Effect of erlotinib combined with cisplatin on IL-6 and IL-12 in mice with Lewis lung cancer

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Abstract. Effect of erlotinib combined with cisplatin on tumor growth, interleukin-6 (IL-6) and interleukin-12 (IL-12) in mice with Lewis lung cancer (LLC) was investigated. Forty-four pure inbred SPF C57BL/6J mice were modeled for LLC and randomized into groups A, B, C and D (n=11 each group). Mice in group A were given normal saline, group B was given erlotinib, group C was given cisplatin injection and group D erlotinib combined with cisplatin. Tumor growth of the mice was observed and the tumor mass was measured. Serum IL-6 and IL-12 levels were measured by enzyme-linked immuno-sorbent assay (ELISA) 40 days later. At different time-points after medication, tumor volume in group D was significantly lower than that in groups A, B and C (P<0.05), and that in groups B and C was significantly lower than that in group A (P<0.05), whereas there was no significant difference between groups B and C (P>0.05). Tumor mass in groups B, C and D was significantly lower than that in group A (P<0.05), and that in group D was significantly lower than that in groups B and C (P<0.05), whereas there was no significant difference between groups B and C (P>0.05). Compared with groups B and C, mice in group D had significantly lower IL-6 level (P<0.05), but significantly higher IL-12 level (P<0.05). There was no significant difference in IL-6 and IL-12 levels between groups B and C (P>0.05). In conclusion, erlotinib combined with cisplatin can inhibit the tumor growth of mice with LLC, and inhibition of IL-6 level and upregulation of IL-12 level may be one of its therapeutic mechanisms.

Introduction

As a common malignant tumor threatening human life and health, lung cancer has increasing morbidity and mortality rates (1). Approximately 85% of patients with the disease are patients with non-small cell lung cancer (NSCLC). Early lung cancer mostly treated by surgery has no obvious signs and symptoms, so the patients diagnosed have been in the middle and advanced stages when the tumor tissue has metastasized. That is to say, the patients lose the best surgical treatment time and have a low overall survival rate (2,3). Clinically, lung cancer has been treated by platinum-based chemotherapy. Although dose-limited platinum drugs cause toxic and side effects such as nephrotoxicity and neurotoxicity, cisplatin improves the overall survival of patients with lung cancer metastasis (4). With the development of molecular biology, targeted therapy is gradually used for the treatment of lung cancer (5,6). Epidermal growth factor receptor (EGFR) mutations are common drivers of lung cancer, and EGFR-tyrosine kinase inhibitors (TKIs) are considered as the best choice for the first-line treatment of advanced or recurrent non-squamous NSCLC carrying activating EGFR mutations (7). EGFR-TKIs are more effective for patients with EGFR mutant lung cancer than platinum-based chemotherapy (8). Erlotinib as a kind of EGFR-TKIs, is clinically effective for the treatment of NSCLC (9). According to Korytowsky et al (10), there is no significant difference in efficacy between erlotinib and chemotherapy (docetaxel or pemetrexed) for patients with advanced NSCLC who have received platinum-based chemotherapy for no more than 4 cycles and have disease progression during or after chemotherapy.

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In previous treatment, tumor cells were given high level of consideration, while the influence of tumor microenvironment on the efficacy was ignored (11). Interleukin-6 (IL-6), a cytokine that exists in tumor microenvironment, is closely related to cancer cell proliferation, angiogenesis and metastasis (12). IL-12 is one of the most effective cytokines for mediating antitumor activity and has a pleiotropic effect on immune cells forming tumor microenvironment. As a therapeutic target for tumors, it establishes a link between innate and adaptive immunities and plays a key role in shaping antitumor or tumor immunity (13).

Previous studies have shown obvious benefits of erlotinib and cisplatin in advanced lung cancer (14), but few studies exist on the specific application of erlotinib combined with cisplatin and its effects on IL-6 and IL-12. Lewis lung cancer mouse model is one of the tumor models frequently used in studies. As a common model for studying drug treatment of lung cancer, it is easy to model and has high tumor formation rate (15). Therefore, a mouse model of lung cancer was established in this study to explore the effects of erlotinib combined with cisplatin on the tumor growth, IL-6 and IL-12 of mice with Lewis lung cancer (LLC).

Materials and methods

Animals and cells. Forty-four pure inbred SPF C57BL/6J mice [Shanghai SLAC Laboratory Animal Co., Ltd., license no. SCXK (2003-0003)], aged 6-8 weeks with a body weight of 20.13±2.16 g, were fed with SPF granular chow in well ventilated clean facility. They have free access to water and food. Indoor humidity was 45-64% and indoor temperature was 20-24°C, with 12-h light (500 lx)/12-h dark (0 lx). This experiment was carried out 1 week after acclimatization and was approved by the Ethics Committee of the Hospital, with the process following Guide for the Care and Use of Laboratory Animals (16,17). LLC cell line of the mice was introduced by Beina Chuanglian Biotechnology Research Institute (item no. ATCCCRL-1642) and stored in liquid nitrogen.

Cell culture and preparation of animal models. LLC cells were inoculated into a culture dish, added with RPMI-1640 medium containing 10% fetal bovine serum (both from Gibco BRL) and 1% mycillin/streptomycin, and then cultured in an incubator (Thermo Electron Corporation) at 37°C with 5% CO_2. The culture fluid was changed once/2 days, and the LLC cell line was digested with 2.5% pancreatin and then passaged. Cells in logarithmic phase were taken to prepare a suspension with a cell concentration of 2.80×10^7 ml^{-1} for subsequent experiments.

Modeling, grouping and medication. The mice were fixed on the operating console for routine skin disinfection then 0.2 ml (~2×10^6 living cells) of the LLC cell suspension was subcutaneously injected into the right axilla of mice with a 1 ml syringe, during aseptic operation. The tumor formed around the 8th day and then grew to ~8 mm. At that time, 44 mice were randomized into groups A, B, C and D. Mice in group A were given 30 mg/kg of normal saline, group B was given 30 mg/kg of erlotinib (Roche Medical Electronics), group C was given

3 mg/kg of cisplatin injection (Qilu Pharmaceutical Co., Ltd., batch no. ALA1206023) and group D erlotinib (30 mg/kg) combined with cisplatin (3 mg/kg). From the 2nd day after modeling, the drugs were intraperitoneally injected once daily for 21 consecutive days.

Observational indexes and methods. The longest diameter (a) and the shortest diameter (b) of the tumor were measured with vernier calipers at 1 day, 5 days, 9 days, 13 days, 17 days and 21 days after medication. The tumor volume was estimated with reference to $V = ab^2/2$ (18), and the tumor growth curve was plotted.

Forty days later, the mice were sacrificed through Cervical dislocation, with the eyeballs enucleated. The eyeballs were removed and 0.5 ml of blood was taken with the EP tube. The serum was separated by centrifugation at 1,500 x g at 4°C for 10 min. Upper serum (50 μl) was collected and stored in a refrigerator at -80°C. The tumor was excised and measured for mass (average tumor mass in group A - average tumor mass in each group after medication)/average tumor mass in group A x100% = tumor inhibition rate (TIR).

Serum IL-6 and IL-12 levels were detected by enzyme-linked immunosorbent assay (ELISA) according to the instructions of mouse IL-6 and IL-12 ELISA kits (Shanghai Hengfei Biotechnology Co., Ltd., CSB-E04639m-1, CSB-E07360m-1). All samples and reagents were taken out in advance to balance with room temperature. The ELISA plate was washed twice with 300 μl of washing liquid, and discarded, the wells were dried with absorbent paper. A well for the sample to be tested, a standard well and a blank well were set up, in which 50 μl of the sample, standard substance and sample diluent was respectively added, and then 50 μl of biotin-labeled antibody was added. The plate was sealed with a microplate sealer and incubated at 37°C for 1 h. After the liquid was discarded, each well was added with 300 μl of the washing liquid to wash the plate 5 times, and the wells were dried each time after the washing. Each well was added with 100 μl of streptavidin, and then the plate was sealed with a microplate sealer and incubated at 37°C for 1 h. Each well was added with 300 μl of the washing liquid to wash the plate 5 times, and the wells were dried each time after the washing. Each well was added with 50 μl of A and 50 μl of B working solutions, and then incubated in dark at room temperature for 20 min. After that, 100 μl of stop solution was added to each well. OD values of each well were detected at 450 nm using a 680 fully automatic microplate reader (Bio-Rad), to calculate IL-6 and IL-12 levels.

Statistical methods. SPSS 18.0 (IBM Corp.) was used for statistical analysis, GraphPad Prism 7 for plotting figures. Count data were expressed by the number of cases/percentage (n/%), and Chi-square test was used for comparison of the count data between groups. Measurement data were expressed by mean ± standard deviation (mean ± SD), and one-way analysis of variance (ANOVA) was used for comparison of mean between multiple groups. After that, Dunnett t-test was used for pairwise comparison, repeated measures ANOVA for comparison of different time-points, Bonferroni for pairwise comparison between different time-points within groups. P<0.05 indicates a statistically significant difference.

Table I. Comparison of general conditions [n(%)]/(mean ± SD).

Categories	Group A (n=11)	Group B (n=11)	Group C (n=11)	Group D (n=11)	F/χ^2 value	P-value
Sex					0.786	0.853
Male (%)	7 (63.64)	8 (72.73)	7 (63.64)	6 (54.55)		
Female (%)	4 (36.36)	3 (27.27)	4 (36.36)	5 (45.45)		
Age (weeks)	7.02±0.58	6.94±0.46	7.12±0.51	6.85±0.63	0.483	0.696
Body weight (g)	20.13±2.16	21.08±1.93	20.47±2.09	20.54±2.12	0.394	0.758
Indoor humidity (%)	50.18±2.93	49.76±3.67	50.26±3.52	51.64±3.46	0.727	0.542
Indoor temperature (˚C)	22.05±1.24	22.67±0.94	22.38±1.05	22.03±1.27	0.792	0.505

Table II. Comparison of tumor mass and TIR (mean ± SD).

Groups	n	Tumor mass/g	TIR (%)
Group A	11	5.57±0.27	-
Group B	11	3.42±0.53[a]	38.60
Group C	11	3.37±0.24[a]	44.88
Group D	11	1.89±0.13[a,b]	66.07
F value	-	242.000	-
P-value	-	<0.001	-

[a]P<0.05 compared with group A; [b]P<0.05 compared with groups B and C. TIR, tumor inhibition rate.

Table III. Comparison of IL-6 and IL-12 levels (mean ± SD).

Groups	n	IL-6 (ng/ml)	IL-12 (ng/ml)
Group A	11	3.19±0.35	4.73±0.54
Group B	11	2.59±0.37[a]	5.39±0.66[a]
Group C	11	2.51±0.43[a]	5.34±0.62[a]
Group D	11	2.08±0.29[a,b]	5.94±0.41[a,b]
F value	-	17.390	8.415
P-value	-	<0.001	<0.001

[a]P<0.05 compared with group A; [b]P<0.05 compared with groups B and C. IL, interleukin.

Results

Comparison of general conditions. There were no statistically significant differences between groups A, B, C and D with respect to sex, age, body weight, indoor temperature and indoor humidity (P>0.05) (Table I).

Comparison of tumor volume. According to the tumor growth curve, the tumor volume of mice in the four groups increased with time, and the growth rate of group A was the fastest, followed by groups C, B and D. According to repeated measures ANOVA, there was a statistically significant difference in tumor volume between the four groups after medication (P<0.05). At different time-points after medication, tumor volume in group D was significantly lower than that in groups A, B and C (P<0.05), and that in groups B and C was significantly lower than that in group A (P<0.05), whereas there was no significant difference between groups B and C (P>0.05) (Fig. 1).

Comparison of tumor mass. There was a statistically significant difference in tumor mass between the four groups (P<0.05). Tumor mass in groups B, C and D was significantly lower than that in group A (P<0.05), and that in group D was significantly lower than that in groups B and C (P<0.05), whereas there was no significant difference between groups B and C (P>0.05) (Table II and Fig. 2).

Comparison of IL-6 and IL-12 levels after treatment. There were statistically significant differences in IL-6 and IL-12
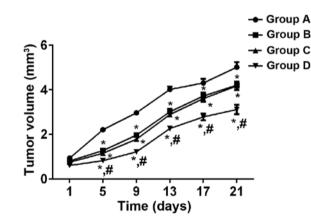

Figure 1. Comparison of tumor volume. According to repeated measures ANOVA, there was a statistically significant difference in tumor volume between the four groups after medication (P<0.05). At different time-points after medication, tumor volume in group D was significantly lower than that in groups A, B and C (P<0.05), and that in groups B and C was significantly lower than that in group A (P<0.05), whereas there was no significant difference between groups B and C (P>0.05). *P<0.05 compared with group A at the same time-point; #P<0.05 compared with groups B and C at the same time-point.

levels between the four groups (P<0.05). Compared with group A, mice in groups B, C and D had significantly lower IL-6 levels (P<0.05), but significantly higher IL-12 levels (P<0.05). Compared with groups B and C, mice in group D had significantly lower IL-6 level (P<0.05), but significantly higher IL-12 level (P<0.05). There were no significant differences in IL-6 and IL-12 levels between groups B and C (P>0.05) (Table III and Fig. 3).
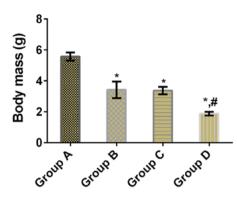
Figure 2. Comparison of tumor mass. Tumor mass in groups B, C and D was significantly lower than that in group A (P<0.05), and that in group D was significantly lower than that in groups B and C (P<0.05), whereas there was no significant difference between groups B and C (P>0.05). *P<0.05 compared with group A; #P<0.05 compared with groups B and C.

Discussion

Most patients with lung cancer are in the advanced stage when diagnosed. Those who undergo chemotherapy and radiotherapy for the treatment of unresectable advanced lung cancer have poor median progression-free survival time and 5-year overall survival time (19). Patients with advanced lung cancer are mostly treated by platinum drugs, but some of the patients have poor tolerance and therapeutic effects. Therefore, lung cancer has no cure although chemotherapeutics are continuously updated (20).

First-line treatment decisions for advanced lung cancer are currently based on sensitive EGFR mutations (21). In recent years, EGFR-TKIs represented by erlotinib, which specifically inhibits EGFR signaling pathway and further inhibits tumor growth are increasingly valued in the comprehensive treatment of lung cancer (22). According to Scagliotti et al (23), erlotinib combined with gefitinib is a tolerable regimen and has better clinical efficacy than erlotinib alone in chemotherapy for patients with EGFR-mutant NSCLC. Therefore, the combination of erlotinib and other drugs is effective for patients with advanced lung cancer. It has been shown that erlotinib-cisplatin combination is effective for erlotinib-resistant cancer by targeting (down-regulating) Atg3-mediated autophagy and inducing apoptotic cell death (24). However, scarce research exists on the effects of erlotinib combined with cisplatin for lung cancer in vivo. Lewis lung cancer is a commonly used model for studying drug therapy for lung cancer, so Lewis lung cancer mice were used in this study. Tumor volume in group D was significantly lower than that in groups A, B and C at different time-points after medication, and tumor mass was significantly lower than that in groups A, B and C, suggesting that erlotinib combined with cisplatin significantly inhibits the tumor growth of mice with lung cancer. Therefore, erlotinib combined with cisplatin may become a new therapeutic regimen for lung cancer.

IL-6 is a main cytokine in tumor microenvironment, and its high level shows the correlation of inflammations with cancers. It promotes tumorigenesis through regulating markers and signal transduction pathways (including apoptosis, survival, proliferation, angiogenesis, invasion and metastasis) of cancers (25). As a cytokine that stimulates cellular immunity, IL-12 exerts effective antitumor activity through immune stimulation and antiangiogenic mechanisms, and promotes rapid reversal of
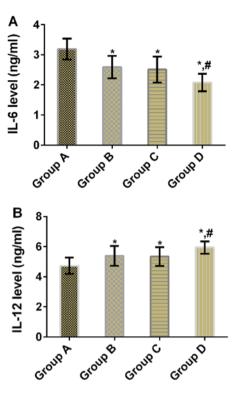

Figure 3. Comparison of IL-6 and IL-12 levels after treatment. ELISA was used to detect IL-6 level (A) and IL-12 level (B). *P<0.05 compared with group A; #P<0.05 compared with groups B and C. ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

immunosuppression in tumor microenvironment (26). In a study by Caetano et al (27), IL-6 was used as a therapeutic target for tumors, and its blocking not only directly inhibited tumor cells, but also redirected the lung microenvironment to antitumor phenotypes through changing the ratio of tumor-promoting to antitumor immune cells. According to Li et al (28), the antitumor activity of IL-12 increases the immunoregulation of cytokines, inhibits the growth of human lung adenocarcinoma and acts on normal bronchial epithelial cells near tumors. In this study, mice in group D had significantly lower IL-6 level but significantly higher IL-12 level, indicating that inhibition of IL-6 level and upregulation of IL-12 level through improvement of the microenvironment may be a therapeutic mechanism of erlotinib combined with cisplatin for lung cancer. However, the specific regulatory mechanism remains to be further studied.

This study confirmed the inhibitory effect of erlotinib combined with cisplatin on the tumor growth of mice with lung cancer, and preliminarily discussed its therapeutic mechanism.

In conclusion, erlotinib combined with cisplatin can inhibit the tumor growth of mice with LLC, and inhibition of IL-6 level and upregulation of IL-12 level may be one of its therapeutic mechanisms.

Availability of data and materials

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

Authors' contributions

XZ conceived the study and wrote the manuscript. JC and HJ were responsible for ELISA. WZ, ZC and HW contributed to analysis of observation indexes. The final version was read and adopted by all the authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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