the epidermal growth factor superfamily, plays an important role in the occurrence and development of some tumors. However, the role of EGFL6 in the EC has never explored.

Methods

Immunohistochemical staining was used to evaluate the expression level of EGFL6 protein in human EC and its adjacent non-tumor tissues, and analyzed the correlation between the expression level of EGFL6 protein and clinical pathological indexes and survival rate. In vitro, by constructing EGFL6 silence and overexpressed EC cells, used CCK-8, clone formation, wound healing assays, transwell experiment and flow cytometry to explore the effects of EGFL6 on the proliferation, invasion, migration and apoptosis of EC. By using real-time PCR or western blot to detect the related marker genes of epithelial-mesenchymal transformation (EMT), tumor stem cells (TSCs) and Wnt/ β -catenin. In vivo, established a nude mouse EC transplantation tumor model.

Results

The results showed that the expression level of EGFL6 in EC is significantly higher than that in adjacent non-tumor tissues, and is related to poor prognosis of patients. In vitro, CCK-8, clone formation, wound healing assays, transwell experiment and flow cytometry results show that EGFL6 overexpression can promotes proliferation, invasion and migration of EC cells and inhibits apoptosis. EGFL6 silencing inhibits proliferation, invasion and migration of EC cells and promotes apoptosis. Real-time PCR and Western-blot detection of EMT-related markers found that EGFL6 can induce EC cells EMT. Real-time PCR detection of esophageal cancer stem cell-related genes showed that EGFL6 may maintain the expression of esophageal cancer stem cell-like cell population. Western-blot detection of Wnt/ β -catenin signaling marker genes showed that EGFL6 participated in the expression of Wnt/ β -catenin signaling pathway. In vivo experiments found that knockout of EGFL6 could inhibit the formation of subcutaneous tumors in nude mice.

Conclusion

Taken together, our study identified a novel role and mechanism of EGFL6 in EC and provided epigenetic therapeutic strategies for the treatment of EC.

Background:

Esophageal cancer (EC) is a common digestive system tumor that occurs in esophageal squamous epithelium or glandular epithelium. Its global incidence rate ranks 9th among all malignant tumors and its mortality rate ranks 6th [1]. China has a particularly high incidence of EC; squamous cell carcinoma is the main pathological type. Shanxi, Henan, and Hebei provinces are "high-incidence bands for EC." According to a report on the global trend of EC in 2017, half of the patients with EC in the world are Chinese [2]. However, because early symptoms are not obvious, many patients have entered the local advanced stage at the time of starting treatment. At this stage, the treatment effect of surgical resection alone is poor. Therefore, efforts to characterize the molecular mechanism and develop targeted treatment of EC have become a current research hotspot.

EGFL6 (Epithelial growth factor like domain, multiple 6), also known as MAEG and W80, is a secretory protein belonging to the EGF superfamily. It was first discovered using high-throughput screening of DNA molecular hybridization [3]. EGFL6 is involved in the regulation of the cell cycle, cell proliferation, and development [4]. Further, EGFL6 participates in a series of other physiological processes including regulating cell adhesion, proliferation and migration of vascular endothelial cells, and mediating angiogenesis [5–7]. EGFL6 expression can also regulate cell movement, reduce contact inhibition and make cell anchoring independent; these processes can enhance the invasion and metastasis of tumor cells [8]. The biological functions of EGFL6 suggest that EGFL6 plays an important role in the occurrence and development of cancer, and may become a new target for cancer diagnosis and treatment. Although EGFL6 plays an important role in cancer development, the functional role of EGFL6 in EC has not been previously studied.

The purpose of this study is to determine the expression of EGFL6 in EC, the role of EGFL6 in the development of EC, and the effect of EGFL6 on the migration, invasion, proliferation, apoptosis and Epithelial-mesenchymal transitions (EMT) of EC cells. In addition, this study elucidates elements of the regulatory mechanism of stem cells and EMT, focusing on Wnt / β - Catenin pathway. The results of this study indicate the potential for EGFL6 as a drug target for EC treatment and prevention of metastasis.

Materials And Methods:

2.1 Patients and clinical tissue specimens

Clinical tissue specimens consisted of 120 primary EC tumor tissues with corresponding adjacent non-tumor tissues (ANT) samples and clinical data collected from the First Hospital of Shanxi Medical University from 2017 to 2019. EC patients receiving preoperative radiotherapy, chemotherapy, and other anti-tumor treatments were excluded from our study. All clinical experiments were performed with the consent of patients and obtained the approval from the Ethics Committee of the First Hospital of Shanxi Medical University. The clinicopathological features assessed in this study included age, sex, histological type, differentiation, lymph node metastasis, metastases (TNM) stage, and tumor size. At the same time,

combined with clinical and pathological data, we used The Cancer Genome Atlas (TCGA) Websites (<https://tcga-data.nci.nih.gov/tcga/>) to further analyze the expression of EGFL6 in EC and normal tissues, and to further carry out survival analysis.

2.2 Immunohistochemistry And Scoring

For immunohistochemistry, 4 μm sections were obtained from paraffin-embedded samples, baked, treated with xylene and gradient alcohol dewaxing, and treated with 0.6% H_2O_2 to eliminate endogenous peroxidase activity. Then, the slides were immersed in boiling 10 mmol/l sodium citrate (pH 6.5) in a heated pressure cooker for the antigen retrieval step. Next, slides were blocked with 5% normal goat serum in 0.01M PBS for 30 min at 37 °C. Then, they were incubated overnight at 4 °C in a humidified chamber with primary antibodies, anti-EGFL6 antibodies produced in rabbit (Cat.HPA001838, Sigma), diluted at 1:100 dilutions in blocking solution. After washing with phosphate buffered saline (PBS), the samples were incubated with a secondary antibody (Cat.SA1052, Boster, Beijing, China) and incubated at 37°C for 20 minutes. Then samples were washed with PBS again, developed with DAB, re-dyed by hematoxylin, and treated with gradient alcohol, xylene dehydration, neutral resin sealing. Finally, Two pathologists independently evaluated the staining and excluded false-positive results, and we used the Scanscope digital pathological scanning system (Aperio, USA) to analyze and calculate the OD value ($\text{OD} = \text{LogA/B}$, $A = 240$, $B = \text{Gray value}$).

2.3 Cell Culture And Transfection

The normal esophageal epithelial Het-1A cell line and three esophageal cancer cell lines (EC9706, KYSE150 and KYSE450) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in RPMI 1640 medium (Gibco, USA) or DMEM high glucose (Gibco, USA) containing penicillin, streptomycin (Solarbio, China), and 10% fetal bovine serum (FBS) (Gibco, USA). Cell cultures were kept in an incubator maintained at 37°C in a humidified atmosphere with 5% CO_2 . When the cells reached the logarithmic growth phase, two EC cell lines were constructed, one with EGFL6 gene silenced and one with EGFL6 overexpressed. EGFL6 gene expression was silenced in KYSE450 cells and overexpressed in KYSE150 cells. To silence the EGFL6 gene, small interference RNAs (siRNA) of EGFL6 were labeled with siRNA-1 and siRNA-2 (Genepharma, Shanghai, China) and used to transfect KYSE450 cells with Lipofectamine®3000 Reagent (Invitrogen, Carlsbad, CA, USA) transfection reagent. An siNC transfection group was used as the negative control group for EGFL6 silencing. KYSE150 cells were transfected with PcDNA3.1-EGFL6 plasmid (Genepharma, Shanghai, China) using Lipofectamine®3000 Reagent for overexpression of EGFL6. Transfection with PcDNA3.1 was performed to create a negative control for EGFL6 overexpression. RNA was collected 48 hours after transfection. Proteins were collected and assessed 72 hours after transfection. To confirm the transfection efficiency.

Finally, in EGFL6 gene silenced cells, we screened out the one with the highest interference efficiency from siRNA1 and siRNA2 through Real-time PCR and Western-blot experimental results, and the following experiments were mainly carried out by using the sequence with the highest interference efficiency (labeled siRNA). The follow-up experiments were divided into five groups: Blank group, without any treatment; siNC, the silencing control group; siRNA, the EGFL6 gene silencing group; PcDNA3.1 + EGFL6, the EGFL6 overexpression group; and PcDNA3.1 empty, the overexpression control group.

The sequence design of EGFL6 gene silencing is as follows:

siRNA1: sense 5'-GGAAGCUACUACUGCAAUUTT-3'

and antisense 5'-AUUUGCAGUAGUAGCUUCCTT-3';

siRNA2: sense 5'-GCAGGGAUAUAAAGGCAAUUTT-3'

and antisense 5'-AUUGCCUUUAUAUCCCUGCTT-3';

control siNC: sense 5'-UUCUCCGAACGUGUCACGUTT-3'

and antisense 5'-ACGUGACACGUUCGGAGAATT-3'.

2.4 Cell Proliferation Assay

The assay of cell proliferation was performed using Cell Counting Kit-8 (CCK-8; Beyotime Biotechnology, Haimen, China). Briefly, EC cells were seeded into 96-well plates at a density of 5×10^3 cells per well and cultured overnight. KYSE150 and KYSE450 cells were transfected according to the above grouping and cultured for 12, 24, 36, 48, 60, and 72 hours. CCK8 reagent was added to each well at designated timepoints following manufacturer's instructions. Cells were then cultured for 2 h at 37 °C. Optical density values were read using a 450 nm wavelength microplate reader (Bio-Tek Company, Winooski, VT, USA). Each sample was subjected to three multiple wells, and each experiment was repeated three times.

2.5 Migration And Invasion Assay

Cells were transfected in groups as above and incubated for 48 hours. Then 50000 cells were trypsinized then migration was measured. Briefly, cells were seeded in serum-free medium in transwell plates (24 wells, 8 µm pores,) (Corning, USA) with complete medium in the lower chamber. After 24 hours of incubation at 37 °C, cell migration was measured. Similarly, for the invasion experiment, cells were seeded in transwells with precoated matrixgel (BD Biosciences, USA). After 24 hours of culture, cells on the upper side of the membrane were removed with the cotton swabs and fixed in 4% paraformaldehyde for 30 minutes. Cells on the lower surface were stained with 0.5% crystal violet for 30 minutes. Images were captured using a digital camera (Olympus C-5060, Japan). Five images were taken for each well.

Replicate wells were included in each experiment condition (n = 3). The number of migrating cell in each image was quantified using Image J 8.0 software.

2.6 Wound Healing Assays

EC cells were used to inoculate a 6-well culture plate (Corning Inc, USA). When cells reached 70–80% confluence, transfection reagent was added, 8 hours later, the cell monolayer was scraped with a sterile plastic tip (10 μ l). Then, they were cultured in FBS-free medium. At 0, 12, and 36 hours after scraping the monolayer, five randomly selected areas were observed using an optical microscope ($\times$ 100 magnification) to assess cell migration. images were captured using a digital camera (Olympus C-5060, Japan) and duplicate wells were included for each experimental condition (n = 3). The area of wound healing in each image was quantified with Image J 8.0 software and used to calculate the wound healing percentage.

2.7 Colony Formation Assay

EC cells were seeded onto 6-well plates, and the cells were transfected according to the above grouping. After 24 hours, cells were collected. Cells (200 per well) were seeded in 6 well plates and cultured in a humidified incubator at 37 °C and 5% CO₂ for two weeks. The culture medium was renewed every 3–4 days during the experiment. When colony formation was apparent, cells were fixed with 4% paraformaldehyde for 20 minutes, then stained with 0.1% crystal violet solution for 30 minutes. Images were captured.

2.8 Flow Cytometry Analysis

For apoptosis analysis, after 48 hours of transfection of EC cells, the cells were collected and washed with ice-cold PBS. The analysis was carried out with Annexin V-FITC reagent kit (GenStar, China), according to the manufacturer's instructions, Cells were resuspended in Annexin binding buffer to a final concentration of 1×10^6 cells / ml, AnnexinV-FITC conjugate and propidium iodide (PI) buffer were added in turn, and cultured at room temperature in dark for 15 min, then 400 μ l of Annexin binding buffer was added, samples were collected by BD FACSC alibur flow cytometer (BD Biosciences) within 1 hour. Subsequent analyses were performed with software (FlowJo 10.5).

2.9 RNA Extraction And Quantitative Real-time PCR

Total RNA was extracted with Trizol (Invitrogen, USA) according to manufacturer's instructions. Total cDNA was synthesized by reverse transcription of 1 μ g of total RNA with Prime Script RT Reagent Kit with gDNA Eraser (Takara, Beijing, China). Quantitative real-time PCR was performed using an Applied Biosystems 7900 quantitative PCR system. We measured expression of each gene in a total volume of 10

μL total volume containing SYBR Green (TB Green Premix Ex Taq II Takara, China). The expression of each gene was calculated as relative expression using the $2^{-\Delta\Delta C_t}$ method normalized to GAPDH. All primers were designed and synthesized by Sangon Biotech and listed in Table 2.

Table 1
Correlation between EGFL6 expression and clinicopathological variables of 120 EC patients

Variables	Cases	EGFL6 expression		P value
		High(63)	Low(57)	
Gender				0.506
Male	79(0.66)	42	37	
Female	41(0.34)	21	20	
Age				0.541
< 65	51(0.425)	27	24	
≥ 65	69(0.575)	36	33	
Tumor size				0.042
≤ 5 cm	53(0.442)	33	20	
> 5 cm	67(0.558)	30	37	
T stage				0.014
T1	2(0.02)	0	2	
T2	15(0.125)	3	12	
T3	60(0.50)	33	27	
T4	43(0.355)	27	16	
Lymph node metastasis				0.002
Absent	62(0.49)	24	38	
Present	58(0.51)	39	19	
Distant metastasis				< 0.001
Absent	68(0.57)	25	43	
Present	52(0.43)	38	14	
Histological differentiation				0.01
Well/moderate	101(0.84)	48	53	
Poor/undifferentiated	19(0.16)	15	4	
The relationship between EGFL6 expression and various clinicopathological parameters were analyzed by a Chi-squared test. A <i>P</i> value of < 0.05 was considered to be statistically significant.				

Table 2
synthesis list of Real-time PCR primers (F:Forward primer;
R:Reverse primer)

Gene Name		Primer Sequence 5'- 3'
EGFL6	F	CTCCTACCTGACCTGCAACC
	R	GCCAGGGCATTGTTACTGTT
E-cadherin	F	GTCTCTCTCACCACCTCCACAG
	R	CTCGGACACTTCCACTCTCTTT
Vimentin	F	GAAGAGAACTTTGCCGTTGAAG
	R	GAAGGTGACGAGCCATTTC
Twist	F	GCAAGAAGTCGAGCGAAGAT
	R	GCTCTGCAGCTCCTCGAA
N-cadherin	F	TGCTACTTTCCTTGCTTCTGAC
	R	TAACACTTGAGGGGCATTGTC
Fibronectin	F	AGGAAGCCGAGGTTTTAACTG
	R	AGGACGCTCATAAGTGTCACC
Snai1	F	TCGGAAGCCTAACTACAGCGA
	R	AGATGAGCATTGGCAGCGAG
BMI1	F	CGTGTATTGTTTCGTTACCTGGA
	R	TTCAGTAGTGGTCTGGTCTTGT
CD44	F	CTGCCGCTTTGCAGGTGTA
	R	CATTGTGGGCAAGGTGCTATT
SOX2	F	GAGAGAAAGAAGAGGAGAGAGAAAG
	R	GCCGCCGATGATTGTTATTATT
NANOG	F	CTCCCTAACAGCTGGGATTTAC
	R	GACGGCAGCCAAGGTTATTA
OCT4	F	GGAGGAAGCTGACAACAATGA
	R	CTCTCACTCGGTTCTCGATACT
Bcl-2	F	GACTTCGCCGAGATGTCCAG
	R	GAACTCAAAGAAGGCCACAATC

Gene Name		Primer Sequence 5'- 3'
Bax	F	CGAACTGGACAGTAACATGGAG
	R	CAGTTTGCTGGCAAAGTAGAAA
Caspase 3	F	GTGGAGGCCGACTTCTTGTATGC
	R	TGGCACAAAGCGACTGGATGAAC
GAPDH	F	TGAACGGGAAGCTCACTGG
	R	TCCACCACCCTGTTGCTGTA

2.10 Protein Extraction And Western Blotting

RIPA lysis buffer (Beyotime, China) and protease inhibitor were mixed according to a ratio of 100:1 and added to each group of EC cells. Protein concentrations were measured using a BCA Protein Assay kit (KeyGEN Biotech, China). Proteins from lysed cells were fractionated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Blocked with 5% non- fat milk for 1 hour at room temperature, the membranes were incubated with antibodies overnight at 4°C. The antibodies used were anti-EGFL6 (Cat.SAB1412824,Sigma), anti-E-cadherin(Cat.ab15148,Abcam),

anti-Vimentin(Cat.ab137321,Abcam), anti-Snail(Cat.ab53519,Abcam), anti-Fibronectin(Cat.ab2413,Abcam), anti-Twist(Cat.ab175430,Abcam), anti-N-cadherin(Cat.ab18203,Abcam), anti-p-β-catenin(Cat.9567S,Cell Signaling Technology), anti-β-catenin(Cat.8480S,Cell Signaling Technology), anti-GSK3β(Cat.8213S,Cell Signaling Technology), anti-c-myc(Cat.ab32072,Abcam),anti-Bcl-2(Cat.12789-1AP,Proteintech Group In), anti-Bax(Cat.50599-2-Ig,Proteintech Group In), anti-Caspase3(Cat.19677-1-AP,Proteintech Group In) and anti-β-actin (Cat.PR-0255,ZSGB-Bio). The membranes were then washed and incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG (Cat.ZB-2305; Cat.ZB-2301, ZSGB-Bio) at 1:5000. Signals were visualized with super enhanced chemiluminescence (ECL) detection reagent (BOSTER, China) and detected using a Quantity One analysis system (Bio-Rad,USA). Quantitative analysis of bands was performed using Image J 8.0 software.

2.11 Mouse Tumor Models

In order to establish a nude mouse xenograft model, firstly, we purchased EGFL6 knockout lentivirus and corresponding controls (Genepharma, Shanghai, China), shEGFL6 and shNC. We transfected KYSE450 cell line using Lipofectamine®3000 Reagent. By puromycin (Solarbio, China) screening, a stable EGFL6 knockdown KYSE450 cell line was obtained and labeled as shEGFL6-KYSE450. This cell line was verified by Real-time PCR and Western-blot. After 3 generations of culture, for later use. Then we purchased 32 3-

week-old BALB/c Nude mice (Vitalriver, Beijing, China) were fed in a specific pathogen-free (SPF) animal center at 25°C, 70% humidity. Four mice were fed in each cage, sterile feed and sterile drinking water were added every two days, and the padding was changed every three days. After feeding for one week, the test was started when the nude mice were in good growth condition, and divided into two groups, shEGFL6 group and shNC group, with 3 mice in each group, then approximately 1×10^7 shEGFL6-KYSE450 cells and shNC-KYSE450 cells were injected subcutaneously into two groups of BALB/c Nude mice once a week. And the length and width of visible tumor were measured twice a week. Tumor size was measured and calculated as $V = (L \times W)^2 / 2$ (V = Tumor volume, L = Length, W = Width). After 4 weeks, all animals were sacrificed by cervical decapitation under anesthesia, tumor samples were separated and weighed. And immediately frozen in liquid nitrogen or fixed in formalin. All the operations comply with the regulations of "Regulations on Management of Experimental Animals" and the approval of the Animal Ethics Committee of Shanxi Medical University, and Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Statistical Analyses

The distribution of EGFL6 expression among different groups, stratified by clinicopathological factors was analyzed using Chi squared test. Data from two groups were analyzed by t-test. Data from more than two groups were analyzed by one-way ANOVA. All data were expressed as the mean $\pm$ SD. A P -value of < 0.05 were considered to be statistically significant. All data analyses were performed using the statistical software SPSS 21.0 (SPSS, Chicago, USA) and GraphPad Prism 8.0 (San Diego, CA).

Results:

1. The expression level of EGFL6 protein in EC tissues is significantly higher than that in adjacent non-tumor tissues, and it is related to the poor prognosis of EC. In order to verify the expression of EGFL6 in EC and the correlation between EGFL6 expression and the malignant degree of EC, we collected EC samples with different malignancies. According to the TNM stage of EC, they were marked as T2N0M0, T2N2M0, T3N0M0, T3N2M0, T4N0M0, and T4N2M0. Samples of the adjacent non-tumor tissues (ANT) were collected and labeled as ANT1, ANT2, ANT3, ANT4, ANT5, and ANT6. Immunohistochemistry showed that the expression of EGFL6 in EC tissues was significantly higher than that in the adjacent non-tumor tissues (Fig. 1A). Immunohistochemistry OD score showed an even great difference between the expression of EGFL6 in EC samples and that in samples of adjacent non-tumor tissues (Fig. 1B). The expression of EGFL6 increased with increasing TNM stage of malignancy (Fig. 1C). Combined with the clinicopathological data, we found that the expression level of EGFL6 protein was related to the tumor size ($P = 0.042$), invasion depth (T stage) ($P = 0.014$), lymph node metastasis ($P = 0.002$), distant metastasis ($P < 0.001$), and cancer differentiation degree ($P = 0.01$). In contrast, we found EGFL6 expression in human EC were significantly with age ($P = 0.541$) or gender ($P = 0.506$) (Table.1). Combined with TCGA data, EGFL6 gene expressions in human EC were significantly higher. The overall survival rate

of patients with high EGFL6 protein expression in EC was lower than that of patients with low EGFL6 protein expression ($P < 0.05$) (Fig. 1D, E, F).

2. The expression of EGFL6 in different EC cell lines. Next, we sought to compare the expression of EGFL6 in normal esophageal epithelial cell lines and that in different EC cell lines. We first selected a normal esophageal epithelial cell line (Het-1A) and three EC cell lines (EC9706, KYSE150, KYSE450). Through real-time PCR, we found that the expression of EGFL6 in Het-1A was significantly lower than that in EC cell lines (Fig. 2A). The results of Western-blot were consistent with those of real-time PCR (Fig. 2B, C). EGFL6 was expressed differently in each EC cell lines. The highest level of expression was measured in the KYSE450 cell line; the lowest level of expression was measured in the KYSE150 cell line.

In order to further explore the role of EGFL6 in the occurrence and development of EC, two small interfering RNA (siRNA) sequences and one unintentional sequence were synthesized using interfering RNA technology. These were labeled siRNA1, siRNA2, and siNC. Then, the KYSE450 cells were transfected using Lipofectamine®3000 to obtain EGFL6 silenced KYSE450 cells and a corresponding control group. The silencing efficiency was verified by real-time PCR and Western-blot (Fig. 2D, E, F). The siRNA1 with the highest transfection efficiency was selected for further study. The EGFL6 overexpression plasmid of PcDNA3.1 + EGFL6 and PcDNA3.1 empty plasmid were also constructed and transfected into the KYSE150 cell line with Lipofectamine®3000 to obtain the EGFL6 overexpression KYSE150 cells and a corresponding control group.

3. Effect of EGFL6 on invasion and migration of EC cells

We observed the migration ability of EC cells when EGFL6 was silenced at 0 h, 12 h, and 36 h using wound healing assays. The cell migration ability of siRNA group was significantly lower than that of the siNC group; the cell migration rate at 36 h was only about 20% (Fig. 3A,B). A consistent result was observed when the mixture of overexpression plasmid and transfection reagent and its corresponding control were added to KYSE150 cells. In that case, we found that the migration ability of cells in the PcDNA3.1 + EGFL6 group was significantly enhanced (Fig. 3C, D). Further, the results of the Transwell migration experiment are consistent with the wound healing assay. EGFL6 silencing can inhibit the migration of EC cells (Fig. 3Ea, Eb). EGFL6 overexpression can promote the migration of esophageal cancer cells (Fig. 3Fa, Fb). The transwell invasion experiment showed that the invasion ability of EC cells decreased when EGFL6 was silenced (Fig. 3Ec, Ed) and the invasion ability of EC cells increased when EGFL6 was overexpressed (Fig. 3Fc, Fd). Collectively, these results indicate that EGFL6 expression in EC cell lines can promote the invasion and migration of EC cells. This, in turn, indicates that EGFL6 expression in EC may be related to EC metastasis.

4. Effect of EGFL6 on proliferation and apoptosis of EC cells

To determine the effect of EGFL6 expression on the proliferation and apoptosis of EC cells, Group as above, the cell viability was assessed by CCK-8. When EGFL6 gene expression was silenced, the activity of siRNA group was lower than that of the siNC and blank groups (Fig. 4A). When EGFL6 gene was

overexpressed, the activity of KYSE150 cells in PcDNA3.1 + EGFL6 group was higher than that in the PcDNA3.1 group (Fig. 4B). Consistently, in the colony formation experiment, it was also found that when EGFL6 gene was silenced, the colony formed by KYSE450 cells were significantly reduced (Fig. 4C). When EGFL6 gene was overexpressed, colony formation by KYSE150 cells significantly increased (Fig. 4D), indicating EGFL6 plays an important role in the proliferation of EC cells.

Next, we analyzed the effect of EGFL6 gene on the apoptosis of EC cells. KYSE450 and KYSE150 cells were used to detect the apoptosis of EC cells corresponding to EGFL6 gene silencing and overexpression. Apoptosis detection was conducted by flow cytometry 48 hours after adding transfection reagent. The flow cytometry results showed that the apoptosis rates of KYSE450 cells was 31.07% when EGFL6 were silenced, significantly higher than that of the siNC group (12.98%) (Fig. 4E,F). The apoptotic rates of KYSE150 cells was 5.35% when EGFL6 were overexpressed, significantly lower than that of the PcDNA3.1 group (15.25%) (Fig. 4G, H). Next, we detected the apoptosis related marker genes (Bcl-2, Bax, Caspase 3) by real-time PCR and Western-blot, and further verified the effect of EGFL6 on the apoptosis of EC cells. After adding transfection reagent at 48 hours and 72 hours, we extracted RNA and protein from each group of cells respectively. The real-time PCR results showed that when EGFL6 gene was silenced, the expression of Bax and Caspase-3 increased. The expression of anti-apoptotic gene Bcl-2 decreased (Fig. 5A). When EGFL6 was overexpressed, the expression of Bax and Caspase-3 decreased and the expression of Bcl-2 increased (Fig. 5B). Western-blot results were consistent with real-time PCR results (Fig. 5C,D,E,F). Taken together, these results show that EGFL6 is involved in the regulation of apoptosis of EC cells, and inhibition of EGFL6 gene expression can promote apoptosis.

5. EGFL6 can induce the EMT process in EC cells.

In the process of malignant evolution of tumor, EMT triggers tumor cell invasion and metastasis. EMT may also make tumor cells escape apoptosis induced by some factors. Occurrence of EMT phenotype indicates the malignant process of tumor. Previously, we found that EGFL6 participates in the proliferation, invasion, migration and apoptosis of EC cells. Next, we sought to identify the mechanisms of EGFL6 gene inducing the proliferation, invasion, migration and apoptosis of EC cells. We sought to answer the question of whether EMT plays an important role in EGF6 promotion of metastasis. We know that in EMT, the polarity of epithelial cells is lost, the contact with surrounding cells and stromal cells is reduced, the interaction between cells is reduced, and the ability of cell migration and movement is enhanced. At the same time, the cell phenotype is changed and the epithelial phenotype is lost. There will be a decrease of E-cadherin expression level, facilitating cell invasion and metastasis. Therefore, the loss of E-cadherin expression has been considered the most significant feature of EMT. At the same time, the cells obtained stromal phenotypes, such as vimentin, snail, fibronectin, twist, and N-cadherin, we detected EMT phenotype related markers through real-time PCR and Western-blot. We found that when EGFL6 was silenced, the transformation of tumor cells to EMT was inhibited, the expression of E-cadherin related markers increased, and the expression of vimentin, snail, fibronectin, twist, and N-cadherin decreased (Fig. 6A). When EGFL6 was overexpressed, tumor cells were promoted to EMT and the expression of E-cadherin, vimentin, snail, fibronectin, twist, and N-cadherin decreased, as they would in EMT (Fig. 6B).

Western-blot results were consistent with real-time PCR results (Fig. 6C, D, E, F). Therefore, results indicate EGFL6 plays an important role in promoting the EMT of EC cells.

7. EGFL6 Maintains Esophageal Cancer Stem Cell-like Cell Population

Tumor stem cells (TSCs) are closely related to cancer treatment, recurrence and metastasis. TSCs are stem cells with the same self-renewal and pluripotency as normal stem cells^[9]. Self-renewal is the ability to produce at least one daughter cell identical to the mother cell in order to maintain its stem cell properties. The pluripotency of stem cells allows them to differentiate into a variety of specialized cell types^[10, 11]. TSCs may emerge from normal somatic stem cells in the affected tissue or organ system. After obtaining genetic changes in the DNA replication process, TSCs is thought to be related to the occurrence of cancer through various microenvironment factors. TSCs may be caused by gene changes of cancer cells from the tumor body, thus activating one or more major signal pathways, obtaining the characteristics of self-renewal. We sought to address the questions of whether EGFL6 affects the esophageal cancer stem cell like cell population and whether it is related to the activation of that signaling pathway. Through a review of the literature, we selected the marker genes^[12] (Bmi1, Oct, Sox2, Nanog, CD44) related to the characteristics of esophageal cancer stem cells. We used real-time PCR to assess the expression of related genes in EGFL6 gene silencing and overexpression. The results showed that with EGFL6 gene silencing, the expression of esophageal cancer stem cells was low, and when EGFL6 gene overexpression, the expression of esophageal cancer stem cells increased (Fig. 7A, B). This result suggests that EGFL6 may maintain stem cell like cells in EC.

8. EGFL6 modulates Wnt/ β -Catenin signaling to drive EC metastasis and proliferation

According to the above results, we know that EGFL6 can induce EMT and maintain TSCs expression. The characteristics of EMT and TSCs in tumor cells are closely related to tumor invasion, metastasis and proliferation. Studies have shown that there are many signal pathways leading to EMT, among which Wnt/ β -catenin signal pathway is the key signal transduction pathway for epithelial tissue to induce EMT^[13]. It is reported that the characteristics of tumor stem cells are affected by the Wnt/ β -catenin, an evolutionarily conservative signaling pathway^[10, 11]. Therefore, we detected the expression of p- β -Catenin, β -Catenin, GSK3 β and c-myc, which are the marker genes of Wnt/ β -Catenin signal pathway by Western-blot. Expression of p- β -catenin, total β -Catenin and c-myc protein was lower and GSK3 β protein was higher in the siRNA group than in the siNC group and blank group. However, there were no significant difference in expression levels of these genes between the siNC group and blank group (Fig. 7C,D). The expression levels of p- β -catenin, total β -Catenin, and c-myc protein in PcDNA3.1 + EGFL6 group were higher and the expression level of GSK3 β protein was lower compared than those in the PcDNA3.1 group (Fig. 7E, F). Therefore, EGFL6 participates in the regulation of Wnt/ β -Catenin signaling pathway.

9. EGFL6 Inhibits Tumor Growth In Vivo

Finally, we sought to determine whether EGFL6 can inhibit the growth of tumor by in vivo experiments. First, we assessed the infection efficiency of lentivirus by real-time PCR and Western-blot (Fig. 8A,B,C). Then, we measured the volume and mass of subcutaneous tumor in nude mice at a specific time point. We found that the volume and mass of subcutaneous tumor in the shEGFL6 group were significantly smaller than those in the shNC-KYSE450 group. This result is consistent results of our in vitro experiments. (Fig. 8D,E,F,G). These results suggest that EGFL6 knockdown can inhibit the growth of EC. Based on this nude mouse model, we will further explore the effect and mechanism of EGFL6 on the occurrence and development of EC.

Discussion:

EC is a malignant tumor of the digestive tract. Invasion and metastasis are the primary obstacles in the treatment of cancer and the main cause of death. Many studies have explored the invasion and metastasis of tumor, but mechanisms are not yet fully understood. Our study revealed the role of EGFL6 in the proliferation, migration, invasion and apoptosis of EC and determined characteristics of the mechanisms EGFL6 uses to influence these processes.

Tumor invasion and metastasis are related to a series of complex processes, including cell adhesion, migration, invasion, angiogenesis and growth independent of adherent wall^[14, 15, 16, 17]. In addition, the degradation of extracellular matrix (ECM) is a key process for cancer cells to enter the blood vessels and lymphatic vessels. EGFL6, as a member of epidermal growth factor repeat superfamily, has a common feature of epidermal growth factor family, binding of EGFL6 receptor triggers a wide range of biological functions, including differentiation, apoptosis, adhesion and migration^[18]. It also plays an important role in the regulation of cell cycle, proliferation and development^[19, 20, 21]. It is up-regulated in a variety of tumor tissues and promotes tumor angiogenesis. EGFL6 is expressed at lower levels or is absent in normal tissues; inhibition of EGFL6 does not affect the healing of normal wounds^[22]. This draws attention to the potential of EGFL6 in anti-tumor strategies. It has been reported that EGFL6 can enhance the invasion and metastasis of ovarian and breast cancer cells and stimulate tumor angiogenesis^[23, 24]. However, the role and mechanism of EGFL6 in EC have not been reported. Through wound healing assays and transwell experiments, our study demonstrates that EGFL6 plays an important role in the invasion and migration of EC cells.

The invasion and metastasis of tumors are related to EMT. EMT arises from a loss of polarity of epithelial cells, the loss of connections with basement membrane and other epithelial phenotypes. EMT is a transformation to interstitial phenotype associated with a higher ability for migration and invasion, anti-apoptosis and degradation of ECM; features closely related to the invasion and metastasis of tumors. Therefore, the ability of tumor cells to spread around and invade normal tissues is the key for tumor cells to develop into malignant tumors^[25]. The decrease of E-cadherin expression level facilitates cell invasion and metastasis, so the loss of E-cadherin expression has been considered the most significant feature of EMT. Genes such as Vimentin, Snail, Fibronectin and N-cadherin are also important markers of EMT. The

down regulation of E-cadherin, usually tightly associated with β -catenin in normal epithelium, triggers the nuclear localization of β -catenin activity of canonical Wnt/ β -Catenin signaling^[13, 26]. Twist, a strong activator of EMT is another putative β -catenin target^[27]. EMT and Wnt/ β -Catenin interact in the process of tumor invasion and metastasis. Results of our assessment of the expression levels of EMT markers with EGFL6 silencing suggest that this silencing inhibit the EMT of EC cells to EMT and inhibits Wnt/ β -catenin signaling pathway. At the same time, overexpression of EGFL6 stimulates the transformation of EC cells to EMT and activates the Wnt/ β -catenin signaling pathway. It can be seen that EGFL6 affects the invasion and metastasis of EC cells, which is generated by stimulating EMT of tumor cells, and then activating Wnt / β - catenin signaling pathway. EGFL6 may induce EMT in EC cells by activating Wnt/ β -catenin signaling pathway, thus promoting invasion and migration of EC cells.

With EMT, tumor cells can exhibit characteristics of stem cells. EMT plays an important role in the development of TSCs. the source of tumor proliferation, recurrence, metastasis, and drug resistance. The proportion of TSCs is positively related to the malignancy of tumor^[28, 29]. TSCs may arise from gene changes of cancer cells from the tumor body, thus activating one or more major signaling pathways, of which Wnt/ β -catenin signal pathway is the most important one^[30]. Our research shows that EGFL6 participates in maintaining the tumor stem cell-like cell population, thus we suspect that EGFL6 may maintain the tumor stem cell-like cell population by activating WNT signaling pathway. Therefore, we speculate that EGFL6 may play a role by activating Wnt/ β - catenin signaling pathway, thus promoting proliferation of EC cells.

Conclusion

EGFL6 has the potential to promote the invasion and migration of EC cells. In vitro and in vivo experiments show that knockdown of EGFL6 can significantly inhibit the growth of tumor. EGFL6 can also regulate the apoptosis of EC cells. Therefore, EGFL6 is a promising target in the diagnosis and treatment of EC. Further definition of the mechanism of EGFL6 in EC and development of corresponding antibody drugs will provide new approaches to tumor diagnosis, prognostic markers or therapeutic targets.

Abbreviations

EGFL6
Epidermal growth factor-like domain-containing protein 6
EC
Esophageal cancer
EMT
Epithelial-mesenchymal transitions
TSCs
Tumor stem cells

ANT
Adjacent non-tumor tissues
TCGA
The Cancer Genome Atlas
OD
Optical density
siRNA
small interference RNAs
ECM
Extracellular matrix
Sox2
SRY (sex determining region Y)-box 2
Bmi1
Polycomb ring finger
Oct4,
Octamer-binding transcription factor 4
c-Myc
bHLH transcription factor
E-cadherin
Epithelial cadherin
ABCG2
ATP binding cassette subfamily G member 2
Twist
Twist family bHLH transcription
F
Forward
R
Reverse
SPF
Specific pathogen-free
GAPDH
Glyceraldehyde-3-phosphate dehydrogenase

Declarations

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Authors' contributions

All authors participated in the design of the study, the interpretation and analysis of the data, and the review of the manuscript. Y.W., J.K., J.T., H.J., J.W., R.S., S.C., H.J., J.Y., Y.G., G.W., and X.R. conducted the experiments and analyzed the data. Y.W. and J.K., wrote the manuscript, and J.K., J.T., H.J. contributed constructive suggestions to the writing of the manuscript.

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Availability of data and materials

All data during this study are included within this published article and additional files. Any material described in the article can be requested directly from corresponding author on reasonable request.

Ethics approval and consent to participate

This study was performed with approval from the Ethics Committee at the Shanxi Medical University. Written informed consent was obtained from all patients. All Animal experiments complied with the national guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

EGFL6 is highly expressed in EC tissues. (A): In EC tissues with different degrees of malignancy and adjacent non-tumor tissues (ANT), the expression of EGFL6 in cancer tissues is significantly higher than in ANT, and higher levels of EGFL6 correspond with the increasing degree of malignant degree of the tumor. (B,C): The immunohistochemical results were analyzed quantitatively. * $P < 0.05$; ** $P < 0.01$.

(D,E,F):According to the TCGA database, the high expression of EGFL6 is related to the low survival rate of esophageal cancer patients, $P<0.05$.

Figure 2

EGFL6 were differentially expressed in normal esophageal epithelial cells and EC cells. (A): Real-time PCR showed that the expression of EGFL6 gene in EC cell lines (EC9707, KYSE150, KYSE450) were significantly higher than that in normal esophageal epithelial cell line(Het-1A). (B,C): Western-blot results were consistent with real-time PCR results. The expression of EGFL6 protein in EC cell lines (EC9706, KYSE150, KYSE450) were significantly higher than that in a normal esophageal epithelial cell line(Het-1A). (D,E,F):Western-blot and real-time PCR showed, the expression levels of EGFL6 in the siRNA1 and siRNA2 groups were significantly lower than that in the siNC group; the silencing efficiency of siRNA1 group was about 70% lower. There was no statistical difference between EGFL6 expression levels in the siNC and blank groups. (G,H,I):Western-blot and real-time PCR showed the EGFL6 expression of PcDNA3.1+EGFL6 group were significantly higher than that in the PcDNA3.1 group. All assays were repeated three times ($n=3$), ** $P<0.01$. mean $\pm$ SD.

Figure 3

EGLF6 stimulates mobility of EC cells. (A,B):Wound healing assays showed that the cell migration ability of siRNA group was significantly lower than that of siNC group, and the cell migration rate of siRNA group was only about 20% at 36h. (C,D):Wound healing assays showed that the migration ability of pcDNA3.1+EGFL6 group was significantly higher than that of PcDNA3.1 group. (E,G,H): Transwell migration and invasion assay showed compared with siNC group, the number of cell migration and

invasion in siRNA group is significantly reduced. (F,I,K):Transwell migration and invasion assay showed compared with PcDNA3.1 group, the number of cell migration and invasion in PcDNA3.1+EGFL6 group is significantly increased. All assays were repeated three times (n=3), **P<0.01. mean ± SD.

Figure 4

EGFL6 can enhance growth and reduces apoptosis of EC cells. (A):CCK-8 assay EGFL6 silencing can inhibit the activity of KYSE450 cells. The proliferation ability of siRNA group was lower than that of the

siNC group. There was no significant difference between proliferation abilities of the siNC group and the blank group. (B):Overexpression of EGFL6 can enhance the viability of KYSE150 cells. The proliferation ability of PcDNA3.1+EGFL6 group is higher than that of the PcDNA3.1 group. (C,D):Colony formation analysis shows that EGFL6 silencing inhibits the growth of KYSE450 cells, and EGFL6 overexpression promotes the growth of KYSE150 cells. (E,F):Flow cytometry showed that the apoptosis rate of siRNA group was 31.07% when EGFL6 was silenced significantly higher than that of siNC group (12.98%). (G,H): the apoptosis rate of PcDNA3.1+EGFL6 group was 5.35%, which was significantly lower than that of the PcDNA3.1 group (15.25%).All assays were repeated three times (n=3), *P<0.05;**P<0.01. mean $\pm$ SD.

Figure 5

EGFL6 is involved in the regulation of EC cell apoptosis, and inhibition of EGFL6 expression can promote apoptosis. (A,B):Real-time PCR assays showed that the expression of pro-apoptotic genes Bax and Caspase-3 in siRNA group were higher than in the siNC group, and the expression of anti-apoptotic gene Bcl-2 decreased. There was no significant difference between expression levels in the siNC group and the blank group. When EGFL6 is overexpressed, the result is exactly the opposite. (C,D,E,F):The results of

western-blot were the consistent with those of real-time PCR. All assays were repeated three times (n=3), *P<0.05. mean ± SD.

Figure 6

EGFL6 can induce EMT process in EC cells. (A,B): Real-time PCR detected expression of EMT related markers. When EGFL6 gene was silenced, E-cadherin expression increased; Vimentin, Snail, Fibronectin, Twist, N-cadherin expression decreased. There was no significant difference between siNC group and Blank group. When EGFL6 gene was overexpressed, the expression of E-cadherin decreased and the expressions of Vimentin, Snail, Fibronectin, Twist and N-cadherin increased. (C,D,E,F):The results of the western-blot assay were consistent with those of real-time PCR. All assays were repeated three times (n=3), *P<0.05. mean ± SD.

Figure 7

EGFL6 participates maintaining esophageal cancer stem cell-like cell population and involves WNT/ β -catenin signaling pathway. (A,B): Real-time PCR was used to detect the expression of esophageal cancer related stem cell genes. The expression of esophageal cancer stem cell genes in the siRNA group was lower than that in the siNC group. There was no significant difference between expression levels in the siNC group and the blank group. PcDNA3.1+EGFL6 group showed higher expression levels of esophageal

cancer stem cell genes than did the PcDNA3.1group. (C,D,E,F):Western-blot assays showed the expression of WNT/ β -catenin signaling pathway related marker proteins. When EGFL6 is overexpressed, the WNT/ β -catenin signaling pathway is activated; the expression of P- β -catenin, β -catenin, c-myc protein increases and the expression of GSK3 β protein decreases. All assays were repeated three times (n=3), *P<0.05. mean $\pm$ SD.

Figure 8

The knockout of EGFL6 inhibited the growth of xenograft tumor in nude mice. (A,B,C):Real-time PCR and western-blot to verify the efficiency of EGFL6 knockdown in KYSE450 cells. (D,E,F,G):The subcutaneous xenograft tumor model of nude mice was successfully established. The mass and volume of transplanted tumor in shEGFL6 group were significantly lower than those of the shNC group. All assays were repeated three times (n=3), *P<0.05. mean $\pm$ SD.