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Effective Combined Treatment with Aqueous Extract of Taxus chinensis var.mairei (AETC) and Gefitinib in Inhibiting Non-Small Cell Lung Cancer PC9, PC9/R Cell Lines *In Vitro* and *In Vivo*

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Research Article

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Keywords: AETC, Gefitinib, Drug resistance, Non-small cell lung cancer, *In vitro* and *In vivo*.

ABSTRACT

Objective: Explore the effects and mechanism of AETC combined with Gefitinib in inhibiting non-small cell lung cancer PC9/R cell lines *in vitro* and *in vivo*.

Methods: Culture human lung adenocarcinoma cell lines PC9/R, PC9 and establish nude mice models. MTT was used to measure the inhibition of cell proliferation. Xenografts' volume of each treatment group was measured every 3 days and the weight of tumors was measured after sacrifice of mice. Western-blot was used to test the expression of EGFR, p-EGFR, ERK1/2, p-ERK1/2, PI3K, AKT, p-AKT, STAT3, p-STAT3 protein in cells and xenografted tumors. PCR was used to test the expression of EGFR, ERK1, ERK2, PI3K, AKT, STAT3 mRNA. One-way analysis of variance of each group was performed by SPSS 19.0 software.

Results: AETC in combination with certain concentration of gefitinb manifests better affection than single drug (compared with Gefitinib, $P<0.05$) in PC9 and PC9/R cells, which shows the 2 drugs have synergistic effect. AETC plus Gefitinib can achieve better inhibitory effect in the growth of PC9/R induced xenografts than Gefitinib alone ($P<0.05$) through decreasing the expression of EGFR and its downstream pathways related proteins and mRNAs *in vivo* and *in vitro*. AETC alone can inhibit EGFR and its downstream pathways to reach anti-tumor effects in PC9 and PC9/R cell lines.

Conclusions: AETC plus Gefitinib is effective in overcoming Gefitinib resistance in PC9/R cell lines *in vivo* and *in vitro* through suppressing EGFR and its downstream pathways. AETC alone can reach anti-tumor effects in PC9 and PC9/R cell lines through inhibiting EGFR and its downstream pathways.

INTRODUCTION

Non-small cell Lung cancer (NSCLC) has become the cancer type with the highest mortality around the world [1]. The majority of NSCLC patients are diagnosed with the advanced stage of the disease, leading to a poor prognosis, despite the use of chemotherapy. In recent years, advances in the molecular characterization of NSCLC, have resulted in the use of novel drugs that are able to target oncogenic mutations.

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The epidermal growth factor receptor (EGFR) is one of the most important targets in NSCLC. The inhibition of EGFR signaling by Gefitinib, Elotinib, Icotinib is an effective clinical therapy in patients with advanced NSCLC and EGFR-activated mutations. However, all patients will eventually develop acquired resistance to these drugs, with the median progression- free survival times of 9.5-13.7 months [2].

Taxus chinensis (Pilger) Rehd is a kind of evergreen trees distributed in the southeastern region of China. In TCM theory, the aqueous extract of *Taxus chinensis Rehd* (AETC) (especially its leaves, barks and small branches) is bitter in flavor, neutral in nature, slightly toxic and is belong to the heart meridian. AETC has been widely used for hundreds of years in TCM formulas and is considered effective in treating diseases caused by the accumulation of poisonous factors or dampness, such as cancers and kidney diseases. A combination with AETC and EGFR-TKIs is common in China and provides superior clinical effectiveness. Moreover, our previous studies had verified that AETC has a synergetic effect with erlotinib in antitumor activity, with the different mechanism from paclitaxel in A549 cell line [3,4]. So we hypothesize AETC in combination with EGFR-TKIs has the effect of attenuating EGFR-TKI resistance via EGFR and its downstream signaling pathways. Therefore, through *in vitro* and *in vivo* experiments in PC9 (Gefitinib sensitive) cell line and PC9/R (Gefitinib resisitant) cell line, we explored the curative effect and mechanism of AETC in combination with Gefitinib in overcoming Gefitinib resistance.

MATERIALS AND METHODS

Cell Culture and Reagents

EGFR mutant human lung adenocarcinoma cell lines PC9 (EGFR exon 19 del E746-A750, Gefitinib sensitive) and PC9/R (EGFR exon 19 del E746-A750, Gefitinib resistant). Dulbecco's Modified Eagle Medium (DMEM) media, fetal bovine serum (FBS), and trypsin-EDTA (Ethylene Diamine Tetraacetic Acid) were purchased from Gibco BRL (Gaithersburg, MD, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, and streptomycin, were purchased from Sigma Chemical Co. (St. Louis, MO). PC9 and PC9/R cell lines were all cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Taxus chinensis (Pilger) Rehd (8 g/bag) was purchased from the Pharmacy of Zhejiang Provincial Hospital of Traditional Chinese Medicine, produced by Ningbo Taikang Bio-engineering Company (Ningbo, China. Batch number: 100513). Gefitinib (250 mg/tablet) was provided by AstraZeneca UK Limited (London, UK. Approval number: J20100014).

Preparation of AETC

The preparation of AETC used in this study was based on the TCM processing method [5]. Dry *Taxus Chinensis* (Pilger) Rehd (1000 g) was immersed in distilled water overnight. This raw solution was boiled in distilled water for 3 times, in total for 70 min, filtered by vacuum suction filter and finally concentrated to 500 ml. The concentrates were then centrifuged at 12000 rpm for 15 min; the supernatant extracts were then filtered through a 0.22 μm micropore filtrate for sterilization and dried to powders. AETC were stored at -20°C for use. Based on our previous study, AETC diluted to 0.25, 0.5, 1, 2, 4, 8, 16 mg/ml for *in vitro* experiments, AETC diluted to the concentration of 104 mg/ml for *in vivo* experiments [5].

Cytotoxicity Assay

During logarithmic phase, cells were trypsinized and harvested to test the cytotoxicity of AETC and Gefitinib. Cells were seeded in flat-bottomed 96-well plates with concentration of 5×10^4 cells/ml and cultured for 24 h in a humidified atmosphere of 5% CO_2 at 37°C. Then, PC9 cells and PC9/R cells were randomized to 3 groups (AETC group, Gefitinib group, AETC plus Gefitinib group respectively. In AETC group, both PC9 and PC9/R cells were cultured for 72 h more with different concentrations of AETC (0.25, 0.5, 1, 2, 4, 8, 16 mg/ml) respectively. In Gefitinib group, different concentrations of Gefitinib were given (the concentrations of PC9 cells: 0.01, 0.02, 0.04, 0.1, 0.5, 1, 5, 10 μmol/l, the concentrations of PC9/R cells: 1, 3, 6, 9, 12, 15, 18, 30 μmol/l) to cells, respectively. In AETC plus Gefitinib group, same concentration of AETC (0.25 mg/ml) plus different concentration of Gefitinib (the concentrations of PC9 cells: 0.01, 0.02, 0.04, 0.1, 0.5, 1, 5, 10 μmol/l, the concentrations of PC9/R cells: 1, 3, 6, 9, 12, 15, 18, 30 μmol/l) were given to cells. 20 μl MTT dye (5 mg/ml) were added to each well in order to determine the drug effects. Wavelength was measured by an ELX808 Micro Plate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) and was assessed from 3 independent experiments with absorption at 490 nm. Relative cell proliferation inhibition rate (IR) and Combination index (CI) were then measured. IR=(1-average A490 of the treatment group/average A490 of the control group) × 100%. CI<1 implies synergistic effect, CI>1 shows antagonistic effect, CI<0.5 means highly synergistic effect.

Xenograft Tumor Models

80 BALB/c male nude mice aged 4-8 weeks obtained from Hercynian poole-Rubicam Experimental Animals Co. Ltd. (Production license number: SCXK, Shanghai, China, 2013-0016) were used to establish xenograft tumor models. During logarithmic phase, 0.2 ml suspension of PC9 or PC9/R cells ($1\text{-}2 \times 10^6$/ml) was injected subcutaneously into the right axilla of each mouse. Nude mice bearing PC9 or PC9/R tumors were randomized to 4 groups respectively (10 mice each group). (a) oral AETC group (10.4 mg/10 g daily), (b) oral Gefitinib group (0.5 mg/10 g daily), (c) combined group (oral AETC 10.4 mg/10 g daily+Gefitinib 0.5 mg/10 g daily), (d) control group (oral NS 0.1 ml/10 g daily). Tumor width and length were measured every 3 days during the

treatment and tumor volume was calculated as width^2 × length/2 in mm^3. After 4 weeks treatment, all mice were sacrificed and the xenograft tumors were stripped. The weight of tumors and the inhibitory rate were then measured. The xenograft tumors were stored in liquid nitrogen and were used to do the experiments of western blotting, RT-PCR and immunohistochemistry. All the animal experiments in this study were carried out in Zhejiang University of Traditional Chinese Medicine and approved by the Animal Ethics Committee.

Antibodies and Western Blotting

Protein 30 μg each extracted from cells or xenograft tumors were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membrane (Immunobilon-P, 0.45 mm; Millipore Co., Billerica, MA, USA). The membranes were blocked with 5% skim milk in TBST at room temperature for 1 h, followed by incubation with primary antibodies to β-Acitne, EGFR, p-EGFR (Tyr1068), ERK1/2, p-ERK1/2 (Tyr202/Tyr204), PI3K, p-AKT (Ser473), AKT, STAT3, p-STAT3 (Tyr705) (Cell Signaling Technologies, Beverly, MA, USA) at 37℃ for 2 h or at 4 ℃ overnight. The membranes were washed three times with TBST for 10 min each and incubated with HRP-conjugated secondary antibody for 1-2 h at room temperature. The membranes were then rewashed three times with TBST. The proteins were visualized using ECL and MP4+Chemidoc XRS protein mark detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-Acitne was used as the internal loading control. The densitometry readings of the bands were normalized to β-Acitne expression. The band intensity was analyzed by using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed at least 3 times independently.

RT-PCR

After extracting the total RNA from cells or tissues, RNA concentration was measured for 3 times by ultraviolet spectrophotometer. Based on the required amount of RNA, different volumes of RNA samples were used for reverse transcription in order to synthesize cDNA, after reverse transcription, primers were joined. Primers of GAPDH, EGFR, ERK1, ERK2, PI3K, AKT, STAT3 were synthesized by Shanghai PuDi Biotech Co.,Ltd (Shanghai, China). (GAPDH forward and reverse primers: 5′-GGAGCGAGATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTTCTCATGG-3′, EGFR forward and reverse primers: 5′-ACCACGTACCAGATGGATGTGAAC-3′ and 5′-AGCCGTGATCTGTCACCACATAA-3′, ERK1 forward and reverse primers: 5′-CTACACGCAGTTGCAGTACAT-3′ and 5′-CAGVAGGATCTGGATCTCCC-3′, ERK2 forward and reverse primers: 5′-CAGVAGGATCTGGATCTCCC-3′ and 5′-CATGTCTGAAGCGCAGTAAGATT-3′, PI3K forward and reverse primers: 5′-CCACGACCATCATCAGGTGAA-3′ and 5′-CCTCACGGAGGCATTCTAAAGT-3′,AKT forward and reverse primers: 5′-AGCGACGTGGCTATTGTGAAG-3′ and 5′-GCCATCATTCTTGAGGAGGAAGT-3′, STAT3 forward and reverse primers: 5′-ATCACGCCTTCTACAGACTGC-3′ and 5′-CATCCTGGAGATTCTCTACCACT-3′). Amplification of targets was carried out by using Power SYBR green PCR mix with Step One Plus Real-time PCR system (Applied Biosystems). PCR was performed for 40 cycles in 10 μl reaction mixture at 95℃ (5 seconds), 60℃ (30 seconds). Quantification of gene expression was calculated by the delta Ct method. Data represent the mean ± SD of three independent experiments. Each experiment was performed at least 3 times independently.

Statistics

All the data were expressed as mean ± SD. Statistical analysis was performed by SPSS 19.0 software. Comparisons between 2 groups were tested for statistical differences by the t-test, comparisons among mutiple groups were tested for statistical differences by Analysis of variance (one-way ANOVA), Statistical significance was taken as $P<0.05$.

RESULTS

***In vitro* Experiment**

AETC combination with Gefitinib on the proliferation of PC9 and PC9/R cell lines: To investigate the effect of AETC alone and in combination with Gefitinib, we examined the differential corresponding sensitivity to EGFR-TKIs: PC-9 (sensitive), PC9/R (resistant). We first tested the inhibitory rate and IC50 value of different doses of Gefitinib or AETC alone effecting on PC9 and PC9/R cell lines **(Figure1 and Table 1)** by MTT assay. After Gefitinib effecting on cells for 72 hrs, the IC50 value of PC9/R is 14.245 ± 0.243 μmol/L, the IC50 value of PC9 is 0.044 ± 0.005 μmol/L, which demonstrates PC9/R is resistant to Gefitinib, while PC9 is sensitive to Gefitinib. Different doses of AETC can inhibit PC9 and PC9/R cells in a concentration-dependent manner and the values of optical density of experimental groups significantly decreased compared with control group($P<0.05$) **(Figures 1A and 1D).** We used AETC with concentration of 0.25 mg/ml in combination of gefitinb in different concentrations to test the inhibitory rate of PC9 and PC9/R cell lines. This concentration of AETC is of minimum cytotoxicity in this experiment. The combination of 2 drugs manifests better effect than single drug **(Figure 1G)** in PC9 and PC9/R cell lines ($P<0.05$). The combination index of of PC9 and PC9/R is 0.278 and 0.645 respectively, which shows AETC combined Gefitinib can achieve synergistic effect. Therefore, PC9 and PC9/R cell lines can be attenuated by AETC in combination with Gefitinib.

Combined Effects on Protein Levels of EGFR Signaling Pathways in PC9 and PC9/R Cell Lines

To determine the effect of AETC in combination with Gefitinib in PC9 and PC9/R cell lines *in vitro*, we tested the expression of protein in EGFR and its downstream signaling pathways including ERK signaling pathway, PI3K/AKT signaling pathway and STAT3 signaling pathway by Western-blot assay. As shown in **Figure 2** and **Figure 3,** in PC9 cell lines, the combined treatment

group can significantly decrease the expression of p-EGFR, p-AKT, p-STAT3 (P<0.05) compared with Gefitinib alone. These results indicate that AETC combined with Gefitinib can suppress the activation of PI3K/AKT and STAT3 pathway. In comparison with control group, AETC alone can decrease the expression of EGFR, p-EGFR, p-ERK1/2, PI3K, AKT, p-AKT, STAT3, p-STAT3 (P<0.05), which demonstrates AETC can inhibit the expression of EGFR and its downstream molecules in PC9 cells.

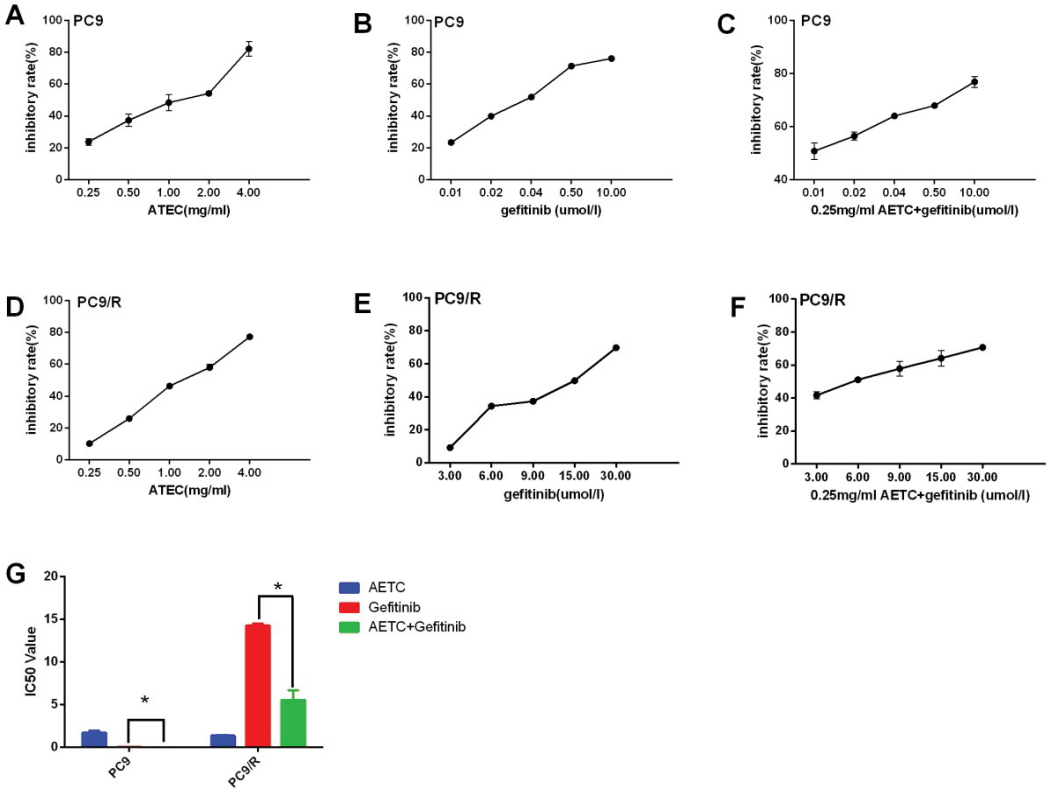

Figure 1. Effects of AETC, Gefitinib or AETC plus Gefitinib on the proliferation of PC9 and PC9/R cell lines.

A: Inhibitory rates of different concentrations of AETC (0.25, 0.5, 1, 2, 4 mg/ml) effect on PC9 cells; B: Inhibitory rates of different concentrations of Gefitinib (0.01, 0.02, 0.04, 0.5, 10 μmol/l) effect on PC9 cells; C: Inhibitory rates of 0.25 mg/ml AETC plus different concentrations of Gefitinib (0.01, 0.02, 0.04, 0.5, 10 μmol/l) effect on PC9 cells; D: Inhibitory rates of different concentrations of AETC (0.25, 0.5, 1, 2, 4 mg/ml) effect on PC9/R cells; E: Inhibitory rates of different concentrations of Gefitinib (3, 6, 9 , 15, 30 μmol/l) effect on PC9/R cells; F: Inhibitory rates of different concentrations of 0.25 mg/ml AETC plus Gefitinib (3, 6, 9, 15, 30 μmol/l) effect on PC9/R cells; G: IC50 value of AETC (mg/ml), Gefitinib (μmol/l) and 0.25 mg/ml AETC plus Gefitnib (μmol/l) in PC9, PC9/R cell lines.

Table 1. IC50 value of AETC, Gefitinib or AETC plus Gefitinib and combination index (CI) in PC9 and PC9/R cell lines, Compared with Gefitinib, *P<0.05.

Cell lines	IC50 value			Combination index(CI)
	AETC (mg/ml)	Gefitinib (μmol/l)	0.25 mg/ml AETC+Gefitinib (μmol/l)	
PC9	1.693 ± 0.25	0.044 ± 0.005	0.005 ± 0.001*	0.278
PC9/R	1.344 ± 0.075	14.245 ± 0.243	5.516 ± 1.145*	0.645

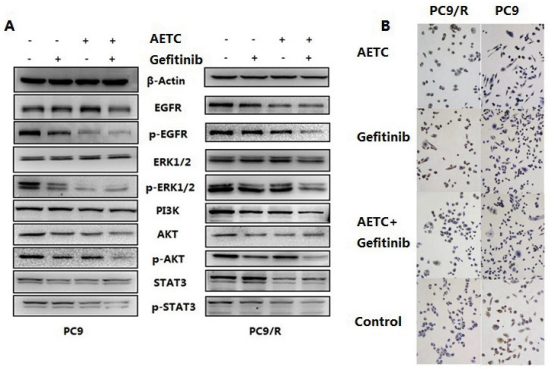

Figure 2. Effects of AETC, Gefitinib or AETC plus Gefitinib on the expression EGFR and downstream signaling pathways related proteins in PC9 and PC9/R cells. The AETC group was treated with 0.25 mg/ml AETC for 72 hrs; the Gefitinib group was treated with 3 μmol/L Gefitinib in PC9/R cells for 72 hrs, 0.02 umol/L Gefitinib in PC9 cells for 72 hrs; the combined group was treated with 0.25 mg/ml AETC plus 3 μmol/L Gefitinib in PC9/R cells for 72 hrs, 0.25 mg/ml AETC plus 0.02 μmol/L Gefitinib in PC9 cells for 72 hrs; the control group was treated with cell culture medium.

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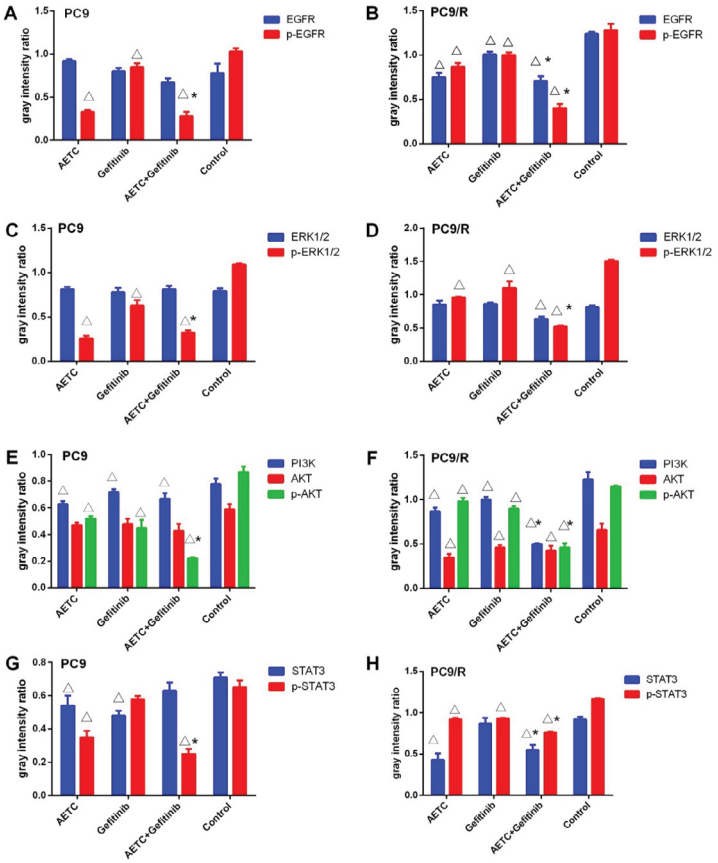

Figure 3. Gray intensity ratio of AETC, Gefitinib or AETC plus Gefitinib effecting on the expression EGFR and downstream signaling pathways related proteins in PC9 and PC9/R cells Compared with Gefitinib group, *P<0.05; Compared with control group, △P<0.05.

In PC9/R cell lines, compared with Gefitinib, AETC combined Gefitinib can significantly decrease the expression of p-EGFR, p-ERK1/2, PI3K, p-AKT, p-STAT3 (P<0.05), which demonstrates that the combination of two drugs may restrain Gefitinib resistance in PC9/R cell line by means of suppressing the activation of ERK, PI3K/AKT and STAT3 signaling pathway. In comparison with control group, AETC alone can decrease the expression of p-EGFR, p-ERK1/2, PI3K, AKT, p-AKT, STAT3, p-STAT3 (P<0.05), which demonstrates AETC can inhibit the expression of EGFR and its downstream molecules in PC9/R cells.

Combined Effects on mRNA Levels of EGFR Signaling Pathways in PC9 and PC9/R Cell Lines

To explore the effects AETC combined Gefitinib can achieve in gene levels, we tested the expression levels of EGFR and downstream signaling pathways related mRNA in PC9 and PC9/R cell lines. As **Figure 4A** shows, compared with Gefitinib, AETC in combination with Gefitinib can significantly decrease the expression of EGFR, ERK1, ERK2, AKT mRNA in PC9 cell line (P<0.05), while no significant changes were found in the expression of PI3K and STAT3 mRNA.

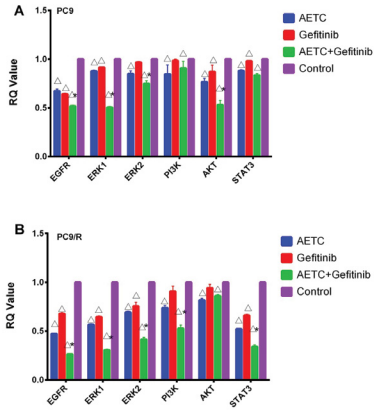

Figure 4. Effects of AETC, Gefitinib or AETC plus Gefitinib on the expression EGFR and downstream signaling pathways related mRNAs in PC9 and PC9/R cells.

The AETC group was treated with 0.25 mg/ml AETC for 72 hrs; the Gefitinib group was treated with 3 μmol/L Gefitinib in PC9/R cells for 72 hrs, 0.02 μmol/L Gefitinib in PC9 cells for 72 hrs; the combined group was treated with 0.25 mg/ml AETC plus 3 μmol/L Gefitinib in PC9/R cells for 72 hrs, 0.25 mg/ml AETC plus 0.02 μmol/L Gefitinib in PC9 cells for 72 hrs; the control group was treated with cell culture medium.Compared with Gefitinib group, *P<0.05; Compared with control group, △P<0.05.

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In PC9/R cell line, the expression level of EGFR, ERK1, ERK2, PI3K, STAT3 mRNA significantly decrease in combination group (P<0.05) in comparison with Gefitinib group **(Figure 4B).**

AETC group can decrease the expression level of EGFR, ERK1, ERK2, PI3K, AKT, STAT3 mRNA in PC9 and PC9/R cell lines compared with control group.

***In vivo* Experiment**

AETC could inhibit the growth of PC9 and PC9/R xenografts in the nude mice: Based on *in vitro* experiments, the anti-tumor effect of AETC *in vivo* was further explored. Nude mice were given daily treatment after inoculation. All xenografts were palpable about 5 days after inoculation, the tumor formation rate is 100%. **Figure 5A and Table 2** show the growth and weight of PC9 xenografts, since PC9 is Gefitinib sensitive cell line, xenografts in nude mice receiving Gefitinib or Gefitinib plus AETC disappeared about 10 days after treatments. Compared with control group, xenografts in nude mice receiving AETC treatment grew slower, and the weight of xenografts in AETC group (0.921 ± 0.405 g) is significantly lighter than control group (1.398 ± 0.417 g) (P<0.05). As shown in **Figure 5B and Table 2**, AETC plus Gefitinib can achieve better inhibitory effect in the growth of PC9/R induced xenografts than Gefitinib alone (P<0.05), the inhibitory rate of AETC plus Gefitinib and Gefitinib group is 71.9% and 63.1%, respectively. In addition, in comparison with control group, the inhibitory rate in AETC group is 56.9%, which means AETC alone can remarkably inhibit the growth of tumor (P<0.05).

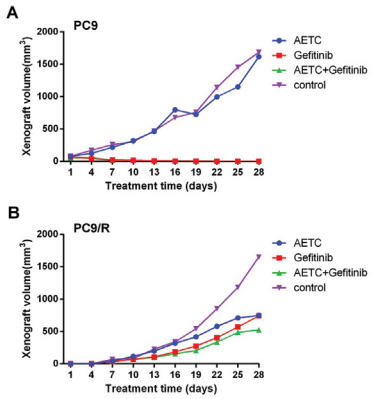

Figure 5. Effects of different treatments on the growth of PC9 and PC9/R xenografts AETC group was treated with 10.4 mg/10 g AETC daily; Gefitinib group was treated with 0.5 mg/10 g Gefitinib daily; combined group was treated with 10.4 mg /10 g AETC daily+0.5 mg/10 g Gefitinib daily); control group was treated with 0.1 ml/10 g NS daily.

Table 2. Effects of different treatments on the growth of PC9 and PC9/R xenografts Compared with Gefitinib group, *P<0.05; Compared with control group, ΔP<0.05.

Group	n	PC9		PC9/R	
		Weight of xenografts (g)	Inhibitory rate (%)	Weight of xenografts (g)	Inhibitory rate (%)
AETC	8	0.921 ± 0.405Δ	34.1	0.604 ± 0.239Δ	56.5
Gefitinb	8	0.000 ± 0.000Δ	100	0.513 ± 0.259Δ	63.1
AETC+Gefitinib	8	0.000 ± 0.000Δ	100	0.391 ± 0.095*Δ	71.9
Control	8	1.398 ± 0.417	0	1.390 ± 0.720	0

Notes: the weight of the primary subcutaneous xenografts and the inhibitory rate of the weight were measured 24 h after the last administration, when all the mice were sacrificed.

Effects of AETC on Expression Levels of EGFR and Downstream Signaling Pathways Related Proteins in PC9 and PC9/R Xenografts

Figure 6 and Figure 7 manifests that in PC9 induced tumors, AETC can significantly decrease the protein expression level of EGFR, p-EGFR, p-ERK1/2, PI3K, p-AKT, p-STAT3 (P<0.05), which indicates AETC achieve anti-tumor effect in PC9 induced tumors by suppressing EGFR and its downstream ERK, PI3K/AKT, STAT3 signaling pathways.

In PC9/R induced tumors, compared with Gefitinib group, AETC in combination with Gefitinib can remarkably decrease the expression level of EGFR, p-EGFR, ERK1/2, p-ERK1/2, PI3K, AKT, p-AKT, p-STAT3 (P<0.05). In comparison with control group, AETC alone can decrease the expression level of EGFR, p-EGFR, p-ERK, PI3K, p-AKT, STAT3, p-STAT3 (P<0.05). This result demonstrates that the mechanism combination group attains in inhibiting Gefitinib resistance in PC9/R induced tumors is closely related to the suppression of ERK, PI3K/AKT, STAT3 signaling pathways. AETC achieve anti-tumor effect in PC9 induced tumors by suppressing EGFR and its downstream ERK, PI3K/AKT, STAT3 signaling pathways.

Effects of AETC on Expression Levels of EGFR and Downstream Signaling Pathways Related mRNA in PC9 and PC9/R Xenografts

In PC9 induced tumors, AETC group achieves significant effect in decreasing the expression level of EGFR, ERK1, ERK2, PI3K, AKT mRNA (P<0.05) **(Figure 8A)**.

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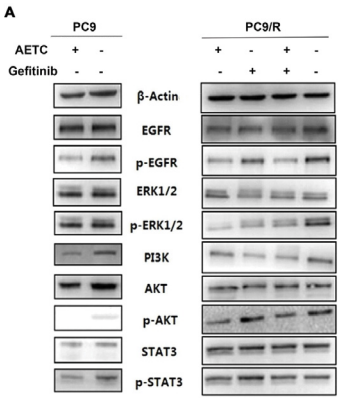

Figure 6. Effects of AETC, Gefitinib or AETC plus Gefitinib on the expression EGFR and downstream signaling pathways related proteins in PC9 and PC9/R xenografts.

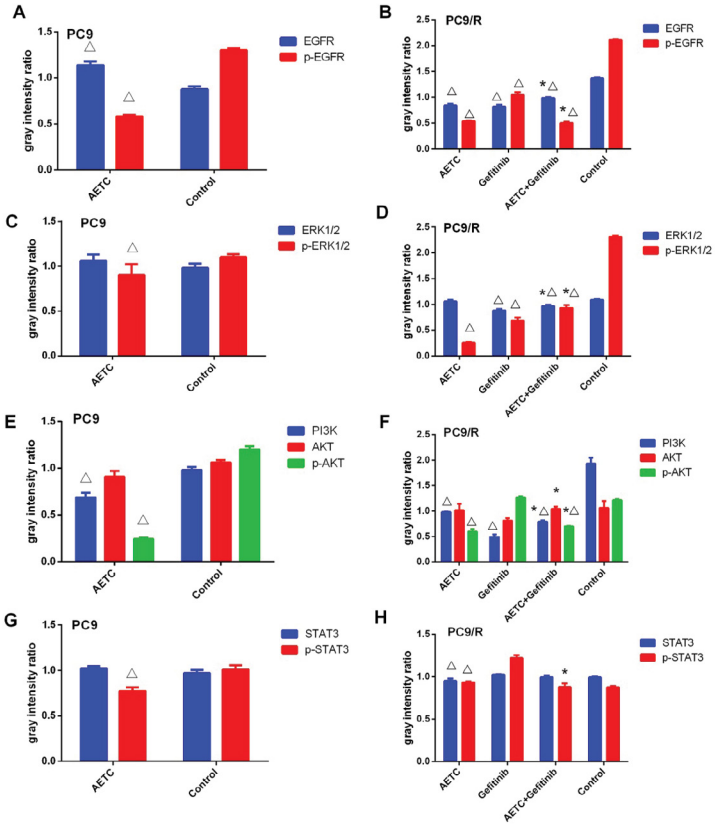

Figure 7. Effects of AETC, Gefitinib or AETC plus Gefitinib on the expression EGFR and downstream signaling pathways related proteins in PC9 and PC9/R xenografts. Compared with Gefitinib group, *P<0.05; Compared with control group, ΔP<0.05.

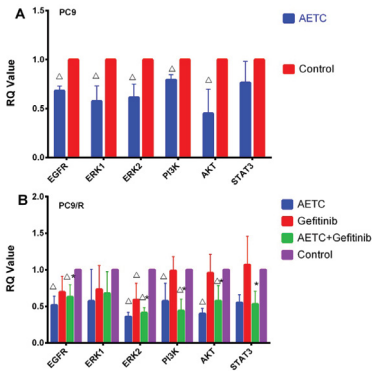

Figure 8. Effects of AETC, Gefitinib or AETC plus Gefitinib on the expression EGFR and downstream signaling pathways related mRNAs in PC9 and PC9/R xenografts Compared with Gefitinib group, *P<0.05; Compared with control group, ΔP<0.05.

To further research the mechanism of AETC plus gefitinb in restraining gefininb resistance in PC9/R xenografts on mRNA level. We tested the mRNA expression level of EGFR, ERK, PI3K, AKT, STAT3 in PC9/R xenografts. In PC9/R induced tumors, PI3K, AKT, STAT3 mRNA remarkably decreased in comparison with Gefitinib group (P<0.05) while EGFR, ERK don't show significant

difference **(Figure 8B).** AETC alone can inhibit the expression level of EGFR, ERK2, PI3K, AKT mRNA in PC9/R induced tumors ($P<0.05$).

DISCUSSION

Recently, targeted anticancer drugs, including EGFR-TKIs, have been approved for the treatment of NSCLC. However, not all patients benefit from this therapy due to primary or acquired resistance. Fatinib improvement in Gefitinib or Erlotinib-resistant patients. Although Afatinib was expected to overcome EGFRT790M-mediated acquired resistance to first generation reversible EGFR TKIs, and ALK inhibitor crizotinib was used with alterations in the EML4-ALK gene [2,6-8]. But these two drugs failed to demonstrate overall survival improvement in Gefitinib or Erlotinib-resistant patients. Therefore, the treatment options for NSCLC remain unsatisfactory.

The mechanism of EGFR-TKI acquired resistance can be classified to the following points. (1) Secondary mutation in EGFR: T790M is the most frequent secondary mutation in EGFR, it accounts for approximately 50% Gefitinib and erlotinib acquired resistance in clinic [9]. T790M increases ATP's affinity to EGFR, which inhibit the affinity of EGFR-TKI to EGFR [10]. (2) Activation of HGF/MET pathway: Overexpression of HGF, MET amplification, MET mutation, MET overexpression is factors that can activate HGF/MET pathway [11,12]. Researches demonstrate about 20% patients with EGFR-TKI acquired resistance get HGF/MET pathway activated [13]. Activation of HGF/MET pathway lead to the activation of downstream pathway such as PI3K/AKT pathway, which can promote tumorigenesis [12,14]. (3) Activation of IGF-1R pathway. (4) HER2 mutation [15]. (5) Activation of HER3 [16,17]. (6) SCLC phenotypic transforming. (7) EMT. The first two mechanisms account for more than 70% EGFR-TKI resistance in clinic.

Currently, several extracts from herbs have shown their effects in overcoming EGFR-TKIs resistance by inhibiting EGFR and its downstream pathways. Extract of epimedium koreanum Nakai in combination with Gefitinib shows synergistic inhibitory effects through PI3K/Akt/mTOR pathway in H1975 and PC9/GR cell lines [18]. Hong found luteolin has significant anti-tumor activity in elotinib-resistant EGFR T790M mutant NSCLC at cell and animal levels by blocking PI3K/AKT/mTOR signaling [19].

Our previous studies have shown AETC has anti-tumor effect *in vivo* and *in vitro* and the mechanism of AETC's anti-tumor effect is different from paclitaxel. In A549 cell lines, AETC block A549 cells by inducing early stage apoptosis while paclitaxel block cells by inducing necrosis and late stage apoptosis [3]. Meanwhile, AETC can inhibit the activation of EGFR downstream signaling pathway- MAPK/ERK pathway in A549 cell lines and A549 xenografts [5]. In our present study, The MTT result indicates AETC and Gefitinib have synergistic effect in PC9/R cell lines the animal experiment shows AETC plus Gefitinib can remarkably inhibit the growth of PC9/R induced tumors. These results demonstrate AETC in combination with Gefitinib can reach more inhibitory effect in PC9/R cell lines than Gefitinib alone *in vitro* and *in vivo*. In addition, compared with control group, AETC alone can reach anti-tumor effects in PC9 and PC9/R cell lines *in vivo* and *in vitro*. Based on results above, we further explore the mechanism of AETC plus Gefitinib in inhibiting Gefitinib resistance by testing EGFR and its downstream pathways.

EGFR and its downstream extracellular signal-regulated kinase 1/2 (ERK1/2), phosphatidy linositol 3-kinase (PI3K)/AKT and signal transducers and activators of transcription3 (STAT3) pathways are involved in the survival, proliferation and/or progression of tumors with the activation of EGFR, FGFR or other growth factor receptors M [20-22].

PI3K is activated by some growth factor receptors such as EGFR, c-MET, c-KIT, IGF-IR at the membrane. Activated PI3K subsequently catalyzes PIP2 into PIP3 [23]. Afterwards, AKT is activated by phosphorylation at amino acids Thr308 and Ser473 [22]. Activated AKT not only plays an important role in anti-apoptotic signaling and cellular glucose metabolism, but also a good predictor in NSCLC harboring EGFR mutation [21,24]. Continuous activation of PI3K/AKT is closely associate with Gefitinib resistant NSCLC [25]. Thus, PI3K and AKT inhibitors in combination with EGFR-TKIs are thought to have a bright future in overcoming EGFR-TKIs resistance [26,27]. Our study manifests AETC combined with Gefitinib can significantly downregulate the expression level of PI3K, phospho-AKT protein and PI3K mRNA in comparison with Gefitinib alone *in vitro* and *in vivo*. According to this, AETC combined with Gefitinib can possibly inhibit PI3K/AKT pathway.

ERK1/2 is activated by mitogenic agents and plays an important role in cellular actions such as proliferation, transformation and survival. The amplification of ERK2 induces epithelial-to-mesenchymal transformation and is identified as a mechanism that leads to EGFR-TKI resistance in NSCLC [28]. Researches indicate reactivation of ERK induces Gefitinib resistance, dual inhibition of ERK and EGFR may be a strategy in overcoming Gefitinib resistance [29,30]. In addition, there are many cross talks between ERK and PI3K/AKT pathways, ERK1/2 can sustain AKT phosphorylation when PI3K/AKT pathway is blocked [23,31]. Chetram et al. study found that the activation of ERK1/2 signaling pathway in (NSCLC) had high expression of ERK1/2 and its phosphorylation increased significantly. Inhibition of ERK1/2 phosphorylation can inhibit cell proliferation and induce apoptosis and play a role in the inhibition of lung cancer. In our study, apart from blocking PI3K/AKT pathway, AETC combined with Gefitinib can remarkably inhibit the expression level of phospho-ERK1/2 and ERK2 mRNA at cell and animal level compared with Gefitinib group, which implies AETC can inhibit ERK1/2 signaling pathway.

STAT3 is a member of STAT family, STAT3 forms dimers when activated by various cytokines such as EGF, IL-6 and interferons [32,33]. STAT3 holds an important position in tumorigenesis, it deregulates cell growth and regulates cell apoptosis, tumor angiogenesis and metastasis [32]. Researches demonstrate that activated EGFR/STAT3 promotes NSCLC survival, Gefitinib's

efficacy can be impaired by STAT3 activation, and suppression of STAT3 activity can sensitize Gefitinib-resistant non-small cell lung cancer [34-37]. Moreover, activation of STAT3 is associate with AKT, Wu found Gefitinib resistant NSCLC results from STAT3 mediated AKT activation [34]. Indeed, the strength and duration of JAK/STAT3 promoted the creation of an immunosuppressive microenvironment and abrogating anti-tumor responses, which involved the inactivation of dendritic cells, T cells and natural killer (NK) cells. Also, activated JAK/STAT3 can in turn transcriptionally regulate the expression of inflammation cytokines [14]. Moreover, STAT3 activation regulates the expression of vascular endothelial growth factor and finally progression to infiltration and metastasis [15]. Our research suggests that combined group can significantly suppresses the expression of p-STAT3 and STAT3 mRNA in comparison with Gefitinib group, which means AETC can inhibit STAT3 pathway.

Above mentioned results and discussion suggest AETC plus Gefitinib can inhibit Gefitinib resistance, the effect targets of AETC towards PC9/R cells and xenografts is EGFR and its downstream pathways. AETC alone can reach anti-tumor effects in PC9 and PC9/R cell lines through inhibiting EGFR and its downstream pathways. Based on the results above, we believe AETC is an effective anti-tumor agent that can be used worldwide.

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