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Chronopharmacology of Antitumor Effect of Gefitinib in Tumor-Bearing Nude and Its Mechanism1

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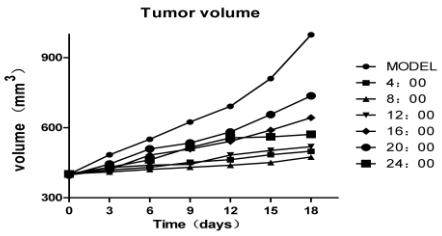
Abstract: **The aim of this study was to investigate the influence of different time points on the efficacy, and investigate its underlying mechanism. We established the non-small cell lung cancer model by BALB/c Nude, and then nude were randomly divided into gefitinib groups and model group. Group gefitinib were given 1mg/kg gefitinib intragastrically at 4:00, 8:00, 12:00, 16:00, 20:00, 24:00, the model group received the same volume of 1%Tween-80 solution. The change of tumor volume in 20 days and tumor weight on the 21th day of mice were measured. The mRNA expressions of EGFR, AKT, ERK1/2 and mTOR in tumor tissue were detected. Compared with the model group, the tumor of group gefitinib mice grow slower(P <0.05), in which the group 8:00 is the slowest, group 20:00 is the fastest (P <0.05); the tumor mass of groups gefitinib were lower than the model group(P <0.05), the inhibition rate of group 8:00 was the highest, 20:00 group was the lowest; the lowest mRNA expression of EGFR, AKT, ERK1/2 and mTOR was group 8:00 group 20:00 was the highest. Gefitinib can inhibit the tumor of bearing mice, and have time rhythm. The best time of inhibitory effect is 8: 00, and the worst time of inhibitory effect is 20:00.**



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1. Introduction

Lung cancer has become the cancer has the higher rates of morbidity and mortality, threat to human health seriously, the non-small cell lung cancer (NSCLC) accounts for about 80% [1]. As people continue to learn of biological characteristics and behaviors of tumor, molecular targeted drugs started coming into view, and gradually replace traditional medicines treatment, become the fourth treatment except surgery, radiotherapy, chemotherapy [2-3]. Gefitinib as a new generation targeted small molecule anticancer drugs, has a wide range of research, with the low toxic side effects, and high sensitivity [4].

Chronopharmacology is the study about time-related physiological response of the body to the drug, including pharmacological effects and toxicity, pharmacokinetics and bioavailability vary by time [5]. Chronopharmacology has gain more and more attention and attracted widespread attention now.

Epidermal Growth Factor Receptor (EGFR) has tyrosine kinase activity, is an important transmembrane receptor. Studies have shown that abnormal expression of EGFR gene can be activated associated gene with tumor proliferation differentiation. Occupy an important position in the process of tumor formation and development [6]. Gefitinib effect on the tyrosine kinase domain of the adenosine triphosphate (ATP) binding site through EGFR/AKT/mTOR and EGFR/ERK1/2 pathway, and then prevents the growth of tumor cells [7].

This topic studies the differences of pharmacodynamic and gene expression in different points of the hour, to show the Chronopharmacology characteristics of Gefitinib. This paper attempts to reveal the molecular mechanism of Gefitinib's anti-tumor effect, and provides theoretical reference for clinical hour administration.

2. Materials and methods

2.1 Material and Reagents

Gefitinib (Trade name Iressa, batch KN058, specifications 250 mg/tablets, AstraZeneca Pharmaceutical Co., Ltd.) RPMI-1640 medium (batch NYL1029, specifications 500 mL/bottle), Trypsin (batch J130049, specifications 100 mL/bottle), class FBS (lot NYE0873, specifications 500 mL/bottle), penicillin /streptomycin dual anti-(batch J130043, specifications 100 mL/bottle) were purchased in the United States HyClone the company. mRNA extraction kit (batch number: AK7806), cDNA extraction kit (batch number: AK2501), RNA amplification kit (batch number: AK5305) were purchased from Dalian Takara Company; real-time quantitative reverse

transcription-polymerase chain reaction (RT-PCR), detection kit (Takara biotechnology Dalian Co., Ltd.).

2.2 Apparatus

CB150 type CO2 incubator (Germany Binder Company); TGL16 type desktop-speed refrigerated centrifuge (Changsha Intentia Instrument Co., Ltd.); DW-86WHO type -86 ℃ cold storage box (China Aucma Company); TL988 fluorescent quantitative polymerase chain type reaction (PCR) instrument (West Tianlong company); inverted microscope (Shanghai optical Instrument Factory); 80-1 type of small centrifuge (Jintan is based Instrument Co., Ltd.).

2.3 Animals

Mice, BALB / c-nu, SPF level, body mass (10 ± 2) g, ♀, 4 weeks old, provided by Beijing Vital River Laboratory Animal Technology Co., license number: SCXK (Beijing) 2012-0001. Two weeks before the experiment to the end of the experiment, the mice were placed in a standard light-dark period (light period 7: 00-19: 00, dark period 19: 00-7: 00). The light period was with illumination 500 lx, and dark period with light 0. Free access to water consumption, room temperature was maintained for 21± 2℃, humidity was 30-70%.

2.4 Cell

HCC827 human non-small cell lung cancer cells were purchased from Chinese Academy of Sciences Cell Bank, Item 31800022.

2.5 Cell culture and non-small cell lung cancer mouse model

HCC827 non-small cell lung cancer with RPMI-1640 medium containing 15% fetal bovine serum and 1% antibiotic were placed in 37 ℃, 5% CO2 incubator. When cell adhesion rate was up to 85% to 95%, then digested and passaged with 0.25% trypsin. Logarithmic growth phase cells, adjusting the cell concentration to $1\times107ml^{-1}$. Mice were injected subcutaneously in the inner side of the left forelimb of about 0.2 ml of cell suspension. 3d after inoculation visible tumor growth, that successful model. When tumor growth to be around 400 mm^3, the randomized experiments were started.

2.6 Grouping and administration

Take 80 successful modeling of tumor-bearing mice, and then nude was randomly divided into Gefitinib groups（A, B, C, D, E, F）and model group. Group A-F were given 1mg/kg gefitinib intragastrically at 4:00, 8:00, 12:00, 16:00, 20:00, 24:00, and the model group received the same volume of 1% Tween-80 solution.

2.7 Pharmacodynamic index detection

Measured the mice tumor diameter and short diameter once every three days, calculated tumor volume [Formula: volume = 2 ×short axis diameter / 2]. Draw tumor volume growth curve. At 21th, sacrificed mice. Peel the tumor, weighed the tumor mass. Calculated inhibition rate [Formula: inhibition rate = (control group mean tumor mass - the experimental group mean tumor mass) / control group mean tumor mass×100%]. The tumor tissue stored in liquid nitrogen immediately.

2.8 qRT-PCR Analysis

100 mg frozen tumor tissue quickly transferred to a pre-cooled mortar with liquid nitrogen. RNAiso Plus was added to the fully grinded tissue according to the amount of homogenized tissue. Chloroform was added to the homogenate solution, mixed well, and then centrifuged to separate the solution into three layers. The top liquid layer was removed into a new tube. An isopropanol precipiation was performed to extract the total RNA, which was reversely transcribed into cDNA according to the instruction of PrineScript RT reagent Kit with gDNA Eraser. The expressions of EGFR, AKT, mTOR and ERK1/2 in tumor tissue were detected by qRT-PCR according to the instruction of SYBR PrimeScript RT reagent Kit.

EGFR primer:

F:5-CATCCAGGCCCAACTGTGAG-3,R:5-CATGGAAGCCTTGAAGCAGAA-3;

GAPDH primer:

F:5-GCACCGTCAAGGCTGAGAAC-3,R:5-TGGTGAAGACGCCAGTGGA-3;

ERK1/2 primer:

F:5-CGTTGGTACAGGGCTCCAGAA-3,F:5-CTGCCAGAATGCAGCCTACAGA-3;

AKT primer:

F:5-GGGCTGAAGAGATGGAGGTG-3;mTOR:F:5-AGAAACTGCACGTCAGCACCA-3,R:5-CCATTCCAGCCAGTCATCTTTG-3.

Reaction parameters were: 95℃ denaturation 30s, 95℃ denaturation 5s, 55℃ annealing 30s, 72℃ extension 30s, 40 cycles. Each sample was repeated for three times and the mean Ct was calculated. The gene expression was estimated with theformulate:

$\Delta\Delta Ct$ = (Target gene Ct of experimental group
- Referencegene Ct of expermental group)
- (*T*arget gene Ct of control group
- Referencegene Ct of control group)

The relative changes in target gene in different treatment group were determined by the formula 2-Δ Δ Ct.

2.9 Statistical analysis

All data were represented with mean ($\bar{x}$) ± standard deviation(SD). The statistical data of the differences among groups was analyzed by one-way ANOVA and LSD (Least-significant difference) in SPSS19.0. The 5% level of probability was considered to be significant.

3. Results

3.1 Changes in tumor volume

Figure 1 shows that as the experiment progresses, the tumor volume of each group gradually increased, which tumor growth is most rapid in the control group of mice, compared with the control group, model group of mice tumor growth slower (P＜ 0.05), group 8:00 tumor growth slowest, group 20:00 tumor growth fastest (P<0.05). Other group growth rates were between those two groups.

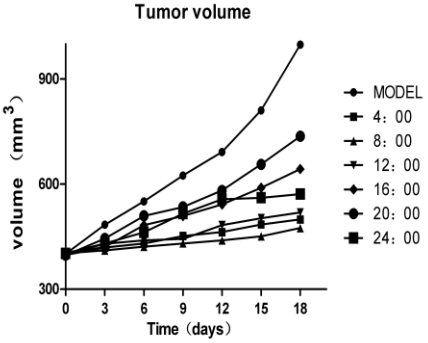

Figure 1. Tumor growth curve of miceat different dosing time

3.2 The results of gene expression in tumor tissue

Compared with the model group, 4: 00, 8: 00, 12: 00 relative amount of mRNA expression in mice tumor tissue of EGFR, AKT, ERK1/2 and mTOR was significantly reduced (*P*<0.05). The index of group 4: 00, 8: 00, 12: 00 were lower obviously than the group 16: 00, 20:00 and 24:00. The mRNA expression data of EGFR, AKT, ERK1/2 and mTOR of each group were in Table 1.

Table 1 Comparison of mRNA expression of EGFR，AKT，ERK1/2 and mTOR in each group（$\bar{x}$ ±s，n＝10）

Group	EGFR	AKT	ERK1/2	mTOR
Model	1	1	1	1
4：00	0.39±0.05*	0.63±0.04*	0.49±0.05*	0.46±0.05*
8：00	0.33±0.06*	0.51±0.08*	0.42±0.06*	0.30±0.06*
12：00	0.71±0.05	0.57±0.04*	0.56±0.05*	0.56±0.05*
16：00	0.81±0.14	0.82±0.11	0.71±0.06	0.80±0.04
20：00	0.78±0.07	0.98±0.07	0.91±0.11	1.04±0.09
24：00	0.62±0.07	0.87±0.08	0.82±0.16	0.82±0.09

vs. model group：*p<0.05

4. Discussion

In the past ten years, Gefitinib as first targeted drug for the treatment of non-small cell lung cancer small molecules, has been extensively studied [8]. Apply to have received at least one chemotherapy failure locally advanced or metastatic after non-small cell lung cancer. The binding sites of gefitinib is tyrosine kinase domaina denosine triphosphate(ATP), interference ATP-binding, thereby preventing tumor growth, metastasis and angiogenesis, and increased apoptosis of tumor cells [9]. Inhibition of EGFR mediated intracellular protein tyrosine phosphorylation at the cellular level, blocking the downstream signal transduction pathways. Thereby inhibiting the proliferation and metastasis of tumor cells and Induce apoptosis, inhibit tumor [10].

This study constructed a non-small cell lung cancer mice model. One hour was divided into six different points of administration, detection of drugs on tumor volume changes. Differences in the quality and impact of cancer-related gene expression were observed to

determine an optimal point in time of administration. As the experimental results displayed, gefitinib can inhibit tumor of bearing mice, and has an hour rhythm. The results show 4:00 and 8:00 in the expression of tumor volume changes result, tumor mass, the inhibition rate and related genes is superior to 16:00 and 20:00. This result shows gefitinib inhibit tumor in an hour rhythm. The mechanism may be related to EGFR-AKT-mTOR and EGFR-ERK-MAPK pathway, and further research is needed.

5. Conclusion

The results of this experiment can be used to choose the best time of administration and provide the basis for clinical that can maximize the therapeutic effect, and make the toxicity of drugs to a minimum at the same dose. Our research provides a reference for further clinical study. The chronopharmacology mechanism of action is possibly associated with in vivo enzyme metabolism and DNA synthesis [11-13].

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