

Induced IGF-1R activation contributes to gefitinib resistance following combined treatment with paclitaxel, cisplatin and gefitinib in A549 lung cancer cells

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Abstract. Gefitinib demonstrates excellent performance in the treatment of lung adenocarcinoma patients; yet, there was no added benefit in combination with chemotherapy as reported in a phase III clinical trial. For exploring the mechanism of the failed combination therapy in lung cancer, in the present study, four therapy assessment groups, including a control group, a chemotherapy group [paclitaxel + cisplatin (TP)], a gefitinib monotherapy group (G) and a combination group [paclitaxel + cisplatin + gefitinib (TP+G)], were established in an A549 cell line and mouse xenotransplanted tumor models. By HPLC, we found that the gefitinib concentration was significantly higher in the combination group when compared to that in the G group in the non-small cell lung cancer cell line, A549 ($p < 0.05$). Following the treatment time extension, an increased cell growth rate was observed in the combination group, while the cellular concentration of gefitinib was not decreased. The expression levels of P-IGF-1R, P-SRC and P-ERK in the fourth combination treatment group were significantly higher than levels in the fourth G treatment and control groups ($p < 0.05$). Following downregulating of IGF-1R

in the fourth combination treatment group, drug sensitivity was recovered *in vitro*. In the mouse model, compared with the gefitinib monotherapy group, the combination group exhibited a smaller tumor volume, lower body weight and reduced survival rate ($p < 0.05$). Gefitinib concentrations in the serum and tumor tissues in the combination therapy group were also decreased when compared with these concentrations in the gefitinib alone group. The present study is the first to demonstrate that the decreased gefitinib concentration in serum and tumor tissues is one of the reasons resulting in the failed combination treatment (chemotherapy + gefitinib) *in vivo* study. Frequent use of the combination treatment in A549 lung cancer cells induced IGF-1R activation which contributed to gefitinib resistance and gave rise to the failure of the combination therapy.

Introduction

Lung cancer still remains the leading cause of cancer-related mortality in the world, and the majority of patients present with advanced disease at diagnosis or develop recurrence, neither of which is amenable to curative approaches (1). The epidermal growth factor receptor (EGFR) is expressed in a number of tumors, including non-small-cell lung cancer (NSCLC). EGFR is a member of the EGFR family that includes HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (2-4). Activation of the EGFR leads to receptor associated tyrosine kinase activity that initiates a cascade of events leading to downstream signaling and a variety of changes characteristic of malignant progression including upregulation of ras, raf, mitogen-activated phosphorylated (MAP) kinase, and phosphatidylinositol 3-kinase (PI3K) and the downstream protein-serine/threonine kinase Akt. In turn, cellular growth and invasive capacity are enhanced. Preclinical data indicate that small molecules that compete with adenosine triphosphate for delivery of phosphate groups to critical tyrosine residues could block signal transduction through EGFR. Studies have demonstrated that gefitinib is a promising agent for the treatment of a wide range of tumors, including NSCLC. Gefitinib can enter cells and inhibit the autophosphorylation of EGF-stimulated EGFR in a variety of EGFR-expressing human cancer cell lines (5). Several

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Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IGF-1R, insulin-like growth factor receptor 1; G, gefitinib monotherapy; TP, paclitaxel + cisplatin; TP+G, paclitaxel + cisplatin + gefitinib; G4, the fourth gefitinib treatment; (TP+G)4, the fourth TP and gefitinib treatment; HPLC, high performance liquid chromatography

Key words: chemotherapy, gefitinib, lung cancer, drug resistance, IGF-1R

phase I studies have shown that gefitinib is generally well tolerated, with evidence of antitumor activity in NSCLC, and two large phase II gefitinib monotherapy studies [Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and 2] in patients with pretreated advanced NSCLC further confirmed that gefitinib was generally well tolerated and demonstrated clinically significant antitumor activity (6-8). Cetuximab is an IgG1 subclass chimeric mouse-human antibody that plays roles similar to gefitinib. In addition, cetuximab binds to the extracellular portion of EGFR and induces destruction of the receptor (9). Addition of cetuximab to platinum-based chemotherapy results in a moderate increase in the overall survival of patients with advanced NSCLC as confirmed in clinical research (10). There is a strong rationale for combining gefitinib with standard chemotherapeutic agents. Unfortunately, this exciting, high-level of preclinical activity has not transpired in most clinical studies reported to date. In fact, the addition of gefitinib has offered minimal to no additive or synergistic clinical activity to the standard antineoplastic regimens which has led to its limited availability (11-15). Likewise, two phase III trials have not shown gefitinib to be effective when combined with chemotherapy in the treatment of NSCLC (16,17). Paclitaxel + cisplatin (TP) chemotherapy regimens are used for the treatment of NSCLC (18). We hypothesized that one of the reasons resulting in the failure of combination therapy was that chemotherapy drugs affect the cellular gefitinib concentration. Thus, the present study investigated the effects of combining gefitinib with TP on growth factor signaling, cell cycle progression, and apoptosis *in vitro* and *in vivo*. Subsequently, we evaluated the difference in cellular gefitinib concentration after gefitinib monotherapy and after combination therapy with gefitinib and TP to confirm our hypothesis.

Materials and methods

Chemical preparation. Gefitinib was purchased from AstraZeneca (Macclesfield, UK). A 10 mM working solution in DMSO was prepared and stored at -20°C. Recombinant human EGF was purchased from PeproTech (London, UK). Fetal bovine serum (FBS) was purchased from Gibco Invitrogen Co. (Carlsbad, CA, USA). The primary antibodies used for western blot assays were as follows: rabbit monoclonal for anti-EGFR antibody, anti-phospho-EGFR (PY1068) antibody, anti-IGF-1R antibody, anti-phospho-IGFR (Tyr1135/1136) antibody, anti-SRC antibody, anti-phospho-SRC (Tyr416) antibody, anti-ABCG2 antibody, anti-ERK1/2 antibody, anti-phospho-ERK (PY204) antibody, anti-Akt antibody, anti-phospho-AKT (PS473) antibody (all from Abcam Co.); and mouse polyclonal anti-P53 antibody, anti-BAX antibody (from Cell Signaling Technology).

Cell culture. The lung adenocarcinoma A549 cell line was maintained in RPMI-1640 medium with 10% FBS at 37°C in a 5% CO₂ atmosphere. For this study, we divided the A549 cells into four groups: group one remain in normal growth; group-two (G) was administered gefitinib monotherapy and TP was added to group-three; group-four was administered the combination of gefitinib and TP. Group two, three and four received four repeated doses to simulate clinical situations.

Detection of gefitinib concentration in NSCLC A549 cells by high performance liquid chromatography (HPLC). The cells were cultured according to the experimental design and harvested. By thawing and refreezing five times in liquid nitrogen, the A549 cells were destroyed, added to a mixture of mobile phase methanol:water (70:30, 500 µl), and the supernatant was directly injected into HPLC. The objective of this study was to compare the differences in the cellular gefitinib concentration between the G and TP+G groups. The gefitinib concentration of one cell was equal to the concentration obtained by HPLC/total cell number. Assay performance was monitored using quality control samples, and the limit of quantification for each analysis was 0.05, 0.1 and 0.2 µM for gefitinib. The linear relationship between the peak area and the concentration of gefitinib was good; it was convenient for calculation and comparison with the intracellular gefitinib concentration.

Cell growth inhibition assay. CCK-8 assays were used to evaluate the growth inhibitory effects of gefitinib, paclitaxel and cisplatin. The cells were seeded on 96-well plates at a density of 3,000 cells/well, incubated for 24 h, and then treated for 48 h with the drugs at 37°C. After drug treatment, CCK-8 solution was added to each well and incubated for 4 h at 37°C. Cell viability was determined by measuring the absorbance at 540 nm in a microplate reader (micro-ELISA). Six replicate wells were used for each analysis, and at least three independent experiments were conducted. The data from the replicate wells are presented as the mean numbers of remaining cells, with 95% confidence intervals. To determine the effects of the combined drug treatments, any potentiation was estimated by multiplying the percentage of remaining cells (percent growth) for each drug.

Growth curves. Culture media were replaced every 48 h following drug treatment. The cells in the G and TP+G groups were cultured for 5-10 days to allow cells to return to the logarithmic phase. Then cells were harvested and seeded on 96-well plates at a density of 800 cells/well. Three replicate wells were used for each analysis. Every three wells were select from two groups of cells on day 1, 2, 3, 4 and 5. CCK-8 solution was added to each well and incubated for 4 h at 37°C. Cell viability was determined by measuring the absorbance at 540 nm in a microplate reader (micro-ELISA).

Analysis of the cell cycle and apoptosis. The cells were exposed to the different drug treatments for 48-72 h and were harvested. The cells were then washed with PBS and stained by PI at 4°C for 30 min using a cell cycle detection kit (KeyGen, Nanjin, China) and analyzed using a flow cytometer (BD FACSCalibur). Cells were stained using Annexin V-FITC and PI. Briefly, cells were suspended in binding buffer, followed by incubation with 1X Annexin V-FITC binding buffer (195 µl) and Annexin V-FITC (5 µl) for 10 min at room temperature in the dark. Expression of Annexin V and PI was determined using a fluorescence-activated cell sorting (FACS) flow cytometer (BD FACSCalibur). Apoptosis in the A549 cells was analyzed by Hoechst 33258 staining according to the manufacturer's instructions (Beyotime). Stained nuclei were observed under a fluorescence microscope.

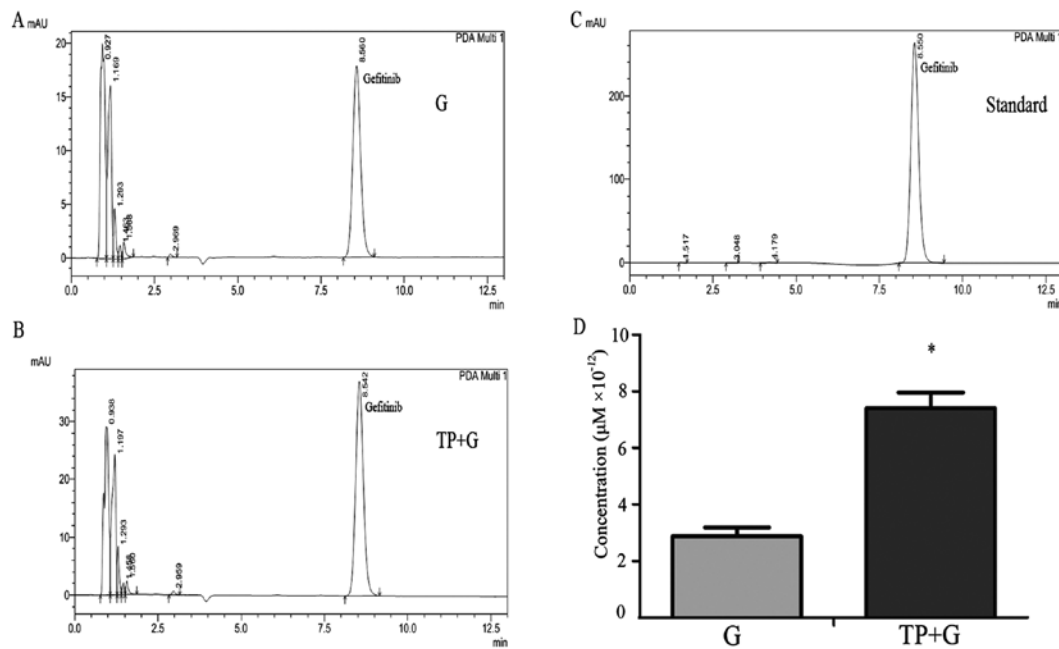


Figure 1. Cellular gefitinib concentration is significantly increased in the combination group. The data represent means \pm SEM from 3 independent experiments performed in triplicate. (A-C) Gefitinib chromatographic profiles in the G group, TP+G group, and standard (gefitinib; 0.1 μ M) chromatographic profiles. x-axis is retention time (min); y-axis is intensity (arbitrary units). The gefitinib concentration of one cell was equal to the concentration obtained by HPLC/total cell number. (D) Gefitinib concentration in the NSCLC A549 cells was significantly higher in the TP+G group than that in the G group. * $p < 0.05$, compared with the G group.

Mutational analysis of the EGFR (exon 18-21) gene. Genomic DNA was extracted from the A549 cells. Primers were designed using Primer 5, and the sequences were as follows: 18 exon forward, 5'-GATGCCAAAGAAGTAGAATGAGAAA-3' and reverse, 5'-CAGGAAAGCATCTTCACCCA CAG-3'; 19 exon forward, 5'-ATGGAATCTGTCAGCAAC CTCACCCTT-3' and reverse, 5'-CAACTGCTAATGGCCCG TTCTCG-3'; 20 exon forward, 5'-AACATGGTGAGGGC TGAGGTGAC-3' and reverse, 5'-ATGCCTTGGTCTGTGA ATTGGT-3'; 21 exon forward, 5'-AAATTACTATGCAGCA TGTGGCACC-3' and reverse, 5'-TAGGATGTGGAGATGAG CAGGGT-3'. All mutations were confirmed at least twice from independent PCR isolates.

Western blotting. A549 cells and tumor samples were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 mM deoxycholic acid and 1 mM EDTA) containing a cocktail of protease inhibitors and phosphatase inhibitors (Calbiochem, Darmstadt, Germany). Equal amounts of the protein samples (30-50 μ g) were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA) using the Bio-Rad Semi-Dry Transfer system.

Knockdown of IGF-1R by RNA interference and inhibitor for SRC. siRNA (sense, 5'-GAAGAGGUAUUGAAUGCUAdT dT-3' and antisense, 5'-UAGCAUUCAAUACCUCUUCT dTd-3') against IGF-1R was purchased from Guangzhou RiboBio Co., Ltd. Cells were transfected with short interfering RNAs at a final concentration of 40 nmol/l using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Cell lysates were harvested 48 h

after transfection. The SRC inhibitor, saratinib (5 nM) was added to the culture medium for 48 h, and the cell lysates were collected.

In vivo studies. Male athymic nude mice (4- to 6-weeks old) were purchased from Beijing HFK Bioscience Co., Ltd. Gefitinib was suspended at 50 mg/kg in 1% Tween-80 for administration by oral gavage. A549 cells were injected subcutaneously into the right dorsal flank of the mice. Tumor volumes were calculated using the formula: $\text{Width}^2 \times \text{length} \times 0.52$. Tumor growth was monitored and when tumors reached an average of 200 mm³, 40 mice (injected with A549 cells) were randomized into 4 groups (10 mice/group). One group of female nude mice (control group) received 1% Tween-80 alone (0.2 ml volume delivered as an oral gavage). The second group (bearing A549 tumor xenografts) was dosed orally with gefitinib at a dose level of 50 mg/kg. The third group received paclitaxel (injected in the abdominal cavity at a dose level of 5 mg/kg) and cisplatin (injected in the caudal vein at a dose level of 2 mg/kg). The fourth group received gefitinib and TP (cisplatin and paclitaxel). Treatment was performed once daily. Tumor size and mouse weight were measured at the same time. After 14 days, the mice were sacrificed and the tumors were dissected. All animals were treated in accordance with National Institutes of Health guidelines for animal care and use, and experimental protocols were approved by the Institutional Animal Care and Treatment Committee of Sichuan University.

Determination of gefitinib concentration in the tumor samples. Twenty mice were sacrificed by inhalation of halothane at 2 h after the final dose, and blood samples were collected into heparinized tubes. The tumor samples were flash-frozen and

stored at -70°C before processing. Plasma and tumor samples were immediately assayed for concentrations of gefitinib by HPLC as previously described (19). Tumor extracts were processed in an identical manner, except that samples were diluted using methanol:water (70:30, v/v).

Statistical analysis. The data are presented as the means \pm SEM. $p < 0.05$ was considered to indicate a statistically significant result. Calculation of the survival rate of the nude mice was by the Life Table method. The statistical significance was determined by the log-rank test. The statistical significance of the tumor size and weight was determined by the Student's *t*-test for two groups or by the Kruskal-Wallis test followed by Dunn's post-test for multiple groups. All data were analyzed using SPSS 19.0.

Results

Cellular gefitinib concentration is significantly increased in the combination group. The gefitinib concentration in the NSCLC A549 cells was detected by HPLC. Representative chromatographic profiles from the G and TP+G groups are shown in Fig. 1A and B. Fig. 1C shows standard (gefitinib; $0.1 \mu\text{M}$) chromatographic profiles; peaks at ~ 8 min, maximum absorption wavelength of 246 nm. After repeating the test, the results showed that the cellular gefitinib concentration was significantly higher in the combination (TP+G) group as compared with that in the G group (Fig. 1D).

Combination drug treatment greatly inhibits tumor cell growth following the first three treatments but not after the fourth treatment. To confirm the effect of the combined treatment and the single-drug exposure, we simulated the clinical treatment model by administering gefitinib alone or in combination with TP for four times.

After the first drug treatment, cell proliferation was inhibited (Fig. 2A), the cell cycle was arrested in the G2/M phase (Fig. 2F) and marked cell death induction was observed in the TP+G group (Fig. 2G). Compared with the normal control and TP group, the expression levels of P-EGFR, P-AKT and P-ERK protein were decreased in the G and TP+G groups. Compared with the normal control and the G group, p53 and BAX were increased in the TP and TP+G groups (Fig. 2B). The expression of ABCG2 displayed no obvious difference among the four groups. During treatment, the IC_{50} values of gefitinib increased (Fig. 2E), and the cellular gefitinib concentration was always significantly higher in the combination (TP+G) when compared with that in the G group (Fig. 2D). The second and third treatment had a similar effect on cell viability in comparison to the first treatment. However, after the fourth drug treatment, cells in the TP+G group exhibited increased growth rate, reduced cell cycle arrest in the G2/M phase, and decreased apoptosis (Fig. 2F and G).

Protein levels of P-IGF-1R, P-SRC and P-ERK in the fourth (TP+G) treatment group are significantly increased (Fig. 3). The expression of cell growth-associated proteins was detected after the fourth drug treatment. The levels of P-EGFR, P-IGF-1R, P-SRC and P-ERK were higher in the fourth treatment (TP+G)4 group than these levels in the control group

and the fourth G4 treatment group. The results of P-HER2, P-MET, P-PTEN are not shown due to the absence of expression in the three groups.

IGF-1R mediates drug resistance in the late combination treatment group. We aimed to determine the molecular mechanism by downregulating expression of IGF-1R and SRC in the fourth (TP+G)4 treatment group. Results showed that cell proliferation was inhibited (Fig. 4C), and the levels of P-AKT, P-ERK, P-SRC were reduced after IGF-1R silencing with specific siRNA (Fig. 4A). Moreover, the percentage of cell apoptosis increased following exposure to gefitinib (Fig. 4B). However, all these changes were not observed after SRC was inhibited by saratinib.

TP+G treatment fails in the lung cancer mouse model. The TP+G group exhibited a significant reduction in tumor volume and mouse body weight when compared to the other groups (Fig. 5A and B). The tumor inhibitory rate (IR) in the TP+G group was 52.7%, and this rate in the G group was 34.28%. However, the body weight of mice in the TP+G treatment group was markedly decreased. Additionally, this group exhibited poor body condition and the shortest survival time (Fig. 5C). The concentrations of gefitinib in the serum and tumor tissues were measured by HPLC. The gefitinib concentrations in the serum and tumor tissues were higher in the gefitinib alone group when compared to the concentrations in the combination therapy group (Fig. 5D and E). The gefitinib relative absorption rate was calculated according to the following formula: Relative absorption rate = (gefitinib concentration in the serum)/(gefitinib concentration in the tumor tissue). The relative absorption rate in the G group was higher than that in the TP+G group (Fig. 5F).

In the course of treatment, there was no mutation in 18, 19, 20, 21 exons of EGFR as determined by sequencing. During the treatment period, we extracted total DNA from A549, G4-A549, (TP+G)4-A549, cells designed and synthesized the primers for EGFR exons 18, 19, 20 and 21. Mutation analysis was performed by direct sequencing of the four exons. No mutation was detected throughout the treatment process.

Discussion

The present study results demonstrated that the use of a combination treatment (chemotherapy + gefitinib) resulted in a failed antitumor effect *in vitro* and *in vivo*. In the *in vitro* studies, the gefitinib concentration in the cells was significantly higher in the paclitaxel + cisplatin + gefitinib (TP+G) group than that in the G group. Following the treatment time extension, the TP+G treatment increased the cell viability while the cellular concentration of gefitinib was not reduced. In the A549 xenograft model, the gefitinib concentrations in the G group in the serum and tumor tissues of the nude mice were higher than these levels in the combination therapy group. However, the body weight of mice in the TP+G group was markedly decreased with a poor condition and the shortest survival time. Previous studies on the combination of gefitinib and chemotherapy for initial treatment of advanced NSCLC also found the failure for an improved response or prolonged

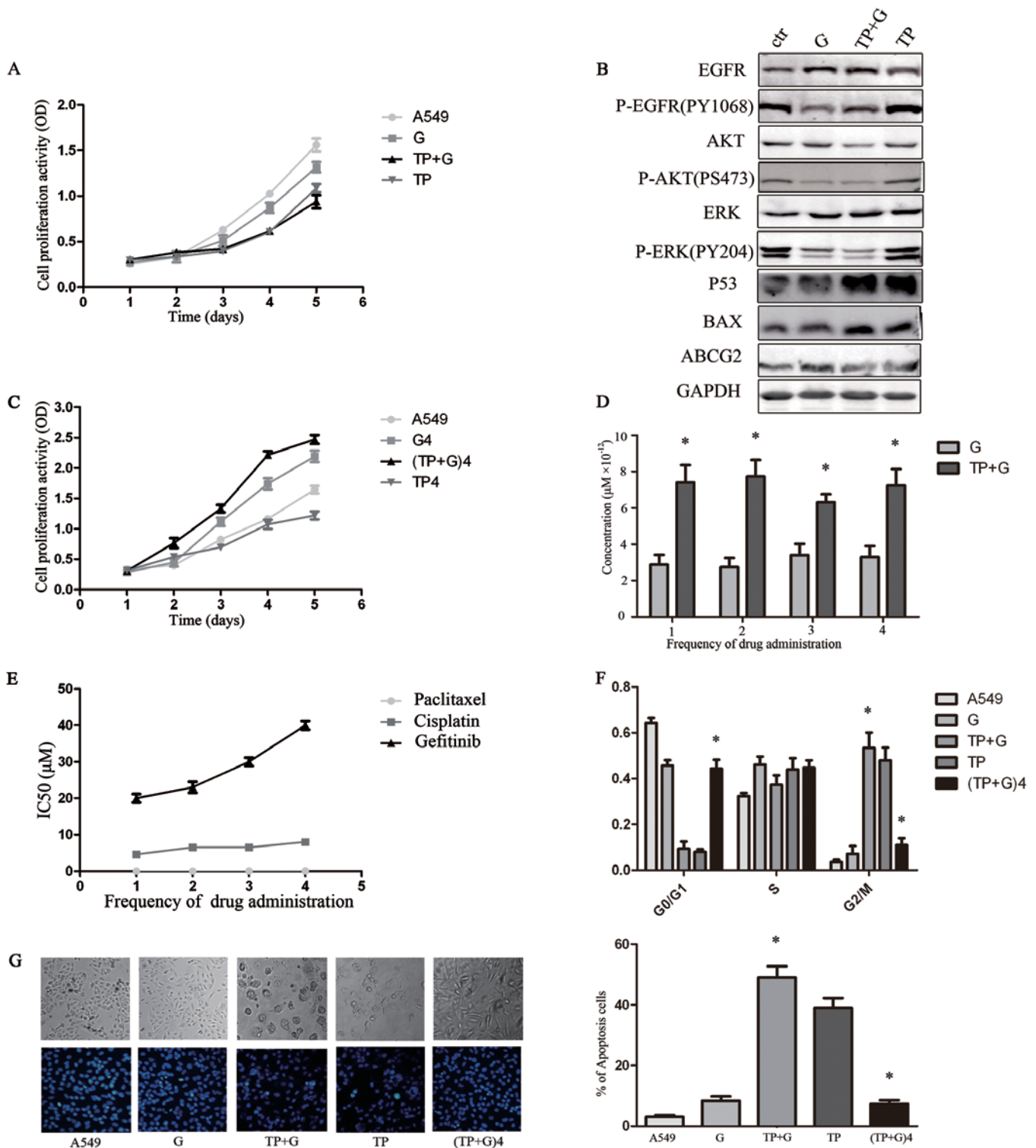


Figure 2. Effect of different drug treatments on NSCLC A549 cells following 4 treatments. Values are expressed as the means \pm SEM at each time-point and represent three individual experiments. (TP+G)4, the fourth TP and gefitinib treatment group. (A) CCK-8 assay of the growth-inhibitory effect of different treatments on the various groups including A549 (control), G (gefitinib), TP+G (paclitaxel, cisplatin and gefitinib) and TP. The control group exhibited the most rapid proliferation rate while the TP+G group had the slowest. (B) Different protein levels in the cells were detected by western blotting. Compared with the control and TP group, the expression levels of P-EGFR, P-AKT and P-ERK proteins were decreased in the G and TP+G groups, while p53 and BAX were increased in the TP and TP+G groups. (C) Cell proliferation was analyzed after the fourth drug treatments. After the fourth drug treatment, cells in the (TP+G)4 group exhibited an increased growth rate ($p < 0.05$). (D) Cellular gefitinib concentration was significantly higher in the TP+G group when compared with that in the G group ($p < 0.05$). (E) The IC₅₀ value of paclitaxel, cisplatin and gefitinib after each treatment in the TP+G group. After the fourth drug treatment, cells in the (TP+G)4 group exhibited improved gefitinib IC₅₀ values ($p < 0.05$). Results are the average of duplicate experiments. (F) Cells in the TP+G group exhibited cell cycle arrest in the G2/M phase ($p < 0.05$), compared with the G and control groups. Following the time extension, cell cycle in the fourth treatment (TP+G)4 group was arrested in the G0/G1 phase ($p > 0.05$), compared with the control group. (G) Hoechst staining images of cells. The percentage of Hoechst-positive cells were counted as apoptotic. The TP+G group showed an increase in apoptosis compared with the control, G and TP groups ($p < 0.05$). The fourth time (TP+G)4 treatment group showed a decrease in the percentage of apoptotic cells when compared with the TP+G treatment group ($p < 0.05$).

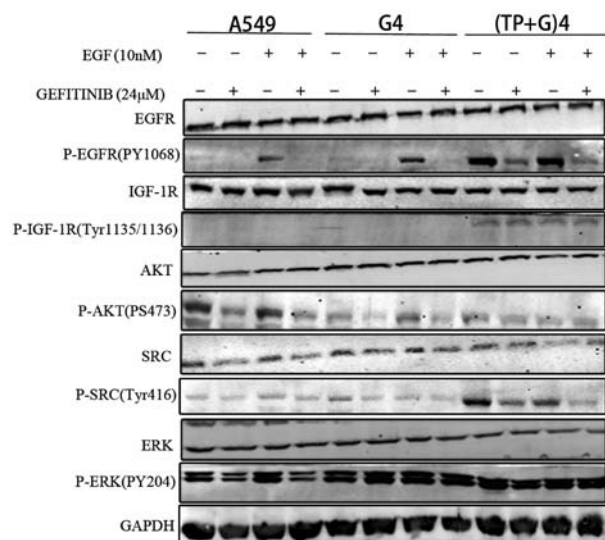


Figure 3. Protein expression levels in the cells following 10 nM EGF or/and 24 μ M gefitinib stimulus for 30 min. A549, control cells; G4, the fourth gefitinib alone treatment group; (TP+G)4, the fourth TP and gefitinib treatment group. P-EGFR, P-IGF-1R, P-ERK and P-SRC levels were increased in the (TP+G)4 group following gefitinib alone treatment. Densitometric analysis was performed, and the results were normalized to the GAPDH level. The data illustrated are representative of 3 experiments.

survival over chemotherapy alone (20). Similar results were also reported in animal models (21,22). In regards to the failed combination therapy, we hypothesized that the possible reason may be that the chemotherapy drugs disturbed the cell membrane transport of gefitinib consequently resulting in failure. A previous study reported that one mechanism for resistance to EGFR-TKI is associated with the downregulation

of ABCG2 expression (23). In the present study, the results did not demonstrate a decrease in the expression of ABCG2 following the combination therapy. Furthermore, several studies have reported that EGFR mutations or amplification can identify patients more likely to benefit or cause drug-resistance from gefitinib monotherapy (7,24,25). To dismiss these confounding factors, including EGFR mutations, a high EGFR copy number, we chose the A549 cell line which is known to be EGFR^{WT}. Moreover, a fluorescent derivative of Tasisa was synthesized to study the cellular Tasisa concentration (26). However, the fluorescent derivative of Tasisa has a lower efficiency that could be attributed to the structural alteration of the molecule, resulting in decreased affinity to its target. The isotope derivative has the same problem. In this study, we established an HPLC method for determination of the content of gefitinib in the A549 cells.

In the *in vivo* studies, the gefitinib concentration in the gefitinib group in the serum and tumor tissues of nude mice were higher than these levels in the combination therapy group. The major human P450 enzyme involved in the metabolism of gefitinib is CYP3A4, demonstrating that CYP3A4 is primarily responsible for the overall metabolism of gefitinib. However, in the present study, there were no significant differences in CYP3A4 protein expression in the liver tissues of the nude mice. The results of such an analysis may be explained by the fact that the chemotherapy seriously affected the gastrointestinal function in the nude mice, resulting in a reduction in gefitinib absorption, which eventually led to a higher gefitinib concentration of serum and tumor tissues in the gefitinib alone group. In addition, metabolism of gefitinib was fairly rapid and complex (27). The content of gefitinib could not be detected for >3 h by HPLC. Following treatment of gefitinib, the mice were sacrificed no later than 2 h afterwards and the gefitinib

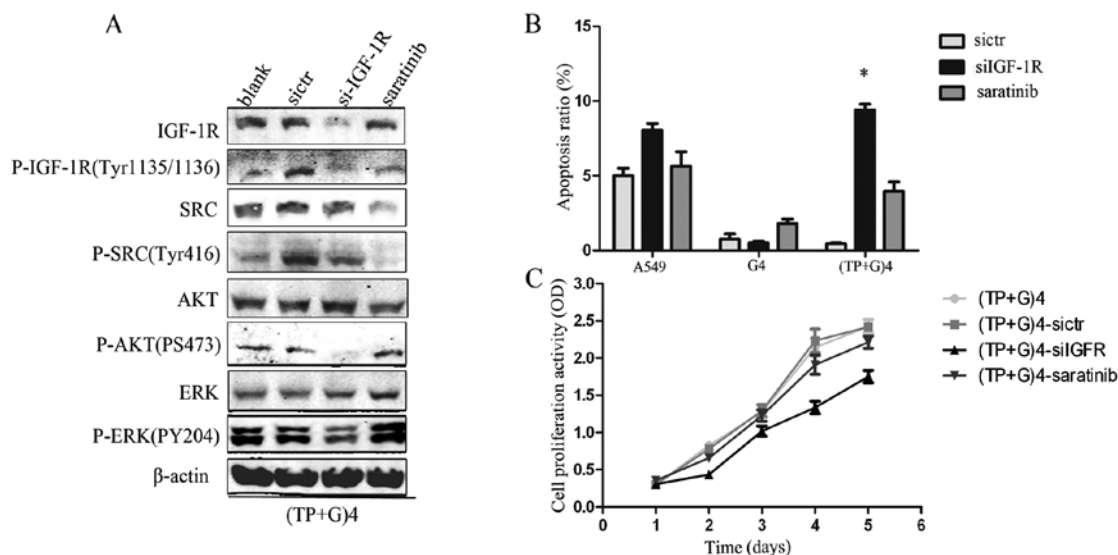


Figure 4. IGF-1R induces drug resistance in the fourth TP+G treatment group. Data represents the means \pm SEM (n=3). (A) IGF-1R and SRC expression in the (TP+G)4 group was determined. A549 cells were transfected with siCTR, siIGF-1R and SRC inhibitor (5 nM, saratinib). Following downregulation of IGF-1R with siRNA, the levels of P-AKT, P-ERK, P-SRC were reduced in the (TP+G)4-A549 cells. β -actin was used as a loading control. (B) A549, G4-A549 and (TP+G)4 group cells were treated with siCTR, siIGF-1R and saratinib for 48 h, respectively. Gefitinib (24 μ M) was then added to continue culture for 24 h. Cell apoptosis was increased in the (TP+G)4-siIGF-1R cells (* p <0.05) compared with the control and saratinib-treated cells. (C) (TP+G)4 A549 cells were separately treated by siCTR, siIGF-1R and saratinib and then analyzed by CCK-8 for cell proliferation. After downregulation of IGF-1R, cells in the (TP+G)4 group exhibited a decreased growth rate (p <0.05).

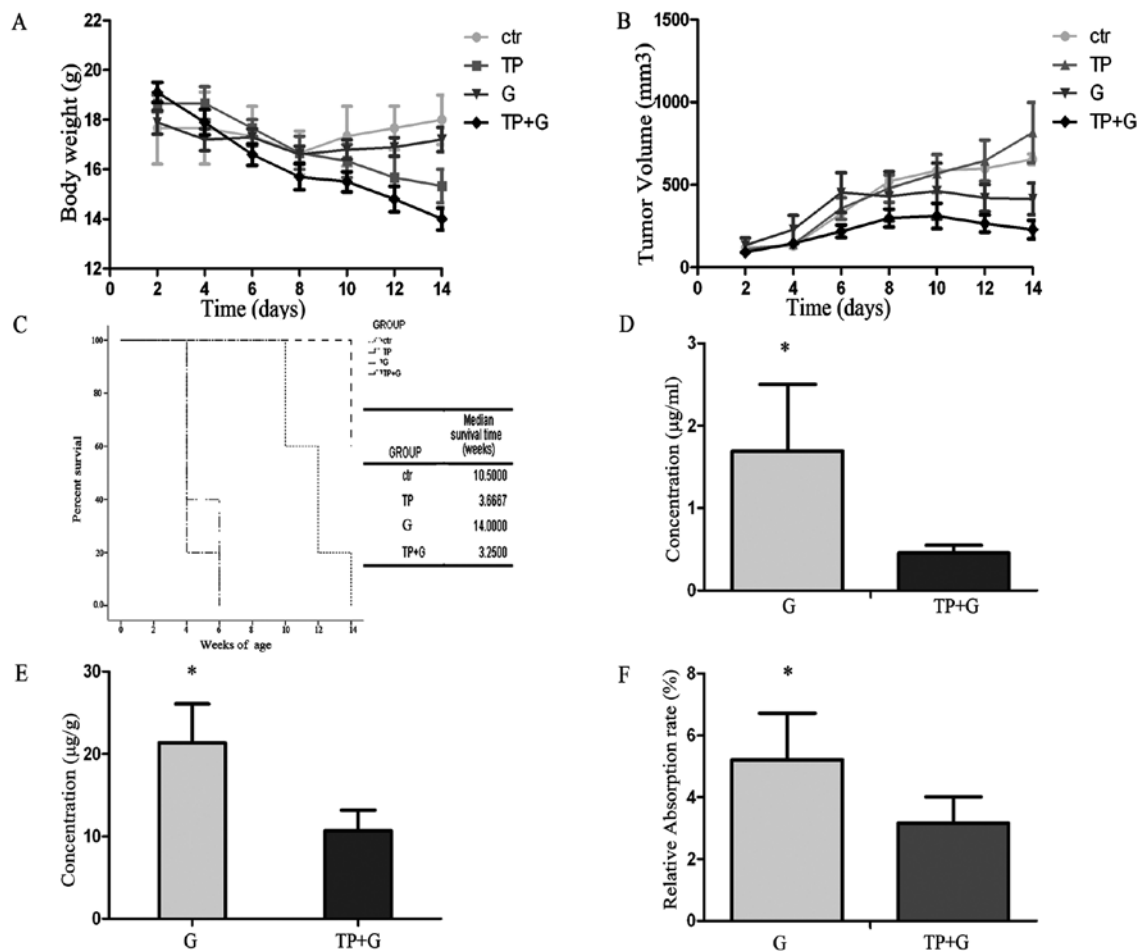


Figure 5. Failed combination treatment in a lung cancer xenograft model after 14 days of treatment. Each data value represents the mean tumor weight of 5 mice. Inhibition rate (IR) = (the weight of tumors in the control group - the weight of tumors in the experimental group)/the weight of tumors in the control group \times 100%. (A) The TP+G group had the most decreased body weight compared to the control, G and TP groups ($p < 0.05$). (B) The TP+G group had the smallest tumor volume compared to the control and G group ($p < 0.05$). (C) Survival rate in the G group was longer than the rate in the control, TP and TP+G groups ($p < 0.05$). (D) Gefitinib concentration in the serum of the G group was higher than that in the TP+G group ($*p < 0.05$). (E) Gefitinib concentration in tumor tissues of the G group was higher than that in the TP+G group ($*p < 0.05$). (F) Gefitinib relative absorption rate = (gefitinib concentration in serum)/(gefitinib concentration in tumor tissue). Result showed that the G group had a higher gefitinib relative absorption rate than the TP+G group ($*p < 0.05$).

concentration was immediately detected in the serum and tumor tissues. Although there are many reasons for the failed combination therapy *in vivo*, decreased gefitinib concentration in serum and tumor tissues could also have been a reason.

For the *in vitro* assay, following the first three treatments, the combination group demonstrated an obviously inhibitory effect on cell proliferation compared to gefitinib monotherapy. However, this effect was reversed after the fourth treatment. Further analysis of the protein expression found that levels of P-EGFR, P-IGF-1R, P-SRC and P-ERK were higher in the fourth TP+G group than levels in the fourth gefitinib alone treatment and control groups. A previous study reported that SRC can activate EGFR in colorectal cancer cell lines, and EGFR overexpression is correlated with SRC activation (28). Through transactivation, as described above, it appears that the effect of EGFR on tumorigenesis extends beyond ligand-mediated effects. However, in the present study, we were unable to detect any effect of SRC. Studies have found that IGF-1R also activates P-AKT and P-ERK leading to increased cell growth. By downregulating the expression of IGF-1R in the fourth TP+G treatment group, drug resistance was

successfully reversed. Thus, we conclude that IGF-1R activation contributed to the drug resistance in the fourth TP+G group. IGF-1R activation resulted in a marked phosphorylation level of ERK and Akt. Both of them transduce signals that trigger a cascade of cell viability responses. The signals involved in cell growth possibly reversed the effects of the high cellular concentration of gefitinib resulting in final failure of the combination treatment. IGF-1R is a transmembrane TK receptor which is activated by binding of its ligands IGF-I and IGF-II. Extensive studies have established that the receptor signaling plays an important role in tumorigenesis, metastatic potential of tumor cells and neoplastic growth (29). The consequences of IGF-1R activation by its ligands result in the recruitment of major adapter signaling proteins which lead to interaction with Grb/SOS and ultimately RAS/MAPK (ERK) signaling cascades and PI3-kinase/AKT activation (30). Evidence suggests that IGF-1R plays a role in maintaining the malignant phenotype (31), and disruption of IGF-1R activation has been shown to inhibit growth and motility of a wide range of cancer cells *in vitro* and in mouse models (32). In our study, we first reported that frequent use of the combined

treatment with chemotherapy and gefitinib in the A549 cell line induced IGF-1R activation which resulted in the failed treatment. Furthermore, through paclitaxel, cisplatin, gefitinib, paclitaxel + cisplatin (TP) and (TP+G) separately stimulated, we confirmed that the treatment schedules of TP+G caused IGF-1R activation. Although IGF-1R activation normally requires the presence of insulin-like growth factor-1, there is some precedent for IGF-1R activation without its cognate ligand (33).

In summary, the combination therapy (TP+G) appears to be ineffective for NSCLC treatment. In the *in vivo* assay, decreased gefitinib concentrations in the serum and tumor tissues could have been a reason for the failure of the combination therapy. More importantly, frequent use of the combination treatment in A549 lung cancer cells induced IGF-1R activation which contributed to gefitinib resistance and gave rise to the failure of the combination therapy. Although the exact molecular mechanism of IGF-1R activation remains unclear, the present study provides foundation for further study concerning the IGF activation pathway.

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