BASIC RESEARCH

Anti-tumor activity of erlotinib in the BxPC-3 pancreatic cancer cell line

Ying-Ying Lu, Da-Dao Jing, Ming Xu, Kai Wu, Xing-Peng Wang

Ying-Ying Lu, Da-Dao Jing, Kai Wu, Xing-Peng Wang, Department of Gastroenterology, Affiliated First People's Hospital, Shanghai Jiao Tong University, Shanghai 200080, China

Ming Xu, Department of Gastroenterology, Wuxi Second People's Hospital, Wuxi 214002, Jiangsu Province, China

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Correspondence to: Dr. Da-Dao Jing, Department of Gastroenterology, Shanghai First People's Hospital, Shanghai Jiao Tong University, 100 Haining RD, Hongkou District, Shanghai 200080, China. jingdadao@medmail.com.cn

Telephone: +86-21-63240090-3141 Fax: +86-21-66302029

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Abstract

AIM: To investigate the effect and mechanism of action of erlotinib, an epidermal growth factor receptor (EGFR) small molecule tyrosine kinase inhibitor (TKI), in the human pancreatic cancer cell line BxPC-3 both *in vitro* and *in vivo*.

METHODS: *In vitro*, human pancreatic cancer cell line BxPC-3 was exposed to varying concentrations of erlotinib, and its effects on proliferation, cell cycle distribution, apoptosis and the expression of pro- and antiapoptotic factors such as bcl-2, bcl-xl, bax and bak, and the expression of vascular endothelial cell growth factor (VEGF) were measured with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometric analysis, terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL), and reverse transcription-polymerase chain reaction (RT-PCR). Potential effect of erlotinib on angiogenesis was examined by tube formation assay. Tumor growth suppression was observed in xenografted nude mice with pancreatic cancer *in vivo*. Immunohistochemical (IHC) staining for EGFR and factor Ⅷ-related antigen was undertaken to detect the microvessel density and VEGF expression in tumor tissue in xenograft nude mice.

RESULTS: Erlotinib, as a single agent, repressed BxPC-3 cell growth in a dose-dependent manner, triggered G_1 arrest and induced cell apoptosis, and suppressed capillary formation of endothelium *in vitro*. Expressions of VEGF were significantly down-regulated at a high concentration of 200 μmol/L, however, the expressions of bcl-2 and bcl-xl were decreased at 50 μmol/L. *In vivo*, Erlotinib-treated mice demonstrated a reduced tumor volume, weight and microvessel density as compared to the control. IHC staining showed decreased expression of EGFR and RT-PCR had lower VEGF expression in treated mice.

CONCLUSION: The *in vitro* and *in vivo* findings provide evidence that BxPC-3 cells are inhibited with erlotinib treatment. Inhibition of EGFR may be a promising adjuvant chemotherapy strategy in pancreatic cancer treatment.

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem[1,2]. Recently, several orally bioavailable compounds aimed at specific molecular targets have been developed in hopes of improving survival in this dismal disease. Tumor development and progression depend on cellular changes like overexpression of oncogenic tyrosine kinase receptors. Many gastrointestinal tumors, including pancreatic cancer, have been shown to overexpress the epidermal growth factor receptor (EGFR)[3,4]. The overexpression of the EGFR and its ligands correlates with rapidly progressive disease and resistance to chemotherapy.

EGFR is a 170-kDa transmembrane protein with intrinsic tyrosine kinase activity. Stimulation of the EGFR results in activation of multiple intracellular signaling cascades that increase cellular proliferation and prevent programmed cell death[5]. Multiple therapeutic strategies designed to manipulate this receptor have been developed, including specific antibodies and low molecular EGFR tyrosine kinase inhibitors (TKIs). Erlotinib is a small molecule TKI that efficiently blocks EGFR. Preliminary results of a phase III trial of gemcitabine with or without erlotinib in pancreatic cancer revealed a modest improvement in survival with the addition of erlotinib. Treatment with anti-EGFR agents is used as a potential therapeutic strategy for pancreatic cancer, but the mechanisms are not yet precisely understood.

The aim of this study was to investigate the growth inhibitory effects of erlotinib in pancreatic cancer cells *in vitro* and *in vivo*, to determine the mechanisms involved and to examine the effects of erlotinib on the regulation of angiogenesis.

MATERIALS AND METHODS

Cell culture and reagents

Human pancreatic cancer cell lines BxPC-3, obtained from Shanghai Institute of Biochemistry and Cell Biology, and ECV 304, a cell line derived from human umbilical vein endothelial cells from ATCC were maintained in RPMI-1640 (Gibco) medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37℃. The EGFR-selective TKI erlotinib was provided by DeBioChem (Nanjing, China). The agent was dissolved in DMSO (Sigma) or carboxymethylcellulose sodium at appropriate concentrations for *in vitro* or *in vivo* studies, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted with TRIzol (Life Technologies, INC) following the manufacturer's instructions. Reverse transcription was performed starting with 2 μg of total RNA, using oligo (dT) primer and other reagents, and procedures contained in the MMulv RT-PCR kit (Promega) to form cDNA. cDNA (2 μL), 2 μL of 50 pmol/L of each primer, 10 mmol/L dNTP Mix 1 μL, 1 μL of Taq DNA polymerase (Sangon, China) were used for PCR analysis. The PCR amplification cycles consisted of denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 60 s, annealing [54℃ for *bcl-2*, *bcl-xl*, *bax*, *bak*, vascular endothelial cell growth factor (VEGF), 56℃ for GAPDH] for 60 s, extension at 72℃ for 60 s, and a final elongation at 72℃ for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized by UV light. The primer sequences are listed in Table 1.

Tube formation assay

A well established method was used for the process of *in vitro* angiogenesis assay[6] with a kit from Chemicon (Temecula, California, USA). A 96-well tissue culture plate was coated with Matrigel (50 μL/well). After matrix solution gelled, ECV304 cells were premixed with RPMI-1640 (control), erlotinib (100 μmol/L) and then seed at a concentration of 1×10^4 per well onto the surface of the polymerized gel. Four wells were used for each treatment. After 18 h of incubation at 37℃ and 5% CO_2, the status of capillary tube formation by ECV304 cells was recorded using a CCD camera attached to an inverted light microscope (40 × objective lens).

Cell viability assay

The viability of BxPC-3 cells treated with erlotinib was determined by the standard 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BxPC-3 cells were plated (5×10^3 per well) in 96-well plates and incubated overnight at 37℃. Erlotinib was dissolved in DMSO and added to the cell culture medium at a concentration not exceeding 0.1% (v/v). The effects of erlotinib on cell proliferation were studied at various concentrations (0, 1, 5, 10, 50 and 100 μmol/L) and at different time points (24, 48, 72 and 96 h) with a certain concentration (50 μmol/L). The MTT assay was done in quadruplicate for each drug concentration used. At appropriate intervals, 100 μg MTT solution was added to each well and incubated for 4 h at 37℃, 5% CO_2. The supernatant was removed, and 150 μL of DMSO was then added. Plates were then read at 490 nm wavelength using a microplate reader (BIO-RAD550, USA). Percentage of inhibition was determined by comparing the cell density in the drug-treated cells with that in the untreated cell controls in the same incubation period [percentage of inhibition = (1-cell density of a treated group)/cell density of the control group]. All experiments were repeated three times.

Cell cycle analysis and apoptosis assays

The effects of EGFR TKIs erlotinib on both cell cycle and apoptosis in BxPC-3 cells were analyzed using flow cytometry. Cells were plated into 12-well plates and the following day, erlotinib (50 μmol/L) was added and kept for 48 h. Cell floating in the medium combined with adherent layer were trypsinized and fixed with 2 mL of Citrate buffer for 1 h. Cells were then incubated with RNase A (1500 μL) and stained with propidium iodide (1500 μL). Samples were immediately analyzed by flow cytometry for cell cycle and apoptosis assays. Immunocytochemical (ICC) detection of apoptotic cells was carried out with terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL), in which residues of digoxigenin-labeled dUTP were catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase II. After treatment with erlotinib (50 μmol/L) for 48 h, slides were fixed and washed thrice in 0.01 mol/L PBS, the following procedures were performed according to the manufacturer instructions (Boster, Wuhan, China). The positive particles of DAB staining were viewed under microscope (Olympus Japan). The number of apoptotic cells was viewed and counted

Table 1 PCR primers

Target genes		Primer sequence	Size (bp)	Annealing temperature (℃)	Cycles
Bcl-2	Sense	5'-GGTGCCACCTGTGGTCCACCT-3'	458	54	35
	Antisense	5'-CCTCACTTGTGGCCCAGATAGG-3'			
Bax	Sense	5'-CTGACATGTTTTCTGACGGC-3'	289	54	35
	Antisense	5'-TCAGCCCATCTTCTTCCAGA-3'			
Bcl-xl	Sense	5'-TTGGACAATGGACTGGTTG-3'	765	54	35
	Antisense	5'-GTAGAGTGGATGGTCAGTG-3'			
Bak	Sense	5'-TGAAAAATGGCTTCGGGGCAAGGC-3'	642	54	35
	Antisense	5'-TCATGATTTGAAGAATCTTCGTACC-3'			
GAPDH	Sense	5'-CATGCCAGTGAGCTTCCCGTT-3'	408	56	35
	Antisense	5'-GTGGAGTCTACTGGCGTCTTC-3'			
VEGF	Sense	5'-ATGAACTTTCTGCTGTCTTG-3'	382	54	35
	Antisense	5'-TGCATGGTGATGTTGGAC-3'			

under microscope (40 × objective lens, Olympus Japan) and expressed as the Apoptotic Index (AI = number of apoptotic body/1000 cells).

Development of nude mice xenografts of pancreatic cancer

BALB/C nu/nu female mice, aged 4-6 wk, weighing about 20 g, were maintained pathogen free at the Shanghai Experimental Animals Centre of Chinese Academy of Sciences. BxPC-3 cells (1×10^7, suspended in 200 μL of PBS) were implanted *s.c.* in the hind flank of each mouse. Once palpable tumors were established, animals were randomly divided into two groups so that all groups had similar starting mean tumor volumes of 100-150 mm^3. The mice in each group were orally gavaged with the vehicle control (0.5% CMC-Na, $n = 6$) and erlotinib (100 mg/kg, $n = 6$) for 4 wk. The tumor size was measured with a linear caliper twice a week up to 4 wk, and the volume was estimated using the equation $V = (a \times b^2)/2$, where a is the large dimension and b the perpendicular diameter. After all the mice were sacrificed, part of the tissue was fixed in formalin and embedded in paraffin, and some parts were frozen in liquid nitrogen. Hematoxylin and eosin staining confirmed the presence of tumors. Total mRNA was prepared and RT-PCR analyses were performed as described previously.

Immunohistochemistry (IHC) of tumor xenografts

To assess EGFR expression and microvessel density (MVD) in xenograft tumors, rabbit polyclonal anti-EGFR antibody (diluted to 1:100, Boster, Wuhan) and rabbit polyclonal factor VIII antibody (Boster, Wuhan) were used in IHC. Paraffin-embedded tissue sections (4 μm) were dried, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with 3% hydrogenperoxide in ion free water for 30 min. After nonspecific binding sites were blocked with 10% goat serum, slides were incubated at 4℃ overnight with 1:100 dilution of primary antibody directed against EGFR and factor VIII followed by a 30-min incubation in a Horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG secondary antibody. Sections were rinsed with PBS and developed with the DAB kit (Boster, Wuhan)

and then counterstained with haematoxylin. Each slide was scanned at a low power (× 100) and the area with a higher number of new vessels was identified (hotspot). This region was then scanned at × 400 magnification. For individual tumors, microvessel count was scored by averaging the five field counts[7].

Statistical analysis

Quantitative results were expressed as mean ± SEM. Statistical analysis was performed using a two-tailed unpaired t test (between two groups) or a one way analysis of variance (ANOVA) (among three or more groups) with the computer software SAS 8.02. $P < 0.05$ was considered statistically significant.

RESULTS

Growth inhibition of BxPC-3 pancreatic cancer cells by erlotinib

Overall cell growth of BxPC-3 pancreatic cancer cells treated with erlotinib at different concentrations ranging from 1 to 100 μmol/L was determined by MTT assay. The results showed that erlotinib inhibited cell growth in a dose-dependent manner and significant growth inhibition was demonstrated at 72 h ($P < 0.01$). A similar result was obtained after erlotinib treatment for 96 h. We also treated BxPC-3 cells with erlotinib at 24 and 48 h. The results showed a trend toward growth inhibition, but it was not statistically significant among different concentrations ($P > 0.05$). Cell proliferation was again determined by MTT at 24, 48, 72 and 96 h. No significant difference in cell growth was noted between 24 h and 48 h, 72 h and 96 h at a concentration of 50 μmol/L. Percentage of survival cells is illustrated in Figure 1.

Effect of erlotinib treatment on cell cycle progression and apoptosis in BxPC-3 cells

To elucidate potential mechanisms of erlotinib-induced growth inhibition previously shown by MTT assay, we examined the distribution of cell cycle and apoptotic cells by flow cytometry and TUNEL. The concentration of the drug was 50 μmol/L which inhibited BxPC-3 cell growth by approximately 53.5% after 3 d of exposure. The results showed that erlotinib led to the accumulation of BxPC-3
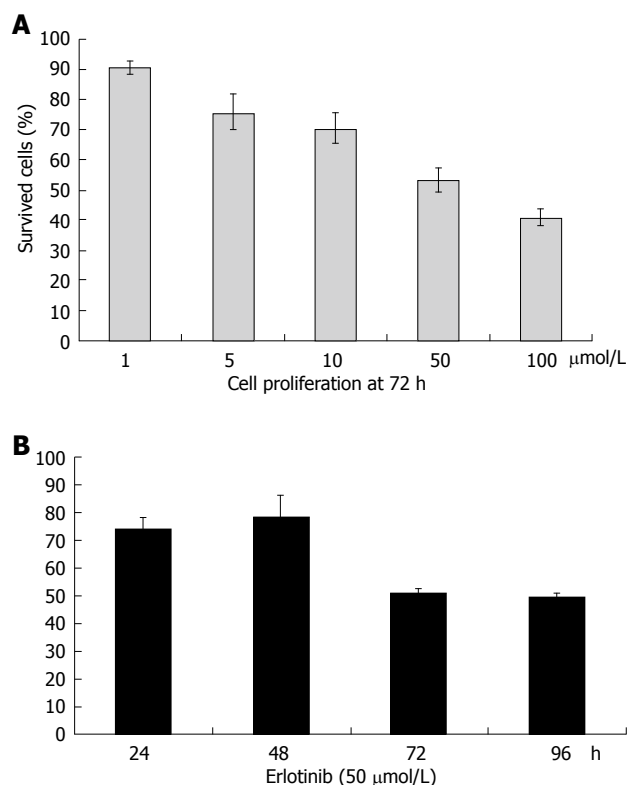
A

B

Figure 1 **A**: Growth percentage of BxPC-3 cell line after 72 h of exposure to erlotinib at varying concentrations ranging from 1-100 μmol/L. BxPC cell proliferation was significantly suppressed by erlotinib at different concentrations; **B**: Growth percentage of BxPC-3 cell line after exposure to erlotinib at a concentration of 50 μmol/L at different time points.
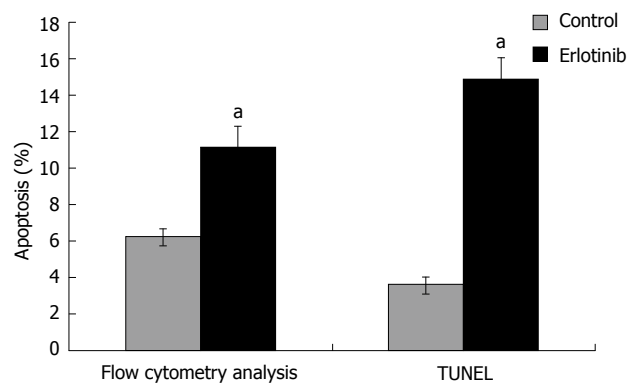

Figure 2 Induction of apoptosis in BxPC-3 cell line. There was a significant apoptosis in erlotinib treated group both in flow cytometry analysis and TUNEL assay after 48 h treatment ([a]$P < 0.05$).

Table 2 Cell cycle analysis (%, mean ± SD)

Group	G_0/G_1	S	G_2/M
Control	63.31 ± 0.99	25.28 ± 0.88	11.40 ± 1.68
Erlotinib	73.40 ± 1.34[b]	14.15 ± 0.99[b]	12.44 ± 1.95

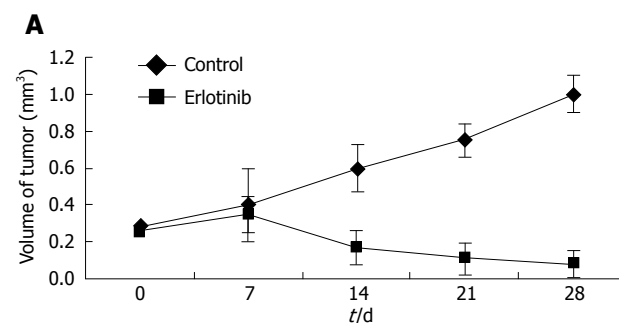[b]$P < 0.01$ *vs* control group.

A

B

C

Figure 3 **A**: Effect of erlotinib on mean tumor volume in the BxPC-3 xenograft model. Mice were implanted with BxPC-3 cells. When palpable tumors were established, animals were randomly divided into two groups. The animals were continuously gavaged with the agents for 4 wk as described in Matericals and Methods. Tumor size was measured twice per week. Values are means, n = 6. **B**: Mice of control group; **C**: Mice of erlotinb group.

cells in G_0/G_1 phase, thereby decreasing the proportion of cells in the S phase (Table 2). To assess the effects of erlotinib on induction of cell apoptosis of pancreatic cancer cells, we performed a PI apoptosis and TUNEL assay after 48 h. Flow cytometric analysis showed an induction of apoptosis (11%) compared with the control (6%) ($P < 0.05$), which was further confirmed by TUNEL (AI 14.86 ± 1.20 to 3.60 ± 0.45) ($P < 0.05$) (Figure 2). The cell cycle alterations and cell apoptosis increase indicated that cell cycle arrest and increase in apoptosis are one of the mechanisms responsible for the antiproliferative action of erlotinib in BxPC-3 cells *in vitro*.

Effect of treatment with erlotinib on the growth of mouse xenografts

A nude mouse model of pancreatic cancer was used for the *in vivo* portion of the study to assess the *in vivo* antitumor activity of erlotinib. Heterotopic murine pancreatic carcinoma was successfully established in the flank of BALB/C nude mice. Erlotinib at a dose of 100 mg/kg per day was administered to mice bearing established BxPC-3 tumors. The results indicated that erlotinib significantly inhibited tumor growth. As shown in Figure 3, at day 14, the control group of mice showed an increased tumor volume by 114.3% of the initial tumor while the group treated with erlotinib showed a decreased tumor volume by 34.6%. At the end of the
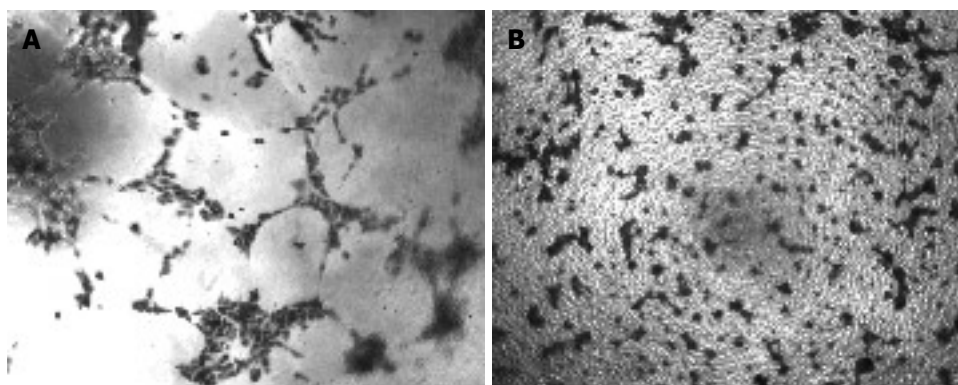
Figure 4 Effects of erlotinib on capillary formation by ECV304 were examined *in vitro*. **A**: When cultured on ECMatrix, ECV304 cells rapidly aligned with hollow tube-like structures; **B**: ECV304 cells were treated with erlotinib (100 μmol/L), and significant inhibition of tube formation was achieved compared with the control.

study in the BxPC-3 xenograft (day 28), the growth inhibition rate was 74.5% ($P < 0.05$) in the erlotinib treated group.

In vitro angiogenesis

EGFR TKIs have been reported to inhibit angiogenesis of pancreatic carcinoma. To determine whether erlotinib suppressed tumor vessel formation *in vitro*, we examined the effects of erlotinib on the ability of ECV304 to form capillary tube structures. ECMatrix is a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor and consists of laminin, collagen type IV, heparin sulphate proteoglycans, entactin, and nidogen. It also contains various growth and proteolytic enzymes that occur normally in EHS tumors. When cultured on ECMatrix, ECV304 cells rapidly aligned and formed tube-like structures (Figure 4A), however, the length of the tubes in the erlotinib treated cells was markedly reduced compared with the control which demonstrated that erlotinib significantly inhibited ECV304 tube formation (Figure 4B).

In vivo inhibition of EGFR and MVD after treatment with erlotinib

The EGFR expression pattern in the BxPC-3 tumors was examined by IHC staining. IHC analysis confirmed that the tumor xenograft tissues maintained both cell membrane and cytoplasmic EGFR expression. The expression of EGFR was decreased in the erlotinib treated groups (2.45 ± 0.81) compared with the control (10.65 ± 1.26) ($P < 0.05$). MVD was also assessed by immunostaining with factor VIII-related antigen in the most intense areas of neovascularization. Representative images of the two groups are shown in Figure 5. MVD of erlotinib treated tumors (1.86 ± 0.43) was significantly lower than that of the control (5.98 ± 1.27) ($P < 0.05$).

Modulation of apoptosis-associated gene and VEGF expression in BxPC-3 cells and xenografts treated with erlotinib

The effect of erlotinib on the expression of EGFR, apoptosis-associated factors such as *bcl-2*, *bak*, *bax*, *bcl-xl* and *VEGF* mRNA was determined by RT-PCR. BxPC-3 cells were treated with erlotinib at different concentrations ranging from 5-200 μmol/L for 48 h. The results showed that the expression of *EGFR* and

VEGF mRNA in the BxPC-3 cell line seemed to be down-regulated, the highest suppression was detected at the highest concentration used, 200 μmol/L. Densitometric analysis revealed the relative expression of VEGF at 200 μmol/L concentration of erlotinib ($1.2\% \pm 0.68\%$) was significantly lower than that of the control ($2.67\% \pm 0.13\%$) ($P < 0.05$). Apoptosis-associated gene including *bcl-xl* and *bcl-2* was suppressed by erlotinib in a concentration dependent manner; however, bax appeared to be up-regulated following erlotinib treatment at various concentrations; but it did not apparently affect bak expression. In the tumor xenograft tissues, the expression of *VEGF* mRNA was significantly lower (almost disappeared) in the erlotinib-treated group compared with that in the control ($P < 0.01$) (Figure 6).

DISCUSSION

Treatment options of pancreatic adenocarcinoma are unsatisfactory, and the prognosis of patients with pancreatic cancer is poor. Considering that the cure rate for these patients with surgery alone is low[8], development of potentially effective treatment is urgently needed. Many features of the pancreatic malignant phenotype are associated with the signaling networks that involve the EGFR. EGF and its receptor, EGFR, are over-expression in many human pancreatic cancers[9,10]. Recently, there is increasing evidence demonstrating the therapeutic potential of EGFR blockade in the management of pancreatic cancer and other malignancies[11-13]. EGFR inhibitors have shown activity in clinical trials in pancreatic, colorectal and non-small cell lung cancers[14-16]. Erlotinib is an orally available low-molecular-weight quinazolinamine that acts as a potent and reversible inhibitor of EGFR-TK activity. Single agent activity was observed in patients with non-small cell lung cancer, head and neck carcinoma and ovarian cancer[17-20]. A randomized phase III placebo-controlled trial has shown that the combination of gemcitabine and erlotinib is associated with a modest but statistically significant survival benefit compared with gemcitabine and placebo and this represents an EGFR-targeted agent conferred benefit in addition to chemotherapy[21]. In this study, we evaluated the efficacy of erlotinib, as a single agent, on pancreatic cancer cells grown *in vitro* and *in vivo*
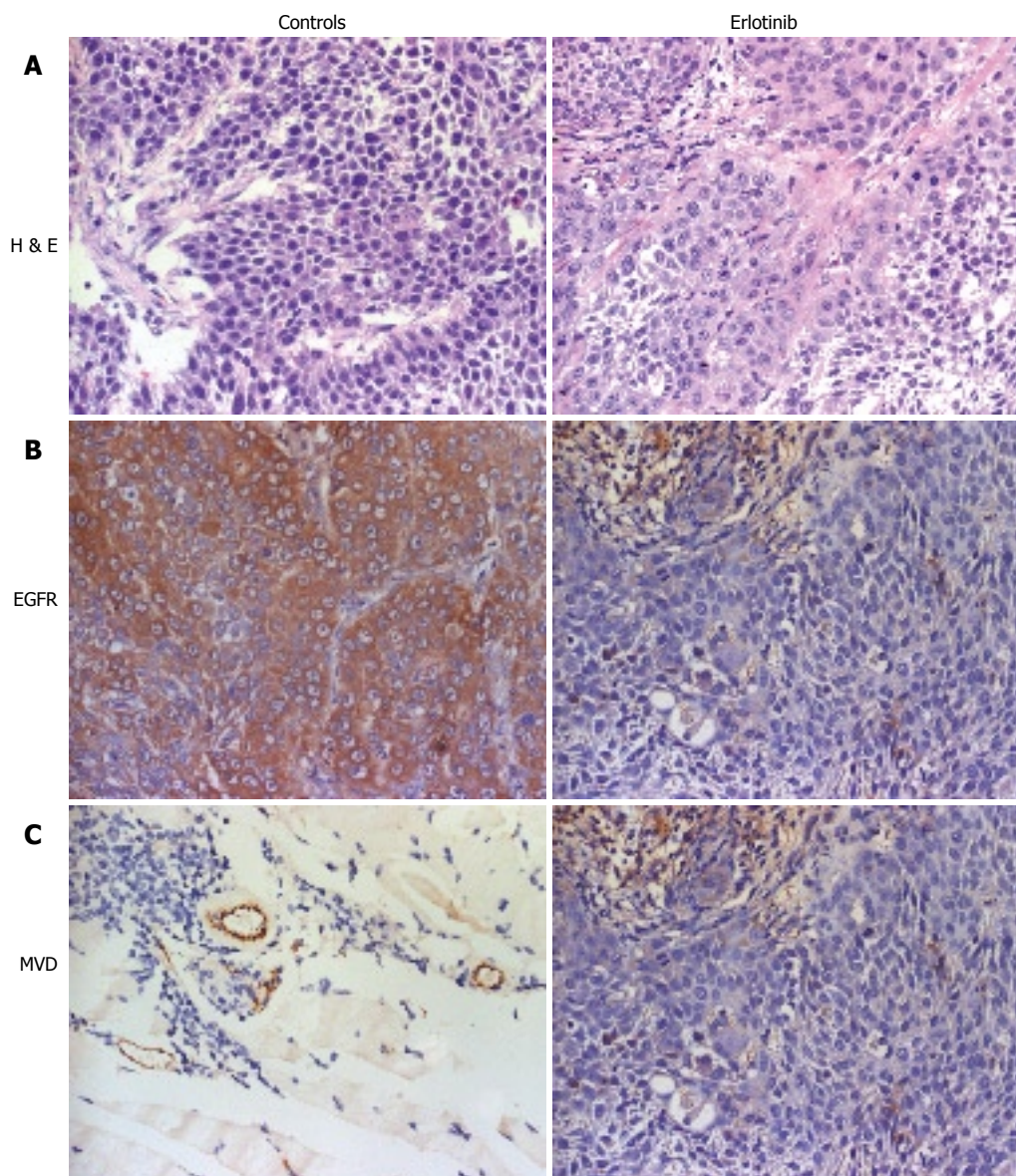
Figure 5 Expression of EGFR and the blood vessel endothelial cells in different treatment group in BxPC-3 mouse xenograft tissues. IHC was used to determine expression levels of EGFR and evaluate tumor microvessel density. **A**: HE staining for each sample (x 400); **B**: Expression of EGFR in treatment group was decreased compared with the control (x 400); **C**: Microvessel density of erlotinib treated group was lower than that of the control group.

using a nude mice xenograft model and explored the mechanisms involved as well. *In vitro* results showed an inhibition efficiency of 53.5% by erlotinib at the concentration of 50 μmol/L in the growth of cultured BxPC-3 pancreatic carcinoma cells as determined by MTT assay. The cell viability of BxPC-3 pancreatic cells was 53.5% with erlotinib treatment at a 50 μmol/L concentration by the MTT assay *in vitro*. There was no difference between 50 μmol/L and 100 μmol/L concentrations of erlotinib when cells were treated for 48 h (data not shown). We performed cell cycle analyses to characterize the underlying mechanisms of erlotinib's mode of action. Upon erlotinib treatment for 48 h, the proportion of cells in the G_0/G_1 phase increased to 73.4%, being significantly higher than that of the control. The antineoplastic effect of erlotinib is not solely due to cell cycle arrest. Induction of apoptosis by EGFR-TK inhibition has recently been reported[22,23]. In our study, we also observed cell apoptosis induced by erlotinib following cell cycle arrest. As we know, Bcl-2 members are crucial regulators of apoptotic cell death.

We also checked the expression of anti-apoptotic factors such as bcl-2, bcl-xl as well as pro-apoptotic factors bax and bak. Erlotinib induced a moderate decrease in the expression of bcl-2 and bcl-xl at a concentration of $\geqslant$ 50 μmol/L. Our study *in vitro* provides evidence that the growth of BxPC-3 cells can be suppressed by erlotinib and at least two mechanisms are involved, i.e. cell cycle arrest and apoptosis. We used a nude mouse xenograft model to further evaluate the antitumor efficacy of erlotinib in pancreatic cancer *in vivo*. Erlotinib was very effective in suppressing the growth of BxPC-3 tumors in a subcutaneous tumor model. Tumor inhibition of approximately 74.5% was observed after 4 wk of treatment with erlotinib at a dose of 100 mg/kg per day. IHC staining of sections of subcutaneously implanted tumors showed constitutively high EGFR expression, however, the mice treated with 100 mg/kg erlotinib daily showed lower levels of EGFR. These results showed that erlotinib as monotherapy has strong antitumor activity in human BxPC-3 xenograft models expressing EGF receptors.
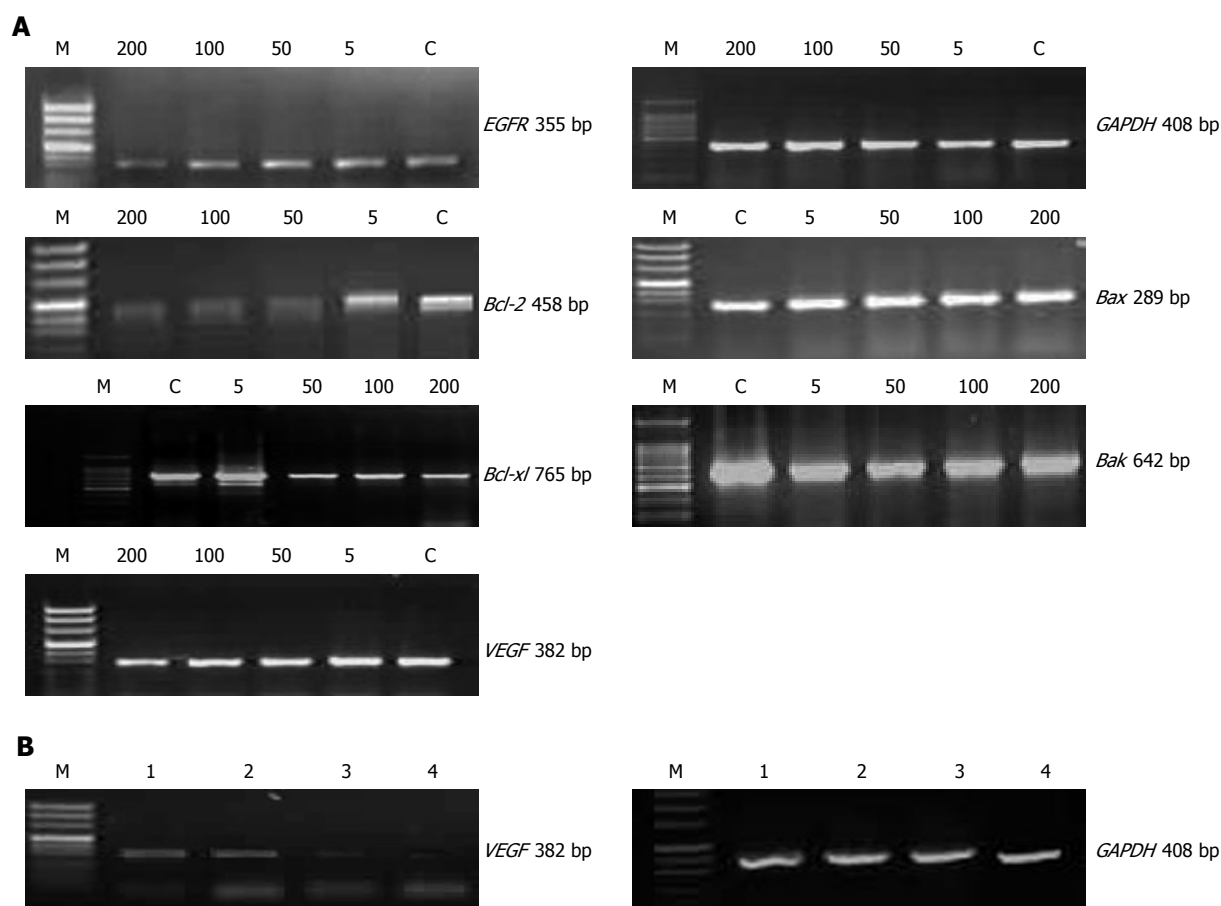
Figure 6 Expressions of *EGFR*, *Bcl-2*, *Bcl-xl*, *Bax*, *Bak* and *VEGF* mRNA in BxPC-3 cells treated with different concentrations (μmol/L) of erlotinib for 48 h and xenograft tissues were detected by RT-PCR. **A**: Effects of erlotinib on the expressions of EGFR, apoptosis-associated factors and *VEGF*. Lane M: DNA marker; Lane C: Control; other lanes: Different concentrations of erlotinib. **B**: Effects of erlotinib (100 mg/kg daily) on expression of VEGF in tumor tissues. Lane M: DNA marker; Lane 1, 2: Control group; Lane 3, 4: Treatment group. RT-PCR for *GAPDH* was performed in parallel and showed an equal amount of total RNA in the sample.

Numerous lines of evidence have shown that angiogenesis plays a significant clinicopathological role in tumors. Although pancreatic cancer is not a grossly vascular tumor, it often exhibits enhanced foli of endothelial cell proliferation. Moreover, several studies have reported a positive correlation between blood vessel density and disease progression in cases of pancreatic cancer, supporting the important role of angiogenesis in this disease[24-26]. EGFR signaling has previously been shown to play a role in angiogenesis[27]. In this study, we used the expression of VEGF, the MVD and tube formation assay to investigate the effects of erlotinib on angiogenesis both *in vitro* and *in vivo*. At the molecular level, VEGF is believed to be critical for pancreatic cancer angiogenesis[28]. Erlotinib exhibited a moderate decrease in VEGF expression *in vitro*, especially at a high concentration (200 μmol/L). Similarly, in xenograft tissues, there were marked reductions in the amounts of VEGF present in the treated group as compared with the control. As a unique tool, an *in vitro* capillary formation assay has been used to verify specific antiangiogenic activities of many agents with a good correlation to blood vessel formation *in vivo*[29]. Using this method, we observed that erlotinib as a single agent inhibited capillary tube formation by ECV304. Our immunohistochemical analysis of tumor specimens revealed that the treatment of mice with erlotinib produced a significant decrease in the number of tumor-associated blood vessels (MVD). The decrease in MVD could be attributable to a decrease in endothelial cell proliferation, as we proved *in vitro*.

In summary, our study showed that blockade of the EGFR signaling pathway by erlotinib has provided significant treatment in the BxPC-3 cell line *in vitro* and in nude mice and it may have a potential for the treatment of human pancreatic carcinoma. However, it is apparent that the use of a single signal transduction inhibitor cannot antagonize all the potentially relevant survival pathways in pancreatic cancers. As an adjuvant use in chemotherapy, targeting EGFR pathway seems to be a promising approach in the prevention and/or treatment of pancreatic cancer.

ACKNOWLEDGMENTS

COMMENTS

Background

The activity of the epidermal growth factor receptor (EGFR), a tyrosine kinase

receptor of the ErbB family, is abnormally elevated in most human solid tumors, including pancreatic cancer, and is associated with progression and poor prognosis. There are two main categories of therapeutic strategy for targeting the EGFR pathway, specific anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors (TKIs). Blockage of EGFR activity may lead to growth inhibition in pancreatic cancer.

Research frontiers

Molecularly targeted therapies have recently expanded the options available for patients with gastrointestinal tumors. Low weight EGFR TKIs such as erlotinib may play an important role in the treatment of human pancreatic carcinoma. Administration of erlotinib inhibits the BxPC-3 human pancreatic cancer cell line growth and induces antiangiogenic effect both in vitro and in vivo.

Innovations and breakthroughs

Small-molecule inhibitors of the EGFR tyrosine kinase, such as erlotinib, have shown promise in phase III trials in non-small-cell lung cancer (NSCLC). But the anticancer mechanism has not been clearly elucidated especially in gastrointestinal tumors, including pancreatic cancer. The results indicate that the blockade of EGFR may provide a rationale for translating this therapeutic strategy into a clinical setting in some pancreatic cancer patients.

Applications

This study showed that blockade of the EGFR signaling pathway by erlotinib suppressed the BxPC-3 human pancreatic cancer cell line growth and induced antiangiogenic effects both in vitro and in vivo. As an adjuvant used in chemotherapy, targeting the EGFR pathway seems to be a promising approach in the prevention and or treatment of pancreatic cancer.

Terminology

The epidermal growth factor (EGF) receptor (or ErbB1) and the related ErbB4 are transmembrane receptor protein tyrosine kinases which bind extracellular ligands of the EGF family. ErbB2 and ErbB3 are "co-receptors" structurally related to ErbB1/ ErbB4, but ErbB2 is an "orphan" receptor and ErbB3 lacks tyrosine kinase activity. They transduced biological signals from the extracellular to the intracellular compartment. These families of ligands and receptors have been firmly linked to proliferative signaling and oncogenesis.

Peer review

In this paper, the effect of erlotinib on the proliferation and apoptosis of the human pancreatic cancer cell line BxPC-3 was studied. A dose-dependent inhibition of BxPC-3 cell growth in vitro, with a block of G_1-S transition, and rise in apoptosis were observed in erlotinib-treated cells. A decrease in tumor volume and microvessel density was also found in erlotinib-treated nude mice carrying BxPC-3 cell xerography. This is a very interesting manuscript with some minor points needing further addressing.

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