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Original Article

Attenuation of EGFL7 inhibits human laryngocarcinoma cells growth and invasion

Xiao-Xia Wang[1,2], Xiao-Bao Yao[1], Zhang-Shao Qiang[1], Hong-Liang Zhu[1]

[1]Department of Otolaryngology-Head and Neck Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, P. R. China; [2]Department of Otolaryngology, 451 Hospital of People's Liberation Army, Xi'an 710054, Shaanxi, P. R. China

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Abstract: Aim: To investigate its effect on the proliferation and invasion of laryngeal carcinoma and understand the potential underlying mechanisms to provide new targets for the diagnosis and treatment of recurrent laryngeal cancer metastasis. Methods: We constructed a lentiviral vector expressing EGFL7 specific shRNA, and introduced it in EGFL7 functions were attenuated by a lentiviral vector harboring shRNA targeting at EGFL7 in laryngeal carcinoma cell line Hep-2. Prolifereation and invasion assays were carried out in vitro. And in vivo tumor burden assay was done in nude mice. Results: The expression of EGFL7 was knocked-down by 80% in hep-2 cells transfected by the lentiviral EGFL7 shRNA vector and EGFL7 gene expression was detected by realtime PCR and Western blotting analysis respectively. The flow cytometric analysis showed that arrested the cell cycle in G1 phase, In tumor burden assay, to parental And vector control cells, the survival rates Of nude mice in EGFL7 shRNA group dropped down from the first day after implantation as indicated by MTT assay ($P < 0.05$). The formation and growth rate of xenograft tumor in mice transfected with siRNA against Bmi-1 slowed down significantly. Conclusion: Attenuation of EGFL7 function significantly suppresses tumor growth and induces apoptosis, both in vitro and in vivo. EGFL7 may be play a key role in invasion and metastasis of Laryngeal squamous cell carcinoma (LSCC), thus would to be a new target for gene therapy in LSCC.

Keywords: Apoptosis, laryngeal cancer, short interfering RNA, EGFL7, invasion and metastasis

Introduction

Laryngeal cancer is one of the most common malignant tumors of head and neck, although surgical procedure and radiation extend patient survival laryngeal cancer, the radiation and chemotherapy can make a small part of the late recurrence cases or prolong survival time or save lives, but there are still 30% to 40% of the patients with laryngeal cancer died of recurrence or distant metastasis [1]. Tumor invasion and metastasis were the main cause of Laryngeal cancer patients died within five years, the exact metastasis mechanism is still not very clear. Research [2] shows that the invasion and metastasis of laryngeal cancer occurrence is a extremely complex and multistep process, involved in cancer cells attaching, degradation, angiogenesis and mobile multiple events. Despite the most aggressive current treatment (concurrent chemo-radiotherapy), recurrence and distant metastasis remain the major causes for laryngeal carcinoma deaths [3]. Together with the undesirable side-effects from conventional therapies, development of a more specific targeted therapy for laryngeal carcinoma is needed.

The development of novel strategies is required for prevention and early detection, and to reduce cancer incidence and overcome problems associated with treatment of late-stage tumors is necessary. Improved prediction of outcome will encourage making the treatment decisions that prolong patients' survival and quality of life.

Tumor cell malignant transformation is closely related to abnormal cell proliferation, differential inhibition and apoptosis blocked. Tumor growth, invasion and metastasis depend on angiogenesis [4]. Regulating angiogenesis me-

chanisms which need further research is still lack of enough understanding, although known many factors directly or indirectly influence the formation of blood vessels. Recent studies have found a kind of specific growth factors secreted by endothelial cells analogues, named as epidermal growth factor domain 7 (EGFL7) [5] preliminary studies suggest that the main function may be play an important role in the process of vascular development [6], but the specific mechanism needs further research. Studies have shown that EGFL7 high expression in tumor [7], whether or not to participate in the head and neck tumor growth form has not been reports. Invasion and metastasis of tumor is the key factor in patients with malignant tumor with poor prognosis, at the same time it is the main cause lead to death of patients [8, 9]. Tumor cell invasion and metastasis mechanism research has become one of the hot spots of basic and clinical research. Targeted blocking the invasion and metastasis of tumor cells will be a target to solve the problem of tumor recurrence and metastasis.

Eexpression of EGFL7 in tumor, its function and relationship with tumor metastasis remains to be further study. At the same time, it also can provide a new potential biological treatment to finally overcome cancer recurrence, metastasis and poor prognosis.

Our previous studies also demonstated that EGFL7 was overexpressed in laryngeal cancer [10].Therefore, EGFL7 constitutes an important therapeutic target for molecular therapy of laryngeal cancer. Up to date, a few reports using RNAi based approach for treatment of laryngeal cancer had published elsewhere. In human cancer, this specificity of expression as Egfl7 has been detected in tumor cells themselves and endothelial cells. Its expression levels correlate with a higher tumor grade in glioma [11] and colon cancer patients [12], and with a poorer prognosis and higher metastatic score in hepatocarcinoma patients [13]. Although these observations suggested a role for Egfl7 in cancer progression, its direct role in tumor development has not still been studied clearly.

The few previous studies that have described the expression of Egfl7 in human cancers all suggested that Egfl7could promote tumor growth and metastasis [14], but no experimental study had addressed the direct role of Egfl7 in tumor development. Data [15] indicate that the effects of Egfl7 on tumor growth and metastasis are rather indirect.

Materials and methods

Reagents

Anti-EGFL7, anti-MMP-9, anti-MMP2, anti-VEGF, anti-PIK3CA, anti-cyclinD1, anti-survivin and anti-AKT was from Santa Cruz Biotechnology (CA, USA), anti-actin antibodies from Cal- biochem (NJ, USA).

Cell culture and samples

Hep-2 cell lines were cultured at 37°C and 5% CO_2 incomplete RPMI with 10% FBS (Hyclone, ThermoFisher Scientific, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin and 1 mM sodium pyruvate (Invitrogen, CA, USA).

Patients

A total 42 laryngeal carcinoma and 42 tumor-adjacent epithalium samples were used in this study. The patients underwent surgical procedure at the Department of Otolaryngology-Head and Neck Surgery, The First Afiliated Hospital, School of Medicine, Xi'an Jiaotong University, from May 2008 to November 2008, and their clinical follow-up records were available. The samples were formalin-fixed and paraffin-embedded for the immunohisto-chenmical examination. Using semi-quantitative RT-PCR and western blot analysis, the differential expression of genes and their protein products between laryngeal cancer tissues and corresponding adjacent normal tissues was verified respectively. The expression levels of these genes and proteins were investigated for associations with clinicopathological parameters taken from patient data. The tumor extent was classified according to the TMN system by UICC [16]. The grade of tumor differentiation was determined according to the criteria by the WHO [17]. Our institutional Human Ethics Committee approved the study. Informed consent was obtained either from the patient or the patient's family.

Immunohistochemistry

Then the sections were deparaffinized with xylene, rehydrated in graded alcohol. Then 3% hydrogen peroxide was applied to block the

Table 1. Association of the expression of EGFL7 genes with patient clinicopathological data

Charateritics	n	EGFL7		*P*
		(-)	(+)	
Gender				
Male	26	11	15	0.651
Female	16	1	15	
Age (y)				
< 60	29	7	22	0.317
≥ 60	13	5	8	
Smoke				
Yes	26	12	24	0.124
No	16	10	6	
Differentitation				
Well/moderate	14	5	9	0.805
Poor	28	7	21	
Clinical stage				
III	24	6	18	0.078
IIIIV	18	1	17	
T classification				
T1 + T2	22	9	11	0.007
T3 + T4	20	1	19	
Lymph metastasis				
Yes	13	1	12	0.033
No	29	13	16	
Tumor site				
Supraglottic	6	1	5	0.065
Glottic	30	8	22	
Subglottic	6	1	5	

endogenous peroxidase activity. After blocking with 5% dry milk, the primary antibody was applied. After rinsing, immunostaining was performed with an Envision system (Dako, Carpinteria, CA) according to the manufacturer's instructions. Peroxidase activity was visualized by applying the diaminobenzidine chromogen, containing 0.05% hudrogen peroxidase. The sections were then counterstained with methy1 green, dehydrated, cleared, and mounted. Negative control staining was carried out by substituting non immune mouse or rabbit serum for the primary antibodies. All of the immunostaining procedures were evaluated in a coded manner without knowledge of the clinical and pathological parameters by three independent observers. For each section, ten high-power fields were chosen, and a total of at least 1000 cells were evaluated. The results were expressed as the percentage of positive cells counted.

For real-time PCR analysis

Total RNA was isolated from hep-2 cells using the RNA queous Microo Kit (Ambion) as permanufacturer's in structions. EGFL7 levels were determined by carrying out reverse transcription as described above, followed by PCR using the following primers: EGFL7 5'-AGCACCTACCGAACCATCTA-3' (forward), 5'-ATCCACATCTGACTGGCAAG-3' (reverse); GAPDH 5'-GAAGGTGAAGGTCGGAGTC-3' (forward), 5'-GAAGATGGTGATGGGATTTC-3' (reverse).

MTT assay

Transfected and control HEp-2 cells (5×10^6/well) were plated into a 96-well plate in octuple. For six consecutive days, 20 μl of MTT (5 mg/ml) were added daily to each well, and the cells were incubated at 37°C for an additional 4 h. The reaction was stopped by lysing the cells with 200 μl of DMSO for 5 min. Quantification was carried out at 570 nm and expressed as a percentage of the control.

Cell cycle analysis

Treated cells were fixed with 70% ethanol, then stained them with propidium iodide (0.05 μg/ml) solution containing RNase A (0.2 mg/ml). Analysis was performed using a FACScan flow cytometer (Becton Dickinson, Bedford, MA).

Western blotting

Western blotting was performed as previously described [18].

Transwell migration and Matrigel invasion assays

Cell migration and invasion were performed using Transwell-chamber or Matrigel-coated modified Boyden inserts (8 μm pore size; Becton Dickinson/Biocoat, MA, USA). Cells were seeded onto the upper chamber (7×10^4 cells/chamber) and maintained in serum-free medium. The cell-containing chamber was immersed in lower chamber containing either serum-free medium or medium supple-mented with 10 ng/ml EGF or 10% FBS, Cells were incubated for 24 h at 37°C. Migrated or invaded cells were stained counted as previously described [19].

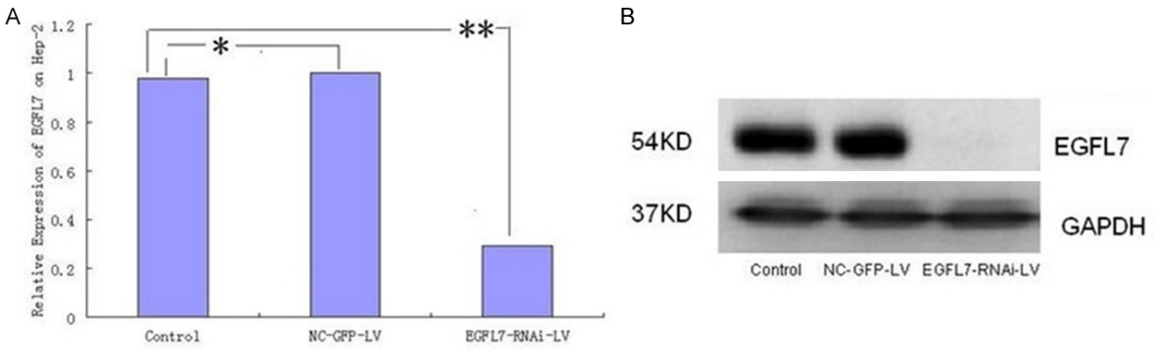

Figure 1. EGFL7-RNAi-LV transfection silenced the mRNA and protein expression of EGFL7 in Hep-2 cells. (A)-ΔΔCt Real-time PCR data were analyzed using the 2 methods. EGFL7 mRNA expression decreased significantly in the cells transfected with EGFL7-RNAi-LV compared to that in the untransfected cells (vs. control group, t = 11.314) or the cells transfected with the negative control (NC) (vs. NC-GFP-LV group, t = 22.776); (B) EGFL7 protein expression detected by western blot analysis. NC, NC-GFP-LV group; KD, cells in which EGFL7 expression was knocked down through EGFL7-RNAi-LV transfection. Data are presented as the mean ± SEM (n = 3, P < 0.01).

Colony formation analysis

Colony formation analysis was performed as previously described [20].

Animal experiments

Allanimal experiments were approved by the institutional Animal Welfare Committee. BALB/c-nu mice (5-6 weeks old; 20 g in weight), provided by the Central Animal Facility of Xi'an Jiaotong University, were bred in an aseptic condition according to standard guidelines. Fifteen mice underwent subcutaneous injection of 100 μL cell suspension of Hep-2 (1 million) in the dorsal scapula region. The size of tumors was blindly measured twice a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid (L × W × 0.5). Once tumors reached approximately 0.5 to 0.6 cm, 5 mice in the experimental group were treated by intratumoral injection of 200 μL EGFL7-RNAi-Lentivirus, 5 mice in the control group were treated with 200 μL of GFP-Lentivirus and other 5 mice in the negative control group were treated with 200 μL of water once a week for 3 weeks. Tumors were harvested from mice 1 week after the end of treatment.

Statistical analysis

SPSS 10.0 was used for statistical analysis. One-way analysis of variance (ANOVA) was used to analyze the significance between groups. The LSD method of multiple comparisons with parental and control vector groups was used when the probability for ANOVA was statistically significant. A *P*-value of 0.05 was considered to indicate a statistically significant difference.

Results

Association of the expression of EGFL7 genes with patient clinicopathological data. Associations between the expression of EGFL7 genes and clinicopathological data from the patients were also established, including age, tumor classification, stage, differentiation and lymph node metastasis (**Table 1**). The higher expression of EGFL7 was no significantly difference with age, gender, tumor differentiation and stage ($P > 0.05$), but was significantly different with tumor classification and lymph node meatastasis ($P < 0.05$).

Our earlier studies have shown that the expressions of Egfl7 mRNA and protein are increased in laryngocarcinoma cell lines and laryngocarcinoma tissues [10]. These constructs reduced expression at the RNA level by 75-95%, and also significantly reduced expression at the protein level (**Figure 1**). To examine our hypothesis, we silenced EGFL7 expression in human Laryngocarcinoma cells, Hep-2, by stable expression of an EGFL7-specific siRNA. In this study, three pairs of shRNA were designed according to EGFL7 sequence in the Genebank (NM_0111045) (**Table 2**), and they demonstrated striking differences in silencing efficiency. The knockdown of EGFL7 expression by short hairpin RNA (shRNA) reduced the cell invasion activity in vitro and tumorigenicity in nude mice. Western blotting for deter-

Table 2. Sequences of siRNAs from human EGFL7 cDNA

NO.	5'	STEMP	Loop	STEMP	3'
Target 1#-1	Ccgg	aaGGAAGAAGTGCAGAGGCTG	TTCAAGAGA	CAGCCTCTGCACTTCTTCCtt	TTTTTg
Target 1#-2	aattcaaaaa	aaGGAAGAAGTGCAGAGGCTG	TCTCTTGAA	CAGCCTCTGCACTTCTTCCtt	
Target 2#-1	Ccgg	gaGTCGTTCGTGCAGCGTGTG	TTCAAGAGA	CACACGCTGCACGAACGACtc	TTTTTg
Target 2#-2	aattcaaaaa	gaGTCGTTCGTGCAGCGTGTG	TCTCTTGAA	CACACGCTGCACGAACGACtc	
Target 3#-1	Ccgg	aCCGAACCATCTATAGGACC	TTCAAGAGA	GGTCCTATAGATGGTTCGGt	TTTTTg
Target 3#-2	aattcaaaaa	aCCGAACCATCTATAGGACC	TCTCTTGAA	GGTCCTATAGATGGTTCGGt	

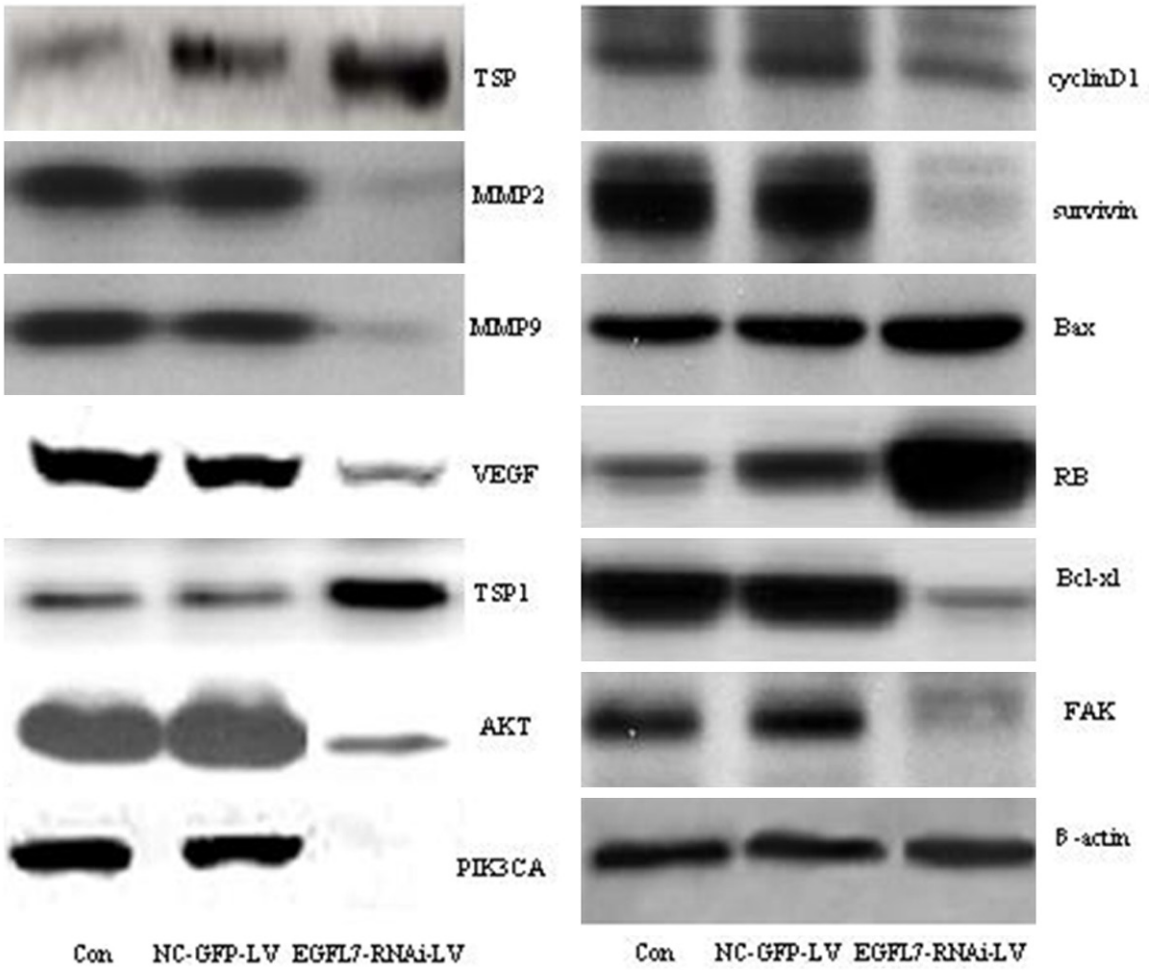

Figure 2. Western blotting for determining levels of TSP and other molecules in hep-2 cells after the treatments. Representative western blots showing levels of TSP, MMP2, MMP9, VEGF, TSP1, PIK3CA, survivin, Bax, RB, Bcl-xl, FAK and AKT. The western blots were reprobed for levels of β-actin to confirm that equal amounts of protein were loaded in all lanes.

mining levels of TSP and other molecules in hep-2 cells.

Bax, RB, TSP and TSP1 levels were increased significantly ($P < 0.05$) in the cells treated with EGFL7 siRNA. MMP2, MMP9, VEGF, PIK3CA, cyclinD1, survivin, Bcl-xl, FAK and AKT expression were decreased significantly ($P < 0.05$) in the cells treated with EGFL7 siRNA (**Figure 2**).

Cell growth was inhibited by down-regulation of EGFL7 expression

The SiRNA transfected cell proliferated at a significantly lower rate than control and parental

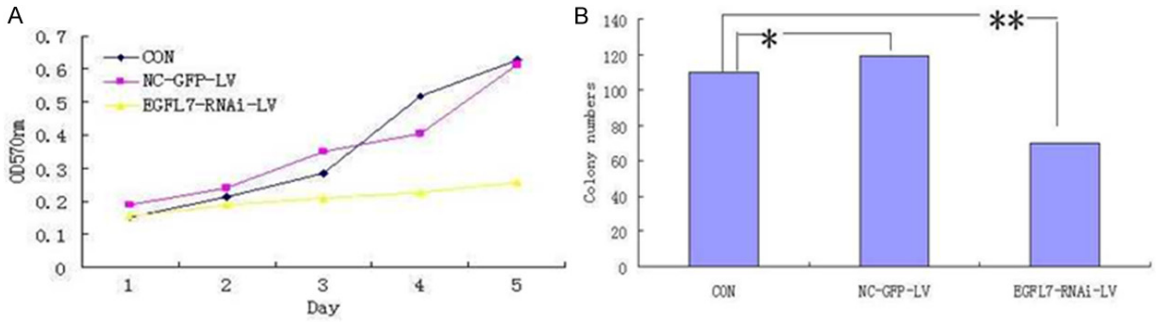

Figure 3. A. Cell growth curves. Cell growth assay was performed demonstrating a significant decrease in cell viability in EGFL7-RNAi cells compared with control cells in a timedepentent manner, and the highest inhibitory rate was 51.25 ± 2.86% for Hep-2; on day 6 ($P < 0.05$). However, there was no obvious difference in cell proliferation between NC group and control group in each cell line ($P > 0.05$). *$P < 0.01$ versus NC-GFP-LV. B. Colony formation. Cells were plated in 6-wells plate at a density of 1,000 cells/well, Number of colonies counted in triplicate with cell number +50 cells on 10th day. The results are presented as mean ± S.E with triplicate measurement.

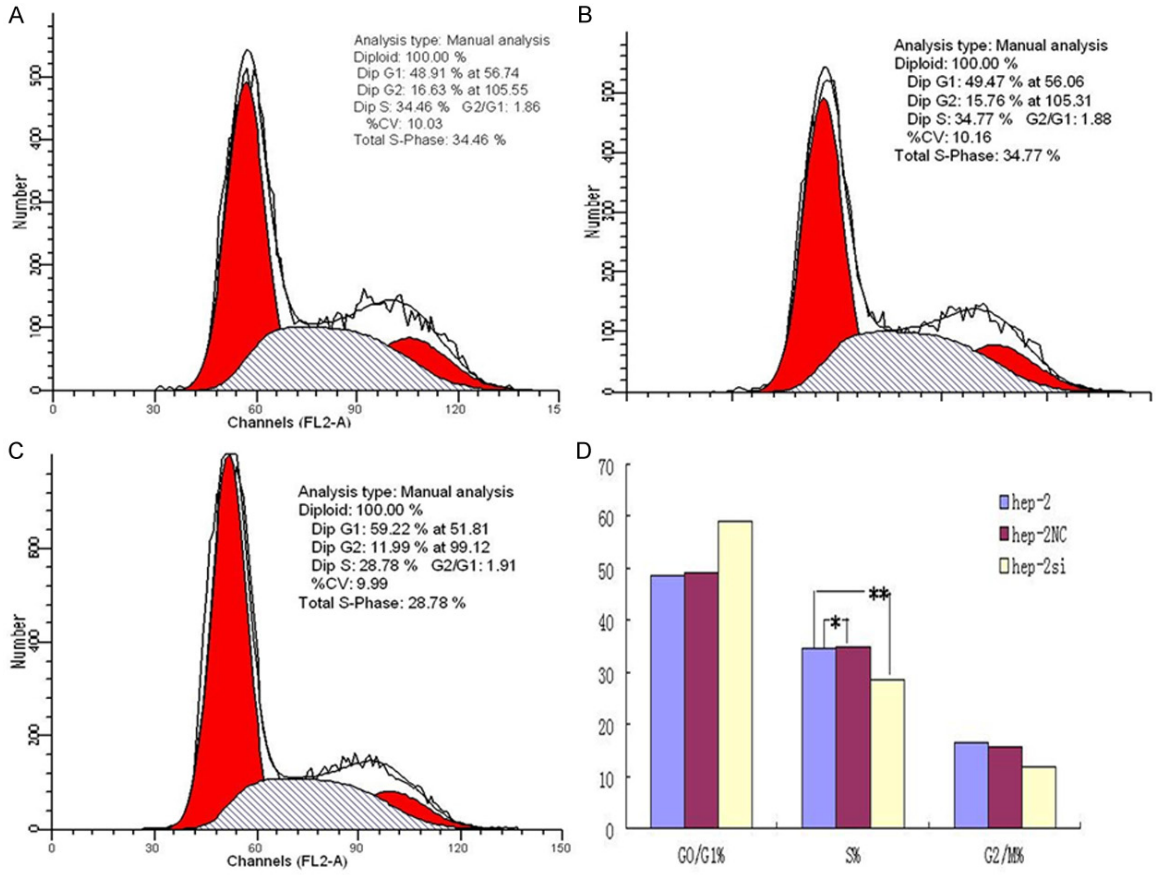

Figure 4. Cell cycle distribution analyses by flow cytometry. DHMEQ-induced G_2/M arrest in Hep-2 cells. Percentage of cells at various phases of cell cycle was shown as mean ± SEM (n = 3). Similar results were obtained in three independent settings for all experiments. Indicates **P < 0.001, EGFL7-si VS Control. *P > 0.05, NC VS Control.

cells measured by MTT assay (**Figure 3A**). Cell growth assay was performed demonstrating a significant decrease in cell viability in EGFL7-RNAi cells compared with control cells in a timedepentent manner, and the highest inhibitory rate was 51.25 ± 2.86% for Hep-2, on day

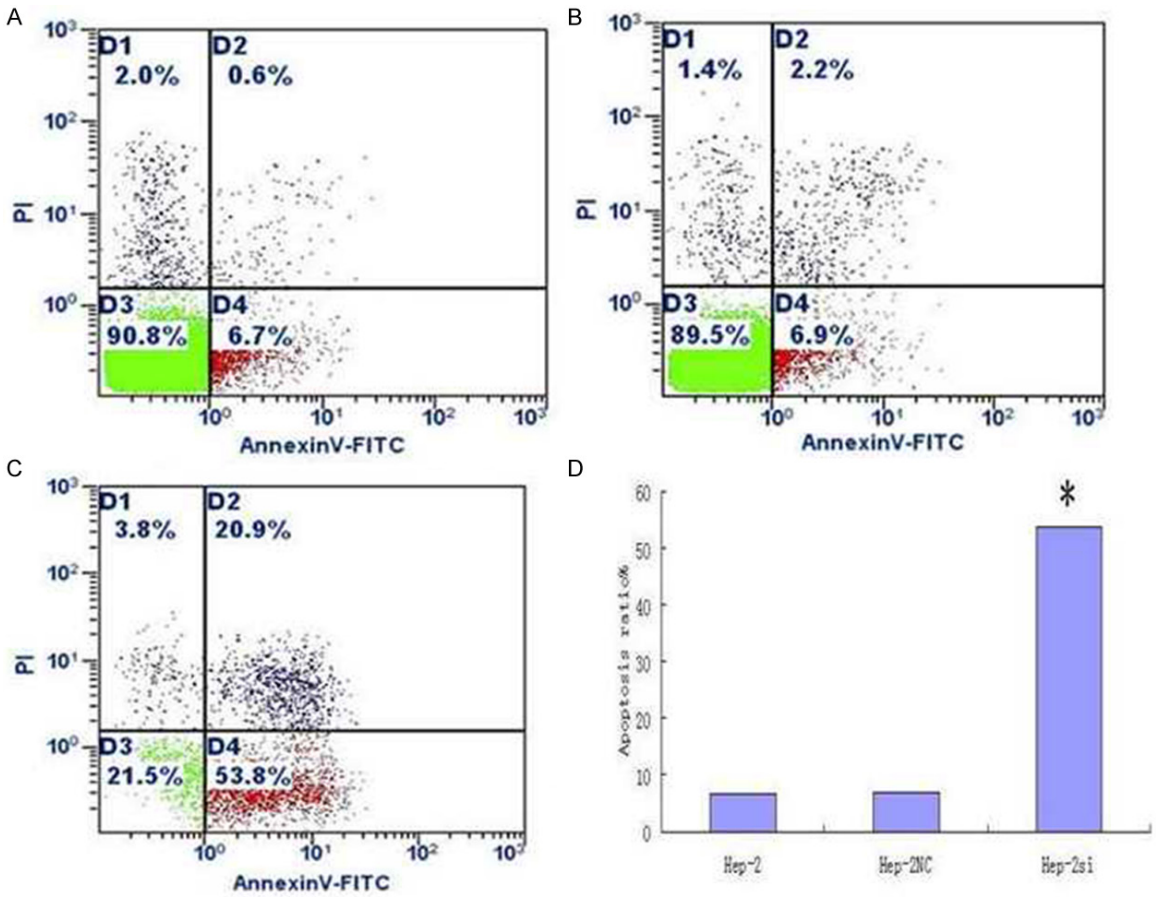

Figure 5. Effects of anti-EGFL7 siRNA on HEp-2 cells discriminated by Annexin V/PI double staining, (A) HEp-2 cells transfected with mock (control), (B) scrambled nucleotide control siRNA (NC-GFP-LV), and (C) HEp-2 cells transfected with EGFL7 siRNA (EGFL7-RNAi-LV). (D) Graph shows the apoptotic ratio in the EGFL7-RNAi-LV group.

6 ($P < 0.05$). However, there was no obvious difference in cell proliferation between NC group and control group in each cell line ($P > 0.05$).

To test potential malignant state of the tumor cell line, colony-forming assay was conducted in Hep-2 cells transfected with mock, Scrambled nucleotide control siRNA (NC-GFP-LV) and Hep-2 cells transfected with EGFL7 siRNA (EGFL7-RNAi-LV) cells. The results showed that the colony numbers of cells transfected with EGFL7 siRNA were decreased compared with parental cells and negative siRNA transfected cells (**Figure 3B**). These results showed that anti-Bmi-1 siRNA had significant growth inhibition effect on Hep-2 cells.

Cell cycle and apoptosis analyses

Cell cycle distribution analysis (**Figure 4**) indicated that significant changes were observed in the EGFL7-RNAi cells, compared with the control group cells; several cells were blocked in the G0/G1 phase by 57.85 ± 0.25% ($P < 0.05$) and reduced in the G2/M phase by 12.98 ± 0.13% ($P < 0.05$), whereas no significant difference was observed in the cell cycle distribution between the negative control and control group ($P > 0.05$). The cells were stained with Annexin V and PI to further evaluate the induction of apoptosis. The proportion of Annexin V stained cells to the total EGFL7 siRNA-transfected cells was increased (**Figure 5**). A small amount of necrotic cells stained with PI, but not Annexin V, was also observed. The apoptotic rates in the experimental group after the cells were treated with RNAi were 53.8, 6. 9 and 6. 7% in the EGFL7-RNAi-LV, NC-GFP-LV group and control group respectively. The apoptotic rate of HEp-2 cells increased to 46.9% ($P < 0.01$) following treatment with Bmi-1-RNAi-LV, whereas no obvious cell apoptosis was observed in the negative control and control group ($P > 0.05$). These results indicated that anti-EGFL7 siRNA induced HEp-2 cells apoptosis.

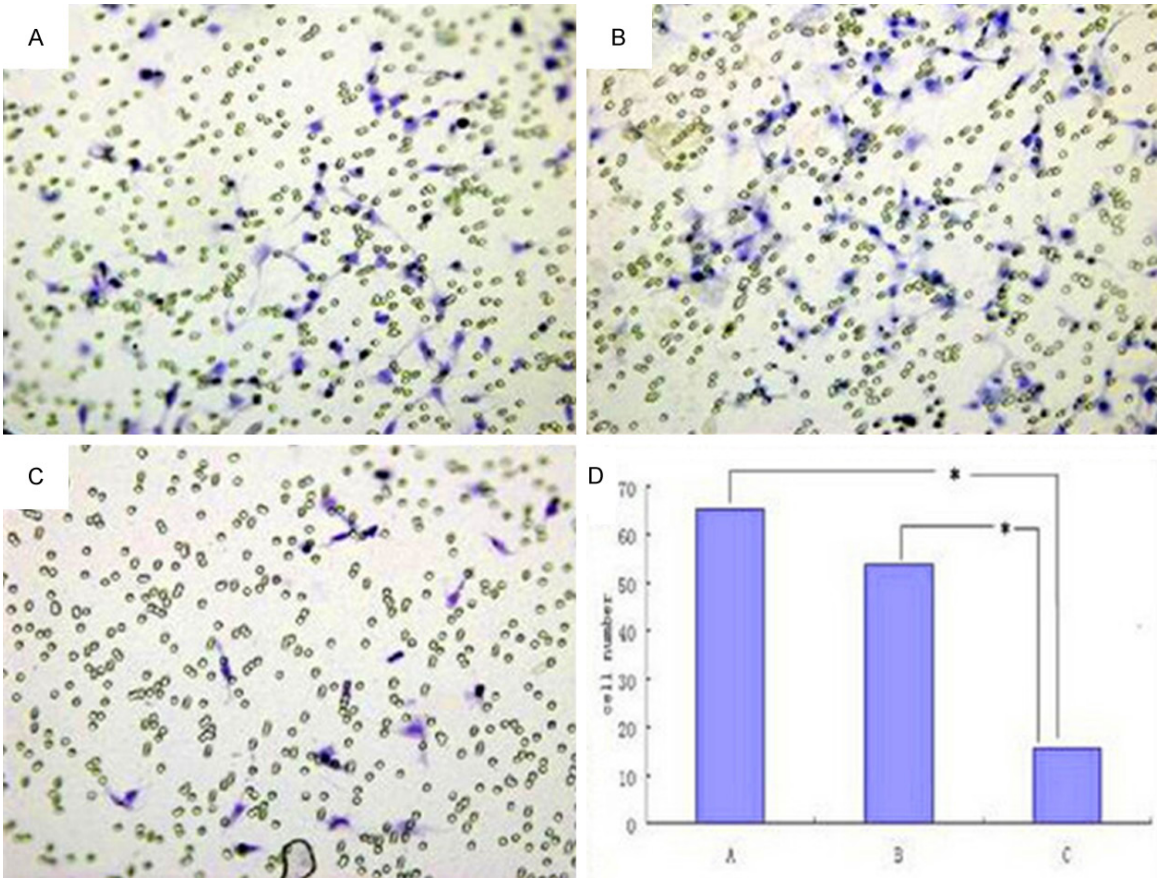

Figure 6. Effect of EGFL7 silencing on the migration of HEp-2 cells, (A) HEp-2 cells transfected with mock (control), (B) scrambled nucleotide control (C) siRNA (NC-GFP-LV), and HEp-2 cells transfected with EGFL7 siRNA (EGFL7-RNAi-LV). (D) Statistical analyses revealed that EGFL7 knockdown by EGFL7-RNAi-LV transfection (EGFL7 KD groups) significantly inhibited cell motility. *$P < 0.05$ EGFL7-RNAi-LV vs HEp-2 cells and NC-GFP-LV.

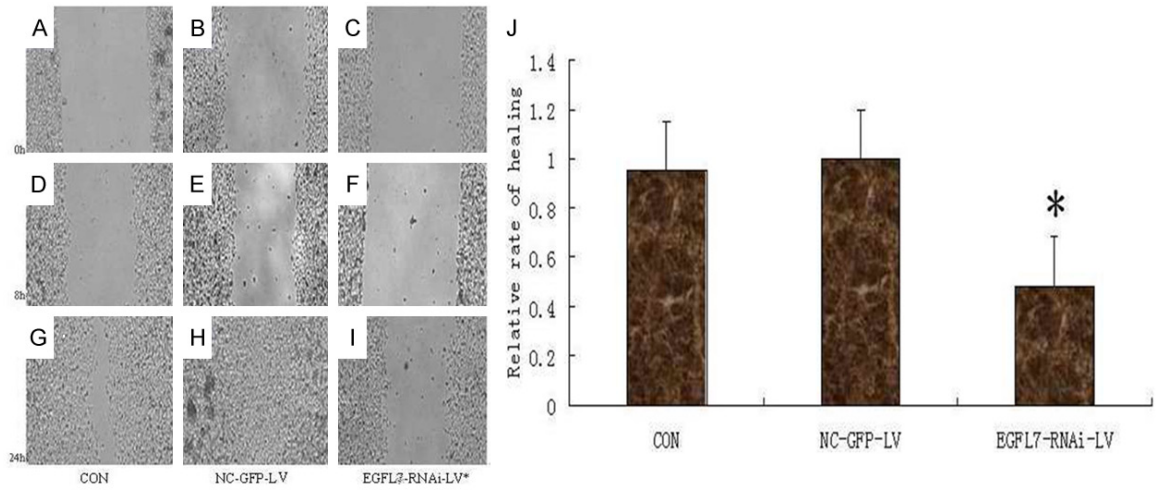

Figure 7. Wound healing assay determined the migration rate of HEp-2 cells transfected with mock, scrambled nucleotide control siRNA (NC-GFP-LV), and HEp-2 cells transfected with Bmi-1 siRNA (EGFL7-RNAi-LV) cells. Migration of the cells to the wound was visualized at 24 h under an inverted Olympus phase-contrast microscope (× 100). The healing rate of the HEp-2 cells transfected with mock cells was normalized to that of the scrambled nucleotide control siRNA cells (*P < 0.05, relative to scrambled nucleotide control siRNA cells).

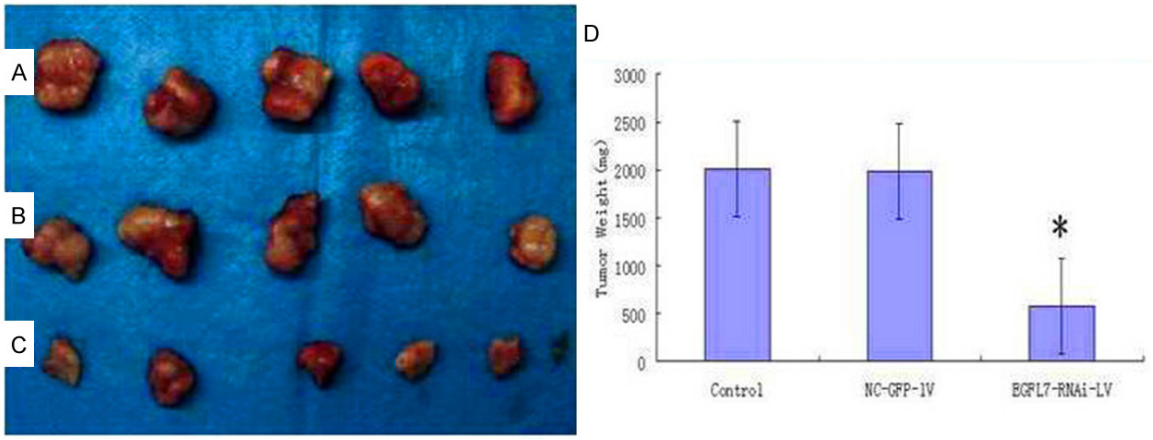

Figure 8. Knockdown of EGFL7 inhibits hep-2 xenograft tumor growth. Three groups cell were injected into right scapular region of nude mice (each group = 5). A. Hep-2 cells transfected with mock (Control). B. Scrambled nucleotide control siRNA (NC-GFP-LV). C. Hep-2 cells transfected with EGFL7 siRNA (EGFL7-RNAi-LV). D. Statistical analyses revealed that the mean tumor weight at the end of the experiment was remarkably lower in the Bmi-1-RNAi-LV group than in the NC group or control group (0.48 ± 0.21 g versus 1.09 ± 0.13 g, 0.99 ± 0.18 g respectively, *P = 0.003 or P = 0.005).

Boyden chamber assay and wound healing assay

On the other hand, we performed an invasion assay in a modified Boyden chamber to examine the levels of the invasive potentials of the Hep-2 cells in vitro. Movement of cells through Matrigel-coated Boyden chambers mimics the early steps of tumor invasion. After cultivation of the cells for 48 h, invasion of 16.5 ± 2.9, 48.4 ± 8.5 and 50 ± 3.5 cells per field of view through the porous transwells was observed for EGFL7-RNAi-LV treated group and control lentivirus respectively (t = 16.44, P < 0.01, **Figure 6**).

Wound healing assay was carried out to test cell migration. Hep-2 cells transfected with EGFL7 siRNA cells had a much slower wound-healing rate compared with Scrambled nucleotide control shRNA (NC-GFP-LV) (0.48 ± 0.046:1, P < 0.05) (**Figure 7**).

Downregulation of EGFL7 inhibit the tumorigenicity of hep-2 cells in vivo

Since EGFL7 confers a growth advantage to hep-2 cells in vitro, its effect in vivo was investigated. hep-2 cells (A), hep-2 cells transfected with Scrambled nucleotide control siRNA (B), EGFL7 knockdown cells (C) were injected into three separate groups of nude mice. Tumor volume was measured every 2 days until the mice were sacrificed on day 21 (**Figure 8**). Group 3 formed substantially smaller tumors in nude mice than the other two groups. At the time of death, the tumor volume of mice injected with EGFL7-RNAi-LV was 247.66 ± 147.39 mm^3 compared to 764.13 ± 224.03 mm^3 or 734.76 ± 265.21 mm^3 of mice injected with hep-2 cells or NC group cells, respectively (P = 0.002 or P = 0.004, respectively). In addition, the mean tumor weight at the end of the experiment was remarkably lower in the EGFL7-RNAi-LV group than in the NC group or control group (0.48 ± 0.21 g versus 1.09 ± 0.13 g, 0.99 ± 0.18 g respectively, *P = 0.003 or P = 0.005). This animal model provided strong support for the role of EGFL7 in Laryngeal carcinoma and suggests that EGFL7 can promote the growth of hep-2 cells. The animal experiment in vivo provides a strong support for the importance of EGFL7 in growth of hep-2.

Tunel assay to detect apoptotic cells in situ

TUNEL method showed that the group A and group B only see scattered apoptotic cells, compared to C group we can see a large number of apoptotic cells (200 ×) (**Figure 9**). Compared to hep-2 group and NC group, P < 0.05.

Expression of Caspase-3 in groups of specimens of tumors

Caspase-3 immunohistochemical results show that the group A and group B only express several positive cells in Caspase 3, group C visible piece of Caspase-3 positive expression cells

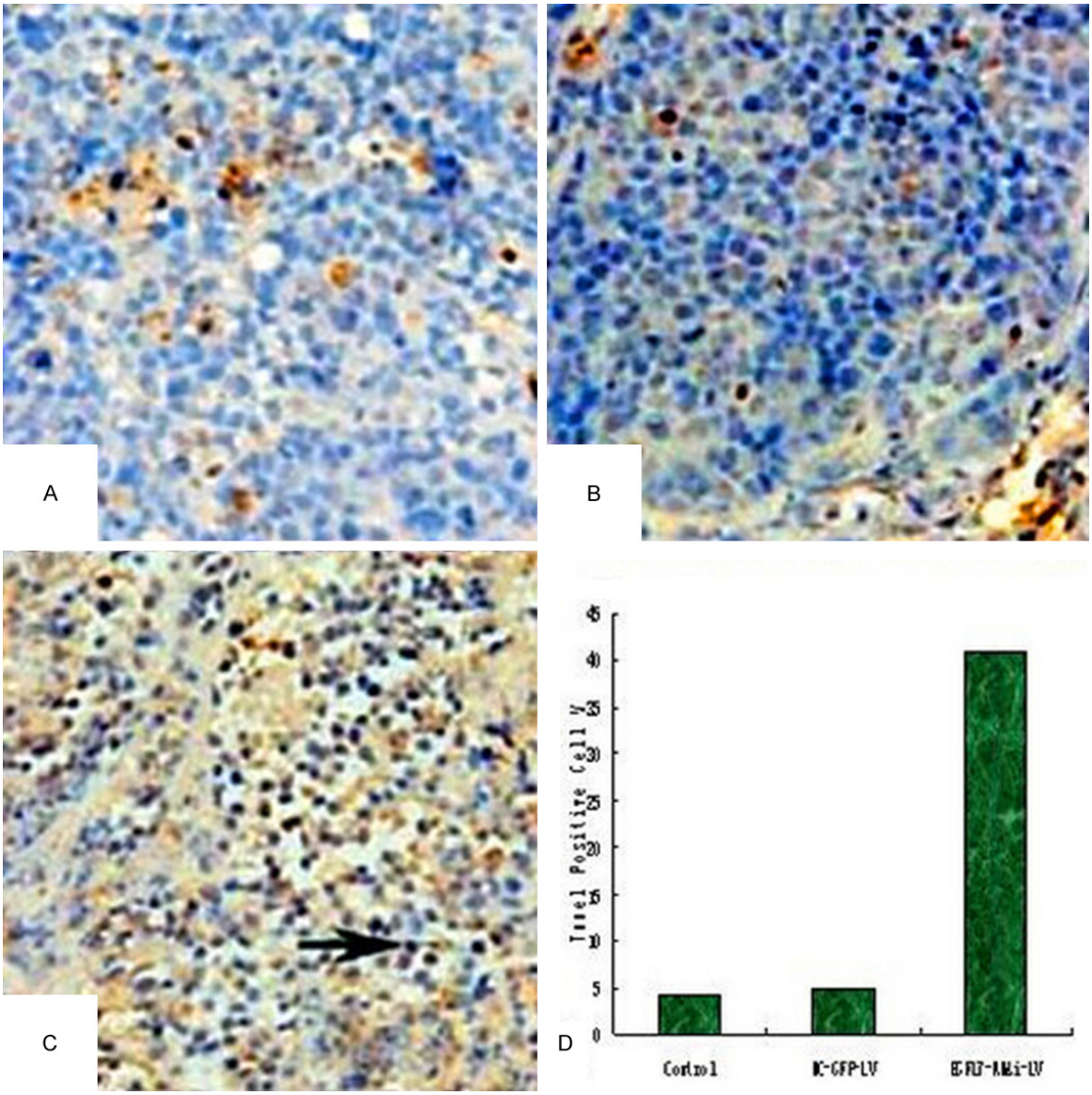

Figure 9. siRNA against EGFL7 increases cell apoptosis in vivo. HEp-2 cells transfected with EGFL7 siRNA, scrambled nucleotide control siRNA or mock (control) were injected into nude mice. TUNEL assay was used to detect apoptotic cells in situ. Dark staining of nuclei indicates apoptosis. Representative TUNEL-positive cells are shown. A. HEp-2 cells transfected with EGFL7 siRNA (EGFL7-RNAi-LV); B. HEp-2 cells transfected with scrambled nucleotide control siRNA (NC-GFP-LV); C. HEp-2 cells transfected with mock (control); D. The percentages of TUNEL-positive cells were scored for HEp-2 cells transfected with EGFL7 siRNA, scrambled nucleotide control siRNA or mock control. *$P < 0.05$ EGFL7-RNAi-LV vs HEp-2 cells and NC-GFP-LV.

(400 ×). *KD group compared with CON group. $P < 0.05$ (**Figure 10**).

Western blot analysis

The western blot analysis results showed the significant inhibitory effect of EGFL7 lentiviral-mediated RNAi on EGFL7, MMP-2, MMP-9, and cyclinD1 protein expression. EGFL7, MMP-2, MMP-9, and cyclinD1 protein expression in the HEp-2 cells trans-fected with EGFL7 lentiviral-mediated RNAi was significant decreased (**Figure 11**).

Discussion

Rapid progress has been made in the use of RNAi and more specifically siRNAs as a means of attenuating the expression of specific proteins both in vitro and in vivo enabling virtually

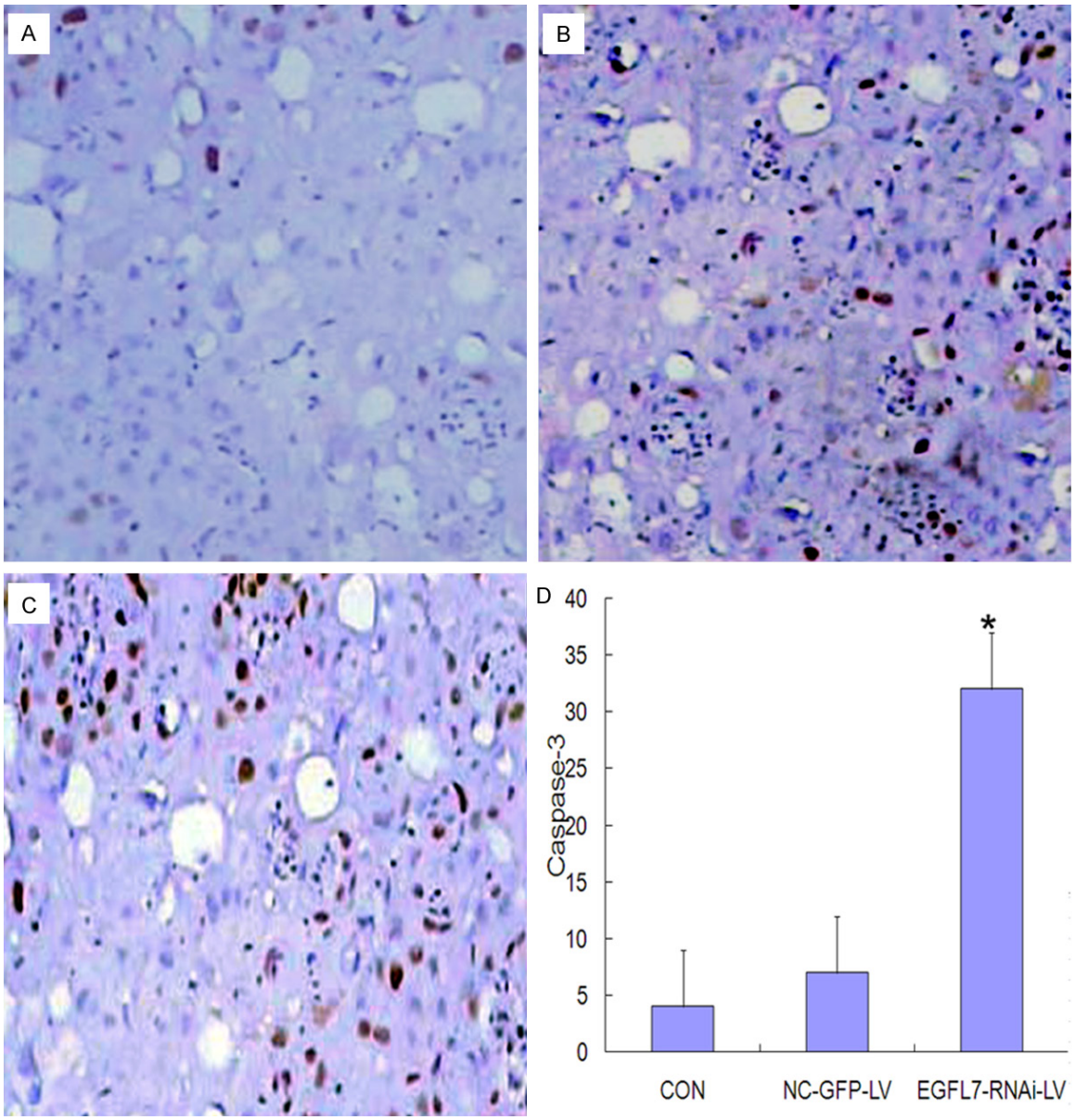

Figure 10. Expression of Caspase-3 in (A) HEp-2 cells transfected with EGFL7 siRNA (EGFL7-RNAi-LV), (B) HEp-2 cells transfected with scrambled nucleotide control siRNA (NC-GFP-LV), (C) HEp-2 cells transfected with mock (control). (D) The Caspase-3 positive cells were scored for HEp-2 cells transfected with EGFL7 siRNA, scrambled nucleotide control siRNA or mock control. *$P < 0.05$ EGFL7-RNAi-LV vs HEp-2 cells and NC-GFP-LV.

any protein target to be inhibited by these sequence-specific, double-stranded RNA molecules [21, 22].

Particularly, patients with LSCC have poor functional outcomes due to the indispensability of the anatomic location, and a 5-year survival rate can be achieved in only about 30-40% of advanced cases [23]. Various treatment strategies have been attempted for managing advanced LSCC according to the tumor location, histological type, clinical stage, and many other biomolecular parameters [24]. Current treatment options (surgery, radiotherapy and/or chemotherapy) are toxic as well as functionally and cosmetically debilitating. Since patients with advanced SCCHN have a high rate of local-regional recurrence and low survival rate with the existing treatment modalities, novel biological therapies, such as gene therapy, have to be developed and tested. Gene therapy may provide an alternative mechanism for controlling the microscopic residual disease with limited or no added toxicity.

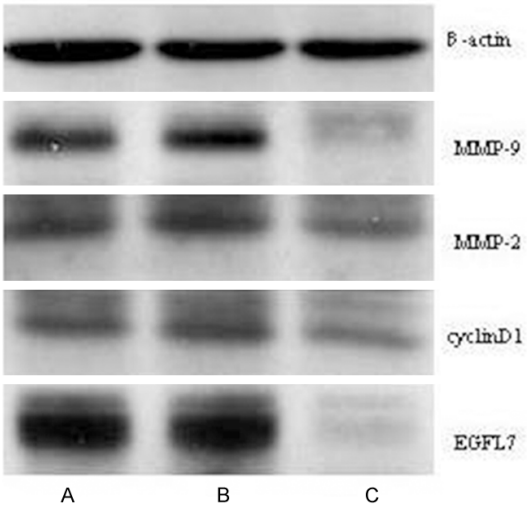

Figure 11. Western blot analysis for determining the levels of EGFL7, MMP-2, MMP-9and cyclinD1 in HEp-2 xenograft tumors. Lane A, control group; lane B, NC-GFP-LV groiup; and lane C, EGFL7-RNAi-LV group.

Cancer invasion and metastasis are complex mechanisms that include cellular transformation and tumor growth, angiogenesis and lymph angiogenesis, and release of cancer cells into the circulation; then attachment, invasion, and proliferation within the new target organ [25]. Tumor cells abnormal enhancement movement ability and organ specificity of metastasis are two key steps in the process of tumor metastasis. The high expression of EGFL7 in tumor tissue and tumor cell lines [26], can significantly affect and enhance the capacity of cell migration, and invasion and metastasis of the tumor is closely related to the key steps [27]. At the same time, the solid tumors especially rich vascular tumors such as liver cancer tissue to resist oxygen pressure, resulting in a large number of new blood vessels, EGFL7 as a relatively specificity protein of new blood vessels endothelial cells is certainly secrete. Although it can also further boost the sports ability of tumour cells of EGFL7 expression in tumor with paracrine way, but its function and relationship with tumor metastasis remains to be further in-depth study. Also it is expected to finally overcome cancer which is easy recurrence, metastasis, poor prognosis and can provides a new potential therapeutic targets.

At the same time, blood vessels are essential for the growth and metastasis of the most solid tumors. Within a tumor, new blood vessels, formed mostly by endothelial cells, sustain the nutriment and oxygen supplies of the developing mass [28]. The tumor endo thelium forms an imperfect, tortuous, and leaky barrier [29]. Metastatic cells enter the blood circulation through this endo-thelium (intravasation) and spread across the organism as an alternative route to lymphatic metastasis [30-32].

Recent study [33] show that incresed EGFL7 protein expression is associated with laryngeal squamous cell carcinoma stage, lymphnode metastasis and poor survival which consistent with the results of our previous study [10].

Our data showed a significant decrease of in vitro invasive abilities and viability of Hep-2 cells after EGFL7 shRNA transfection in a modified Boyden chanmber and MTT assay. This suggests an implication of this regulation of an invasive phenotype in LSCC cells by EGFL7 expression. To investigate in vivo the inhibitive role of EGFL7-RNAi-Lentivirus on LSCC, BALB/c-nu mice with induced tumor of Hep-2 cells had been treated by EGFL7-RNAi-Lentivirus. Results showed that both the average tumor weight and volume were significantly lower in the EGFL7-RNAi-Lentivirus treated group than those in the control group, and the rate of tumor inhibition was about 45%, which gave us the evidence that the EGFL7 gene silencing could partially inhibit the growth of LSCC in vivo. Our results demonstrate that the EGFL7 gene silencing could not the only protease involving the invasion and growth of LSCC.

Colony forming assay and wound healing assay are judging tumor cell proliferation, lateral migration and invasion ability. Our study shows EGFL7-RNAi group cell colony density was 87. 76 ± 9. 35 (*$P < 0.05$ EGFL7-RNAi-LV vs HEp-2 cells and NC-GFP-LV). It means that after EGFL7 gene expression suppression can affect Hep-2 cell proliferation, which is consistent with the results of MTT. Wound healing assay shows EGFL7-RNAi group cell healing rate is far lower than the control group, which is relate with growth suppression and descenting cell in the lateral migrationability.

Recent studies [34] suggested that EGFL7 can up-regulated Bcl-xl and down-regulated Bax expression to inhibit hyperoxia-induced cell death. Therefore, we compared protein expression level of Bcl-xl and Bax between EGFL7-

RNAi group and control group. There is statistical significance, which consistent with the results of Wu fan [13]. Wu fan's research showed [13] Silence EGFL7 of liver cancer cells MTT assay and AnnexinV/PI double staining without significant difference in contrast to our results, which may be related to cell type difference.

Egfl7 promotes tumor escape from immunity, which, in turn, promotes tumor progression. Interestingly, Egfl7 has no effect on the immune cells themselves. Indeed, it does not directly activate dendritic cells, NK cells, or T lymphocytes and does not affect their activation status upon stimulation. The main effect of Egfl7 is to repress the tumor endothelium activation so that immune cells remain sequestered in the blood circulation, thus preventing their infiltration within the tumor mass is important [35].

Although we are not demonstrated clearly the mechanisms underlined the inhibition of EGFL7 gene silencing, but our results concluded that targeted EGFL7 inhibition by shRNA reduced the invasiveness of laryngeal carcinoma cells in vitro. In this study, we show that MMP2, MMP9 in the Hep-2 induced tumors was significantly lower in EGFL7- RNAi-Lentivirus treated group than that in the control group, suggesting that a decreased proliferation of LSCC could be achieved by inhibiting EGFL7. These observations, together with the ability of RNAi targeting for EGFL7 to inhibit invasion and growth of cancer cells, support a main role of EGFL7 in induction of invasiveness, and the knockdown of EGFL7 will result in marked suppression of the proliferation and invasive potential of LSCC cells. Although, we have not demonstrated clearly the mechanisms underlined the inhibition of EGFL7 gene silencing, the tumor cell microenvironment is important in the proliferation and invasion of the tumor [36], matrix metalloproteinase (MMPs), a family of zinc-dependent proteolytic enzymes, play an important role in tumor cell microenvironment. Western blot also indicated that the expression of MMP2 and MMP9 were downregulated in EGFL7- RNAi-Lentivirus groups compared with Hep-2 and Scrambled nucleotide control shRNA groups. These results suggest that the decrease of cell invasion by transfection with shRNA targeting EGFL7 is closely associated with the reduced production of MMP2 and MMP9 [37].

Factors that inhibit or decrease EGFL7 expression may therefore decrease vessel migration, leading to defects in vascular development. Similarly, decreased endothelial cell adhesion in the absence of EGFL7 may lead to apoptosis, just as decreased adhesion in many cells tissues can lead to programmed cell death [38].

Recent studies [39] have found that EGFL7 protein can be used as chemical chemotactic stimuli and significantly enhanced the capacity of cell movement. At the same time, EGFL7 could potentially be important role in tumor invasion and metastasis. These studies will help people to recognize the mechanism of migration of cell movement, and also help to research new "weapons" out of the fight against cancer.

The data suggest that EGFL7 is critical to the invasive process in human laryngeal carcinoma cells and that this occurs via up-regulation of MMP9 expression and activation of ICM [40]. Together, these observations suggest that depending on the conditions and the disruption of the endothelium might lead both to a reduced primary tumor and to an increase in metastasis, which could explain the observed dual anti-primary tumor/prometastatic effects of anti-angiogenic therapies [41, 42].

These antitumor effects were associated with induction of G2/M cell cycle arrest and apoptosis, and down-regulation of EGFL7 target genes (MMP-2, MMP-9, VEGF, cyclinD1 and survivin). This first demonstration of therapeutic benefits of EGFL7 targeting in laryngeal carcinoma implicates the importance of targeting this pathway in laryngeal carcinoma.

Conclusion

In conclusion, our study has shown for the first time that Egfl7 expresses in LSCC carcinoma cells and its over-expression in LSCC tissues and serum significantly correlates to poor prognosis of LSCC. Furthermore, we have demonstrated then novel role of Egfl7 in metastasis of LSCC on the one hand by enhancing cell motility through EGFR-dependent FAK Phosphorylation, on other hand by siliencing MMP-2, MMP-9 and survivin expression, identifying the key molecular regulation mechanism of invasion and metastasis in laryngeal cancer, implicating Egfl7 as an over serum/molecular marker for diagnosis, monitoring after laryngectomy and prognosis of LSCC and a potential therapeutic target for metastasis of LSCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiao-Bao Yao, Department of Otolaryngology-Head and Neck Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, China. Tel: +86 029 8532 3965; E-mail: xiaobao4163@yeah.net

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