# Combined treatment with TNF-α/gefitinib alleviates the resistance to gefitinib in PC-9 cells

Yuan Ji[a], Sheng-lin Ma[c], Yi-ping Zhang[c], Juan-juan Tang[b], Yan-ming Wu[b] and Yan-Jun Lu[b]

**Gefitinib has been approved for the treatment of patients with non-small cell lung cancer. However, its efficiency is limited by the development of drug resistance. Additional treatments for cases of non-small cell lung cancer relapsing with treatment with gefitinib are urgently required. To investigate the mechanisms of acquired resistance to gefitinib, we established PC-9-ZD, a human lung cancer cell line resistant to gefitinib after long-term exposure to the drug. PC-9-ZD cells showed more resistance to gefitinib than their parental PC-9 cells. We show that gefitinib reduces p-Akt levels, concomitant with elevation of p21 levels and suppression of cdk2/4 and cyclinE/D1 activities, which result in impaired cell cycle progression through $G_1$ arrest only in parental PC-9 cells, in which it inhibits growth. Our present data suggested that after long-term exposure to gefitinib, the survival of PC-9-ZD cells with heightened levels of p-Akt and reduced levels of p21 resisted further gefitinib-induced inhibition of cell growth. To explore a new strategy to improve the efficacy of gefitinib, we treated the cells with tumor necrosis factor-α (TNF-α) and found that the cells with acquired resistance to gefitinib showed increasing sensitivity to TNF-α, which correlated with the low activation level of nuclear factor (NF)κB/p65 in PC-9-ZD cells. TNF-α treatment induced an elevated activated NFκB/p65, concomitant with induced p21 levels, which resulted in increased sensitivity to gefitinib in PC-9-ZD cells. Consistent with our earlier observation that p21 is induced in an NFκB/p65-dependent manner, we conclude that p21 plays an important role in mediating cell growth inhibition by gefitinib. Thus, we proposed that combined treatment with TNF-α/gefitinib is an efficient therapeutic strategy for tumors that develop resistance to gefitinib. *Anti-Cancer Drugs* 20:832–837 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Department of Pathology, Zhongshan Hospital, Fudan University, [b]Laboratory of Biochemistry and Molecular Biology, Tongji University, Shanghai and [c]Zhejiang Tumor Hospital, Banshanqiao, Hangzhou, China

Correspondence to Dr Yan-Jun Lu, PhD, Laboratory of Biochemistry and Molecular Biology, Tongji University, 1239 Siping Road, Shanghai 200092, China
Tel: +86 21 65984980; fax: +86 21 65983793; e-mail: yanjunlu@hotmail.com

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## Introduction

Gefitinib is a small-molecule quinazoline derivative that was developed as a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR). EGFR is known to promote the growth of cells, functions as an oncogene and is expressed in up to 80–90% of non-small cell lung cancers (NSCLC) [1]. Gefitinib has been approved for the treatment of patients with NSCLC. However, tumor responses were observed in 12–18% of patients with chemotherapy-refractory advanced NSCLC [2]. Even in patients sensitive to gefitinib, resistance is acquired after long-term exposure to the drug. Additional treatments for cases of NSCLC relapsing with treatment with gefitinib are urgently required.

Tumor necrosis factor-α (TNF-α) is a glycoprotein that possesses antitumor activity against a wide range of tumor cell lines, whereas nonmalignant cells are relatively resistant [3]. However, its clinical application is limited by its severe systemic side effects, before therapeutic doses can be administered. The feasibility of TNF-α as a biological response modifier has recently been established. Clinical phase II and phase III trials have shown that systemic administration of TNF-α in combination with other chemotherapy agents for lung tumors remarkably enhances the antitumor effect, as compared with chemotherapy agent treatment alone. In total, 44% of the 133 patients exhibited complete or partial remission of lung tumors in response to this combination therapy strategy [4–6]. As it has been approved by the Chinese State Drug Administration, this innovative clinical application of TNF-α in combination with chemotherapy agents may offer significant opportunities for cancer patients. Furthermore, in the setting of hyperthermic isolated limb perfusion, TNF-α in combination with melphalan seems to be very active against powerful sarcomas and melanomas, tumors known to be resistant to chemotherapy [7,8]. The results of the combination of TNF-α with other chemotherapy agents suggest synergistic antitumor effects in chemotherapy-resistant tumors.

Alterations in cell cycle control are a universal feature of lung cancers. p21, the product of the WAF1/CIP1/SDI1 gene, is an inhibitor of cyclin-dependent kinases, and is

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activated through p53-dependent or p53-independent pathways [9–13]. Although many experimental studies have clearly indicated that p21 plays an important role in the regulation of the cell cycle, especially in $G_1$ arrest, the clinical significance of p21 expression in NSCLC remains unclear.

In this study, we established PC-9-ZD, an NSCLC cell resistant to gefitinib after long-term exposure to the drug. PC-9-ZD cells showed increasing sensitivity to TNF-α treatment. We observed that the reducing activated nuclear NFκB/p65 was concomitant with reduced p21 levels in PC-9-ZD cells with developed resistance to gefitinb. Combined treatment with TNF-α/gefitinib alleviated the resistance of PC-9-ZD cells to gefitinib.

## Methods

### Cells and cell culture

Human NSCLC PC-9 cells (derived from a patient with adenocarcinoma) and the cells of the gefitinib-resistant cell line PC-9-ZD were cultured in Dulbecco's modified Eagle's minimum essential medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (Hyclone).

### Measurement of cell death

The cells were seeded into a 96-well microtiter plate and treated with gefitinib (0–1000 nmol/l) or TNF-α (500 U/ml). Ten microliters of the CCK-8 solution were added to each well of the plate and then incubated for 2 h (37°C and 5% $CO_2$) according to the procedure of the Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan). The absorbance was measured at 450 nm using a microplate reader (BioTeK, Winooski, Vermont, USA).

### Western blotting

Nuclear, whole-cell, and cytoplasmic extracts from the cells were prepared with the Nuclear Extract Kit (Active Motif, Carlsbad, California, USA). The protein content of the cell lysate was determined by using the Bradford calorimetric assay method (Bio-Rad, Richmond, California, USA). A 40 μg aliquot of the cell lysate was resolved by 12% polyacrylamide–sodium lauryl sulfate gel electrophoresis and transferred to a Hybond-C Super membrane (Amersham, Aylesbury, UK). The antibodies used for detection are as follows: anti-p65 antibody (Upstate, Lake Placid, New York, USA), anti-p21 antibody (Cell Signaling, Beverly, Massachusetts, USA) and anti-Akt/p-Akt antibody (Upstate), CDK2 (CalBiochem, California, USA), CDK4 (Santa Cruz, California, USA), cyclinD1 (Santa Cruz), cyclinE (Santa Cruz), PCNA and β-actin (Beyotime Institute of Biotech., Jiangsu, China). The blot was then incubated with secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) and detected with Odyssey Infrared Imaging System (LI-CDR Biosciences, Lincoln, Nebraska, USA).

### Cell cycle analysis by fluorescence-activated cell sorting

Flow cytometry was performed to determine the cell cycle pattern of NSCLC cell lines in response to gefitinib treatment. The cells were washed twice with PBS, trypsinized and resuspended in PBS containing 0.1% Triton X-100 and RNase (1 mg/ml) (Sigma, St Louis, Missouri, USA). The cell suspension was incubated at 37°C for 30 min. Propidium iodide (Molecular Probes Inc., Eugene, Oregon, USA) was added at a final concentration of 50 μg/ml and the cell suspension was kept at 4°C for 1 h. The cells were filtered and the cell cycle was analyzed by flow cytometry with the FAC Scan system (Becton Dickinson, Franklin Lakes, New Jersey, USA).

## Results

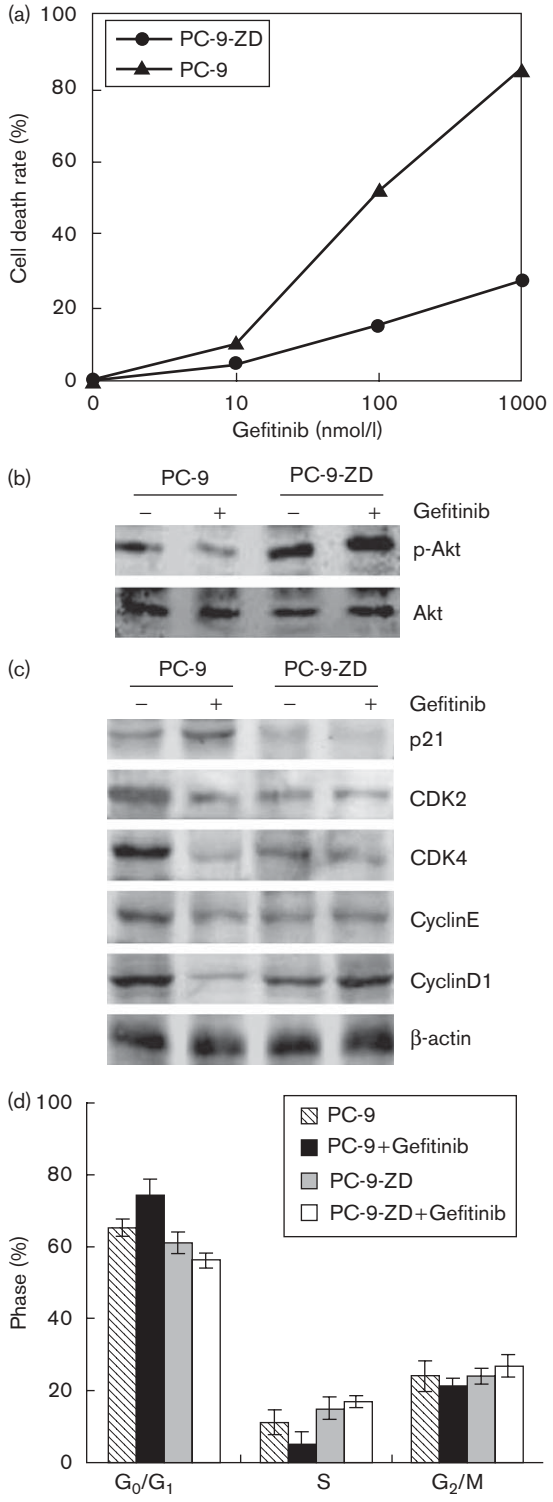
### p21 was elevated by gefitinib in its parental PC-9 cells that are sensitive to gefitinib

Tumor cells that have acquired resistance to gefitinib may complicate future treatment. To elucidate the mechanism of the cells with acquired resistance to gefitinib, we established a gefitinib-resistant PC-9 cell line after long-term exposure to the drug. We chose the clone of a gefitinib-resistant cell line termed 'PC-9-ZD' after it was developed in gefitinib (200 nmol/l) for 3 months. To confirm this point, we treated PC-9 and its gefitinib-resistant derivative PC-9-ZD cells with gefitinib for 24 h. Data from Fig. 1a showed that there were inherent differences between PC-9 and PC-9-ZD as displayed by cell survival curves. The result indicated that PC-9-ZD cells were more resistant to gefitinib than their parental PC-9 cells.

Gefitinib is known to inhibit EGFR autophosphorylation. It also inhibits the V-akt murine thymoma viral oncogene homolog (Akt kinase). The EGFR/Akt signals are down-regulated in response to gefitinib only in cell lines that are sensitive to growth inhibited by gefitinib [14]. Recent studies have shown that Akt and its signal pathway play an important role in mediating gefitinib-induced cell growth inhibition [15–18]. To examine the biochemical events of activated p-Akt in this study, p-Akt activity was assayed by probing western blotting with an antibody that exclusively recognizes serine 473. An elevated level of p-Akt in PC-9-ZD cells was observed (Fig. 1b). Thus, our data suggested that after long-term exposure to gefitinib, the survival of PC-9-ZD cells with high activation levels of Akt resisted further gefitinib-induced cell growth inhibition.

A study has shown that an antitumor agent suppresses cell growth in a manner likely to be related to up-regulation of cell cycle inhibitors, such as p27 and p21 in NSCLC [19]. p21 and p27 are important cell cycle checkpoint proteins. Thus, we asked whether p21 plays a role in response to gefitinib in this case. We examined

**Fig. 1**




p21 was elevated by gefitinib in its parental PC-9 cells that are sensitive to gefitinib. (a) Cell death rate after 24 h of gefitinib treatment in PC-9-ZD cells (●) and parental PC-9 cells (▲). Western blotting analysis was performed to assess p-Akt/Akt (b), p21, CDK2, CDK4, cyclinD1, and cyclinE protein levels (c) in PC-9 or PC-9-ZD cells. (d) Cell cycle pattern of PC-9 or PC-9-ZD cells with or without gefitinib treatment (1 μmol/l) for 72 h. Error bar indicates the standard error of the mean of three independent experiments.

p21 levels in PC-9 and their gefitinib-resistant derivative PC-9-ZD cells with or without gefitinib for 24 h by western blotting analysis. As shown in Fig. 1c, the p21 protein levels were high in gefitinib-sensitive PC-9 cells. Moreover, exposure to gefitinib actually increased p21 expression, an effect that was most pronounced in cells that were sensitive to gefitinib. In contrast, no detectable p21 could be observed in gefitinib-resistant derivative PC-9-ZD cells with or without treatment of gefitinib.

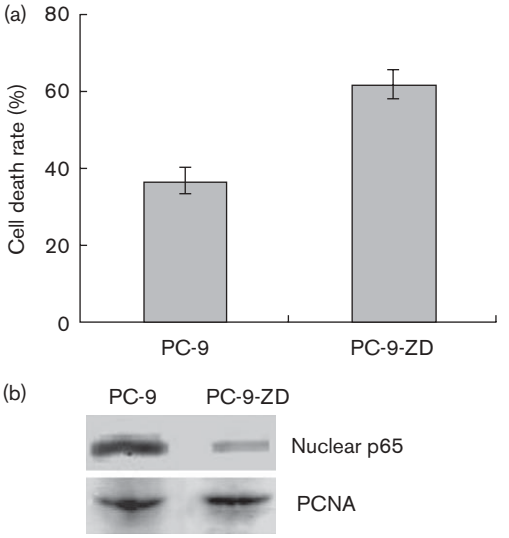
We further sought to test some other key cell cycle regulators, which are shown in Fig. 1c. We observed a significant decrease in the levels of cdk2, cdk4, cyclinE, and cyclinD1 with gefitinib in gefitinib-sensitive PC-9 cells, but this did not occur in PC-9-ZD cells. These data were consistent with our findings on cell cycle analysis of gefitinib-induced retention of cells in the $G_1$ phase, a sharp decrease in the S phase population but no significant change in the $G_2$/M fraction in gefitinib-sensitive PC-9 cells. In contrast, there was no apparent change in cell cycle pattern with gefitinib treatment of in gefitinib-resistant PC-9-ZD cells (Fig. 1d). These data indicated that gefitinib elevated p21 levels and suppressed cdk2/4 and cyclinE/D1 activities, which resulted in impaired cell cycle progression through $G_1$ arrest. Taken together, gefitinib treatment induced cytostasis through multiple mechanisms such as the reduction of phosphorylation of Akt activity and the suppression of cell cycle progression by induction of p21 protein in gefitinib-sensitive PC-9 cells. Thus, we suggested that gefitinib-promoted p21 protein elevation at least partly contributed to negative regulation of cell progression by gefitinib.

### Enhancement of sensitivity to TNF-α treatment in PC-9-ZD cells with acquired resistance to gefitinib

TNF-α bound to and activing its receptor complexes can interact with caspase-8 proteases through TRADD and FADD to induce cell death, whereas NFκB/p65 activation induced by TNF-α inhibits cell death [20]. The prototypical NFκB/p65 complex, which responds to heterodimer of p50 and Rel A subunits, exists as the inactive dimmer sequestered in the cytoplasm. The activated NFκB/p65 heterodimer rapidly translocates into the nucleus, where it engages the κB enhancer elements and alters gene expressions. In fact, NFκB/p65 regulates a set of anti-apoptotic genes including the members of the Bcl-2 family, cellular inhibitors of apoptosis protein C (c-IAP 1 and 2), A20 and superoxide dismutase 2 (SOD2). These anti-apoptotic proteins inhibit the activation of caspases, which results in preventing cytotoxic actions induced by TNF-α stimulation [21].

To explore the conditions required for killing PC-9-ZD cells, we treated PC-9 and PC-9-ZD cells with TNF-α for 6 h. The PC-9-ZD cells were found to be more sensitive

**Fig. 2**




Enhancement of sensitivity to tumor necrosis factor-α (TNF-α) treatment in PC-9-ZD cells with acquired resistance to gefitinib. (a) Cell death rate after treatment with TNF-α (500 U/ml) for 6 h in PC-9 and PC-9-ZD cells. (b) The nuclear factor (NFκB)/p65 levels were determined by western blotting analysis in PC-9 and PC-9-ZD cells.

to the cytotoxic effect of TNF-α than their parental PC-9 cells (Fig. 2a). Our data suggest that PC-9-ZD cells displayed resistance to gefitinib-induced cell growth inhibition and enhanced sensitivity to TNF-α treatment after acquired resistance to gefitinib.

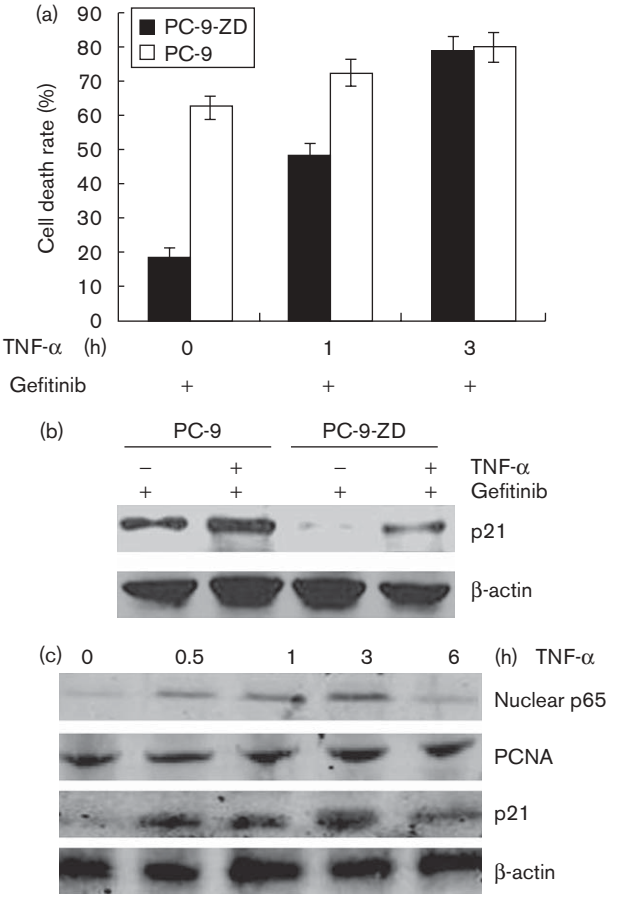
Next, the levels of NFκB/p65 were assessed by western blotting analysis with an antibody against NFκB/p65. As shown in Fig. 2b, NFκB/p65 was lower in PC-9-ZD cells with acquired resistance to gefitinib than their parental PC-9 cells. This correlated with PC-9-ZD cells displaying enhanced sensitivity to TNF-α treatment (Fig. 2a and b). This observation led us to suggest that alternative administration of TNF-α may be a useful effective protocol for the therapeutic treatment of gefitinib-resistant tumors.

### TNF-α alleviated the resistance to gefitinib by inducing p21 levels in PC-9-ZD cells with acquired resistance to gefitinib

The gefitinib-resistant PC-9-ZD cells displayed resistance to gefitinib-induced inhibition of cell growth. To explore a method of treatment for alleviating the resistance of PC-9-ZD cells to gefitinib, we treated cells with TNF-α for 0, 1 and 3 h respectively, and then incubated the cells with gefitinib for 24 h. Interestingly, the PC-9-ZD cells were more sensitive to the cytotoxic effect of this TNF-α/gefitinib treatment than to that of a simple 24 h gefitinib treatment (78.8 vs. 18.9%) (Fig. 3a).

We then examined p21 levels by western blotting analysis. As shown in Fig. 3b, the p21 protein levels were

**Fig. 3**




Tumor necrosis factor-α (TNF-α) alleviated the resistance to gefitinib by inducing p21 levels in PC-9 cells with acquired resistance to gefitinib. (a) Cell death rate after 24 h of gefitinib treatment (500 nmol/l) combined with or without TNF-α treatment (500 U/ml) at the indicated time points for treatment in PC-9-ZD cells (■) and its parental PC-9 cells (□). (b) p21 levels were determined by western blotting analysis in PC-9 and PC-9-ZD cells with or without treatment with 3 h of TNF-α (500 U/ml), then combined with 24 h of gefitinib treatment (500 nmol/l). (c) p21 and nuclear factor (NF)κB/p65 levels were determined by western blotting analysis in PC-9-ZD cells after treatment with TNF-α (500 U/ml) at the time indicated.

higher in PC-9-ZD cells with TNF-α/gefitinib treatment than those with a simple gefitinib treatment. Moreover, exposure to TNF-α/gefitinib actually increased p21 expression, an effect that was most pronounced in PC-9 cells with acquired resistance to gefitinib. Thus, we suggest that combined treatment with TNF-α/gefitinib alleviates the resistance to gefitinib in PC-9 cells with acquired resistance to gefitinib.

In our earlier study, we revealed that NFκB/p65 induced p21 expression [22]. To examine NFκB/p65 and p21 levels in the cells treated with TNF-α, analysis was next performed to detect the NFκB/p65 and p21 after treatment with TNF-α for 0, 0.5, 1, 3 and 6 h. The levels of NFκB/p65 and p21 were assessed by immunoblotting

in the TNF-α-treatment time course. As shown in Fig. 3c, the level of NFκB/p65 and p21 in PC-9-ZD cells was sustained at the peak after 3 h treatment with TNF-α, and then diminished. Taken together, these results indicated that the TNF-α treatment induced the activation of NFκB/p65 and then increased p21 proteins levels in PC-9-ZD cells. The elevation of p21 protein contributed to its negative regulation of cell progression by gefitinib. The induction of p21 by NFκB/p65 or TNF-α treatment may contribute to enhancing sensitivity to combined treatment with TNF-α/gefitinib and alleviating the resistance to gefitinib in PC-9 cells with acquired resistance to gefitinib.

## Discussion

In this study, we established PC-9-ZD as a human lung cancer cell line resistant to gefitinib after long-term exposure to the drug. The PC-9-ZD cells showed increased sensitivity to TNF-α treatment. We observed that reducing activated NFκB/p65 was concomitant with reduced p21 levels in PC-9-ZD cells with developed resistance to gefitinib. Combined treatment with TNF-α/gefitinib alleviated the resistance of PC-9-ZD cells to gefitinib. These data suggested inducing p21 levels by TNF-α treatment contributed to its negative regulation of cell progression by gefitinib in PC-9-ZD cells.

Gefitinib is known for its inhibition of EGFR tyrosine kinase and is used worldwide in lung carcinoma tumors. However, the efficiency of gefitinib treatment is limited by the development of drug resistance after long-term exposure to the drug. Additional treatments have been required to improve drug resistance. TNF-α acts synergistically with cytotoxic drugs against a variety of tumor cells, and the observation that TNF-α is able to do so in drug-resistant tumor cell lines and thereby overcome drug resistance is especially provocative [23–28].

In this study, the PC-9-ZD cell line originally came from human lung cancer cell line PC-9, displaying resistance to gefitinib treatment (Fig. 1a). Current reports suggest that the EGFR/PI3K/Akt pathway is downregulated in response to gefitinib only in cell lines that are sensitive to growth inhibited by gefitinib. In contrast, significant changes in the activation levels of Akt in gefitinib-resistant cell lines could not be detected when they were exposed to gefitinib [14]. Thus, reducing the activation level of Akt plays an important role in mediating cell growth inhibition by gefitinib. Our present data suggested that after long-term exposure to gefitinib, the survival PC-9-ZD cells with high activation levels of Akt resisted further gefitinib-induced cell growth inhibition (Fig. 1b). These findings suggested that heightened activation levels of Akt in PC-9-ZD cells, was involved in augmenting resistance of therapeutic response to gefitinib treatment.

Cell cycle progression is driven by CDKs in association with cyclins. p21 has been considered the most important cell cycle checkpoint protein [22,29,30]. Here, we showed that gefitinib elevated p21 levels and suppressed cdk2/4 and cyclinE/D1 activities, which resulted in impaired cell cycle progression through $G_1$ arrest in the PC-9 cells that are sensitive to growth inhibited by gefitinib, but not in the gefitinib-resistant derivative PC-9-ZD cells (Fig. 1c and d). These results are consistent with our previous findings that p21 suppressed cell growth by arresting the cell cycle at $G_1$ or $G_2$ phase through its binding and inhibition of cdk2/4 activity [11–13], and suggest that p21 plays an important role in mediating of cell growth inhibition by gefitinib.

To explore the conditions required for killing PC-9-ZD cells, we treated the cells with TNF-α and found that PC-9-ZD cells with acquired resistance to gefitinib showed increasing sensitivity to TNF-α as compared to their parental PC-9 cells (Fig. 2a). These data strongly suggest that TNF-α might be an effective anticancer treatment in tumors with acquired resistance to gefitinib after long-term administration of this drug. Furthermore, we showed that combined treatment with TNF-α followed by gefitinib reversed the sensitivity to gefitinib-induced inhibition of cell growth in PC-9-ZD cells (Fig. 3a). This might represent a new strategy to improve the efficacy of therapeutic response to tumors known to be resistant to gefitinib.

The TNF-α-induced cytotoxic action is through death receptor signaling and activation of caspase-8. However, activated NFκB/p65-mediated antiapoptotic gene expression prevents TNF-α-induced cytotoxic action. It is observed that tumors originally have high-level activation of NFκB/p65 after long-term abnormal development. In this study, we found that the treatment with TNF-α induced a modest cytotoxic effect in the parental PC-9 cells with high-level activation of NFκB/p65 that is, about 83% survival rates after 500 U/ml TNF-α treatment (Fig. 2a). This was correlated with the high activation level of NFκB/p65 in PC-9 cells (Fig. 2b). However, the low activation level of NFκB/p65 was detected after acquired gefitinib resistance, which is responsible for the enhanced sensitivity to TNF-α treatment in PC-9-ZD cells (Fig. 2a and b).

In our previous study, we observed that NFκB/p65 was able to bind the p21 promoter to activate its transcription activation, suggesting that p21 is induced in an NFκB/p65-dependent mechanism [22]. Here, our data showed that TNF-α induced nuclear activation NFκB/p65 levels, concomitant with elevation of p21 protein levels in a time-dependent manner (Fig. 3b and c). These results suggested that NFκB/p65 induced p21 expression in response to TNF-α treatment. The elevation of

p21 protein by NFκB/p65 contributed to its negative regulation of cell progression by gefitinib after administration of combined treatment with gefitinib and TNF-α (Fig. 3).

Clinical study of TNF-α as an anticancer treatment has been limited by its side effects, particularly its toxicity to healthy organs. Great effort has been made to reduce the adverse effects of TNF-α. In combination with our data, this strongly suggests that treatment with TNF-α might be effective against tumors that have acquired resistance to gefitinib after long-term administration of this drug. Further analysis is required before clinical application.

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## References

1 Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, *et al.* Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003; **21**:2237–2246.

2 Kris MG, Natale RB, Herbst RS, Lynch TJ Jr, Prager D, Belani CP, *et al.* Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *J Am Med Assoc* 2003; **290**:2149–2158.

3 Frei E III, Spriggs D. Tumor necrosis factor, still a promising agent. *J Clin Oncol* 1989; **7**:291–294.

4 Zhou QH, Hou M, Li L, Ren L, Qiu M, Yang YQ, *et al.* A multicenter randomized phase II trial of domestic product of nrh-TNF in the treatment of non-small cell lung cancer. *Chin J Lung Cancer* 2003; **6**:42–45.

5 Chen Z, Liu ML, Huang WX. A clinical research on rnhTNF for 87 cases with advanced cancer. *Chin J Clin Oncol* 2003; **30**:877–880.

6 Li EX, Li MZ, Nan KJ. Randomized-controlled study of recombinant mutant human tumor necrosis factor in advanced malignant tumor. *Chin Oncol* 2003; **13**:353–355.

7 Lejeune FJ. High dose recombinant tumor necrosis factor (rTNFα) administered by isolation perfusion for advanced tumors of limbs: a model for biochemotherapy of cancer. *Eur J Cancer* 1995; **31**:1009–1016.

8 Eggermont AMM, Schraffordt Koops H, Lienard D, Kroon BB, van Geel AN, Hoekstra HJ. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. *J Clin Oncol* 1996; **14**:2653–2665.

9 Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclindependent kinases. *Cell* 1993; **75**:805–816.

10 Hengst L, Gopfert U, Lashuel HA, Reed SI. Complete inhibition of Cdk/cyclin by one molecule of p21 Cip1. *Genes Dev* 1998; **12**:3882–3888.

11 Lu YJ, Tatsuka M, Takebe H, Yagi T. Involvement of cyclin-dependent kinases in doxorubicin-induced apoptosis in human tumor cells. *Mol Carcinog* 2000; **29**:1–7.

12 Lu YJ, Yamagishi N, Miyakoshi J, Yagi T, Takebe H. Sites and types of UV-induced mutations leading to inactivation of the growth-arresting activity in p21(sdi1/cip1/waf1) cDNA. *Carcinogenesis* 1996; **17**:2343–2345.

13 Lu YJ, Yamagishi N, Yagi T, Takebe H. Mutated p21WAF1/CIP1/SDI1 lacking CDK-inhibitory activity fails to prevent apoptosis in human colorectal carcinoma cells. *Oncogene* 1998; **16**:705–712.

14 Engelman JA, Janne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, *et al.* ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci U S A* 2005; **102**:3788–3798.

15 Lynch TJ, Bell DW, Sordella R, Lynch TJ, Bell DW, Sordella R. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**:2129–2139.

16 Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**:1497–1500.

17 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, *et al.* EGF receptor gene mutations are common in lung cancers from never smokers and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 2004; **101**:13306–13311.

18 Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M, *et al.* Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* 2004; **64**:8919–8923.

19 Sun JZ, Nam S, Lee CS, Li BY, Coppola D, Hamilton AD, *et al.* CEP1612, a dipeptidyl proteasome inhibitor, induces p21WAF1 and p27KIP1 expression and apoptosis and inhibits the growth of the human lung adenocarcinoma A-549 in nude mice. *Cancer Res* 2001; **61**:1280–1284.

20 van der Veen AH, Ten Hagen TLM, De Wilt JHY, van Ijken MJA, Eggermont AMM. An overview on the use of TNF-alpha: our experience with regional administration and developments towards new opportunities for systemic application. *Anticancer Res* 2000; **20**:3467–3474.

21 Whiteside ST, Ernst MK, Lebail O, Laurent-Winter C, Rice N, Israel A. N- and C-terminal sequences control degradation of MAD3/I kappaB alpha in response to inducers of NF-kappa B activity. *Mol Cell Biol* 1995; **15**:5339–5345.

22 Ma S, Tang J, Feng J, Xu Y, Yu X, Deng Q, Lu Y. Induction of p21 by p65 in p53 null cells treated with doxorubicin. *Biochim Biophys Acta* 2008; **1783**:935–940.

23 Safrit JT, Bonavida B. Sensitivity of resistant human tumor cell lines to tumor necrosis factor and adriamycin used in combination: correlation between down-regulation of tumor necrosis factor-messenger RNA induction and overcoming resistance. *Cancer Res* 1992; **52**:6630–6637.

24 Mizutani Y, Bonavida B. Overcoming cis-diamminedichloroplatinum (II) resistance of human ovarian tumor cells by combination treatment with cis-diamminedichloroplatinum (II) and tumor necrosis factor-alpha. *Cancer* 1992; **72**:809–818.

25 Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, *et al.* FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996; **85**:817–827.

26 Cao W, Ma SL, Tang JJ, Shi JQ, Lu YJ. A combined treatment TNF-α/doxorubicin alleviates the resistance of MCF-7/Adr cells to cytotoxic treatment. *Biochim Biophys Acta* 2006; **1763**:182–187.

27 Ando K, Ohmon T, Inoue F, Kadofuku T, Hosaka T, Ishida H, *et al.* Enhancement of sensitivity to tumor necrosis factor α in non-small cell lung cancer cells with acquired resistance to gefitinib. *Clin Cancer Res* 2005; **11**:8872–8879.

28 Cao W, Chi WH, Wang J, Tang JJ, Lu YJ. TNF-a promotes doxorubicin-induced cell apoptosis and anti-cancer effect through downregulation of p21 in p53-deficient tumor cells. *Biochem Biophys Res Commun* 2005; **330**:1034–1040.

29 Tang JJ, Shen C, Lu YJ. Requirement for pre-existing of p21 to prevent doxorubicin-induced apoptosis through inhibition of caspase-3 activation. *Mol Cell Biochem* 2006; **291**:139–144.

30 Xiao GH, Beeser A, Chernoff J, Testal JR. p21-activated kinase links Rac/Cdc42 signaling to merlin. *J Biol Chem* 2002; **277**:883–886.