EGFL6 Promotes Bone Metastasis of Lung Adenocarcinoma by Enhancing Osteoclast Differentiation and Stimulating Cancer Cell Metastasis

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Abstract

Background

Lung cancer is still the most fatal cancer today with approximately 30%-40% of these patients will develop bone metastasis. Lung adenocarcinoma (LUAD) is the most common and aggressive type of lung cancer. The relationship between LUAD and bone metastasis and its underlying mechanism remains unclear. This study proved that epidermal growth factor-like domain multiple 6 (EGFL6) was highly expressed in LUAD specimens of patients, and the expression level was positively correlated with bone metastasis of LUAD.

Method

The expression of EGFL6 in cancer tissues was detected by IHC. CCK-8, colony formation assay, migration and invasion assay, wound healing assay, immunocytochemistry, RT-PCR, Western blotting, ELISA, bone resorption, TRAP staining and H&E staining were performed. A nude mouse model of LUAD-induced bone destruction was established by injecting A549 cells in different EGFL6 expression levels.

Results

EGFL6 is elevated in LUAD and is associated with bone metastasis. *In vivo*, implantation of human adenocarcinoma A549 cells with a higher expression of EGFL6 not only increased tumor growth rate but also bone resorption of tibias in nude mice. *In vitro*, the secretion of EGFL6 from A549 cells increased osteoclast differentiation but had little effect on osteogenic differentiation. To reveal the underlying mechanism, we demonstrated that EGFL6 enhanced osteoclast differentiation through activating nuclear factor-kappa B (NF- κ B) and downstream c-Fos/NFATc1 signaling pathways, and in addition promoted the proliferation, migration and invasion of A549 cells through enhancing the epithelial mesenchymal transformation (EMT) and promoting Wnt/ β -catenin and PI3K/AKT/mTOR signaling pathways.

Conclusions

We unveil EGFL6 as a predictor in bone metastasis of LUAD and underscore the relevance of EGFL6 as a therapeutic target.

Background

Lung cancer is the leading cause of cancer deaths worldwide, among which lung adenocarcinoma (LUAD) is the most common and aggressive type^{1,2}. The skeletal system is one of the most common metastatic sites in lung cancer patients, accounting for approximately 30%-40% of lung cancer patients³.

⁴. Bone metastasis patients often suffer skeletal-related events (SREs) including pathologic fractures, spinal cord compression, bone marrow aplasia, and hypercalcemia⁵. Among all types of lung cancer, LUAD has the highest incidence of bone metastases⁶.

However, the underlying mechanism of bone metastasis in lung cancer, especially in LUAD, remains unclear. Studies were carried out to identify the interaction of lung cancer cells with bone marrow cells. Nakamura ES et al demonstrated that osteoclasts might promote bone metastasis of lung cancer cells by producing macrophage-derived chemokine CCL22, which upregulated receptor activator of the NF- κ B ligand (RANKL) in osteoclast-like cells⁷. The expression of bone remodeling cytokines or receptors, such as RANKL, receptor activator of NF- κ B (RANK), and osteoprotegerin (OPG), were found to be upregulated in bone metastatic tissues from primary non-small cell lung cancer (NSCLC) patients. In addition, the increased expression of RANKL and OPG was correlated with clinical features of the patients⁸. As a collagen receptor, Discoidin domain receptor-1 (DDR1) was found to associate with poor clinical outcome in lung cancer. In a nude mice model of tumor with bone metastasis, the lack of DDR1 showed decreased metastatic activity and osteolytic lesions⁹. A representative study determined that the activation of the WNT/TCF signaling pathway mediates the metastasis of LUAD to bones and brain¹⁰. However, the signaling pathway regulates the LUAD metastasis is still largely unknown.

Tumor growth and metastasis require the formation of new blood vessels, which is termed angiogenesis. Epidermal growth factor-like domain multiple 6 (EGFL6) is an exocrine protein that plays an important role in promoting endothelial cell migration and angiogenesis¹¹. Ectopic expression of *EGFL6* has been shown to promote the invasion of cancer cells in breast, gastric, and nasopharyngeal cancers^{12–14}. In addition, cumulated evidence proved that EGFL6 exerted a pivot role in the proliferation, migration, and invasion of several tumor cells, such as breast, ovarian, and colorectal cancer cells^{15, 16}. Clinically, immunohistochemical staining of 150 LUAD specimens showed that high *EGFL6* expression was associated with poor prognosis of LUAD, especially in young patients (<69 years)¹⁷. However, this study focused on the relationship between the survival rate and the expression level of *EGFL6* in LUAD. It has not been studied whether there is a relationship between the expression of *EGFL6* and the occurrence of bone metastasis.

Here, we proved that *EGFL6* was highly expressed in LUAD samples and was positively correlated with bone metastasis and TNM stage. We demonstrated that EGFL6 enhanced osteoclast differentiation and bone resorption through activating NF- κ B and downstream c-Fos/NFATc1 signaling pathways but exerted little effect on the formation of osteoblasts. Additionally, EGFL6 also promoted the proliferation, migration and invasion of LUAD cells through epithelial mesenchymal transformation (EMT) and Wnt/ β -catenin and PI3K/AKT/mTOR signaling pathways. Therefore, we propose that EGFL6 can not only be used as a predictor of the prognosis of LUAD, but also play a role in promoting LUAD bone metastasis and subsequent bone destruction.

Materials And Methods

Specimen collection

We collected 30 LUAD tissues and matched adjacent normal tissues from the Biological Resource Center of Enze Medical Center, Taizhou Hospital. The specimens were staged according to the 8th manual of the American Joint Committee on Cancer Staging Manual. The time distribution of patients undergoing lung surgery was from 2004 to 2012, and the clinical data and follow-up information of the patients were fully recorded. This study was approved by the Medical Ethics Committee of Taizhou Hospital, and the informed consent of all patients was obtained.

Immunohistochemistry (IHC)

Paraffin sections were baked in a 60°C in an incubator at 60°C for 2h before processing, and then taken out and cooled to room temperature. After deparaffinization and rehydration, the tissue sections were heated with citrate buffer to perform antigen retrieval in an autoclave. Then, 3% H₂O₂ was added to the slices to block endogenous peroxidase. Normal sheep serum (Gibco) was used to block nonspecific binding sites. The sections were incubated with anti-EGFL6 antibody (1:100, ab140079, Abcam, MA, USA) overnight. After that, the secondary antibody was added to the sections. Finally, the tissue sections were sequentially treated with hydrochloric acid, hematoxylin counterstain, dehydration and sealing.

These sections were graded by professional pathologists and were based on the staining intensity of and the percentage of positively stained cells. The intensity was divided into four grades: 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The percentage of positive staining cells was graded as follows: 1 (0–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). We multiplied the two scores, the final score falling in the range of 0-6 was defined as low EGFL6 expression, and 7-12 was defined as high EGFL6 expression.

Cell culture

The human A549 LUAD cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The authenticity of A549 cells (RRID: CVCL_0023) has been verified using Short Tandem Repeat (STR) profiling within the last 3 years. A549 cells were cultured in RPMI 1640 medium containing 1% penicillin/streptomycin, 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Cells were maintained in an incubator at 37°C with a humidity of 5% CO₂. All experiments were performed with cells without mycoplasma.

Cell transfection

The siRNA targeting *EGFL6* (si-EGFL6) and the paired negative control (si-Ctrl) were synthesized by GenePharma (Shanghai, China). A549 cells were seeded in six-well plates and transfected with 5μl siRNA and 5μl Lipofectamine 2000 (Invitrogen). As for the overexpression of EGFL6, the LV-EGFL6-RNAi (sh-EGFL6) was obtained from GeneChem (Shanghai, China). A549 cells were grown in 24-well plates and transfected with lentivirus and 20μl HitransG according to the manufacturer's instructions.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to measure cell proliferation ability. The cells were cultured into 96-well plates, and 10 μ l CCK8 was added to each plate at 0, 24, 48, 72, 96h. Then, after 2h incubation at 37°C, the determination of cell proliferation was replaced by the absorbance value at 450nm.

Colony formation assay

The transfected cells were seeded in 6-well plates at a density of 1000 cells per well. After culturing for 10d, the colonies were fixed with 4% paraformaldehyde for 30min and then stained with 0.1% crystal violet for 30min. The number of colonies was counted and compared in each group.

Migration and invasion assay

A549 cells were transfected on the 24-well transwell plates (Corning Inc., Corning, NY). For migration assays, a serum free cell suspension (100 μ l) containing 5 $\times$ 10⁴ cells was added into the upper chamber, and medium containing 10% FBS was added to the lower chamber. After incubating for 12h at 37°C, the cells at the bottom of the upper chamber were fixed with 4% paraformaldehyde for 30min and stained with 0.1% crystal violet for 30min. Then, a cotton swab was used to remove the non-migrating cells in the upper chamber. For the invasion assay, the upper chamber was pre-coated with Matrigel (BD Biosciences, San Jose, CA), and the cell number of the cell suspension was changed to 1 $\times$ 10⁵. The remaining steps were the same as the migration assay. Finally, we counted and compared the number of cells of each group.

Wound healing assay

The wound healing assay was performed by culturing insert (ibidi GmbH, Martinsried, Germany) in 24-well plates. The cell suspension (70 μ l) at a density of 5 $\times$ 10⁴ cells/ml was placed into each well of the culture-insert. After incubating at 37°C and 5% CO₂ for 24h, we used sterile tweezers to gently remove the culture-insert and photographed under an optical microscope. Then, we added serum free RPMI 1640 medium (300 μ l) to each well and cultured for 24h. Time lapse images were captured at the same position at 0 and 24h.

Immunocytochemistry

A cell suspension containing 1 $\times$ 10⁴ cells was seeded on a coverslip in 12-well plates. After the cells were attached to the coverslips, they were fixed with 4% paraformaldehyde for 30min. The cells were permeabilized in 0.5% Triton X-100 for 10min, then blocked with 10% FBS for 30min. The primary antibodies E-cadherin, N-cadherin and Vimentin (CST, Beverly, MA, USA; diluted 1:250) were used to incubate with the cells overnight at 4°C. On the next day, the cells were cultured with the fluorescently labeled secondary antibody (diluted 1:250) for 1h in the dark. Later, the cells were stained with DAPI for 5min and then mounted. Images were captured under confocal microscope.

RNA isolation, reverse transcription and qPCR

Total RNA was extracted from transfected A549 cells by TRIzol reagent (Invitrogen) following the manufacturer's instructions. Then, the total RNA was reverse transcribed into cDNA using the HiFiScript cDNA Synthesis Kit (CW BIO, Beijing, China). Quantitative real-time PCR analysis was performed by an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) in triplicate. The *GAPDH* gene was utilized as housekeeping gene. All sequences were listed in Table S1.

Western blotting analysis

Total cellular protein of transfected cells was extracted using Radio-Immunoprecipitation Assay buffer (RIPA) containing protease inhibitor PMSF (100:1) and phosphatase inhibitor (Beyotime, Shanghai, China) (100:1). Quantitative of protein concentration was detected by BCA assay (Beyotime, Shanghai, China). The protein samples at the same quantity were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking with Western Blocking Buffer (Biosharp, China) for 2h, the PVDF membranes were incubated with primary antibodies at 4°C overnight. The membranes were washed by TBS-Tween and incubated with HRP-conjugated secondary antibodies for 1h at room temperature. Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA) and ImageQuant LAS 500 (GE Health Care, Fairfield, CT, USA) were used to observe the immunoreactivity. ImageJ software was utilized to quantify the intensity of the bands.

Quantitative analysis of medium EGFL6 level

An ELISA assay was used to verify that EGFL6 play a role through exocrine. The culture medium of the cells cultured for 2d was extracted and the concentration was detected by an ELISA kit (AMEKO, China). The optical density was tested at 450nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA). The standard curve with OD value as the ordinate and standard concentration as the abscissa. The concentration of EGFL6 in each medium could be obtained by the measured OD value against the standard curve.

Osteoclast differentiation *in vitro*

BMMs were extracted from the femur and tibia of C57BL6 mice (8-week-old). After culturing for 4d or reaching 90% confluence in a 10cm cell culture dish, the BMMs were seeded into a 96-well plate at a density of 8000 cells/well in complete α -MEM containing 30ng/ml macrophage colonies stimulating factor (M-CSF). After 24h, we replaced the medium with complete α -MEM containing 30ng/ml M-CSF, 50ng/ml RANKL and supplemented with the culture medium of si-Ctrl, si-EGFL6, sh-Ctrl and sh-EGFL6 respectively. The osteoclast induction medium was changed every 2d until the 7th day. The cells were fixed by 4% paraformaldehyde for 1h, and then stained by TRAP activity kit (Sigma-Aldrich, St. Louis, MO, USA). ImageJ software was used to quantify the area (percentage of a well) of TRAP-positive cells (≥ 3 nuclei).

Osteoblast differentiation *in vitro*

Bone marrow mesenchymal stem cells (BMSCs) were extracted from the femur and tibia of C57BL6 mice (8 weeks old), and cultured in complete α -MEM for 6d or reached 90% confluence in a 10cm cell culture dish. For osteoblast differentiation, BMSCs were re-seeded into a 12-well plate at a density of 1×10^4 cells/well with osteogenic medium (Complete DMEM supplemented with 10mM β -glycerophosphate, 50 μ M ascorbic acid and 100nM dexamethasone). The medium was changed every 2d. On day 7 and 21, the cells were stained with alkaline phosphatase (ALP), and the mineralized bone nodules were stained with Alizarin Red.

Bone absorption assay *in vitro*

Bone marrow mononuclear macrophage (BMMs) were seeded into 100 μ m bovine bone slices (Rongzhi Haida Biotech Co., Ltd, Beijing, China) at a density of 1×10^4 cells/well in α -MEM supplemented with 100 ng/ml RANKL and the culture medium of si-Ctrl, si-EGFL6, sh-Ctrl and sh-EGFL6 respectively. The osteoclast induction medium was changed every 2d until the 14th day. The cells were eliminated by mechanical agitation and sonication. Then, the resorption pits were examined by scanning electron microscope (Field Environmental Instruments Inc., Hillsboro, OR). ImageJ software was utilized to quantify the area of resorption pits.

Nude mouse tumor-induced bone destruction model *in vivo*

All animal experiments followed the guidance of the Institutional Animal Ethics Committee of Taizhou Hospital. 6-week-old female nude mice were randomly divided into si-Ctrl, si-EGFL6, sh-Ctrl and sh-EGFL6 group (6 mice/group). After induction of anesthesia, 20 μ l of transfected tumor cells (1×10^6 cells/ml) suspended in Phosphate Buffered Saline (PBS) were injected into the tibial marrow cavity through the tibial plateau of nude mice. All mice were euthanized on day 14. We removed the hind limbs of nude mice and compared the size of tumor. Then, the hind limbs were fixed in 4% paraformaldehyde and collected for further experiments.

Histological and histomorphometric analysis

After decalcification in 10% EDTA (ethylenediaminetetraacetic acid) for 14d, the fixed hind limbs were embedded in paraffin and then sectioned. The tissue slices were utilized for H&E and TRAP staining. We acquired images of each sample through a high-quality microscope (Olympus, Japan) and counted the number of TRAP-positive cells in each sample.

Statistical analysis

The significant differences between groups were analyzed by Student's t-test or one-way ANOVA or Kruskal Wallis test using GraphPad Prism® version 8.3.1. All *in vitro* experiments were performed independently at least 3 times. The relationship between the expression of EGFL6 and clinicopathological indexes was analyzed by χ^2 or Fisher's Exact Test by IBM SPSS Statistics 26.0 for Mac (IBM Corp, Armonk, NY, USA). Results were presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant.

Results

EGFL6 is elevated in lung adenocarcinoma and is associated with bone metastasis

We collected 30 random cases of LUAD samples and clinical data after surgery. IHC analysis was utilized to evaluate the expression of *EGFL6* between LUAD tissues and adjacent normal tissues. The results showed that, in general, the expression of *EGFL6* in LUAD tissues was higher than that in neighboring normal tissues (Fig. 1A, B). Through statistical analysis of clinical parameters, we found that the expression of *EGFL6* was closely correlated with the TNM stage and bone metastasis of LUAD (Fig. 1C, D). However, the expression of *EGFL6* was not significantly related to the patients' age, histological grade, and lymph node metastasis (Table 1).

Table 1
The relationship between EGFL6 and clinicopathologic characteristics in LUAD

	Number (n=30)	Staining of EGFL6		P value
		High (%)	Low (%)	
Sample type				
LUAD tissues	30	20	10	<0.001*** #
adjacent normal tissues	30	7	23	
Age				
≤62	15	6	9	0.06
>62	15	12	3	
Histological grades				
G1, G2	23	14	9	0.372
G3	7	6	1	
Lymph node metastasis				
Absent	17	10	7	0.440
Present	13	10	3	
TNM stage				
I, II	12	4	8	0.004**
III, IV	18	16	2	
Bone metastasis				
Absent	20	10	10	0.011*
Present	10	10	0	
# χ^2 ; $P<0.05$ are shown in bold, * $P<0.05$, ** $P<0.01$, *** $P<0.001$				

Effects of high and low expression of EGFL6 on the proliferation, migration and invasion of LUAD cells

Through the analysis of clinical samples, we speculated that EGFL6 played a role in promoting the progression of LUAD. In order to explore the biological role of EGFL6 in LUAD, we used human LUAD cell line A549 cells for further study. A549 cells obtained overexpression (sh-EGFL6) and knockdown (si-EGFL6) phenotypes under lentivirus and siRNAs respectively. EGFL6-siRNA and null vector were applied to transfect A549 cells. WB and RT-PCR verified that the knockdown efficiency of EGFL6-siRNA-899 was

the highest (Fig. S1A, B). the lentivirus was transfected into A549 cells and verified by fluorescence (Fig. S1C). Finally, we proved the knockdown and overexpression efficiency by WB and RT-PCR (Fig. S1D, E).

We performed CCK8 assays to test the relationship between the proliferation ability of A549 cells and the expression of *EGFL6*. The enhanced expression of *EGFL6* significantly promoted the proliferation of A549 cells, while silencing *EGFL6* weakened the viability of A549 cells (Fig. 2A). Besides, the colony formation assays also showed the same trend with CCK-8 (Fig. 2B). In order to explore the migration and invasion abilities mediated by *EGFL6*, we conducted a transwell assay and a scratch test *in vitro*. As shown in Fig. 2C, the number of migrated and invading cells in *EGFL6* overexpression group was significantly greater than that in the control group. On the contrary, the *EGFL6* knockdown group had the opposite phenomenon. In addition, the cell scratch experiment found that the healing speed of the *EGFL6* overexpression group was significantly faster than that of the control group, and depleting *EGFL6* expression slowed the healing rate to an extent (Fig. 2D).

High expression of EGFL6 promotes EMT of lung adenocarcinoma cells

EMT is an important biological process for LUAD cells to acquire the ability of migration and invasion. It refers to epithelial cells that lost cell polarity and gain mesenchymal characteristics¹⁸. To validate the relationship between *EGFL6* and EMT of LUAD, we detected the levels of mesenchymal markers, E-cadherin, N-cadherin, MMP2, MMP9, Vimentin, Snail1 and Slug in sh-*EGFL6*, sh-Ctrl, si-*EGFL6* and si-Ctrl cell groups by RT-PCR and WB. The results showed that the forced expression of *EGFL6* up-regulated the mRNA level of *N-cadherin*, *Vimentin*, *MMP2*, *MMP9*, *Snail1* and *Slug* while down-regulating the mRNA level of *E-cadherin*. Interference with *EGFL6* indicated the opposite results (Fig. S2). The WB results were consistent with RT-PCR results (Fig. 2E).

To further prove that *EGFL6* can promote the EMT of A549 cells, we performed immunocytochemistry (ICC) to detect the expression of *E-cadherin*, *N-cadherin* and *Vimentin* in the si-Ctrl, si-*EGFL6*, sh-Ctrl and sh-*EGFL6* groups. The results showed that the expression of *N-cadherin* and *Vimentin* in the sh-*EGFL6* group was higher than that in the sh-Ctrl group, while the expression in the si-*EGFL6* group was lower than that in the si-Ctrl group. The expression of *E-cadherin* was opposite to the above results (Fig. 3A).

EGFL6 partially regulates LUAD cells by activating Wnt and PI3K/AKT/mTOR signaling pathways

Wnt/ β -catenin signaling pathway and PI3K/Akt/mTOR signaling pathway both play key roles in regulating the process of proliferation, migration and invasion of tumor cells. Through Western blotting, we observed that overexpression of *EGFL6* increased the levels of GSK-3 and β -catenin compared to the control. Silencing *EGFL6* significantly downregulated the levels of these signaling proteins (Fig. 3B). Moreover, the forced expression of *EGFL6* increased the phosphorylation levels of PI3K, AKT and mTOR, while the low expression of *EGFL6* did the opposite, with no difference in total protein level (Fig. 3C). Taken together, these evidences indicated that *EGFL6* promoted the proliferation, migration and invasion of A549 cells at least in part through Wnt/ β -catenin signaling pathway and PI3K/AKT/mTOR signaling pathway.

EGFL6 increases tumor growth and bone destruction in bone metastasis of LUAD cells in nude mice

To further validate the role of EGFL6 in promoting the migration and invasion of A549 cells, we established a bone metastasis model by implanting LUAD cells in nude mice. The suspensions of A549 cells of *EGFL6* overexpression, knockdown, and their respective control were injected into nude mice through the tibial plateau. After 14d, all nude mice were euthanized, and the differences in tumor appearance size and bone destruction in each group were compared. The tumor growth rate of the sh-EGFL6 group was much faster than that of the sh-Ctrl group, while the si-EGFL6 group exhibited the opposite result (Fig. 4A).

In addition, we observed by X-ray that the sh-EGFL6 group had more severe tibial bone destruction than the sh-Ctrl group, while the si-EGFL6 group showed the opposite result (Fig. 4B). Then we performed paraffin sections on the tibia of nude mice and stained them with H&E and TRAP. H&E analysis supported that the osteolysis in sh-EGFL6 group was more severe than that of sh-Ctrl group. However, the opposite result was observed in the si-EGFL6 group compared with the si-Ctrl group (Fig. 4C). In addition, based on X-ray and H&E results, TRAP staining proved that osteoclasts in the bone surface were enhanced in the sh-EGFL6 group, but decreased in the si-EGFL6 group (Fig. 4D).

Since the results of animal models showed that the bone destruction in the *EGFL6* overexpression group was more serious, in order to study the effect of EGFL6 on bone formation, we extracted the culture medium of each cell group and added them to the BMM and MC3T3 cells. Compared with the sh-Ctrl and si-Ctrl groups, BMM using sh-EGFL6 medium showed enhanced osteoclast differentiation (Fig. 4E), and MC3T3 using sh-EGFL6 medium had no significant change in osteogenic formation (Fig. 4F). According to the existence of the above-mentioned cell experiment, the *in vitro* bovine bone section resorption test showed that the bone resorption capacity of the sh-EGFL6 group was more potent than that of the control, and vice versa (Fig. 4G).

EGFL6 promotes osteoclast differentiation by activating NF- κ B and downstream c-Fos/NFATc1 signaling pathways

In order to clarify the potential mechanism of EGFL6 promoting osteoclast differentiation, we studied several classic signaling pathways in osteoclastogenesis by Western blotting. We first divided BMMs into two groups. One group was treated with RANKL and a partial medium extracted from sh-EGFL6 (EGFL6 medium) to explore the levels of c-Fos and NFATc1. The other group was stimulated with RANKL and the medium extracted in sh-Ctrl for 0, 1, 3, and 5d. The results showed that under the stimulation of RANKL and control medium, the expression of c-Fos and NFATc1 reached the peak on the 5th day. In addition, when RANKL and EGFL6 medium were used together, the expression of c-Fos and NFATc1 was significantly enhanced on the 5th day (Fig. 5A).

Besides, we further studied the short-term signaling pathways of MAPKs, NF- κ B and AKT under the influence of EGFL6 medium and control medium, respectively. Under the pretreatment of EGFL6 medium or control medium, BMMs was induced by RANKL, and the phosphorylation of each protein in the MAPK,

NF- κ B and AKT signaling pathways was detected at 0, 5, 10, 20, 30 and 60min level. As shown in Fig. 5B, there was no significant difference in the activation and phosphorylation levels of AKT and MAPK members ERK, JNK and p38 between BMMs pretreated with EGFL6 medium and BMMs pretreated with control medium. In contrast, when induced by EGFL6 medium, the phosphorylation of NF- κ B member I κ B α increased at 10 min. Then, the phosphorylation of p65 in the trimeric structure was therefore similarly enhanced at 10min (Fig. 5C).

Discussion

It is well accepted that EGFL6 plays an important role in the formation of new blood vessels of epithelial cells, which is a necessary process for tumor growth and metastasis^{11, 16, 19, 20}. In this study, we found that *EGFL6* expression was higher in LUAD tissues than that in adjacent normal tissues and EGFL6 staining in LUAD tissues was positively correlated with TNM stage and bone metastasis. Our results were consistent with a retrospective study of LUAD¹⁷, which indicated that high *EGFL6* expression might serve as a marker of poor clinical outcome of LUAD, especially in younger patients¹⁷. In that study, the focus was mainly on the relationship between the expression level of *EGFL6* and the prognosis of LUAD. Our study further discovered that the high expression level of *EGFL6* in LUAD tissues was associated with bone metastasis. It has been proven that LUAD is the most common pathological type of lung cancer with a survival rate of 4-17%²¹. Compared with patients without bone metastases, the overall survival of LUAD patients with bone metastases was significantly shorter (5.8 months vs 10.2 months, $P < 0.05$)²². Therefore, the poor prognosis in EGFL6-positive LUAD patients may be the result of enhanced bone metastasis. The expression of *EGFL6* has also been shown to be positively correlated with the invasion, metastasis and poor prognosis of other cancers, such as ovarian cancer, breast cancer, colorectal cancer, gastric cancer, oral squamous cell carcinoma, nasopharyngeal carcinoma and benign meningioma^{12-14, 23-26}.

We also found that EGFL6 might promote bone metastasis of LUAD by stimulating bone destruction by osteoclasts. Bone is the preferred metastasis site for many tumor cells, such as breast, lung and prostate cancer cells²⁷⁻²⁹. We first verified that EGFL6 was secreted to the culture medium by A549 cells. The medium containing a higher concentration of EGFL6 enhanced the osteoclast differentiation of BMMs. The bone resorption assay also confirmed that EGFL6 significantly increased the area of bone resorption pits. Our *in vivo* experiments demonstrated that tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts were increased in the tibia of nude mice invaded by A549 cells with high expression of *EGFL6*. Moreover, studies have shown that when osteoblasts and bone metastatic breast cancer MDA-MB-231 cells were co-cultured, they can reduce the secretion of OPG and promote the secretion of monocyte chemotactic protein 1 (MCP1). This effect can be partially blocked by EGFR inhibitors³⁰. EGFL6 may promote bone metastasis and osteolytic lesions in the tumor microenvironment by decreasing *OPG* expression and increasing *MCP1* expression in an EGFR-dependent manner³⁰. In our study, the medium containing EGFL6 had little effect on the osteoblast differentiation of MC3T3 cells. In

brief, our results suggest that the metastatic cells of LUAD may secrete EGFL6 through paracrine, and stimulate bone resorption by stimulating osteoclast differentiation of BMMs.

Furthermore, we explored the underlying mechanism of EGFL6 promoting osteoclast differentiation. After the stimulation of RANKL in BMMs, NFATc1 and c-Fos, which play an essential role in the downstream pathways of osteoclast differentiation, were activated to control the cytokine expression^{31, 32}. We found that EGFL6 increased the expression of c-Fos and NFATc1 in a time-dependent manner. In addition, the MAPKs, NF- κ B and AKT signaling pathways have been called the most classic signaling pathways in osteoclast formation. In the classic pathway of NF- κ B, phosphorylation of I κ B α induces the release of the RelA(p65)/p50 heterodimer, which causes nuclear translocation of p65 and ultimately leads to the activation of NF- κ B³³. In this study, we found that EGFL6 enhanced the phosphorylation of I κ B α and subsequently the phosphorylation of p65, which means that EGFL6 promoted osteoclast differentiation in part by activating NF- κ B and downstream c-Fos/NFATc1 signaling pathway. However, MAPKs members ERK, JNK, p38 and AKT were almost unaffected by EGFL6. It has been shown that EGF-like ligands are related to the bone environment. As a member of EGF-like ligands, EGFL6 is preferentially expressed in osteoblast-like cells, but has no effect on osteoblast proliferation and mineralization¹¹. In addition, it is well known that osteoblasts are the main source of several cytokines responsible for regulating the differentiation and formation of osteoclasts, such as OPG, RANKL, M-CSF, and MCP1^{34, 35}. Studies have shown that EGF-like ligands can stimulate the formation of TRAP-positive osteoclasts by indirectly reducing *OPG* expression and increasing *MCP1* expression, which may contribute to the effect of metastatic breast cancer cells on bone³⁰. However, the role of EGFL6 in bone metastasis of LUAD remains to be resolved.

Our *in vitro* studies have shown that knocking-in *EGFL6* promoted the proliferation, migration and invasion of A549 cells, while silencing *EGFL6* did the opposite. EMT is an important process for tumor cells to acquire the ability of migration, invasion and anti-apoptotic stimulation. EMT gives tumor cells the characteristics of metastasis, accompanied by the decrease of epithelial markers and the increase of mesenchymal markers³⁶. Our study found that the mesenchymal markers N-cadherin and vimentin were significantly enhanced in knocking-in *EGFL6* cells, but inhibited in *EGFL6* silenced cells. This phenomenon indicated that EGFL6 promoted the ability of A549 cells to obtain migration and invasion and metastasize to bone, which might be partly through the enhancement of EMT. Besides, as a complex biological process of tumorigenesis and progression, EMT is regulated by a variety of signaling pathways, like Wnt/ β -catenin³⁷, PI3K/AKT/mTOR³⁸, MAPK³⁹, and Notch⁴⁰. In Wnt/ β -catenin signaling pathway, cytoplasmic β -catenin is degraded by glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), Axin, and adenomatous polyposis (APC). Phosphorylation is then degraded by ubiquitination and proteasome. Western blotting results showed that knocking-in *EGFL6* promoted the phosphorylation levels of GSK3 and β -catenin, while silencing *EGFL6* showed the opposite effect. Furthermore, hyperactivation of PI3K signaling pathway has been extensively studied in human cancers, can regulate cell movement, survival, growth, and metabolism⁴¹. The PI3K signaling cascade has been shown to be involved in ovarian, gastric, breast and prostate cancers^{42–45}. Similarly, we found that knocking-in EGFL6

enhanced the expression of p-PI3K, p-AKT and p-mTOR in LUAD. The above results collectively proved that EGFL6 promoted the proliferation, migration and invasion of A549 cells at least in part through the enhanced EMT process and the activation of Wnt/ β -catenin and PI3K/AKT/mTOR pathways. However, due to the characteristics of PI3K signaling pathway in regulating proliferation, migration and metastasis of tumor cells, the existing evidence was still insufficient to prove that EGFL6 drives the EMT of LUAD exactly through Wnt/ β -catenin and PI3K/AKT/mTOR pathways. Further experiments remain to be carried out to explore the potential molecular mechanism of EGFL6 to promote LUAD metastasis.

Conclusion

Our findings provide evidence that *EGFL6* is highly expressed in LUAD tissues and is positively correlated with bone metastasis in LUAD. Knocking-in *EGFL6* promotes osteoclast differentiation through NF- κ B signaling pathway and promotes bone destruction in nude mice. As attested by the experiments *in vivo* and *in vitro*, we demonstrated that overexpression of *EGFL6* significantly enhanced the proliferation, migration and invasion of A549 cells. On the contrary, the low expression of *EGFL6* attenuated these capabilities. Further mechanism studies have found that EGFL6 promotes EMT, at least in part through the Wnt and PI3K/AKT/mTOR signaling pathways to promote the above-mentioned characteristics of A549 cells. Therefore, we speculate that EGFL6 tends to be a predictor of bone metastasis in LUAD.

Abbreviations

BMMs Bone marrow mononuclear macrophage

BMSCs Bone marrow mesenchymal stem cells

CCK-8 Cell Counting Kit-8

EGFL6 epidermal growth factor-like domain multiple 6

EMT epithelial mesenchymal transformation

IHC Immunohistochemistry

LUAD lung adenocarcinoma

M-CSF macrophage colonies stimulating factor

MCP1 monocyte chemoattractant protein 1

NF- κ B nuclear factor-kappa B

OPG osteoprotegerin

RANKL receptor activator of the NF- κ B ligand

Declarations

Ethics approval and consent to participate

This research was approved by the Medical Ethics Committee of the Taizhou Hospital and obtained the informed consent of all patients. All experiments of animal followed the instruction of the Institutional Animal Ethics Committee of Taizhou Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Conflict of interest

All authors declare no potential conflict of interest.

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Authors' contributions

Xiaoting Song, Dun Hong and Xu Cheng: conceptualization. Xu Cheng: data curation. Xinhui Wu: formal analysis. Mingxuan Feng and Dun Hong: funding acquisition. Xiaoting Song, Xu Cheng, Xiangang Jin and Shengyu Ruan: investigation. Haixiao Chen, Zhenghua Hong and Dun Hong: project administration. Xianquan Xu, Feng Lu and Fangying Lu: resources. Haixiao Chen, Zhenghua Hong and Dun Hong: supervision. Xiaoting Song: writing – original draft. Renshan Ge and Dun Hong: writing – review & editing. All authors read and approved the final manuscript.

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Tables

Table 1. The relationship between EGFL6 and clinicopathologic characteristics in LUAD

	Number (n=30)	Staining of EGFL6		<i>P</i> value
		High (%)	Low (%)	
Sample type				
LUAD tissues	30	20	10	<0.001*** #
adjacent normal tissues	30	7	23	
Age				
≤62	15	6	9	0.06
>62	15	12	3	
Histological grades				
G1, G2	23	14	9	0.372
G3	7	6	1	
Lymph node metastasis				
Absent	17	10	7	0.440
Present	13	10	3	
TNM stage				
I, II	12	4	8	0.004**
III, IV	18	16	2	
Bone metastasis				
Absent	20	10	10	0.011*
Present	10	10	0	

[#] χ^2 ; $P < 0.05$ are shown in bold, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Figures

Figure 1

EGFL6 is up-regulated in LUAD tissues. **a** The images of EGFL6 IHC staining in LUAD tissues and adjacent matched tissues. Scale bar represents 100 μ m. **b** The expression of EGFL6 in LUAD tissues is higher than adjacent normal tissues ($P < 0.001$, χ^2 test). **c,d** The expression of EGFL6 was strongly correlated with the TNM stage ($P = 0.004$) and bone metastasis ($P = 0.011$) of LUAD. Results were presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

EGFL6 promotes the proliferation, migration and invasion of LUAD A549 cells. **a** CCK-8 analysis shows that over-expression of EGFL6 significantly promotes the proliferation of A549 cells, knockdown of EGFL6 weakens this ability. **b** Colony formation assay exhibits the same results of CCK-8. **c** Transwell assay displays that over-expression of EGFL6 increases the migration and invasion of A549 cells while knockdown of EGFL6 reveals the opposite. **d** Testing the rate of wound closure indicates that over-

expression of EGFL6 promotes the cell mobility in A549 cells, knockdown of EGFL6 attenuates the migration of A549 cells. **e** Over-expression and knockdown of EGFL6 influence the EMT markers in A549 cells. Ectopic expression of EGFL6 promotes the expression of N-cadherin, MMP2, MMP9, Vimentin, Snail1 and Slug and down-regulates the expression of E-cadherin. Knockdown of EGFL6 exhibits the opposite results. Results were presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

EGFL6 enhances the EMT process of LUAD partly through Wnt and PI3K/AKT/mTOR signaling pathways. **a** The effect of EGFL6 on EMT markers (E-cadherin, N-cadherin and Vimentin) in A549 cells is detected by ICC. Over-expression of EGFL6 increases the expression of N-cadherin and Vimentin in cytoplasm but attenuates the expression of E-cadherin while knockdown of EGFL6 displays the adverse. **b** Western blotting analysis is utilized to detect the levels of p-GSK-3, GSK-3 and β -catenin under the influence of over-expression and knockdown of EGFL6 in A549 cells. **c** Western blotting analysis is utilized to detect the levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR under the influence of over-expression and knockdown of EGFL6 in A549 cells. Results were presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

EGFL6 moderates the effect of A549 cells on skeleton system of nude mice and enhances the osteoclast differentiation of BMMs. **a** The tumor size in the sh-EGFL6 group is much larger than the sh-Ctrl group, and the si-EGFL6 group exhibited a smaller size than the si-Ctrl group. **b** Compared with each control, X-ray shows that the bone destruction of tibia in sh-EGFL6 group is much severe while in sh-EGFL6 group is slight. **c** The tibial tumor sections of nude mice were stained with H&E. **d** The tibial tumor sections of

nude mice were stained with TRAP. **e** EGFL6 enhances the osteoclast differentiation of BMMs. **f** EGFL6 has no significant influence on osteoblast differentiation of MC3T3. **g** The bone resorption pits assay. Results were presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5

EGFL6 promotes osteoclast differentiation by activating the NF- κ B and the downstream c-Fos/NFATc1 signaling pathway. **a** The expression of c-Fos and NFATc1 is detected by western blotting on 0, 1, 3, 5 day under the effect of medium extracted from sh-EGFL6 group, si-EGFL6 group and each control. **b** The expression of the phosphorylated or nonphosphorylated forms of AKT and MAPKs, including ERK, JNK and p38 kinase is detected by western blotting at different time points. **c** The expression of p-p65, p65, p-I κ Ba and I κ Ba is detected by western blotting at different time points. Results were presented as mean $\pm$ SD. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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