RESEARCH ARTICLE

Simultaneous Blockage of Epidermal Growth Factor Receptor and Cyclooxygenase-2 in a Human Xenotransplanted Lung Cancer Model

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Abstract

The effects of erlotinib combined with celecoxib in a lung cancer xenograft model were here explored with a focus on possible mechanisms. A xenotransplanted lung cancer model was established in nude mice using the human lung cancer cell A549 cell line and animals demonstrating tumour growth were randomly divided into four groups: control, erlotinib, celecoxib and combined (erotinib and celecoxib). The tumor major axis and short diameter were measured twice a week and after 40 days tissues were collected for immunohistochemical analyses of Bcl-2 and Bax positive cells and Western-blotting analyses for the epidermal growth factor recepto (EGFR), P-EGFR, and cyclooxygenase-2 (COX-2). Tumor size in the combined group was smaller than in the others ($p<0.01$) and the percentage of Bcl-2 positive cells was fewer in most cases ($p<0.01$), while that of Bax positive cells was greater than in the erlotinib and celecoxib groups ($P>0.05$). Western blotting showed decreased expression of P-EGFR and COX-2 with both erlotinib and celecoxib treatments, but most pronouncedly in the combined group ($P<0.05$). Simultaneous blockage of the EGFR and COX-2 signal pathways exerted stronger growth effects in our human xenotransplanted lung cancer model than inhibition of either pathway alone. The anti-tumor effects were accompanied by synergetic inhibition of tumor cell apoptosis, activation of p-EGFR and expression of COX-2.

Keywords: Lung cancer - erlotinib - celecoxib - combination therapy

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Introduction

Molecular targeted cancer therapy has been payed more and more attention recently for the patients with cancer. The therapy targeting epidermal growth factor receptor (EGFR) has become relatively mature. So far, the small-molecular EGFR inhibitor such as EGFR-TKI, gefitinib and erlotinib have been approved successively by American FDA for applying to non-small cell lung cancer (NSCLC) treatment followed by the failed chemotherapy (MH Cohen et al., 2003; FA Shepherd et al., 2005). Nevertheless, as tumor happens in different steps and levels, and interactive affects exist in different signal path, it might be less effective if only a single targeted spot is blocked and multi-targets blocking is a tendency for the future treatment.

In the recent years, it was discovered that cyclooxygenase-2 (COX-2) is over expressed in several different kinds of tumors including NSCLC and it is related to tumor malignancy, lymph nodes metastasis and clinical stages (Chan et al., 1999; Renkonen et al., 2002; Dannenberg et al., 2003). It might also indicate another potential targeted spot for tumor treatment. More and more evidence shows that there is interaction between EGFR and COX-2 signal paths, and they share to affect the tumor gene transcription regulation, proliferation of tumor cell, apoptosis resistance, angiogenesis and metastasis etc (Gerber 2008; Katzel et al., 2009). Lots of in vitro experiments also demonstrate the effect of synergetic anti-tumor by signal paths of EGFR and COX-2 being blocked simultaneously. Through blocking these two independent targeted spots, EGFR and COX-2 via celecoxib and Erlotinib, this experiment aims to study whether this kind of treatment on lung cancer and transplantation tumor of nude mouse could lead to coordinate repression and its possible mechanism.

Materials and Methods

Material

Twenty-four SPF male nude mouth BALB/C (4-6week old, 20g) are purchased from Peiking HuafuKang Biotech

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Co., Ltd with certificate No. (sexk Jing 2009-0004); Erlotinib 0.15 g (trade name: TAREVA) and Celecoxib 0.2 g (trade name: Xi Le Bao) are donated by Shanghai Roche Group Co., Ltd and Huirui Pharmaceutical Co.Ltd respectively. Rabbit anti-human Bel-2 (sc-492) polyclonal antibody, Rabbit anti-human Bax (sc-6236) polyclonal antibody, Rabbit anti-human EGFR polyclonal antibody (sc-03), Rabbit anti-human p-EGFR polyclonal antibody (sc-101666) are purchased from Santa Cruz Co., Rabbit anti-human COX-2 polyclonal antibody (CST4842)is purchased from Cell Signaling Technology Co. Mice SP second antibody kit is purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd; BCA protein titer kit is purchased from Shanghai Shenneng Biocolor Technology Co, Ltd.

Prescription

Erlotinib and elecoxib are dissolved into 1% tween 80 solution, the concentration can be erlotinib (3 mg/ml) and celecoxib (10 mg/ml), respectively.

Establishment and group intervention of nude-mouse transplanted tumor model

Twenty-four nude mouth are raised as SPF in an isolated cage, all needed cages, food and water go through autoclaving process. When routine cultured A549 of logarithmic phase human lung cancer cell line has grown into 80% of the bottom of the glass, use 0.25% trypsinization and PBS cleanning, then discard supernatant after 1000r/min centrifugation for 5 min. After rehanging cell by RP-1640 culture medium, use blood counting chamber for counting, densify the cell into 5×10^6 /ml, and inoculated under the armpit of nude mouth, 0.2ml for each. After about a week, tumor come into being, then these 24 mouses are divided into control group (1% tween 80, n=6), group of erlotinib (30 $mg^{-1}.kg^{-1}.d^{-1}$, n=6), group of celecoxib (100$mg^{-1}.kg^{-1}.d^{-1}$, n=6) and the group of erlotinib combined with celecoxib (erlotinib 30 $mg^{-1}.kg^{-1}.d^{-1}$ and celecoxib 100$mg^{-1}.kg^{-1}.d^{-1}$, n=6). Mouse in each group will receive gavage with corresponding drugs mentioned above everyday, then follows the observation of their diet and their appearance. Major axis and minor axis of tumor are measured by vernier caliper twice a week, then approximate volum of tumor can be calculated by formular "$V=ab^2/2$". After about 40 days, nude moses are put to death by cutting their necks, remove their tumor lumps, part of which are stored into solution with 4% paraformaldehyde, the rest parts are cryopreserved within liquid nitrogen.

Bcl-2 and Bax's expression detection in transplantation tumor tissue through immunohistochemical methods

Take out stable tissue from paraformaldehyde, dehydrate conventionally, then follows paraffin embedding, remove 5 um slice. Dewax the slice and dehydrate conventionally

Specimens were subjected to antigen retrieval by covering the sections with a 10 mM sodium citrate buffer (pH 6.0) and heating them in a microwave for 1 minute. Slides were then cooled at room temperature for 20 minutes, rinsed with phosphate-buffered saline (PBS), and blocked with 5% bovine serum albumin for 30 minutes at room temperature. Sections were incubated overnight at 4°C with the primary antibody. Sections were washed three times with PBS and incubated in the dark for 45 minutes at room temperature with donkey anti-mouse immunoglobulin G (IgG) conjugated with Cy2 (1: 200; Jackson Laboratory, Bar Harbor, Maine), donkey anti-goat IgG conjugated with FITC (1: 200; Santa Cruz Biotechnology), and donkey anti-rabbit IgG conjugated with Cy2 (1: 200; Jackson Laboratory), respectively, as secondary antibody. The sections were then washed with PBS and mounted with medium containing 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

Detection of the expression of the EGFR, p-EGFR and COX-2 in transplantation tumor tissue through Western blot method

Take out 100 ug tissue cryopreserved in liquid nitrogen from each group required by kit instruction, and test their total protein concentration respectively. Add 1/4 buffer solution and 100 °C denatured protein, and use 10% separation gel for protein electrophoresis, with loading amount of 50ug, then transfer the protein onto FVDF membrane by wet prints, add 5% milk and store it for about 1h within room tempreture. 4 °C primary antibodies overnight, second antibody for 1 h within room temperature, enhanced chemiluminescence method for imaging, gel imager/LAS4000 system for reading, then follows grey value analysis, relative protein content =β-actin, divided by interest protein. This experiment goes triplicate.

Statistical methods

Statistical analysis is carried out by statistical software SPSS11.0. t-test method is applied for pairwise comparison, intra-class comparison is applied to analysis of variance, there is statistical significance of the difference if $P<0.05$.

Results

Tumor in nude mouse come into being for about 7 days, and tumor formation rate is 100%. No death was found during experiment session and they were in fine condition. When they were put to death, no metastasis was detected within other organs.

Develpoment of transplantation tumors in each treatment group

Until the end of the experiment, we can see that the volum of tumor in combined drug group was much smaller than that of control group, as well as erlotinib group and celecoxib group ($P<0.01$) (Figure 1). Tumor volum changes were presented in Table 1.

Bcl-2 and Bax expression in each group

Under the light microscope, it is observed that there is structural disorder in the transplantation tumor tissue, tumor cells are roundness and oval, with large and uneven dyeing nucleus. As for the dye: Bcl-2's positive

Table 1. The Volume of the Cancer

Group	n	Begin volume (mm^3)	End volume (mm^3)
Control group	6	4.792±0.062	661.208±203.108
Erlotinib group	6	4.719±0.037	223.708±38.934*
Celecoxib group	6	4.490±0.089	450.958±114.086
Combine group	6	4.375±0.047	187.833±26.389**

*p<0.05 VS control group; **p<0.05 VS erlotinib group, celecoxib group and control group

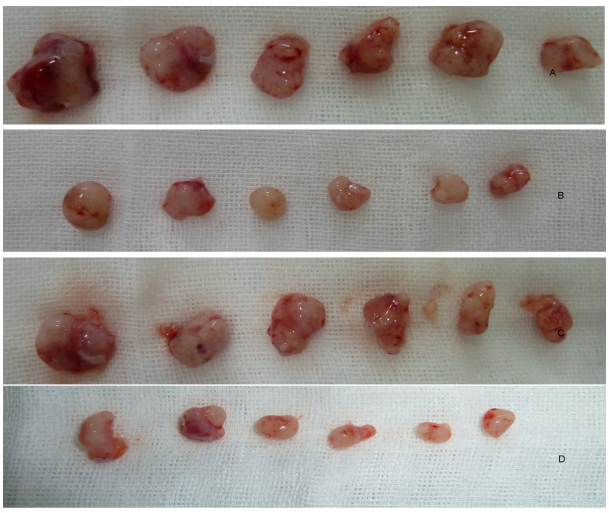

Figure 1. Photograph of Cancers Treated with Diffent Agents. A: control group; B: erlotinib group; C: erlotinib group; D: combine group

expression is shown by that cytoplasm or cell membrane could be dyed into claybank, and colored cytoplasm of Bax (Figure 2). High power (X 400) counting, more than 5 visual fields are counted of each slice, and every visual field for at least 200 cells, which is demonstrated by the positive expression cell rate within the total cells. Positive expression of Bcl-2, and Bax are shown in chart2. Bcl-2' expression of drug combination group was obviously reduced compared with that of control group and erlotinib group (P<0.05), and there wa no statistical significance compared with celecoxib group (P>0.05); there was no statistical significance of the differences btween each group (P>0.05).

Protein expression of P-EGFR, EGFR and COX-2 within each group

Figure 3 and Figure 4 shows the protein expression among each group. Grey value shows there was no statistical significance of EGFR among each treatment group (P>0.05). p-EGFR's expression in combined drug group was reduced compaired with that of control group and erlotinib group (P<0.05), but no statistical significance compared with that of celecoxib (P<0.05). COX-2's expression in combined drug group was reduced obviously compared with that of control group as well as celecoxib group (P< 0.05), but no statistical significance compared with that of erlotinib group (P>0.05).

Discussion

NSCLC occupies 80% of total lung cancer, most of which are discovered advanced stage with unsatisfactory

Table 2. ???

Group	Rate of Bcl-2 expression (%)	Rate of Bax expression (%)
Control	73.15±17.39	11.27±3.49
Celecoxib group	29.45±12.13*	20.28±7.89
Celecoxib group	71.43±19.35	18.39±4.54
Combine group	10.03±2.98**	23.79±13.69

*p<0.05 VS control group; **p<0.05 VS erlotinib group, celecoxib group and control group

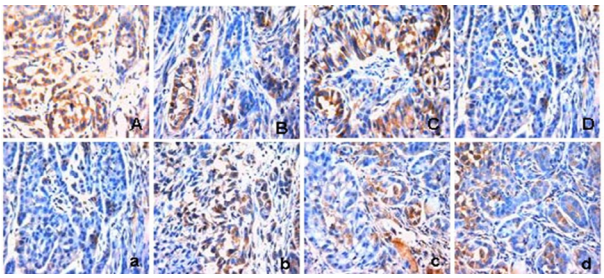

Figure 2. The Express of Protein Bcl-2(×400) A,a: control group; B,b: erlotinib group; C,c: celecoxib group; D,d: combine group

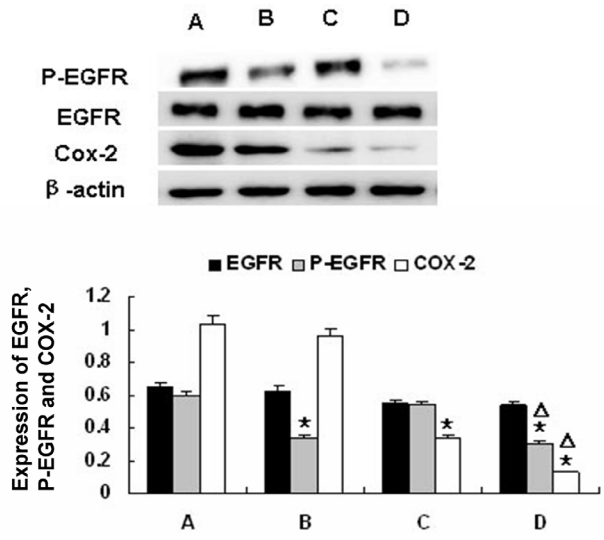

Figure 3. The Expression of COX-2, EGFR and P-EGFR by Western Bolt. A: control group; B: erlotinib group; C: erlotinib group; D: combine group *p<0.05 VS control group **p<0.05 VS erlotinib group, celecoxib group and control group

prognosis. For NSCLC, stage IIIB, there is only 3% to 7% of survival rate within 5 years, and even less than 1% when it comes to stage IV (Jemal et al., 2008). Operation and conventional platinum-based chemotherapy are regarded as the major option for NSCLC but with much side effect. Chemotherapy comes into a stagnation because of problems such as acquired drug-resistance. Molecular targeting treatment for lung cancer becomes a tendency for its strong specificity and less side effect, and its long-term development value

EGFR and its ligand (TGF-α, EGF, and amphiregulin etc.) shows overexpression within different epithelial tumors. Activation of EGFR, is closely related to various activities such as tumor cell apoptosis and differentiation, as well as vascularization (Katzel et al., 2009). EGFR is a transmembrane surface receptor with endogenous

tyrosine kinase (TK) activity, and is of erbB family. And being integrated with its ectoenzyme area, its ligands could launch homodimerization of receptor or heterodimerization with other erbB members, which lead to activation of the TK, launching a series of inferior signal paths, which controls cell growth as well as cell divison. While erlotinib belongs to TKI and could achieve specific binding with intracellular tyrosine domain of EGFR through cell membrane, which will repress the activation of TK, then the inferior signal paths that cause tumor growth and metastasis will be repressed thereafter, all of them share to repress tumor. Erlotinib was approved by American FDA for NSCLC treatment in Nov, 2004 (Shepherd et al., 2005).

Stimulated by inflammation, mitogenic factor etc., COX-2 will catalyze arachidonic acid and result to prostagladin. It is discovered in recent years that COX-2 have overexpression in various tumors and it has been proved that COX-2 inhibitor could reduce tumor growth, transfer and vascularization within multiple tumor cells and xenograft model (Choe et al., 2005; Zhang et al., 2005; Jalili et al., 2008). Nowadays, lots of evidence show that signal paths mediated by EGFR and COX-2 have overlapped field and they join together for the occurrence and development of tumor. Researches show that EGFR combined with its ligand such as TGF-α, EGF etc. It could modify COX-2 expression and its catalysed PGE2 accumulation in either transcriptional or translational level. While combining PGE2 generated by EGFR and GPCRs could facilitate EGFR's lingand separattion from the surface of cell membrane, which leads to more activation about EGFR. And the intersective activation of the two signal paths offers theoratical foundations for combined targeted therapy.

It was first reported in 2000 by Torrance CJ etc. that the combined application of sulindac and EKI-569 (EGFR-TKI) had successfully restrained the occurrence of familial intestinal polyposis (Torrance et al., 2000). And Zhang and his members (Jalili et al., 2008) used celecoxib, the inhibitor of COX-2, combined with gefitinib, the inhibitor of EGFR, to deal with head and neck squamous cell carcinoma of nude-mouse transplanted tumor model, and the synergetic anti-tumor effect demonstrated the feasibility of combined targeted therapy. It was also confirmed that their synergetic anti-tumor effect was shown on pancreatic cancer and head and neck neoplasm by blocking signal paths of COX-2 and EGFR simultaneously (Ali et al., 2005; Fu et al., 2011). But in the study of lung cancer, it only shows at vitro experimental stage, not any in vivo experimental report has been made.

This article aims to study that how blocking COX-2 and EGFR by targeting simultaneously would affect the human lung denocarcinoma and nude-mouse transplantation tumor and its possible mechanisms, which provides theoretical foundation for the targeted treatment of lung cancer. The human lung adenocarcinoma A549 cell line we use was proved to have COX-2 and EGFR expression in our previous researches. Given gavage by using erlotinib combined with celecoxib, transplantation tumor volum of nude-mouse was obviously smaller than that of erlotinib group as well as of celecoxib, showing the effect of synergistic inhibition of tumor growth. Generally speaking, the organism could eliminate non-functional or disordered cells in vivo by launching apoptosis, and less of apopsis could lead to tumor occurrence. There are two ways of organism apoptosis, dependent mitochondria and independent mitochondria. Bcl-2 protein family plays a key role in adjusting apoptosis occurred in mitochondrial pathway. Bcl-2 protein family includs: pro-apoptosis protein such as Bax, Bak and IAP (inhibitor of apoptoasis protein) such as Bel-2, Bel-XL. Apoptosis index could be reflected by Bel2/Bax (Brunelle et al., 2009). According to our experiment, in combination group, there was a clear reduction of Bel-2 protein expression, while Bax's stayed still, which indicats that blocking signal paths of COX-2 and EGFR simultaneously had promoted the apoptosis of transplantation tumor cell. After this blocking, contents of COX-2 and EGFR had obviously reduced, compared with that of single drug group, while EGFR stayed still among each treatment group, which indicates that blocking COX-2 signal path by celecoxib could reduce the activation of EGFR while blocking EGFR signal path by erlotinib could reduce the expression of COX-2, and there is an interactive activation among signal paths between COX-2 and EGFR. So blocking two target spots of COX-2 and EGFR could effectively avoid this kind of intersective activation, then synergetic anti-tumor effect can be achieved. Within their dosage parameter of clinical application, erlotinib and celecoxib can be less side-effect and softer for patients tolerance. Besides, they have different side-effect mechanisms, so there wont be overlapping side-effect when they are combined together, which largely enhances the curative effect as well as the safety. All of that provide the possibility for combined targeted treatment of lung cancer, but this kind of combined treatment for lung cancer needs to go through a stricted clinical trial stage.

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