

ERBB4 promotes the proliferation of gastric cancer cells via the PI3K/Akt signaling pathway

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Abstract. ERBB4 is one of the members of the epidermal growth factor receptor (EGFR) family. ERBB4 is a large transmembrane glycoprotein and has tyrosine kinase activity. Once combined with epidermal growth factor (EGF), ERBB4 can activate the related genes in the nucleus, thus promoting cell division and proliferation. In the present study, we investigated the effect of ERBB4 in the proliferation of gastric cancer cells. We found that high ERBB4 levels were closely related to the poor prognosis of gastric cancer patients. Furthermore, ERBB4 was highly expressed in gastric cancer cell lines when compared to the normal stomach cell line, GES. Clinical samples provided the same results. Two gastric cancer cell lines, SGC-7901 and MNK-45 were used to study the underlying mechanism of ERBB4 in the promotion of cell proliferation in gastric cancer cells both *in vitro* and *in vivo*. It was observed that after the expression of ERBB4 was suppressed, the proliferation of gastric cancer cells was markedly inhibited both *in vitro* and *in vivo*. Moreover, treatment with lentiviral vector siRNA-ERBB4 (Lv-siRNA-ERBB4) or the ERBB4 inhibitor AST-1306, markedly inhibited gastric cancer cell proliferation. Further experiments revealed that inhibition of the expression of ERBB4 could inhibit the activation of the PI3K/Akt signaling pathway. In addition, the use of the PI3K/Akt signaling pathway inhibitor LY294002 demonstrated the aforementioned results. Therefore, we believe that ERBB4 regulates cell proliferation mainly through the PI3K signaling pathway. Finally, nude mice xenografted with gastric cancer cells with low expression of ERBB4 exhibited smaller tumors and longer survival than those engrafted with control gastric cancer cells. These data

indicated that ERBB4 promoted cell proliferation and is thus a potential therapeutic target for the treatment of gastric cancer.

Introduction

Gastric cancer has a high incidence and mortality in China (1). Because the symptoms of early gastric cancer are not obvious, most cases of tumor invasion and metastasis are diagnosed only when patients are hospitalized (2). Low chemotherapy sensitivity results in poor survival rates among gastric cancer patients (2,3). Therefore, finding effective targets to inhibit the growth and metastasis of gastric cancer is particularly important. ERBB4 is a large transmembrane glycoprotein, and has tyrosine kinase activity (4). Once combined with epidermal growth factor (EGF), ERBB4 can activate the related genes in the nucleus, thus promoting cell division and proliferation (5,6). The expression of ERBB4 was revealed to be increased in gastric, breast, and bladder cancer as well as head and neck squamous cell carcinoma (7-10). In addition, ERBB4 has been revealed to contribute to and play an important role in tumor cell survival, proliferation, and motility (11,12). ERBB4 has been demonstrated to be overexpressed or with aberrant expression in a variety of solid tumors especially in gastric cancer (13-16). Therefore, ERBB4 may become a very important drug target for the therapy of gastric cancer. Unfortunately, more research attention has been paid to ERBB2 overlooking ERBB4. In the present study, we investigated the effect of ERBB4 in the proliferation of gastric cancer cells. We found that high ERBB4 levels were closely related to the poor prognosis of gastric cancer patients. ERBB4 was highly expressed in gastric cancer cell lines when compared to the normal stomach cell line, GES. Clinical samples provided the same results. We also found that ERBB4 regulated cell proliferation mainly through the PI3K/Akt signaling pathway. Thus, ERBB4 may become a potential therapeutic target for the treatment of gastric cancer.

Materials and methods

Patients and tissue specimens. Gastric cancer tumor tissues with matched adjacent non-tumor tissues were obtained from 27 patients that underwent surgical treatment at Zhejiang

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Provincial People's Hospital (Hangzhou, China). All patients provided a signed informed consent. None of them had a history of chemotherapy or radiotherapy before sampling, and the diagnosis of gastric cancer was pathologically confirmed. This study was approved by the Institutional Ethics Committee of Zhejiang Provincial People's Hospital.

Reagents and antibodies. RPMI-1640 was acquired from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Gibco (Rockville, MD, USA). Inhibitors AST-1306 and LY294002 were obtained from Selleck Chemicals (Houston, TX, USA). A BCA Protein Assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against β -actin (cat. no. 4970S), ERBB4 (cat. no. 4795S), PI3K (cat. no. 4249S), Akt (cat. no. 9272S), and p70S6K (cat. no. 2708S) and secondary antibodies (cat. no. 7054S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All antibody dilution ratio was 1:1,000. MTT was purchased from Beyotime Institute of Biotechnology. Other chemicals were obtained from commercial sources. An IHC staining kit was purchased from Thermo Fisher Scientific, Inc.

Cell culture and transfection. Gastric cancer cell lines MNK-45 and SGC-7901 were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China). MNK-45 and SGC-7901 cells were cultured in RPMI-1640 containing 10% FBS and maintained at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. MNK-45 and SGC-7901 cells (1.5×10^5) were seeded in 6-well plates, and incubated for 12 h, then transfected with a lentiviral vector encoding small interfering RNA targeting ERBB4 [lentiviral vector siRNA-ERBB4 (Lv-siRNA-ERBB4)] and negative control lentiviral vector (Lv-NC). Lv-siRNA-ERBB4 and Lv-NC were synthesized by Shanghai GeneChem, Co., Ltd. (Shanghai, China). ViralPlus Transduction Enhancer and Polybrene were used for lentiviral vector transfection.

Western blot analysis. Cells or tissues were lysed using RIPA buffer containing 1 mM PMSF protease inhibitor mixture. The total protein concentration was assessed using the BCA assay and was equalized with the extraction reagent. Protein (10 μ g/lane) samples were separated by 10% SDS-PAGE, and electrotransferred onto PVDF nitrocellulose membranes. The membranes were blocked with 5% skim milk for 2 h at 37°C. Then, the membranes were incubated with primary antibodies against β -actin (cat. no. 4970S), ERBB4 (cat. no. 4795S), PI3K (cat. no. 4249S), Akt (cat. no. 9272S) and secondary antibody anti-rabbit IgG (cat. no. 7054S; all from Cell Signaling Technology, Danvers, MA, USA) and diluted 1:1,000. Subsequently, a Gel imaging analysis system and Pierce Western Blot Signal Enhancer (cat. no. 21050; Thermo Fisher Scientific, Inc.) were used to detect the protein bands.

Cell proliferation assay. The cell proliferation of MNK-45 and SGC-7901 cells was analyzed using flow cytometry. Briefly, the cells were plated at a density of 1,000-10,000 cells/well in a 96-well plate, and incubated at 37°C for 12, 24, 36, 48 and 60 h. At 12, 24, 48 or 60 h, the cells were incubated with RPMI-1640 medium containing 0.5 mg/ml MTT at 37°C

for 4 h. Formazan crystals were dissolved with 150 μ l DMSO. The absorbance of each well, including blanks, was measured at 490 nm using an automatic microplate reader subsequent to 10 min of oscillation. The formula of the cell growth inhibition rate was as follows: Inhibition rate = $(1 - \text{absorbance of the COE group} / \text{absorbance of the blank control group}) \times 100\%$.

In vivo imaging technology. In order to further examine the effect of ERBB4 on the growth of gastric cancer cells *in vivo*, the animal imaging technique was used to perform the assay *in vivo*. Four-week-old nude mice were purchased from the Laboratory Animal Center of Zhejiang University. All animal experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Human gastric cancer cell lines MNK-45 were transfected with Lv-siRNA-ERBB4-GFP and Lv-NC-GFP and then transplanted subcutaneously in nude mice. Tumor cells labeled with green fluorescence were used for *in vivo* fluorescence imaging after 7 days. The initial inoculum concentration of MNK-45 was increased to 1×10^7 cells/ml. Tumors were formed ~7 days after the injection. Tumors sizes were measured every 7 days using an *in vivo* imaging system (PerkinElmer, Inc., Waltham, MA, USA). The fluorescence signal reflecting the tumor sizes of the mice were collected and analyzed using the *in vivo* imaging system. This assay was approved by the Institutional Ethics Committee of Zhejiang Provincial People's Hospital.

Immunohistochemistry. All specimens were fixed in neutral buffered formalin and embedded in paraffin. The specimens were cut into 5- μ m sections, deparaffinized in xylene, and rehydrated in graded ethanol. After non-specific binding sites were blocked by exposing them to 10% normal goat serum in PBS for 20 min, the sections were incubated overnight at 4°C using a series of antibodies (ERBB4, 1:200; PI3K, 1:200; Akt, 1:200). Following this incubation, the slides were rinsed with PBS and incubated with biotinylated IgG for 20 min at 37°C. Finally, the sections were slightly counterstained with hematoxylin for 30 sec before coverslip mounting.

Statistical analysis. The data processing was carried out using SPSS 19.0 statistical software. One-way analysis of variance (ANOVA) with Dunnett's test was used to determine statistically significant differences. All data were expressed as the mean \pm standard deviation ($\bar{x} \pm s$). $P < 0.05$ and $P < 0.01$ indicated differences that were statistically significant. All experiments were repeated at least three times.

Results

ERBB4 is downregulated in gastric cancer. We analyzed 378 gastric cancer samples obtained from The Cancer Genome Atlas (TCGA) datasets to identify critical ERBB4 involved in gastric cancer. The analysis demonstrated that high ERBB4 levels in TCGA gastric cancer data were closely related to the poor survival rate of gastric cancer patients suggesting that ERBB4 was a prognostic indicator of gastric cancer (Fig. 1A). Quantification of the western blot analysis was performed. In addition, western blot analysis revealed that ERBB4 was significantly overexpressed in MNK-45 and SGC-7901 gastric cancer cell lines compared to immortalized stomach GES cells (Fig. 1B).

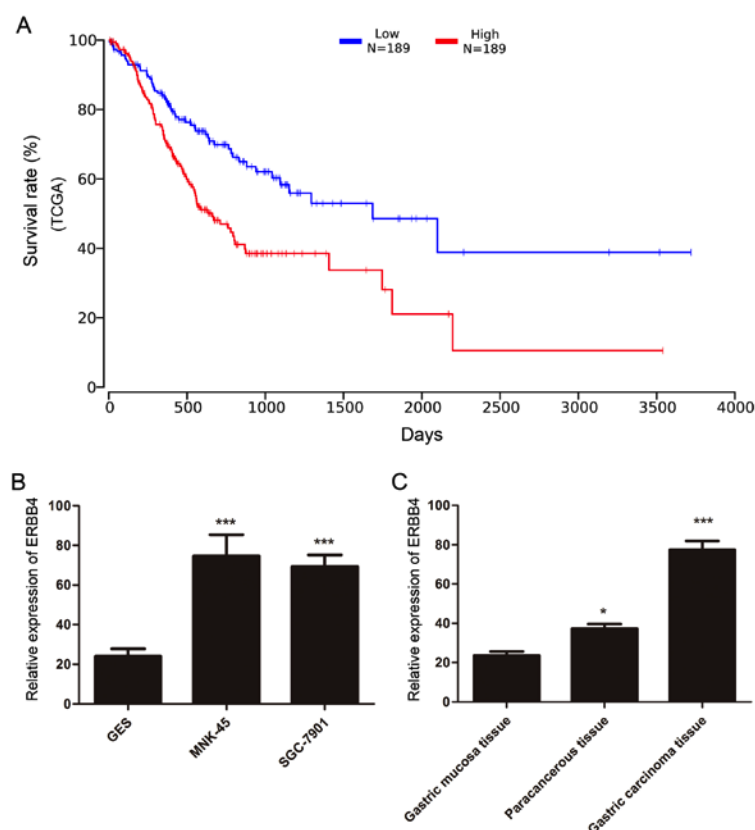


Figure 1. (A) The plot (data from the TCGA database) shows the survival rate (%) of gastric cancer patients with high (red) and low (blue) expression of ERBB4. Low expression of ERBB4 was associated with long term survival. (B) The expression levels of ERBB4 in GES, MNK-45 and SGC-7901 cells. (C) ERBB4 expression in normal stomach, paracancerous and gastric cancer tissues. * $P < 0.05$, *** $P < 0.001$. TCGA, The Cancer Genome Atlas.

Furthermore, western blot analysis also revealed that ERBB4 was markedly upregulated in tumor tissues compared to normal stomach tissues and paracancerous tissue (Fig. 1C).

A lentivirus interferes with the expression of ERBB4. To further study the function of ERBB4 in gastric cancer, lentiviral vectors encoding siRNAs targeting ERBB4 (GenBank accession number NC_000002.12) were constructed by Shanghai GeneChem, Co., Ltd. Three different siRNAs targeting ERBB4 (Lv-siRNA-ERBB4) were designed to ensure the interference effect. The three Lv-siRNA-ERBB4 vectors were transfected into MNK-45 and SGC-7901 cells with ViralPlus Transduction Enhancer and Polybrene so that their specificity for ERBB4 disruption could be determined. The validated Lv-siRNA-ERBB4 was selected for the construction of the lentiviral vector (Fig. 2A-C). A non-silencing sequence was used as a negative control (Lv-NC). Western blot analysis revealed that ERBB4 was markedly silenced by Lv-siRNA-ERBB4 (Fig. 2D and E).

Effects of ERBB4 silencing on gastric cancer cell proliferation and related signaling pathways. The MTT assay revealed that after Lv-siRNA silenced ERBB4 expression, MNK-45 and SGC-7901 cell viability and cell proliferation was decreased. Compared with the control group, the cells after Lv-siRNA silenced ERBB4 expression exhibited a marked growth inhibition (Fig. 3A and B). Western blot analysis revealed that ERBB4 silencing, inhibited the downstream PI3K/Akt signaling. Furthermore, the same results were obtained with the ERBB4 inhibitor, AST-1306. In addition, the PI3K/Akt

signaling inhibitor LY294002 also provided further evidence, consistent with the aforementioned results. (Fig. 3C-F).

Silencing of ERBB4 expression suppresses tumor growth in vivo and prolongs the survival time. To confirm the effects of ERBB4 *in vivo*, we used a xenograft mouse model. The analysis demonstrated that Lv-NC cells formed significantly larger tumors than the Lv-siRNA-ERBB4 group of cells in the nude mice (Fig. 4A). In addition, the body weight of nude mice exhibited no difference (Fig. 4B). Furthermore, the tumor volume was significantly diminished in the Lv-siRNA-ERBB4 group compared to the Lv-NC group (Fig. 4C). Notably, silencing of ERBB4 expression resulted in a significantly enhanced survival time of the tumor-bearing nude mice (Fig. 4D).

Lv-siRNA and AST-1306 markedly inhibit the growth of transplanted tumors in nude mice. Lv-siRNA-ERBB4 significantly inhibited tumor growth in nude mice. The size of the transplanted tumors in nude mice treated with AST-1306 or transfected with the lentivirus was significantly lower than that in the Lv-NC group (Fig. 5).

AST-1306 regulates ERBB4-PI3K/Akt signaling pathways in vivo. To further ascertain the signal transduction pathway mediating the anti-proliferation effects *in vivo*, xenografts of nude mice were prepared as paraffin-embedded sections. The IHC analysis revealed that the inhibitor of ERBB4 (AST-1306) significantly downregulated the expression level of the proliferation-related proteins PI3K and Akt (Fig. 6).

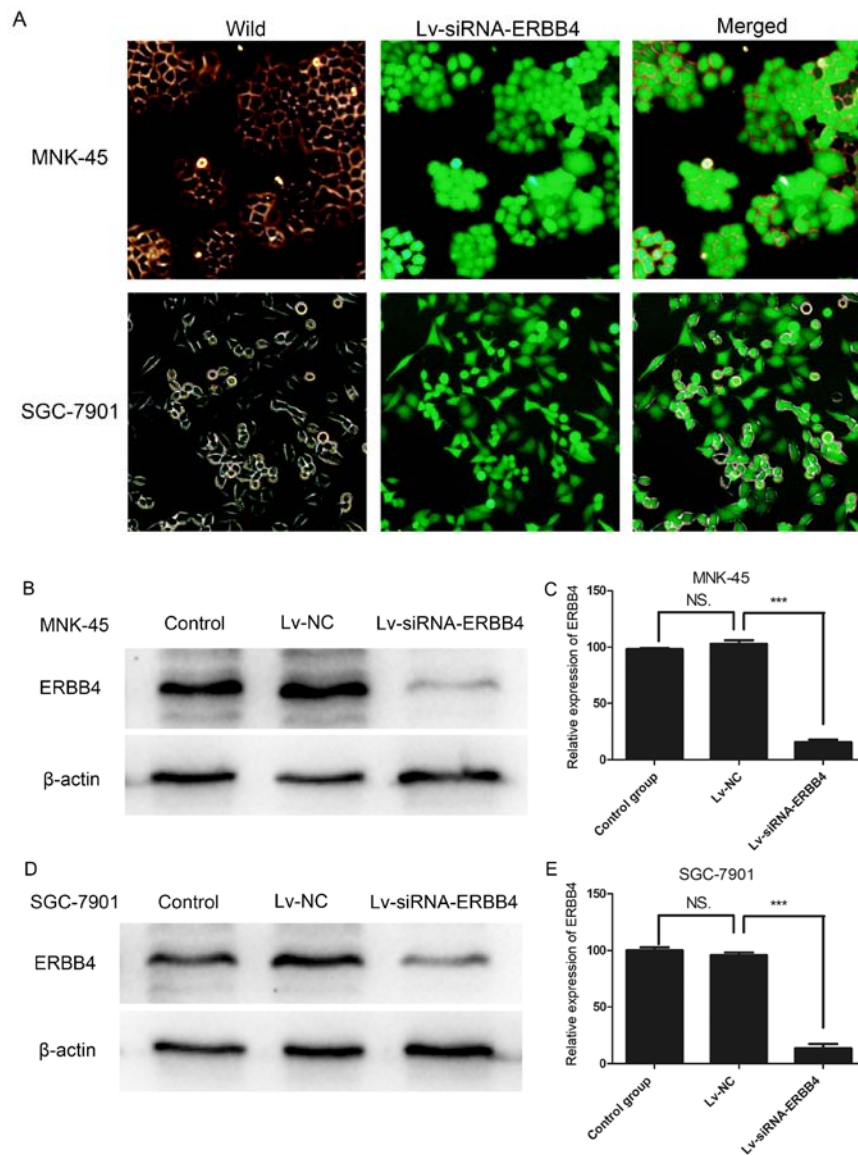


Figure 2. Lv-siRNA-ERBB4 silenced ERBB4 expression (x100). (A) Expression of green fluorescent protein before and after transfection with the lentivirus. (B-E) Lv-siRNA-ERBB4 markedly downregulated ERBB4 expression in MNK-45 and SGC-7901 cells. *** $P < 0.001$. Lv-siRNA-ERBB4, lentiviral vector siRNA-ERBB4; Lv-NC, negative control lentiviral vector.

Discussion

Gastric cancer is one of the most common cancers with the highest morbidity and mortality in China (1). Early screening of gastric cancer is an important method for the treatment and prevention of gastric cancer (17-20). However, there is no reliable and specific molecular marker of gastric cancer. Therefore, it is important to find a specific marker of gastric cancer. ERBB4 belongs to the family of tyrosine kinase receptors and is overexpressed in many tumors especially in gastric cancer. In recent years, the targeted therapy of epidermal growth factor receptor (EGFR) family has achieved fruitful results in the treatment of gastric cancer (21-24). The EGFR family was determined to be highly expressed in gastric cancer, and the positive rate of EGFR (ERBB1/HER1) was 40-60% (25), human epidermal growth factor receptor-2 (ERBB2/HER-2) ranged from 4 to 53% (26), HER-3/ERBB3 and HER-4/ERBB4 were 59 and 86%, respectively (27). The positive expression

rate of ERBB4 in gastric cancer was found to be the highest, but few studies have been performed on ERBB4 in gastric cancer. In the present study, we found that the high expression of ERBB4 was closely related to the poor prognosis of the patients. Inhibiting the expression of ERBB4 contributed to the prolongation of the survival time of gastric cancer patients. Through experiments, we found that ERBB4 was stably expressed in gastric cancer tissues and cells relative to normal gastric mucosa tissues and gastric mucosa cells. Therefore, we hypothesized that ERBB4 may play an important role in the malignant behavior of gastric cancer. It may become a target for predicting the prognosis and treatment for gastric cancer patients. To further investigate the role and mechanism of ERBB4 in gastric cancer, we used molecular cloning techniques and a lentivirus to inhibit the expression of ERBB4. The results revealed that the proliferation of gastric cancer cells was markedly inhibited by silencing the expression of ERBB4. Thus, ERBB4 can promote the proliferation of gastric

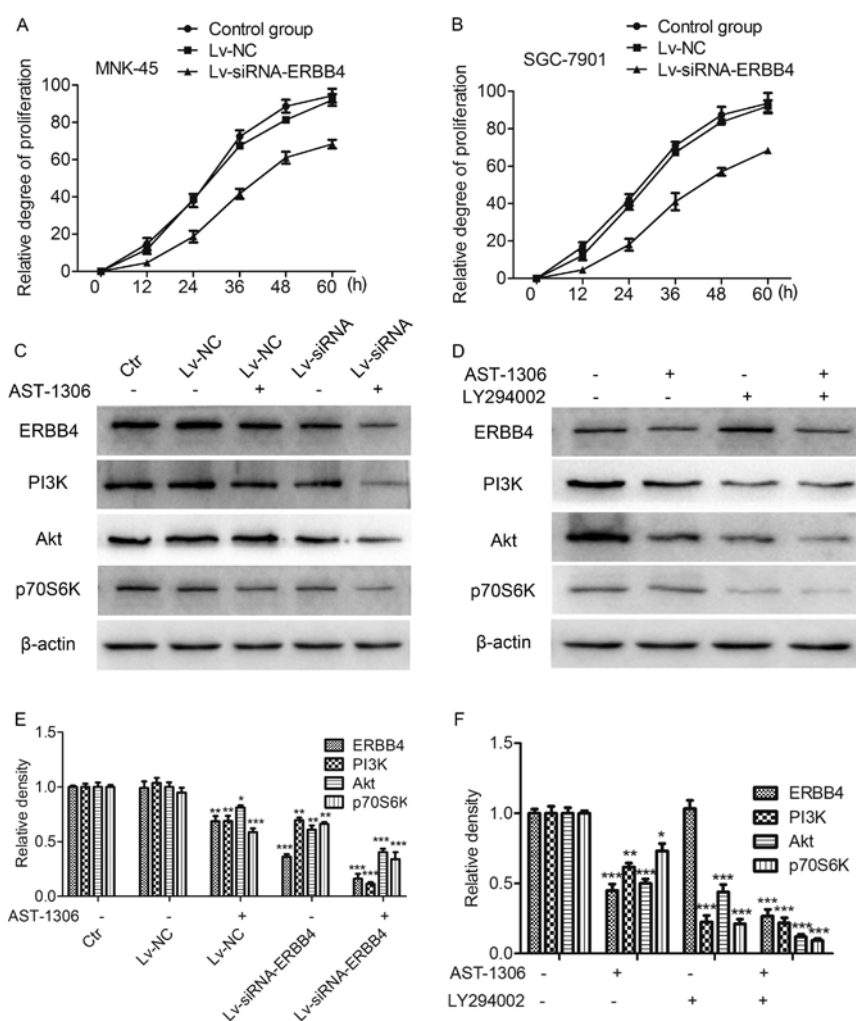


Figure 3. Inhibition of ERBB4 expression on the proliferation of gastric cancer cells (A) MNK-45 and (B) SGC-7901. (C-F) Silencing of ERBB4 expression, inhibited the downstream PI3K/Akt signaling. Two inhibitors upstream and downstream demonstrated the effects of ERBB4 on the PI3K/Akt signaling pathway. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Lv-NC, negative control lentiviral vector; Lv-siRNA-ERBB4, lentiviral vector siRNA-ERBB4.

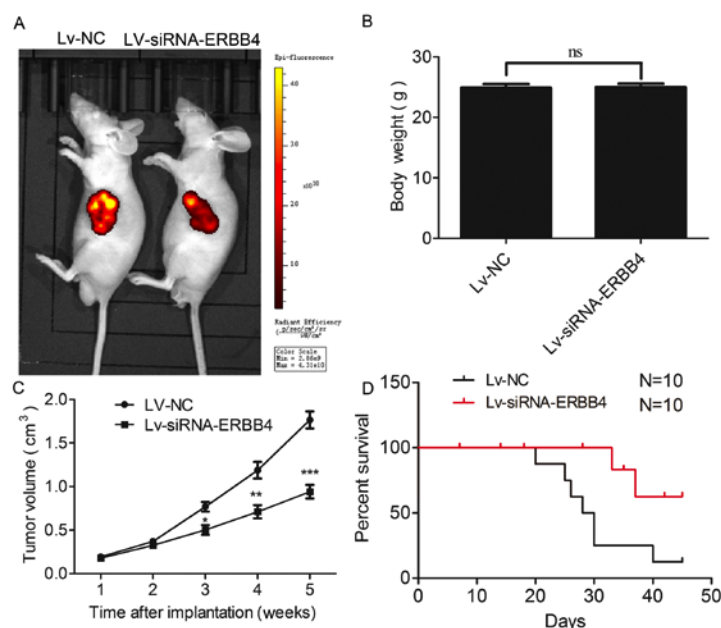


Figure 4. Xenograft mouse model reveals that silencing ERBB4 expression significantly inhibits tumor growth *in vivo*. (A) Live imaging of subcutaneous tumor growth at 21 days in the Lv-NC and Lv-siRNA-ERBB4 groups in nude mice. (B) Body weight of nude mice. (C) Tumor volume curves. (D) Comparison of the survival curves of nude mice injected with gastric cancer cells that are either Lv-NC (black) or Lv-siRNA-ERBB4 (red). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Lv-NC, negative control lentiviral vector; Lv-siRNA-ERBB4, lentiviral vector siRNA-ERBB4.

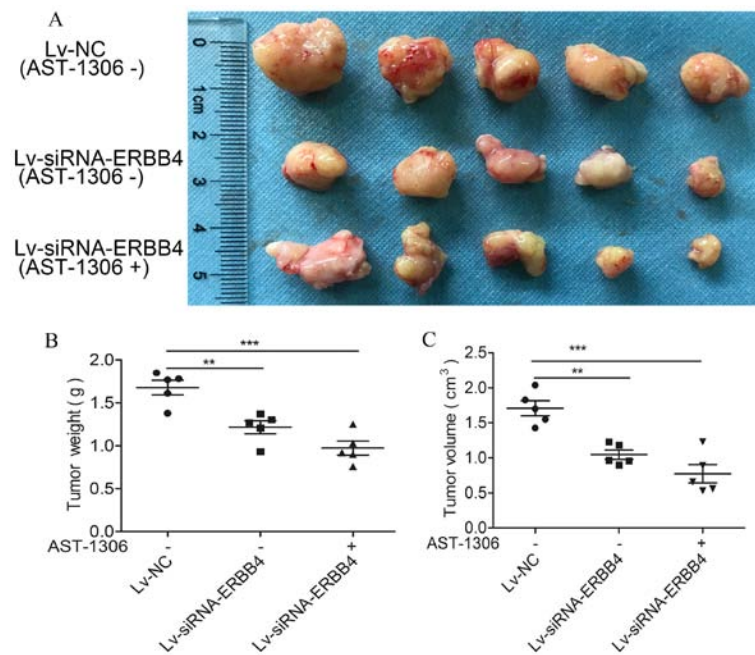


Figure 5. (A) Tumor size of different groups, including Lv-NC group, Lv-siRNA-ERBB4 group and combined use of the lentivirus and AST-1306 group. (B) Tumor weight and (C) volume of nude mice. Lv-NC, negative control lentiviral vector; Lv-siRNA-ERBB4, lentiviral vector siRNA-ERBB4.

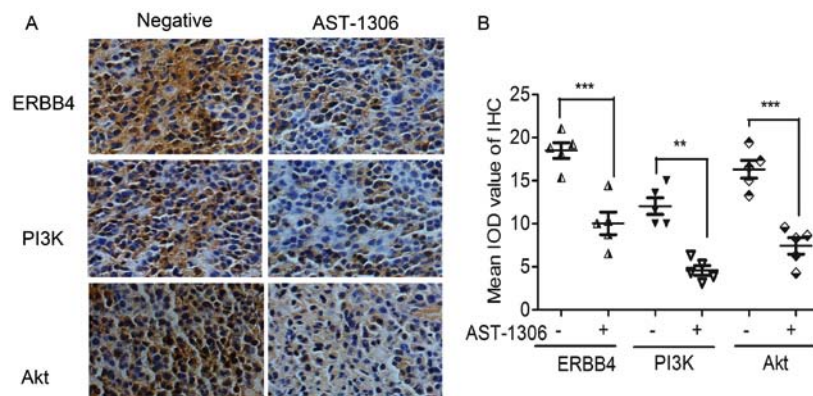


Figure 6. (A and B) AST-1306 downregulates the levels of the proliferation-related proteins ERBB4, PI3K and Akt. ** $P < 0.01$, *** $P < 0.001$

cancer cells. We further detected the key proteins of the downstream signaling pathway of ERBB4 and found that inhibition of ERBB4 expression would directly result in inactivation of the downstream PI3K/Akt signaling pathway. In addition, the PI3K signaling pathway can directly regulate cell proliferation through its downstream proteins m-TOR and p70S6K (28-30). These results revealed that ERBB4 can promote the proliferation of gastric cancer cells through the PI3K/Akt signaling pathway. In addition, the ERBB4 inhibitor (AST-1306) and the PI3K inhibitor (LY-294002) experiments confirmed the aforementioned conclusion. Collectively, these results indicated that ERBB4 mainly promotes the proliferation of gastric cancer cells through the PI3K/Akt signaling pathway. In order to investigate the role and mechanism of ERBB4 on gastric cancer proliferation *in vivo*, xenograft models of gastric cancer and an *in vivo* imaging system (PerkinElmer, Inc.) were used for *in vivo* tests. The xenograft models of the gastric cancer experiments suggested that silencing of ERBB4 can markedly suppress the proliferation of gastric cancer cells *in vivo* and prolong the survival time of tumor-bearing nude mice.

In summary, ERBB4 promoted the proliferation of gastric cancer cells via the PI3K/Akt/mTOR/p70S6K signaling pathway. ERBB4 was significantly correlated with poor survival time. Thus, our data revealed that ERBB4 is a potential prognostic factor and a therapeutic target for gastric cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JX and LG conceived and designed the study. JX, ZQ, LG, GS and JL performed the experiments. JX wrote the paper. GS and JL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by Institutional Ethics Committee of Zhejiang Provincial People's Hospital (Hangzhou, China). All patients provided a signed informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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