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Original Article

Epidermal growth factor-like domain 7 promotes cell proliferation, invasion and serves as a prognostic indicator in human laryngeal squamous cell carcinoma

Jingjia Li[1*], Weixiong Chen[1*], Kai Wang[1], Zhaofeng Zhu[1], Jianli Zhang[1], Fayao He[1], Sucheng Tang[1], Qinglai Tang[2], Xinming Yang[2], Shisheng Li[2], Yuejian Wang[1]

[1]Department of Otolaryngology, The First People's Hospital of Foshan, Foshan, PR China; [2]Department of Otolaryngology-Head and Neck Surgery, The Second Xiangya Hospital, Central South University, Changsha, PR China. [*]Equal contributors.

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Abstract: Background: Epidermal growth factor-like domain 7 (Egfl7), a primary endothelial-specific secreted factor, is involved in tumourigenesis in multiple epithelial tumors. However, the role of Egfl7 in laryngeal squamous cell carcinoma (LSCC) has rarely been reported. Method: We determined the expression levels of Egfl7 from 33 fresh and 116 paraffin-embedded samples, analyzed its correlation with clinicopathological parameters, and further investigated its role in promoting proliferation and invasion in vitro. Results: Both mRNA and protein levels of Egfl7 was up-regulated, with 94 (81.03%) samples staining positive for Egfl7. Moreover, Egfl7 expression was significantly correlated with tumor stage (P=0.001), lymph node metastasis (P=0.002) and microvessel density (r=0.623, P<0.001, Spearman's correlation coefficient). Kaplan-Meier analysis revealed that Egfl7 expression was significantly associated with worse disease-free survival (P=0.011) and overall survival (P=0.004) rates. In multivariable Cox regression analysis, Egfl7 expression (P=0.012) was an independent prognostic factor for LSCC. In vitro, siRNA mediated knockdown of Egfl7 resulted in marked decreases in cell viability and invasion, which was accompanied by the S-phase-specific cell cycle blockage and increases of apoptosis. Conclusion: Egfl7 expression was tightly associated with more aggressive tumor behavior and a poor prognosis, indicating that Egfl7 is a valuable predictive marker and a potential therapeutic target for LSCC.

Keywords: Egfl7, laryngeal squamous cell carcinoma, prognosis, cell viability, invasion

Introduction

Head and neck cancer, including laryngeal squamous cell carcinoma (LSCC), is the sixth most common cancer worldwide [1]. According to the published data, LSCC accounts for 30% to 40% of all malignant head and neck tumors and 1% to 2.5% of all malignant neoplasms in the human body [2, 3], the incidence of LSCC is estimated at around 160000 cases per year [2]. Despite significant progress in therapeutic interventions, including surgery, radiotherapy, and chemotherapy, there have been only modest improvements in the survival of patients with LSCC in the past three decades, especially for those with locally advanced disease [4]. Locoregional recurrence, cervical lymph nodes metastasis and distant metastasis are the factors that significantly affect the prognosis of LSCC [5]. Therefore, early detection and clarification of detailed molecular mechanism of LSCC is of vital importance for prediction of tumor biological behavior and direction of therapeutic strategy.

LSCC, like other solid tumors, develops through a prolonged multistage process involving cancer cell proliferation, migration, invasion as well as tumor angiogenesis. Within a solid tumor, angiogenesis is essential for the growth of primary tumor by sustaining the nutriment and oxygen supply, removing metabolites, and releasing growth factors which further promote tumor progression and metastasis [6]. Increased angiogenesis has been proven to be associated with a poorer prognosis for patients with

LSCC [7, 8]. Recently, Egfl7 (also known as vascular endothelial statin) was identified to be a secreted protein which was specifically expressed during in vitro differentiation and mouse embryogenesis [9-11]. As the vasculatures mature, Egfl7 expression is down-regulated in almost all mature tissues with the exception of the pregnant uterus [10]. Interestingly, in contexts requiring new vessel growth, such as tumorigenesis or vascular injury, Egfl7 levels increase again [10, 12]. Moreover, Egfl7 may act as a chemoattractant for embryonic endothelial cells and fibroblasts migration [13].

Considering the important role of Egfl7 in angiogenesis and cell migration, it is suggested that Egfl7 may participate in tumorigenesis and tumor progression. Recent studies have demonstrated that Egfl7 is significantly upregulated in human epithelial tumors, including hepatocellular carcinoma [14], malignant glioma [15], ovarian carcinoma [16], gastric cancer [17], colon cancer [18] and breast cancer [19]. However, these studies showed distinct results as regard to the correlation between Egfl7 expression and tumor prognosis. In hepatocellular carcinoma [14], Egfl7 expression was closely correlated with poor prognosis, while in breast cancer [19], Egfl7 expression was associated with better prognosis and with the absence of lymph node invasion, indicating the role of Egfl7 protein in different tumors may be highly tissue- and organ-specific. Here, we investigate the expression of Egfl7 in human LSCC tissues, analyze its relationship with clinicopathological parameters as well as microvessel density (MVD), and elucidate the function of Egfl7 in the development and metastasis of LSCC by characterizing its role in vitro.

Materials and methods

Patients and tissue specimens

Tissue samples were obtained from 116 patients with LSCC who underwent surgery at the department of otolaryngology and head and neck surgery, Second Xiangya Hospital of Central South University of China, between March 2008 and May 2010. The 116 cases comprised 107 men and 9 women, with a median age of 55 years (age range, 32-74 years). Among these 116 cases of LSCC, matched fresh specimens of LSCC and adjacent nontumorous laryngeal tissue from 33 cases were collected for real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and western blot detection. The local Ethics Committee had approved the protocol for the study. All participants signed a written informed consent to use biopsies for scientific purposes.

Immunohistochemistry

Formalin-fixed paraffin sections were stained for Egfl7 (1:500 dilution, Abcam, Cambridge, UK) and CD34 (1:500 dilution, Sigma, St. Louis, USA). Then, the sections were incubated with a secondary goat anti-mouse IgG antibody (1:200 dilution, Sigma, St. Louis, USA), followed by incubation with the streptavidin-biotin-peroxidase complex tertiary system (Zymed, San Francisco, California, USA), and allowed to develop a color reaction using the DAB substrate kit according to the manufacturer's recommendations (Sigma, St. Louis, USA).

The immunohistochemical staining of Egfl7 was assessed by observing the incidence and staining intensity of positive cells. The incidence of positive cells was scored as follows: 0, ≤10%; 1+, 11%-25; 2+, 26%-50%; 3+, 51%-75%; 4+, ≥76%. Staining intensity was scored as: 0, none; 1+, weak; 2+, moderate; 3+, strong. The two components were multiplied to obtain an overall expression score, as follows: 0, scores of 0-3; 1+, scores of 4-6; 2+, scores of 7-9; 3+, scores of 10-12. Expression scores of 0 or 1+ were defined as low protein expression, while scores of 2+ or 3+ were defined as high protein expression.

Determination of microvessel density (MVD)

Microvessels were stained by using anti-CD34 antibody. We applied the method proposed by Huang et al. [15] to determine the MVD. Briefly, We selected three neovascular hotspots from each section and counted the number of microvessels from three random high-magnification fields (200×) within each hotspot. Any red-staining endothelial cell cluster that was clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel, but the positive endothelial cells with a thick surrounding muscular layer or a lumen size greater than eight red blood cell diameters were not counted.

qRT-PCR

For the fresh tissue samples, total RNA was extracted using the Trizol reagent (Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was then synthesized using oligo dT as the primer with AMV reverse transcriptase (Yoyobo, Japan). To determine the mRNA level of EGFL7 in each sample, PCR was performed with the following primers: forward, 5'-GATGGCGGGGTGACACTTG-3'; reverse, 5'-CACTGTCC-ACTCC-TGTC-GGG-3'. GAPDH expression was determined as a control using primers: forward, 5'-GGGT-GTGA-ACCA-TGAG-AAGT-ATG-3'; reverse, 5'-GATGGCATGGACTGTGGTCAT-3'. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein was extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membrane (Millipore, Bed ford, MA). The blotted membranes were incubated antihuman Egfl7 antibody (Abcam, Cambridge, UK) and then secondary antibody (Sigma, St. Louis, USA) in order. Beta-actin protein was also determined by using the specific antibody (Sigma, St. Louis, USA) as a loading control.

Follow-up

Post-resection follow-up data were obtained for all 116 patients. The mean follow-up time (from completion of treatment to the last otolaryngological examination) was 36.9 months, with a median of 43.2 months (range, 13-60 months). During follow up, all patients underwent clinical and fibre-laryngoscopic examination every three months in the first post-operative year and every six months in the following year.

Cell lines and cell culture

Hep-2 and 293T cell lines were purchased from the Institute of Medicinal Biotechnology (Shanghai, China). Tu686 and Tu212 cell lines were generously provided by Dr. Zhuo (Georgia) Chen (Emory University Winship Cancer Institute, Atlanta, Georgia) [20, 21]. M2e and M4e cell lines were purchased from Land biological technology corporation (Guangzhou, China). These cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37°C.

Construction of siRNA plasmid vector

The short interfering RNA (siRNA) expressing vector pcDNA6. 2-GW/EmGFP-miR was purchased from Invitrogen (Carlsbad, CA, USA). Four putative candidate sequences were designed by using Invitrogen online RNAi designer (http://rnaidesigner.invitrogen.com/rnaiexpress/) and their specificity confirmed by nucleotide BLAST searches. The sequences of the 4 pairs of the Egfl7 shRNA oligonucleotides are as follows: 5'-TGCTGTTGGGTTGCTCATCCACTG-CTGTTTTGGCCACTGACTGACAGCAGTGGGAGC-AACCCAA-3' and 5'-CCTGTTGGGTTGCTCCCAC-TGCTGTCAGTCAGTGGCCAAAACAGCAGTGATG-AGCAACCCAAC-3', 5'-TGCTGTCATTGCACTGTCC-ACTCCTGGTTTTGGCCACTGACTGACCAGGA-GTGCAGTGCAATGA-3' and 5'-CCTGTCATTGCAC-TGCACTCCTGGTCAGTCAGTGGCCAAAAC-CAGGAGTGGACAGTGCAATGAC-3', 5'-TGCTGAA-GGAAATCTGCTCGCTCAGGGTTTTGGCCACTG-ACTGACCCTGAGCGCAGATTTCCTT-3' and 5'-CC-TGAAGGAAATCTGCGCTCAGGGTCAGTCAGTG-GCCAAAACCCTGAGCGAGCAGATTTCCTTC-3', 5'-TGCTGTTCACAGAGAAGATCCCAGCCGTTTT-GGCCACTGACTGACGGCTGGGATTCTCTGTG-AA-3' and 5'-CCTGTTCACAGAGAATCCCAGCCGT-CAGTCAGTGGCCAAAACGGCTGGGATCTTCTCTG-TGAAC-3'. The designed and synthesised sinle-stranded sequences were annealed to double-stranded oligo and cloned into linear pcDNA6.2-GW/EmGFP-miR vector. Screening positive cloning after transformed into DH5α competent cells and identified by PCR amplification and DNA sequencing.

Transfection

The pcDNA6.2-GW/EmGFP-miR plasmid was linearized by PmeI digestion, cotransformed into DH5α competent cells with the pLenti6.3-MCS/V5-DEST vector, and then transfected into 293T cells with the use of POLO deliverer™ 3000 Transfection Reagent(Invitrogen, Carlsbad, CA, USA) to generate the recombinant lentivirus. Media containing lentivirus were added to the cells supplied with polybrene (8 μg/mL) for 24 hours, then replaced with fresh media on day 2, and the expression levels of green fluorescent protein were examined

under a fluorescence microscope. Blasticidin [22] was used to screen the transfected cells.

Cell viability

Cell viability was measured with CCK8 assay (cell counting kit-8, Dojindo Molecular Technologies, Tokyo, Japan). After transfection, cells were seeded in 96-well plates at a cellular density of 5×10^3 cells per well, and incubated for 6 days. The cell monolayer was washed three times, then a 1:10 diluted CCK8 solution in RPMI 1640 was added to the cells and incubated for 2 h at 37°C. Cell viability was measured with a spectrophotometer at an absorbance of 450 nm.

Flow cytometry

For cell cycle assay, cells were collected and fixed with cold 70% ethanol for 1h, then incubated with ribonuclease A (RNase A) at a final concentration of 3000 units/ml at 37°C for 30 min. A total of 1 mg/ml propidium iodide (Sigma Chemical, St. Louis, MO) was added directly to the cell suspension, and a total of 10,000 fixed cells were analyzed by flow cytometry.

For cell apoptosis assay, cells were trypsinized and washed with serum-containing medium. The samples (5×10^5 cells) were centrifuged for 5 min at 1000×g and the supernatant was discarded. The cells were then stained using a 7-aminoactinomycin D (7-AAD) apoptosis kit (keyGEN bioTECH, Nanjing, China) in accordance with the manufacturer's instructions. The number of apoptotic cells was detected and analyzed using flow cytometry.

Transwell assay

A quantity of 5×10^4 cells were seeded into the chamber of a 24-well Matrigel-coated membrane filter with 8-lm pores (Costar, Lowell, MA, U.S.) for invasion assay. The bottom chamber was filled with RPMI 1640 containing 10% FBS as a chemoattractant. After 48h incubation at 37°C, membranes were fixed and stained with crystal violet. All of the cells invading through the membrane were counted under an inverted light microscope at 100× magnification.

Statistical analysis

All data were analyzed using SPSS 15.0 software and presented as mean ± SD. Student's t test was used to compare the mean values between two groups with equal variances and the Mann-Whitney U test was used for the data with unequal variances. For correlation analysis, Spearman's correlation coefficient was used for non-parametric variables. Survival curves were constructed using the Kaplan-Meier method and evaluated using the log-rank test. In addition, the Cox proportional hazards regression model was established to identify factors that were independently associated with overall survival. A *P*-value of less than 0.05 was considered statistically significant.

Results

Egfl7 was overexpressed in human LSCC tissues

To examine Egfl7 expression in LSCC, 33 cases of LSCC tissues and the corresponding nontumorous tissues (NTs) were measured by qRT-PCR. The results showed that the average expression level of Egfl7 mRNA in LSCC tissues was 2.31 times higher than that in NT tissues (**Figure 1A**). Consistent with the mRNA levels, the Egfl7 protein levels in LSCC tissues were significantly higher than those in NTs (0.98 ± 0.13 versus 0.34 ± 0.08, P=0.001; **Figure 1B**). In immunohistochemical staining, positive Egfl7 expression was mainly localized in the tumor cell cytoplasm (**Figure 1C**) and detected in 81.03 percent of LSCC tissues (94/116).

Egfl7 expression was significantly correlated with tumor stage and lymph node metastasis

We analyzed the correlation between Egfl7 mRNA and protein expression and clinicopathological parameters. As shown in **Table 1**, Egfl7 expression was significantly correlated with tumor stage and lymph node metastasis both in the mRNA (P=0.038 and P=0.014, respectively) and protein levels (P=0.015 and P=0.008, respectively).

Of the 116 LSCC samples analysed immunohistochemically, expression scores were 3+ in 26 cases (22.41 per cent), 2+ in 41 cases (35.34 per cent), 1+ in 27 cases (23.28 per cent) and 0 in 22 cases (18.97 per cent). Using the Mann-Whitney U test, we also found that Egfl7 expression was significantly correlated with tumor stage (P<0.001) and lymph node metastasis (P=0.001), but not significantly correlated with

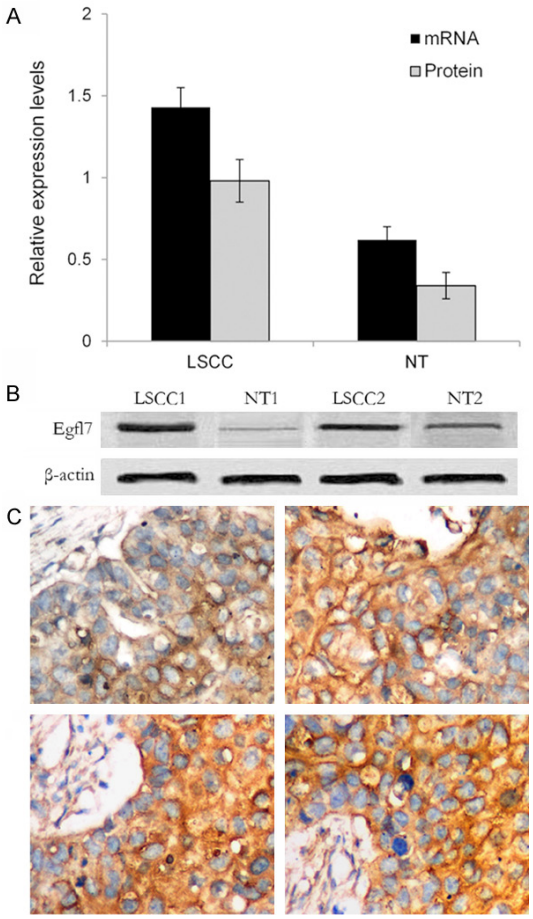

Figure 1. Egfl7 is overexpressed LSCC tissue. A. The qRT-PCR and western blot results show that the average expression level of Egfl7 mRNA and protein in LSCC tissues were 2.31 and 2.88 times higher than that in NT tissues, respectively. LSCC: laryngeal squamous cell carcinoma; NT: nontumorous tissues. B. The representative western blot results showed that Egfl7 protein in LSCC tissues were significantly higher than those in NT tissues (P=0.001, Student's t test). C. Immunohistochemistry of Egfl7 expression in LSCC tissues (Original magnification 400×).

gender, age, tumor primary site or histological differentiation (P>0.05 for all, **Table 2**).

Correlation of Egfl7 expression with MVD

We also analyzed the correlation between tumor MVD and clinicopathological parameters, the results showed that MVD in LSCC tissues was significantly correlated with tumor stage (P<0.001, **Table 2**) and lymph node metastasis (P=0.001). By using the Spearman's correlation coefficient, we found that there was a significant correlation between Egfl7 expression and tumor MVD in the 116 LSCC patients (r=0.623, P<0.001, **Figure 2**). These results suggest that overexpressed Egfl7 may promote tumor angiogenesis.

Correlation of Egfl7 expression with prognosis

According to the immunohistochemistry results, the 116 LSCC patients were divided into two groups: 49 patients with low Egfl7 expression (scores of 0 or 1+) and 67 patients with high Egfl7 expression (scores of 2+ or 3+). Using Kaplan-Meier analysis, we found that that increased Egfl7 expression correlated well with a worse overall survival rate (log-rank test, P=0.004) and disease-free survival rate (log-rank test, P=0.011, **Figure 3**). To further investigate the correlation between Egfl7 expression and prognosis, univariable and multivariable Cox regression analysis were performed. As shown in **Table 3**, Egfl7 expression (relative risk =1.74, P=0.012) and lymph node metastasis (relative risk =1.52, P=0.015) were both found to be independent prognostic factors for LSCC.

Suppression of Egfl7 expression by siRNA

To understand how Egfl7 overexpression contributes to the progression of LSCC, we employed siRNA to suppress the expression of Egfl7. Five selected LSCC cell lines were screened for Egfl7 protein expression by western blot. As shown in **Figure 4A**, Hep-2 cells exhibited the highest expression level of Egfl7 than the other 4 cell lines, thus was selected for the following study. The efficacy of transfection was detected by measuring the percentage of the fluoresc Then, Hep-2 cells were transfected with pLenti6.3-MCS/V5-DEST lentivirus containing sequence 3 and the blank lentivirus, respectively. Blasticidin was used to screen the transfected cells for 2 weeks (**Figure 5A**). The silencing effects were evaluated by using qRT-PCR and Western blot analysis. We observed that the expression of mRNA was suppressed by 94%, and protein by 88% in cells transfected with target lentivirus, while the blank control showed no changes (P>0.05) (**Figure 5B**, **5C**). For convenience, the transfected cells were termed Hep-2$^{\text{Lenti-NC}}$ and Hep-2$^{\text{Lenti-Egfl7}}$, respectively.

Suppression of Egfl7 inhibits cell proliferation by inducing apoptosis and S phase arrest

Since Egfl7 expression correlated well with tumor stage, we doubted whether Egfl7 suppression could impact the ability of cell prolif-

Table 1. Correlations between Egfl7 mRNA and protein expression and clinicopathologic variables of LSCC

Clinicopathologic variables	n	Egfl7 mRNA		Egfl7 protein	
		Expression levels	*P* value	Expression levels	*P* value
Gender					
Male	30	1.46 ± 0.63	0.471	1.03 ± 0.15	0.918
Female	3	1.13 ± 0.23		1.00 ± 0.23	
Age (y)					
≤60	27	1.40 ± 0.50	0.263	1.02 ± 0.33	0.774
>60	6	1.63 ± 0.73		1.04 ± 0.44	
Tumor site					
Supraglottic	11	1.31 ± 0.61	0.754	0.85 ± 0.27	0.386
Glottic	18	1.48 ± 0.63		0.99 ± 0.31	
Subglottic	4	1.56 ± 0.67		1.15 ± 0.34	
Tumor stage					
T1-2	21	1.18 ± 0.41	0.038	1.09 ± 0.44	0.015
T3-4	12	1.53 ± 0.62		1.85 ± 0.35	
Tumor differentiation					
Well	22	1.17 ± 0.44	0.179	1.05 ± 0.35	0.346
Moderate	8	1.23 ± 0.32		1.12 ± 0.13	
Poor	3	1.59 ± 0.64		1.87 ± 0.26	
Lymph nodes					
N0	23	1.21 ± 0.59	0.014	1.20 ± 0.39	0.008
N+	10	1.68 ± 0.62		1.97 ± 0.33	

Table 2. Correlations between Egfl7 expression and clinicopathologic variables, MVD of LSCC

Clinicopathologic variables	n	Egfl7 expression levels					MVD	*P* value
		0	1+	2+	3+	*P* value		
Gender								
Male	107	17	26	39	25	0.485	41.32 ± 13.15	0.647
Female	9	5	1	2	1		40.65 ± 10.63	
Age (y)								
≤60	49	10	11	15	13	0.723	40.79 ± 12.64	0.742
>60	67	12	16	26	13		42.13 ± 14.02	
Tumor site								
Supraglottic	45	7	12	14	12	0.065	46.18 ± 8.49	0.128
Glottic	64	14	13	24	13		34.37 ± 15.23	
Subglottic	7	1	2	3	1		48.28 ± 9.64	
Tumor stage								
T1-2	52	12	16	17	7	0.001	33.24 ± 6.85	<0.001
T3-4	64	10	11	24	19		54.98 ± 12.57	
Tumor differentiation								
Well	95	20	23	31	21	0.731	41.12 ± 12.33	0.256
Moderate	13	1	3	6	3		37.29 ± 10.53	
Poor	8	1	1	4	2		46.18 ± 9.24	
Lymph nodes								
N0	50	13	12	17	8	0.002	36.15 ± 8.13	0.001
N+	66	9	15	24	18		50.72 ± 9.63	

MVD: microvessel density.

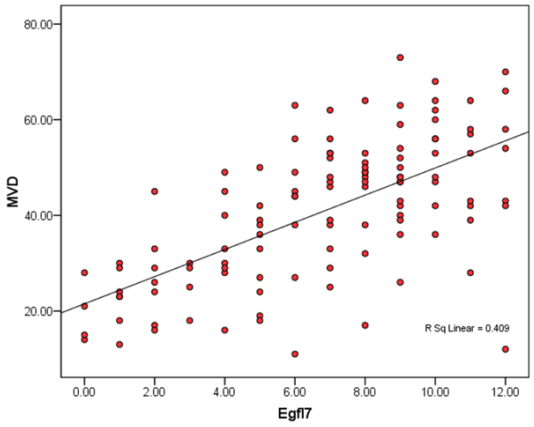

Figure 2. Correlation between tumor MVD and Egfl7 expression levels. Based on the quantifications of immunohistochemistry signals from the 116 LSCC samples, scatter plots were used to depict the correlations between Egfl7 and MVD (r=0.623, P<0.001, Spearman's correlation coefficient).

eration. As shown in **Figure 6A**, CCK-8 cell viability assay showed that the viability of Hep-$2^{Lenti\text{-}Egfl7}$ was significantly decreased than that of Hep-$2^{Lenti\text{-}NC}$ (P<0.01, **Figure 6A**), suggesting a possible role of Egfl7 in promotion of Hep-2 cell proliferation. To further study the growth inhibition mechanism of Egfl7 RNAi, the cell cycle and apoptosis were determined by flow cytometry. When Egfl7 expression was suppressed, there were significant increases in relative numbers of cells in S phase from 17.49% in Hep-$2^{Lenti\text{-}NC}$ to 43.48% in Hep-$2^{Lenti\text{-}Egfl7}$ (**Figure 6B**). Subsequently, cell apoptosis was quantitated using the 7-AAD apoptosis assay. Our results showed that there was a large increase in 7-AAD positive cells (66.2 ± 1.28)% when the Egfl7 expression was suppressed in Hep-$2^{Lenti\text{-}Egfl7}$, which was statistically significantly different compared with Hep-$2^{Lenti\text{-}NC}$ cells (9.86 ± 2.13)% or Hep-2 cells (6.09 ± 3.28)% (P<0.001, **Figure 6C**).

Suppression of Egfl7 inhibits invasiveness of Hep-2 cells

To evaluate the roles of Egfl7 in regulation of cell invasion, Hep-2, Hep-$2^{Lenti\text{-}NC}$ and Hep-$2^{Lenti\text{-}Egfl7}$ cells were examined using a transwell invasion assay. As shown in **Figure 6D**, the numbers of Hep-$2^{Lenti\text{-}Egfl7}$ cells that passed through the Matrigel were significantly lower than that of Hep-$2^{Lenti\text{-}NC}$ cells and Hep-2 cells (P<0.05), suggesting a role for Egfl7 in regulation of cell invasive ability in vitro.

Discussion

Egfl7, which was initially identified as a novel epidermal growth factor (EGF) domain gene and specifically expressed by endothelial cells, was found to involve in the process of gradual separation and proper spatial arrangement of the angioblasts and played a crucial role in vascular tubulogenesis [10]. In mature body, Egfl7 is expressed in highly vascularized adult tissues such as the lung, heart, uterus, and ovary, and is down-regulated in most of the mature vessels in normal adult tissues [9]. As a novel pro-angiogenic factor, Egfl7 is essential for angiogenesis during embryonic development and is up-regulated during tumorigenesis [10].

In the present study, we examined Egfl7 expression by qRT-PCR, western blot and immunohistochemistry in human LSCC tissues. Our results showed that both Egfl7 mRNA and protein levels were significantly higher in LSCC tissues than those in NT tissues (P<0.05). In addition, the level of EGFL7 expression for both mRNA and protein were significantly correlated with tumor stage and lymph node metastasis, indicating the possible role of Egfl7 in LSCC development. Immunohistochemical staining demonstrated the positive expression of Egfl7 in LSCC tissues, these expression pattern has also been observed in hepatocellular carcinoma [14] and malignant glioma [15]. The overexpression of Egfl7 in tumor tissues may be partly due to the heterogeneity of Egfl7 promoter methylation, as described by Azhikina et al. [23] in non-small cell lung cancer.

Analysis of the correlation between Egfl7 expression and the clinicopathologic variables in 116 LSCC patients showed that Egfl7 expression was significantly correlated with tumor stage (P<0.001) and lymph node metastasis (P=0.001), which are widely accepted prognostic factors for LSCC [24]. Using Kaplan-Meier analysis, we found that that increased Egfl7 expression correlated well with a shorter overall survival and disease-free survival period. A multivariate Cox regression model revealed that high Egfl7 expression is an independent risk factor for the prognosis of LSCC patients, suggesting that increased expression of Egfl7 may be a potential biomarker of poor prognosis in LSCC. Since Egfl7 was firstly found as a pro-angiogenic factor, we thus assessed its relationship with MVD, the results showed that

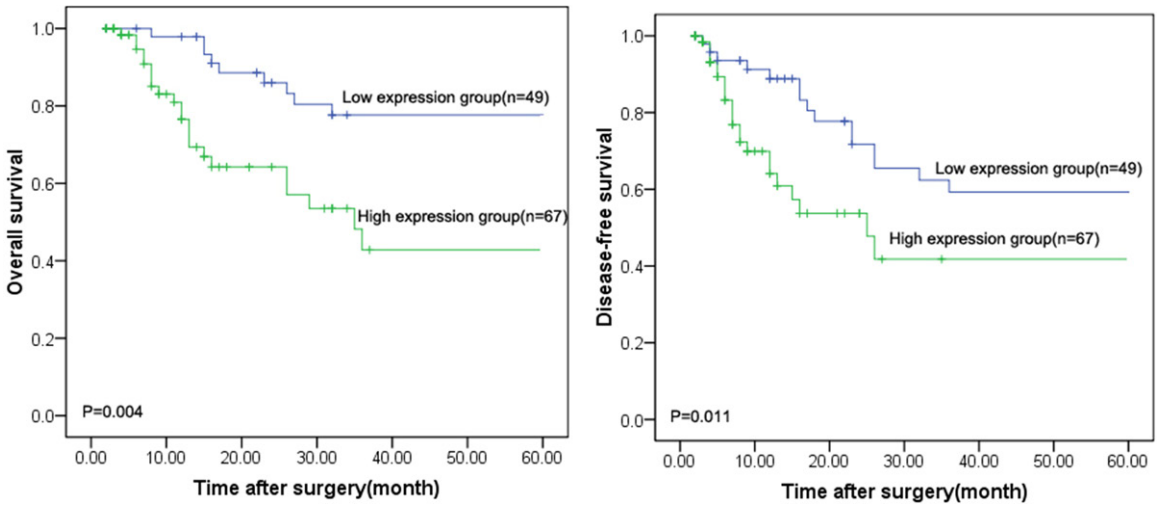

Figure 3. Estimated overall survival and disease-free survival according to the Egfl7 expression in 116 cases of LSCC (Kaplan-Meier method). Cases were classified as either low Egfl7 expression group (score of 0 or 1+, n=49) or high Egfl7 expression group (score of 2+ or 3+, n=67). Log-rank test showed that LSCC patients with the high Egfl7 expression had lower survival than those with the low expression.

Table 3. Multivariate analysis by a Cox proportional hazards regression model

Clinicopathologic variables	n	Univariate		Multivariate	
		RR (95% CI)	*P* value	RR (95% CI)	*P* value
Tumor stage					
T1-2	52	1.00	0.028		
T3-4	64	1.57 (1.03-1.95)			
Tumor differentiation					
Well	95	1.00	0.033		
Moderate and Poor	21	1.42 (0.96-1.49)			
Lymph nodes					
N0	50	1.00	0.027	1.00	0.015
N+	66	1.83 (1.05-2.13)		1.52 (1.08-2.04)	
Egfl7 expression					
Low	49	1.00	0.006	1.00	0.012
High	67	1.94 (1.09-3.05)		1.74 (1.05-2.17)	

there was a significant and positive correlation between Egfl7 expression and MVD, implying that Egfl7 may play a role in promoting tumor angiogenesis. Recently, Delfortrie et al. [25] reported that Egfl7 expression in tumors promote tumor progression by reducing the expression of endothelial molecules that mediate immune cell infiltration, suggesting a novel mechanism through which tumors escape immune control.

To further explore the effect of Egfl7 overexpression on LSCC, we employed human laryngeal carcinoma cell lines to evaluate the correlation between Egfl7 expression and cell viability and invasion in vitro. Our results showed that siRNA mediated knockdown of Egfl7 reduced cell viability and invasion. Moreover, knockdown of Egfl7 resulted in marked increases in relative numbers of cells in S phase as well as apoptotic cells, indicating that Egfl7 siRNA induces cell cycle blockage and apoptosis, thereby inhibiting the proliferation and growth of LSCC cells.

Based on the two centrally located epidermal growth factor (EGF)-like domains included in the structure of Egfl7 [26], it is predicted that Egfl7 may interact with the cell surface receptor like EGF [14]. Schmidt et al. [12] firstly

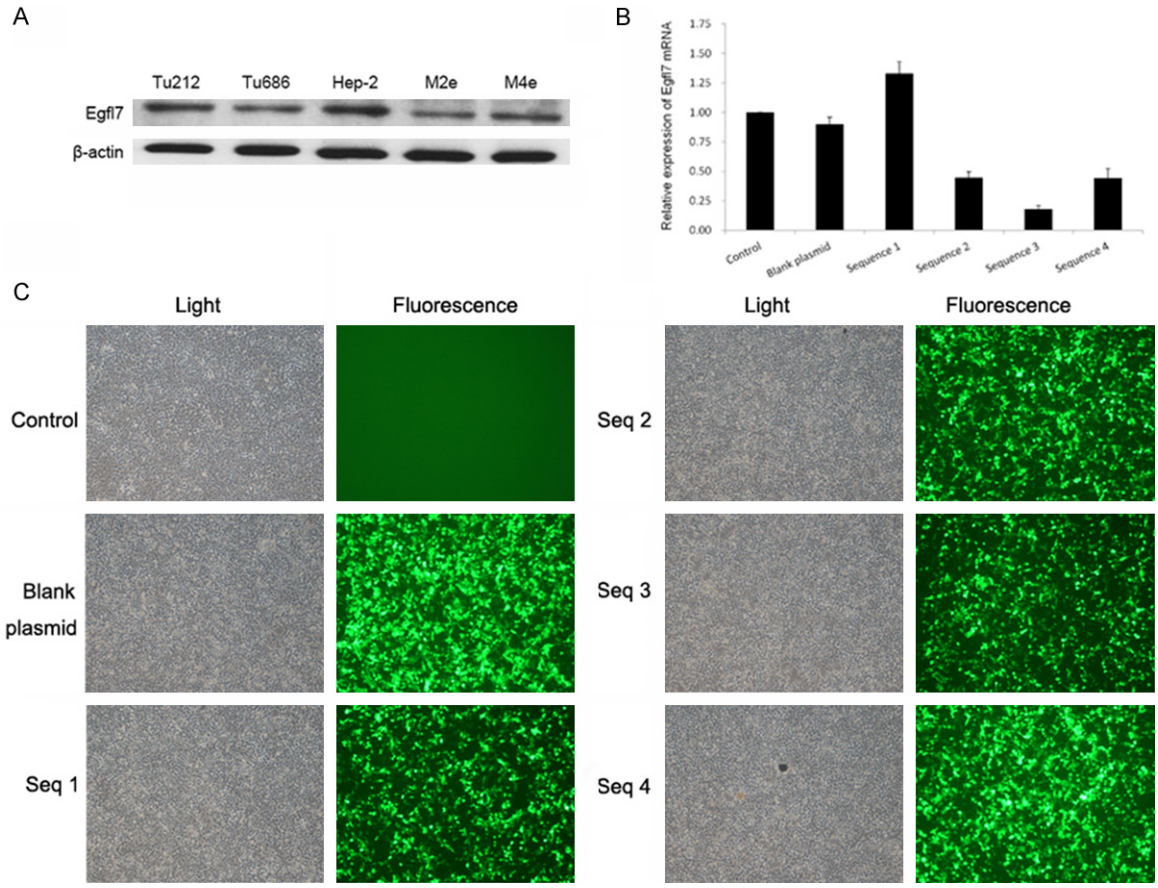

Figure 4. Evaluation of inhibition efficiency of the four candidate sequences. A. Five selected LSCC cell lines were screened for Egfl7 protein expression by western blot. Hep-2 cells exhibited the highest Egfl7 protein expression and were selected for RNA interference. B. The efficacy of transfection was detected by measuring the percentage of the fluorescent cells, which were transfected by the plasmids containing the GFP gene. Results showed the efficacy of transfection of these plasmids to be >80% (Original magnification 10×). C. qRT-PCT was assessed to compare the ability of four candidate siRNA sequences to inhibit Egfl7 expression. Our results showed that sequence 3 inhibited the Egfl7 mRNA more than 80%, while sequence 1 did not cause decreases of Egfl7 expression. The other two sequences, candidates 2 and 4, only resulted in 50%-60% inhibition efficiency.

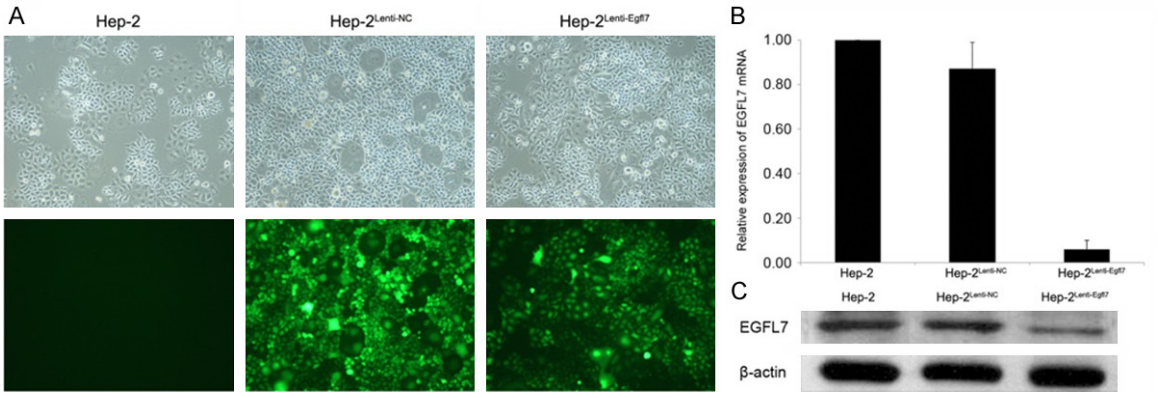

Figure 5. Suppression of Egfl7 by siRNA in Hep-2 cells. A. Hep-2 cells, which were transfected with target lentivirus or blank lentivirus, were screened by blasticidin for 2 weeks (Original magnification 10×). The transfected cells were termed Hep-2$^{Lenti\text{-}NC}$ and Hep-2$^{Lenti\text{-}Egfl7}$, respectively. B. qRT-PCR confirmed that the markedly decreased expression of Egfl7 mRNA in Hep-2$^{Lenti\text{-}Egfl7}$ cells compared with Hep-2$^{Lenti\text{-}NC}$ cells, which showed a 94% inhibitory efficiency. C. Western blot showed that the expression of Egfl7 protein was inhibited by 88% in Hep-2$^{Lenti\text{-}Egfl7}$ cells.

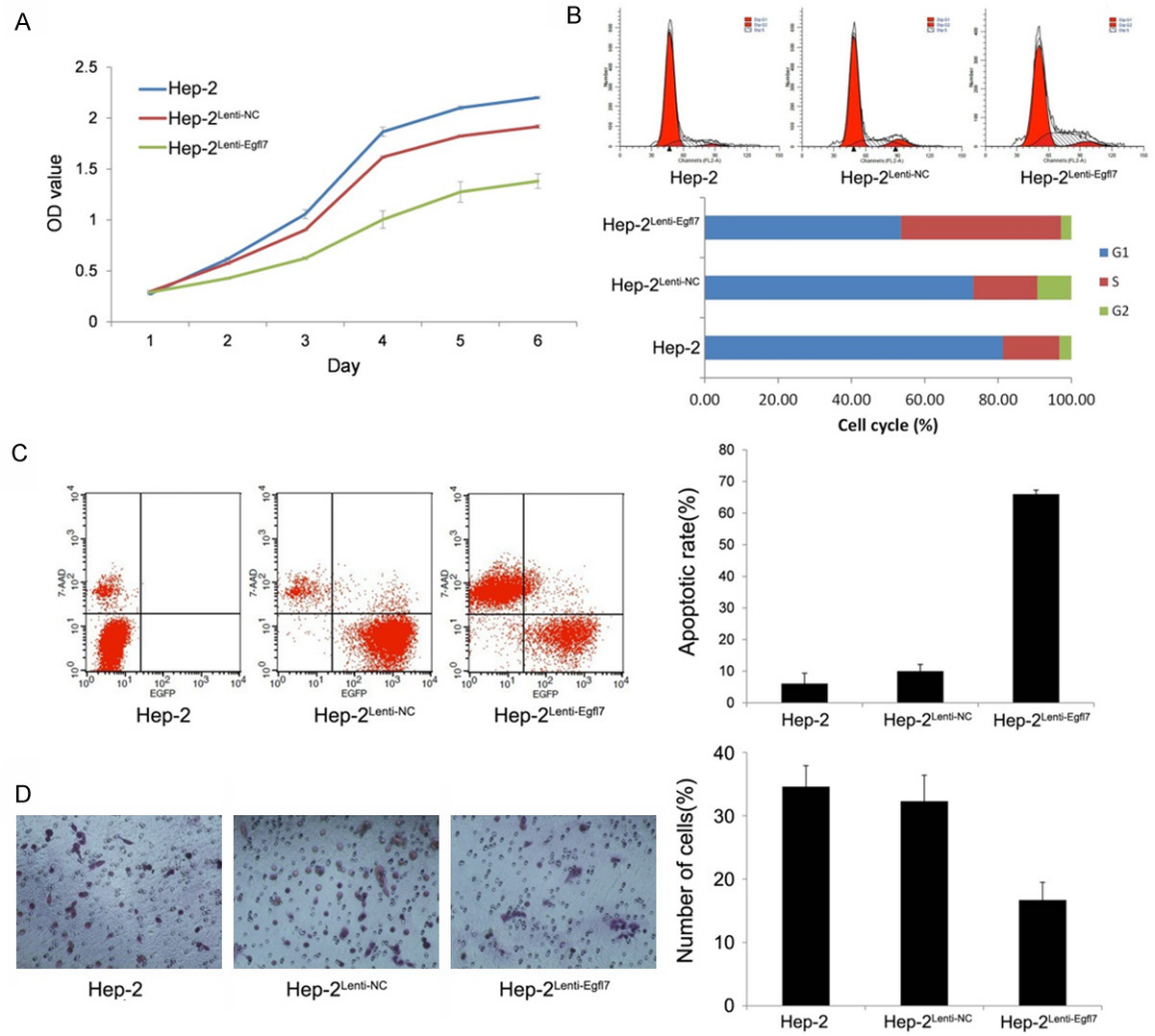

Figure 6. Effect of Egfl7 suppression on the biological characteristics of LSCC cells. A. CCK-8 cell viability assay was performed to investigate proliferation of Hep-2$^{Lenti\text{-}Egfl7}$ and Hep-2$^{Lenti\text{-}NC}$. Every 24 hours, the absorbencies of the test well were read and cell growth curves were drawn. Results showed that the viability of Hep-2$^{Lenti\text{-}Egfl7}$ was significantly decreased than that of Hep-2$^{Lenti\text{-}NC}$ ($P<0.01$). B. Cell cycle analysis by flow cytometry. The average percentage of cells in G1, S and G2 phases is calculated from 3 independent experiments. Suppression of Egfl7 expression resulted in the significant increases in relative numbers of cells in S phase from 17.49% in Hep-2$^{Lenti\text{-}NC}$ to 43.48% in Hep-2$^{Lenti\text{-}Egfl7}$ ($P<0.01$). C. Cell apoptosis assay by 7-AAD method. Apoptotic cell stained by 7-AAD was significantly increased when Egfl7 expression was suppressed ((66.2±1.28)% in Hep-2$^{Lenti\text{-}Egfl7}$ versus (9.86±2.13)% in Hep-2$^{Lenti\text{-}NC}$). D. The number of cells that migrate through the Matrigel into the lower surface of the polycarbonic membrane was determined in a transwell invasion assay. Cell invasion was significantly less pronounced in Hep-2$^{Lenti\text{-}Egfl7}$ cells than in Hep-2$^{Lenti\text{-}NC}$ cells ($P<0.05$).

reported that focal adhesion kinase (FAK) phosphorylation, a critical event in processes of cell migration, adhesion and growth of endothelial cells [27], was significantly reduced in Egfl7-deficient mice, indicating that Egfl7 may promote cell motility through facilitating FAK phosphorylation. Wu et al. [14] further found that, in hepatocellular carcinoma, Egfl7 enhance cell motility through EGFR mediated FAK phosphorylation, indicating that the Egfl7/EGFR/FAK pathway may be critical in controlling cell motility in malignancies. The two EGF-like domains in Egfl7 also comprise a region that is similar to the Delta-Serrate-LAG-2 (DSL) domain, a sequence that is conserved in ligands that bind Notch [9, 28]. Using a transgenic mouse model, Nichol et al. [29] proved that Egfl7 exert its effects on vascular development by signaling to Notch receptors on endothelial cells in an autocrine manner or a paracrine manner. Furthermore, overexpression of Egfl7 represses Notch reporter activity and inhibits Jagged1/

Jagged2-mediated Notch activation in vitro [29, 30]. These findings indicate that Egfl7 could inhibit Notch signaling by binding to the Notch receptor or its corresponding ligand, resulting in blocking ligand/receptor interaction [26]. Since Notch signal pathway has been reported to be involved in the head and neck cancer including LSCC [31-33], further effort will need to be made in identification of the mechanism by which Egfl7 regulate Notch signal in tumors.

In conclusion, our study has shown that there was a tumor grade- and lymph node metastasis-dependent up-regulation of Egfl7 in LSCC, and its overexpression is positively correlated with tumor MVD and a poor prognosis of LSCC, suggesting the importance of Egfl7 in the angiogenesis, proliferation, and progression of LSCC. Furthermore, we have demonstrated knockdown of Egfl7 decrease cell viability and invasion by inducing cell cycle blockage and apoptosis. We conclude that Egfl7 may be used as a predictive marker and a potential therapeutic target for LSCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuejian Wang, Department of Otolaryngology, The First People's Hospital of Foshan, 81 Lingnan North Rd, Foshan 528000, Guangdong Province, PR China. Tel: +(86) 757 83162903; Fax: +(86) 757 83163822; E-mail: wyjian@fsyyy.com

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