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Inhibition of DNA-PK by gefitinib causes synergism between gefitinib and cisplatin in NSCLC

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Abstract. Lung cancer has the highest incidence and mortality rates among the malignant tumor types worldwide. Platinum-based chemotherapy is the main treatment for advanced non-small-cell lung cancer (NSCLC), and epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have greatly improved the survival of patients with EGFR-sensitive mutations. However, there is no standard therapy for treating patients who are EGFR-TKI resistant. Combining EGFR-TKIs and platinum-based chemotherapy is the most popular strategy in the clinical practice. However, the synergistic mechanism between EGFR-TKIs and platinum remains unknown. Therefore, the aim of the present study was to determine the synergistic mechanism of gefitinib (an EGFR-TKI) and cisplatin (a main platinum-based drug). MTT assay, apoptosis analysis, tumorsphere formation and an orthotropic xenograft mouse model were used to examine the combination effects of gefitinib and cisplatin on NSCLC. Co-immunoprecipitation and immunofluorescence were used to identify the underlying mechanism. It was found that gefitinib could selectively inhibit EGFR from entering the nucleus, decrease DNA-PK activity and enhance the cytotoxicity of cisplatin on NSCLC. Collectively, the results suggested that inhibition of DNA-dependent protein kinase by gefitinib may be due to the synergistic mechanism between gefitinib and cisplatin. Thus, the present study provides a novel insight into potential biomarkers for the selection of combination therapy of gefitinib and cisplatin.

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Introduction

Lung cancer is the most common malignant tumor, and its incidence and mortality rates are ~11.6 and ~18.4% worldwide, which are continuously rising (1). Lung cancer is insidious and difficult to detect in the early stage, and this disease seriously threatens patient health. The treatment strategy for advanced non-small-cell lung cancer (NSCLC) is mainly based on platinum-based chemotherapy (2,3). However, the curative effect of chemotherapy is limited, and the effective rate has reached a plateau that has been difficult to overcome (4). Chemotherapy drugs are highly toxic and can cause serious side effects, and thus are intolerable to patients (4). In the past decade, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have influenced the therapeutic strategies for advanced NSCLC, providing notable survival benefits to patients with EGFR-sensitive mutations, including exon 19 deletion and exon 21 L858R mutation (5,6). However, numerous patients cannot benefit or continue to benefit from EGFR-TKI monotherapy due to primary and secondary drug resistance (7).

Currently, there is no standard strategy available for treating patients who are EGFR-TKI resistant. The common

therapeutic strategies usually include: Platinum-based chemotherapy, high-dose EGFR-TKIs, platinum-based chemotherapy combined with EGFR-TKIs, new targeted drugs monotherapy (7), such as AZD929 (EGFR-TKI inhibitor) (8), afatinib (dual EGFR and human EGFR 2 inhibitor) (9), vandetanib (dual EGFR and vascular EGFR inhibitor) (10) and crizotinib (for patients with anaplastic lymphoma kinase mutations) (11). Furthermore, common therapeutic strategies involve new agents combined with EGFR-TKI, such as apatinib (12), AXL receptor tyrosine kinase degradation (13) and Crizotinib (14). However, most of these agents are still in clinical trials, and the combination therapy of EGFR-TKIs and platinum-based chemotherapy is the preferred choice for most patients. Previous studies have reported that such combination therapy could improve the overall survival and progression-free survival of patients (15-18). The synergistic mechanism between EGFR-TKIs and platinum remains unknown, and no biomarkers are currently available to predict the benefit of the combination therapy. If the phenotype of sensitive combinations could be determined, patients may benefit from the combination strategy. However, it is not possible to accurately determine the selection of appropriate patients without understanding the mechanisms of interactions between EGFR-TKIs and chemotherapy agents. Thus, biomarkers could only be identified when the synergistic mechanisms between EGFR-TKIs and platinum-based chemotherapy are understood.

The present study investigated the interaction between gefitinib (an EGFR-TKI) and cisplatin (a main platinum-based drug) to identify the mechanisms of their synergistic effects in NSCLC. Gefitinib was developed to target EGFR (19,20), and cisplatin is a cytotoxic drug that directly targets DNA and promotes the DNA double helix to form hinges that limit DNA unwinding, inhibit DNA replication and induce tumor necrosis (21,22). Previous studies have revealed that some of the agents affecting cisplatin sensitivity are mainly DNA repair proteins (23,24), such as DNA-dependent protein kinase (DNA-PK) (25), ERCC excision repair 1, endonuclease non-catalytic subunit (ERCC1) (26) and BRCA1 DNA repair associated (BRCA1) (27). DNA-PK is the pivotal kinase protein involved in non-homologous end joining pathways of DNA double-strand-break repair (25). Previous studies have also reported that intracellular EGFR could interact with DNA-PK (28,29). Moreover, EGFR in the cytoplasm can enter nucleus and regulate the transcription of DNA repair-related genes (30,31), assisting in the repair of DNA breaks (29,32). The aim of the present study was to determine the synergistic mechanism of gefitinib and cisplatin.

Materials and methods

Chemicals and antibodies. The selective EGFR-TKI gefitinib (GD760; Iressa), kindly provided by AstraZeneca PLC, was dissolved in pure DMSO to a working concentration of 20 mM. Cisplatin, MTT, Nucleopriein Extraction kit and AnnexinV-FITC Apoptosis Detection kit were obtained from Sigma-Aldrich (Merck KGaA). The caspase-3 Activity Assay kit was purchased from Beyotime Institute of Biotechnology. The DNA-PK inhibitor NU7441 (KU-57788) was purchased from Selleck Chemicals. Antibodies against Akt (cat. no. 4691), phosphorylated (p)-Akt (Thr308; cat. no. 13038), ERK1/2 (cat. no. 9102), p-ERK1/2 (Thr202/Tyr204; cat. no. 4370), PTEN (cat. no. 9188), BRCA1 (cat. no. 9010), p-BRCA1 (Ser1524; cat. no. 9009), DNA-PK (cat. no. 38168), p-DNA-PK (Ser2056; cat. no. 68716), EGFR (cat. no. 4267), p-EGFR (Tyr1068; cat. no. 3777), ERCC1 (cat. no. 12345), caspase-3 (cat. no. 9662), Cleaved caspase-3 (cat. no. 9661), β-actin (cat. no. 4970) and Lamin B1 (cat. no. 13435), purchased from Cell Signaling Technology, Inc., which were diluted to 1:1,000 for western blot analysis and Co-immunoprecipitation (Co-IP) analysis.

Cell lines. The human NSCLC cell lines were used: A549, H1299 and H1975 (Cell Bank of Chinese Academy of Sciences) with different *EGFR* and *Kirsten rat sarcoma 2 viral oncogene homolog* (*K-Ras)* gene statuses [exons 18-21 of the *EGFR* gene and exons 2-3 of the *K-Ras* gene were sequenced, the A549 cell line harbors the pathogenic mutation of *K-Ras* (exon 2 G12S), the H1975 cell line carries *EGFR* L858 and T790M and the *EGFR* and *K-Ras* of the H1299 cell line are all wild-type]. Cell lines were maintained in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and were cultured at 37˚C in a 5% CO_2 atmosphere in a humidified incubator.

Sequencing of EGFR and K-ras genes. The exons encoding the intracellular domain of *EGFR* and *K-ras* were amplified by PCR [the source DNA was genomic DNA of cell lines, the DNA polymerase was pfu DNA polymerase (Promega Corproation); thermocycling conditions were as follows: Initial denaturation at 95˚C for 1.5 min, followed by 30 cycles at 95˚C for 40 sec, 52-60˚C for 30 sec and 72˚C for 2 min, with a final extension step at 72˚C for 5 min, and help at 4˚C] and sequenced by bidirectional sequence. The EGFR exon 18-21 and K-ras exon 2-3 were amplified from H1299, A549 and H1975 cDNA, and the PCR products were isolated and sequenced. The primer sequences are listed in Table I. Tests were performed at least twice for every sample.

Cell apoptosis analysis. H1299, A549 and H1975 cells were seeded in 6-well plates at $2x10^5$ cells per well and incubated at 37˚C overnight. After treatment with 10 μM gefitinib or 15 μM cisplatin at 37˚C for 48 h, the cells were collected and assessed using an Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's instructions. Then, cells were detected by a BD FACSCalibur Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (Ver.10 for windows; BD Biosciences). The activation of caspase-3 was detected using the caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Cells were washed twice in PBS and lysed in Lysis Buffer (containing 1 mM DTT, 1 mM PMSF and protease inhibitor) on ice for 20-60 min. Lysates were centrifuged at 15,000 x g for 1 min at 4˚C. Appropriate protein extracts (100-200 μg) of cell lysates was treated with Reaction Buffer and Caspase-3 Substrate 37˚C for 4 h. Absorbance was measured at 400 nm wavelength using a multiwall spectrophotometer (Bio-Rad Laboratories, Inc.). Caspase-3 Activity was expressed as the percentage of agents-treated cells vs. control cells, whose

Table I. Primers for *EGFR* (exon 18, 19, 20 and 21) and *K-Ras* (exon 2 and 3).

Gene	Exon	Forward (5'→3')	Reverse (5'→3')
EGFR	18	CAAATGAGCTGGCAAGTGCCGTGT	GAGTTTCCCAAACACTCAGTGAAA
	19	GCAATATCAGCCTTAGGTGCGGCTC	CATAGAAAGTGAACATTTAGGATGTG
	20	ACTTCACAGCCCTGCGTAAAC	ATGGGACAGGCACTGATTTGT
	21	CTAACGTTCGCCAGCCATAAGTCC	GCTGCGAGCTCACCCAGAATGTCTGG
K-Ras	2	CTTAAGCGTCGATGGAGGAG	CCCTGACATACTCCCAAGGA
	3	TGGGTATGTGGTAGCATCTCA	AATCCCAGCACCACCACTAC

EGFR, epidermal growth factor receptor; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog.

activity was considered 0%. The experiments were independently performed three times.

MTT assay. Cells grown to 70-80% confluence were harvested, seeded in 96-well microtiter plates at 3,000 cells per well and incubated at 37˚C overnight. Cells were then treated with various concentrations (the specific concentration is listed in the corresponding figures) of gefitinib or cisplatin at 37˚C for 72 h. When measuring cell viability, 5 mg/ml MTT was added into the media and cells were cultured at 37˚C for 4 h. Plate centrifugation was performed in the speed of 1,000 rpm for 15 min under normal temperature. Then, the supernatant was removed by pipette and the formazan crystals were dissolved in 200 ml DMSO for 15 min. Absorbance was measured at 570 nm wavelength using a multiwall spectrophotometer (Bio-Rad Laboratories, Inc.). Cell viability was expressed as the percentage of surviving agents-treated cells vs. control cells whose viability was considered 100%. The combination index (CI) values of the two drugs were calculated using the Chou-Talalay median method as previously described (33). To assess the interaction between cisplatin and gefitinib on cell lines, cells were pretreated with 15 μM cisplatin at 37˚C for 72 h before gefitinib was administered or pretreated with 10 μM gefitinib before cisplatin was administered. To examine whether gefitinib could inhibit DNA-PK activation, cells were treated with 20 nM NU7441 (DNA-PK specific inhibitor), 10 μM gefitinib and a combination of cisplatin (the specific concentration is listed in the corresponding figures) at 37˚C for 72 h. These experiments were independently performed in triplicate.

Western blot analysis. Whole cell protein and nuclear protein were obtained by respectively treating cells with RIPA buffer (Beyotime Institute of Biotechnology) and Nucleopriein Extraction kit (Sigma-Aldrich; Merck KGaA), containing protease inhibitor cocktail and phosphatase inhibitor (Bimake.com) for 30 min on ice. Proteins were then determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). Appropriate protein extracts (40-60 μg) of cell lysates were loaded per lane and fractionated through SDS-PAGE (5% blocking gel at 4˚C for 30 min; 8-12% separating gel) and were electro-transferred to PVDF membranes (EMD Millipore). The membranes were immersed in the blocking reagent (5% non-fat milk) and shaken slowly on a shaker at room temperature for 1 h. Membranes were then probed with various primary antibodies (the aforementioned antibodies) and horseradish peroxidase-conjugated secondary antibodies (cat. no. FD-GAR007; 1:5,000; Fude Biological Technology Co., Ltd.), and were visualized using ECL detection reagents (EMD Millipore). The molecular weights of the immunoreactive proteins were estimated based on the PageRuler Prestained Protein ladder (Fermentas; Thermo Fisher Scientific, Inc.). Experiments were repeated ≥3 times.

Tumorsphere formation and growth assays. After adding 200 μl Matrigel (BD Bioscience) per well in 24-well plate (Corning, Inc.) at 37˚C in an incubator for 30 min, cells (100 μl per well) were seeded onto the 24-well plate at 1,000 cells/ml and cultured in the tumorsphere medium: RPMI-1640 medium supplemented with Matrigel at 10% volume ratio. NSCLC cells were treated with low-concentration 6 μM gefitinib and high-concentration 20 μM cisplatin at 37˚C in an incubator according to the following treatment regimens: i) Control group (Control), RPMI-1640 media without drug for 18 days; ii) Cisplatin monotherapy group (cisplatin), RPMI-1640 media without drug for 12 days and 20 μM cisplatin for 6 days; iii) Gefitinib combined with cisplatin group (gefitinib + cisplatin), RPMI-1640 media without drug-treated tumorspheres for 12 days and 20 μM cisplatin combined with 6 μM gefitinib for 6 days; and iv) Gefitinib pretreatment group (gefitinib → cisplatin), RPMI-1640 media without drugs for 6 days, 6 μM gefitinib for 6 days and 20 μM cisplatin for 6 days. The volumes of tumorspheres were measured using a light microscope rule (magnification, x20) once every 6 days and were calculated [Volume (mm^3)=π x length x $width^2$/6]. The experiments were independently performed in triplicate.

Co-IP assays. Cells were washed twice in PBS and lysed in a IP buffer (containing 1 mM DTT, 100 mmol/l NaCl and 1 mM $MgCl_2$) and protease inhibitor cocktails (Bimake.com). Lysates were centrifuged at 12,000 x g for 10 min at 4˚C. The total cell lysates were used for IP with primary antibodies (anti-EGFR or anti-DNA-PK_{CS}) on protein A + G mix beads (Thermo Fisher Scientific, Inc.) at 4˚C overnight The immunoprecipitates were collected and prepared for western blot analysis, which was performed as aforementioned. In total, 100 μl cell lysates were used as the input control for western blot analysis.

Orthotopic xenograft assay. A total of 16 female athymic mice (BALB/$c^{nu/nu}$; age, 5 weeks; weight, 15-18 g) were ordered

from the Experimental Animal Center of Zhejiang Chinese Medical University. Mice were housed in cages with wood chip beddings in a temperature-controlled room (68-72 uF; 24˚C) with a 12 h light-dark cycle and 45-55% relative humidity and were permitted free access to food and drinking water. All of the animal experiments described in this study were approved by the Institutional Animal Care and USE Committee (IACUC) at Zhejiang University. All animals were maintained in accordance with the IACUC guideline.

These mice were randomized into four groups as follows: i) Control group (Control), intraperitoneal injection of normal saline 0.01 ml/g with DMSO; ii) Gefitinib alone group (gefitinib alone), intraperitoneal injection of 35 mg/kg gefitinib; iii) Cisplatin alone group (cisplatin alone), intraperitoneal injection of 5 mg/kg cisplatin; and iv) Combination of gefitinib and cisplatin group (gefitinib + cisplatin): Intraperitoneal injection of 35 mg/kg gefitinib and 5 mg/kg cisplatin. Mice were injected with $2x10^6$ H1299, A549 and H1975 cells suspension in 100 ml RPMI-1640 medium containing 50% Matrigel (BD Bioscience) subcutaneously into the right flank. The mice were randomly assigned to experimental and control groups (4 mice/group) when the tumors reached the size of 50 mm^3. The mice were fasted overnight and then administered 35 mg/kg gefitinib and/or 5 mg/kg cisplatin or 0.9% physiological saline (vehicle) by intraperitoneal injection once every 3 days for 15 day. The treatment was started on day 7 and stopped on day 22. Tumors were measured using calipers once every 3 days, and the tumor volumes were calculated [Volume (mm^3)=π x length x $width^2$/6]. According to the Animal Ethics of IACUC at Zhejiang University, the experiment ended when the largest tumor volume approached 2,000 mm^3. The mice were sacrificed by cervical dislocation under deep inhalation anesthesia with 2% isoflurane at the end of the experiment. At the end of study, tumor tissues were harvested, fixed in 10% formalin at room temperature for 24 h and then embedded in paraffin. Tumor sections (thickness, 2-3 μm) were subjected to standard hematoxylin for 10 min and eosin for 10 sec at room temperature (H&E) staining using a light microscope (magnification, x100). The experiment was conducted in triplicate.

Immunofluorescence. After treatment with 10 μM gefitinib or 15 μM cisplatin at 37˚C for 48 h, cells were fixed in 4% paraformaldehyde at room temperature for 15 min, incubated in 0.1% Triton X-100 at room temperature for 10 min and washed with PBS. Cells were blocked with goat serum (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 60 min, stained with primary EGFR antibody or DNA-PK antibody (aforementioned antibodies; 1:250) at 4˚C overnight and secondary antibodies (cat. no. 102-095-003; Jackson ImmunoResearch Laboratories; 1:50) at 37˚C for 60 min. Then, cells were counterstained by DAPI (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min. The images were acquired by confocal laser-scanning microscopy (magnification, x200; Zeiss LSM710; Zeiss AG).

Statistical analysis. Data are presented as the mean ± standard error of mean obtained from ≥3 experiments. The two-tailed Student's t-tests were used for analyzing statistical differences between two groups and one-way ANOVA was used with a post hoc Tukey for multiple comparisons when comparing >2 groups using SPSS software (Ver.22 for Mac; SPSS, Inc.). Graphs were generated using GraphPad Prism software (Ver.6 for Mac; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Evaluation of the antiproliferative effect of gefitinib and cisplatin on NSCLC cell lines with different EGFR and K-ras mutations. EGFR and K-ras genes were sequenced to identify that H1299 cells are wild-type, that A549 cells carry K-ras G12S mutation and that H1975 cells carry EGFR L858R and T790M mutations (Table II).

A549, H1299 and H1975 cells were treated with 10 μM gefitinib and 15 μM cisplatin for 48 h to investigate the sensitivity of the three types of NSCLC cells with different EGFR and K-ras mutants. Apoptosis was assessed using Annexin V-FITC/propidium iodide (PI) dual staining by FACSCalibur Flow Cytometer. Apoptosis in the treatment groups was markedly higher compared with the control group of each cell line, and no significant difference in sensitivity of gefitinib and cisplatin was found between the three cell lines (Fig. 1A and B). A MTT assay was performed, and the results suggested that the three cell lines were similarly resistant to gefitinib (The IC_{50} values of gefitinib in Fig. 1C are presented in Table II), and the cisplatin IC_{50} values were also similar among the cell lines (The IC_{50} values of cisplatin in Fig. 1D are presented in Table II).

Synergistic and antagonistic antiproliferative effects of gefitinib and cisplatin combination. Each cell line was treated with the combination of cisplatin and gefitinib for 72 h with relatively suitable concentration (Fig. 2A-a, B-a and C-a) based on the IC_{50} ratio of the two agents to investigate the combined effects of cisplatin and gefitinib in the three types of NSCLC with different EGFR and K-ras genotypes. Cell viability was measured by using the MTT method, and the combination index (CI) values of the two drugs were calculated using the Chou-Talalay median method as previously described (33). A marked decrease in cell viability was observed after cisplatin was combined with gefitinib in H1299 cells (EGFR wild-type; K-ras wild-type; Fig. 2A-a). The CI values of cisplatin and gefitinib at any inhibition rate were <1, and the CI value decreased as the inhibition rate increased (Fig. 2A-b). This finding indicates that the combined effect of cisplatin and gefitinib in H1299 cells was synergistic, and the synergistic effect was stronger as the combination concentration of the two agents was increased.

A decrease in cell viability was observed after combination treatment in A549 cells (EGFR wild-type; K-ras exon-2 G12S mutation; Fig. 2B-a). However, the CI value in A549 cells spanned over one (Fig. 2B-b), suggesting that the combined effect in A549 cells was mixed. For instance, the effect was antagonistic when the drugs were combined at low concentrations but was synergistic as the concentrations increased.

The combined effect in H1975 cells (EGFR exon-21 L858R; exon-20 T790M mutation; K-ras wild-type) was antagonistic. No significant decrease in cell viability was found in the combination treatment group in H1975 cells (Fig. 2C-a).

Table II. Cytotoxicity of gefitinib or cisplatin and the mutation status of *EGFR* and *K-Ras* gene in human non-small-cell lung cancer cell lines.

Cell line	Cell type	*EGFR* gene	*K-Ras* gene	Gefitinib IC_{50} (μmol/l)	Cisplatin IC_{50} (μmol/l)
A549	Adenocarcinoma	WT	G12S exon 2	13.2±2.69	11.9±2.77
H1299	Large cell	WT	WT	17.5±4.54	11.6±1.88
H1975	Adenocarcinoma	L858R, T790M	WT	16.6±2.38	14.6±6.45

Data are presented as the mean ± SD of IC_{50} values for each cell line. WT, wild type; EGFR, epidermal growth factor receptor; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog. Cytotoxicity was determined by MTT assay, IC_{50} values were calculated using GraphPad Prism software.

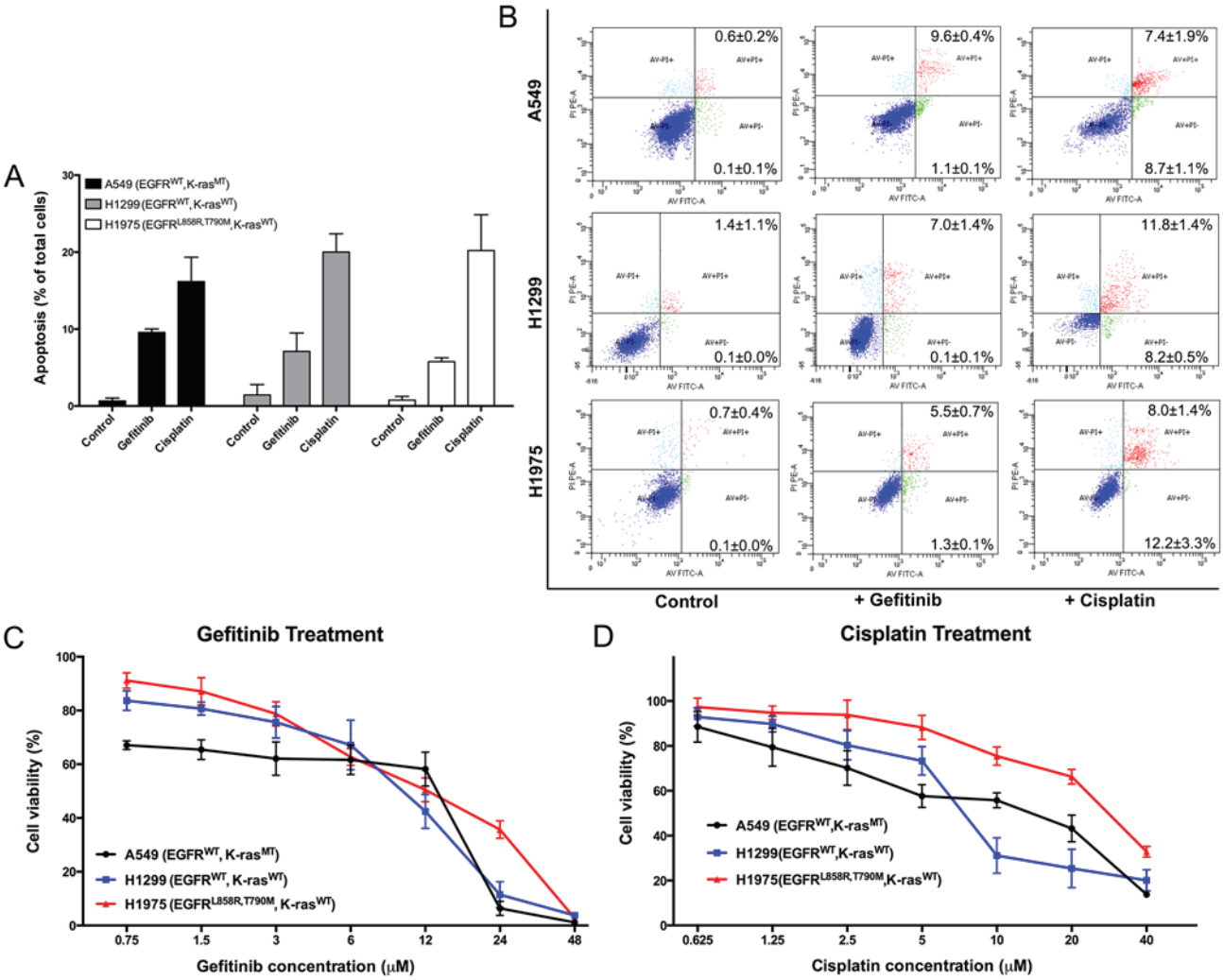

Figure 1. Sensitivity of the three non-small-cell lung cancer cells to gefitinib and cisplatin. (A) Apoptosis was assessed via Annexin V-FITC/PI dual staining using FACSCanto II. (B) Representative flow cytometry plots for all groups. (C) Cell viability after gefitinib treatment was determined via the MTT assay. (D) Cell viability after cisplatin treatment was determined via the MTT assay. WT, wild-type; MT, mutation type; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog; PI, propidium iodide.

The CI value was >1 at most inhibition rate, indicating that the antagonist effect was stronger as the combination concentration of the two drugs increased (Fig. 2C-b). These three different NSCLC cells demonstrated different cisplatin and gefitinib combined effects (Table III).

Different sequential effects of gefitinib and cisplatin on different NSCLC cells. NSCLC cells were pretreated with 15 μM cisplatin for 72 h before gefitinib was administered or pretreated with 10 μM gefitinib before cisplatin was administered to further assess the interaction between cisplatin and gefitinib on H1299, A549 and H1975 cells. No significant difference in gefitinib sensitivity on H1299 cells was observed with or without cisplatin pretreatment (P>0.05; Fig. 3A-a). However, after 10 μM gefitinib pretreatment, the sensitivity of H1299 cells to cisplatin was significantly higher compared with

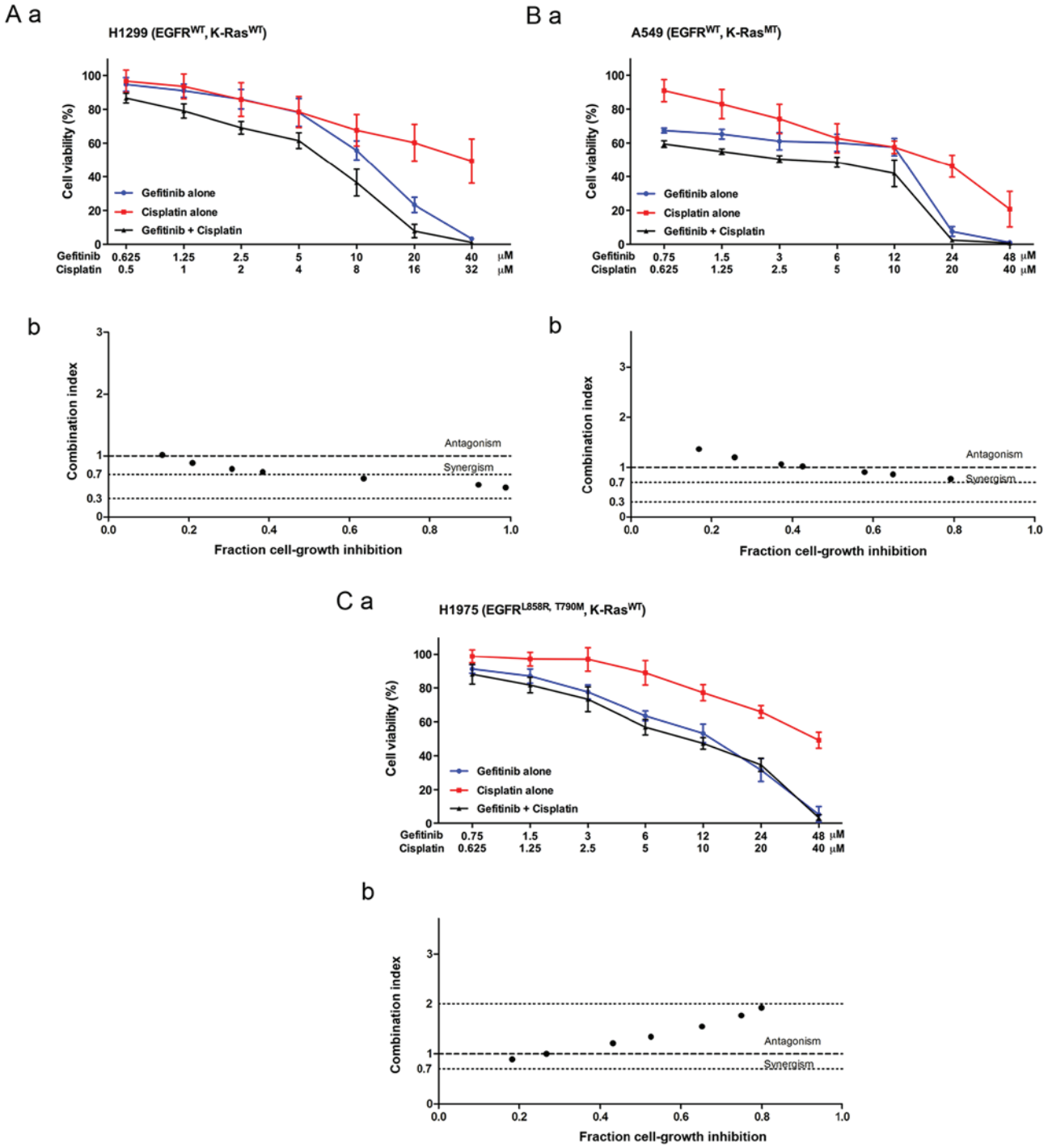

Figure 2. Synergistic, antagonistic and antiproliferative effects of gefitinib and cisplatin combination on non-small-cell lung cancer cells. Cell viability was determined using the MTT assay in (A-a) H1299, (B-a) A549 and (C-a) H1975 cells. CIs defining the proliferation inhibitory effects of the combination treatment of gefitinib and cisplatin in (A-b) H1299, (B-b) A549 and (C-b) H1975 cells. Combinations were antagonistic when CI >1.0, but synergistic when CI <1.0. Points were the mean of three individual treatments. MT, mutation type; WT, wild-type; CI, combination index; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog.

the cells without gefitinib pretreatment (P<0.05; Fig. 3A-b). Thus, in H1299 cells, cisplatin pretreatment did not affect the antiproliferative effect of gefitinib, while gefitinib could enhance the inhibition of cisplatin. The sensitivity of gefitinib on cisplatin-pretreated A549 cells was significantly reduced (Fig. 3B-a), while cisplatin sensitivity was increased after gefitinib pretreatment (Fig. 3B-b). In H1975 cells, cisplatin reduced the sensitivity of gefitinib (Fig. 3C-a), and gefitinib had no significant effect on cisplatin (Fig. 3C-b). Moreover, the three types of NSCLC cells demonstrated different sequential effects of the two drugs (Table III).

Gefitinib selectively promotes cisplatin-induced apoptosis on H1299 and A549 cells. Cells were pretreated with 10 μM gefitinib for 48 h and then treated with 15 μM cisplatin for 48 h to investigate whether gefitinib could promote cisplatin-induced

Table III. Combination and sequential effect of gefitinib and cisplatin on human non-small-cell lung cancer cell lines.

Cell line	*EGFR* gene	*K-Ras* gene	G + C	G→C	C→G
A549	WT	G12S exon 2	Synergistic/antagonistic	Sensitive	Inhibitive
H1299	WT	WT	Synergistic	Sensitive	No effect
H1975	L858R, T790M	WT	Antagonistic	No effect	Inhibitive

WT, wild type; G + C, combination of gefitinib and cisplatin; G→C, gefitinib → cisplatin; C→G, cisplatin → gefitinib; EGFR, epidermal growth factor receptor; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog.

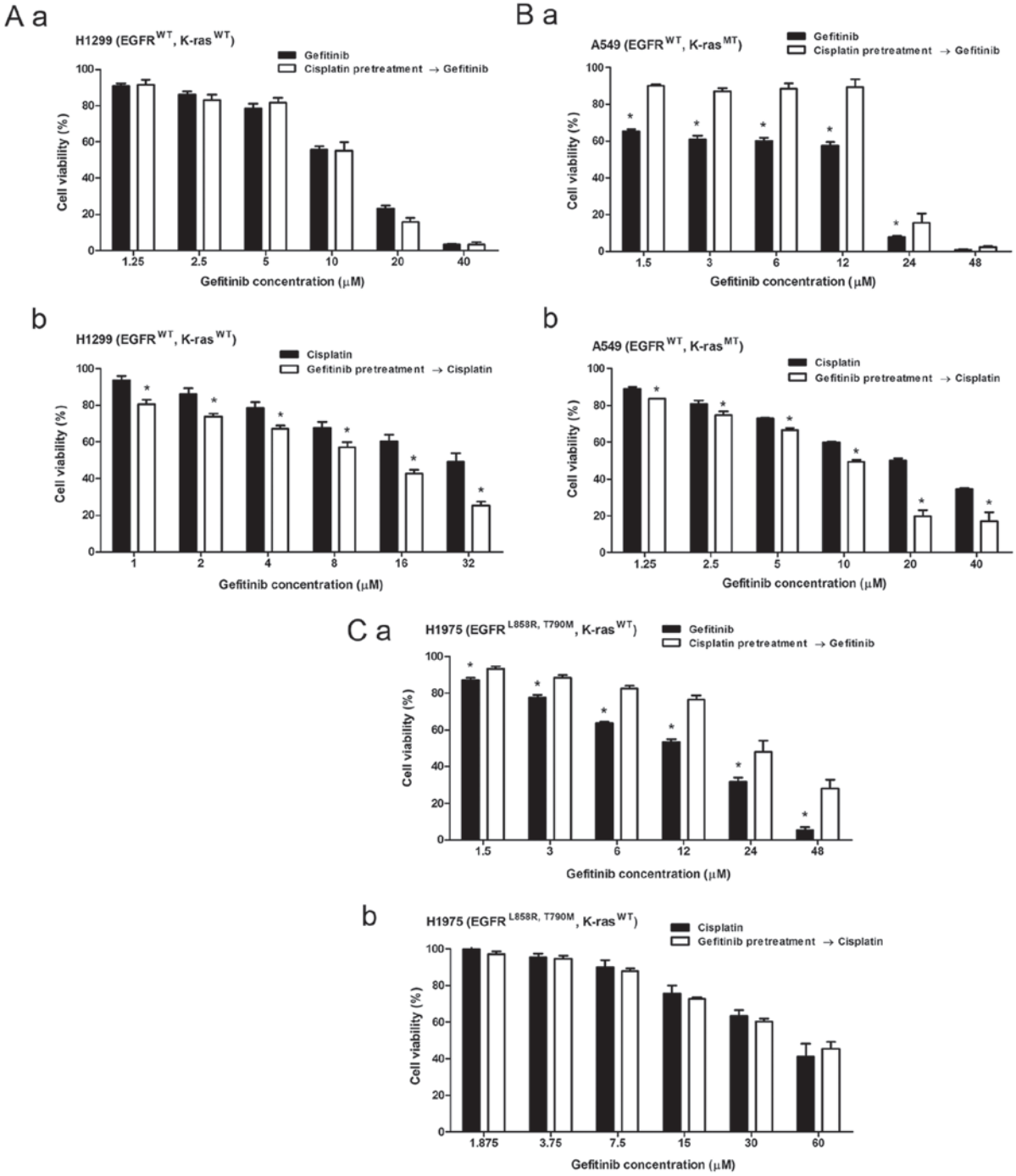

Figure 3. Sequential effects of gefitinib and cisplatin on non-small-cell lung cancer cells. Antiproliferative effect of gefitinib after cisplatin pretreatment for 72 h in (A-a) H1299, (B-a) A549 and (C-a) H1975 cells. Sensitivity of cisplatin after gefitinib pretreatment for 72 h in (A-b) H1299, (B-b) A549 and (C-b) H1975 cell. Cell viability was determined via the MTT assay. Data are presented as the mean ± SEM of three experiments. *P<0.05, single drug groups vs. pretreatment groups. MT, mutation type; WT, wild-type; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog.

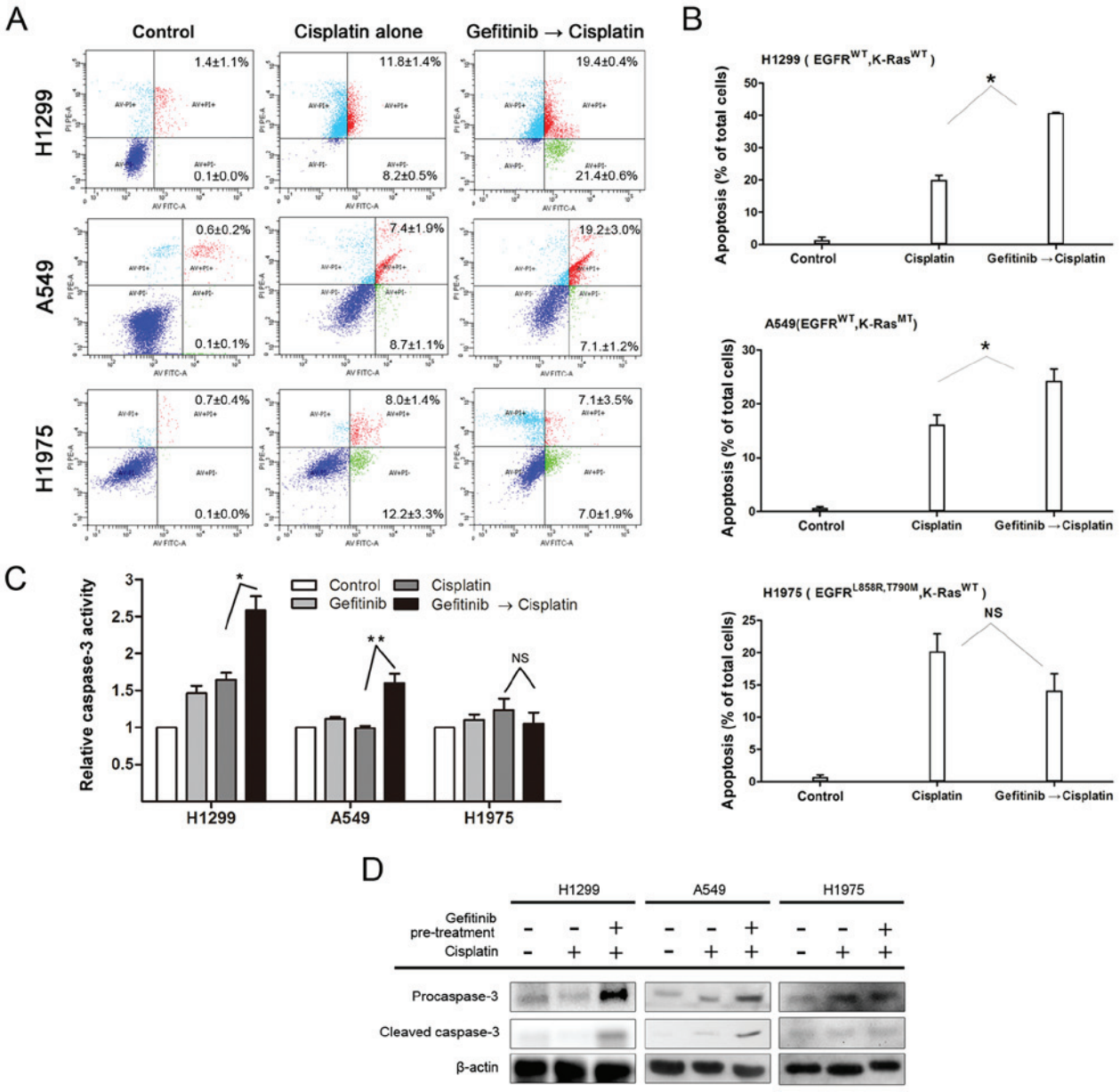

Figure 4. Cisplatin-induced apoptosis after gefitinib pretreatment on non-small-cell lung cancer cells. (A) Cisplatin-induced apoptosis promotion by gefitinib was analyzed using flow cytometry. (B) Statistical analysis of flow cytometry results from (A). (C) Relative caspase-3 activity after cisplatin and/or gefitinib treatment was determined using the caspase-3 Activity Assay kit. (D) Procaspase-3 and Cleaved caspase-3 expressions levels after treatments for 48 h. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. NS, not significant; MT, mutation type; WT, wild-type; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog.

apoptosis on H1299, A549 and H1975 cells. Apoptotic cells were detected via the AnnexinV-FITC/PI flow cytometry assay. For H1299 and A549 cells, the apoptotic rate of gefitinib pretreatment group was significantly higher compared with the cisplatin monotherapy group (P<0.05). However, no significant difference in H1975 cells was observed between the two groups (P>0.05; Fig. 4A and B). Therefore, the results suggested that gefitinib enhanced the apoptotic ability of cisplatin on H1299 and A549 cells.

The activation of caspase-3 was detected via the caspase-3 Activity Assay kit according to the manufacturer's instructions, and the expression levels of caspase-3 precursor protein and cleaved caspase-3 were detected by western blot analysis to determine whether caspase-3 activity after cisplatin administration could be enhanced by gefitinib pretreatment. The results demonstrated that caspase-3 activity was more active and the expression levels of procaspase-3/cleaved caspase-3 proteins were increased after cisplatin treatment in the gefitinib pretreatment group compared with the cisplatin monotherapy group (Fig. 4C and D; P<0.05). However, no enhancement was observed in H1975 cells.

Gefitinib sensitizes the antitumor effect of cisplatin on H1299 and A549 tumorspheres. The 3D tumorspheres were cultured to simulate the cell growth environment *in vitro*, and the combination effect of cisplatin and gefitinib was monitored to assess the promotion of gefitinib on cisplatin-induced tumor inhibition.

Based on previous studies (34), RPMI-1640 medium was replaced with Matrigel at 10% volume ratio every 2 days

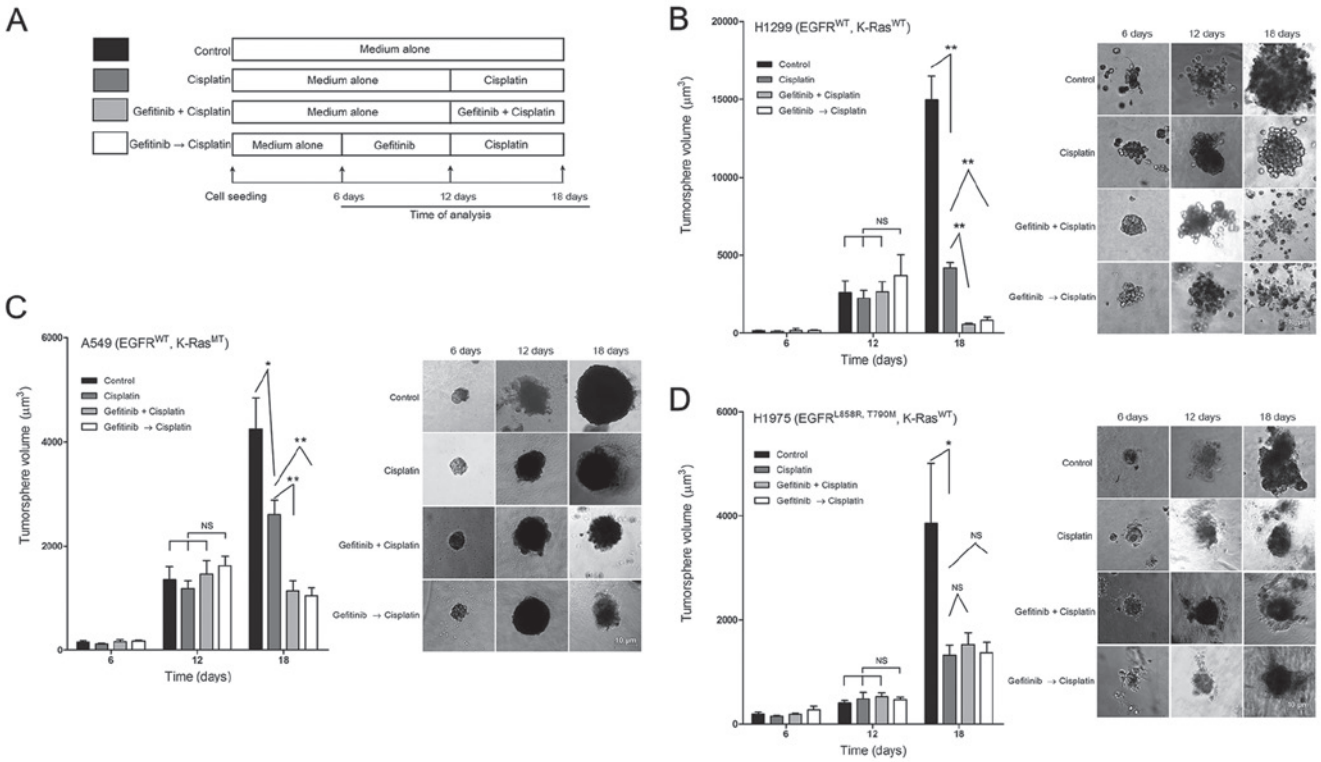

Figure 5. Antitumor effect of cisplatin and gefitinib on non-small-cell lung cancer tumorspheres under different treatment regimens. (A) Schematic diagram of four treatment regimens. (B) Volumes and images of H1299 tumorspheres. (C) Volumes and images of A549 tumorspheres. (D) Volumes and images of H1975 tumorspheres. Magnification, x20. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. NS, not significant; G, gefitinib; C, cisplatin; MT, mutation type; WT, wild-type; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog; d, days.

to ensure that cells could survive >20 days. NSCLC cells were treated with low-concentration 6 μM gefitinib and high-concentration 20 μM cisplatin, according to the treatment regimens (Fig. 5A). According to the growth trend of tumors in control group, the tumor growth was in a logarithmic rise phase on the 18th day (Fig. 5B-D). The tumor lengths were measured on 6, 12 and 18 days, and the tumorsphere volumes were also calculated. No significant difference in the tumorsphere volumes among the four groups were identified on the 6 and 12th day (Fig. 5B-D). However, on the 18th day, the tumorsphere volumes of H1299 in the four groups were as follows: 14,966.4±3,442.1, 4,072.3±561.5, 570.0±203.2 and 871.5±472.6 μm^3, respectively. The tumor volumes of cisplatin monotherapy group were significantly reduced compared with the control group (Fig. 5B; P<0.01). Furthermore, the volumes of G + C and the G → C groups were significantly reduced compared with the cisplatin monotherapy group (Fig. 5B; both P<0.01). The volumes of A549 cells in the four groups were as follows: 4,252.7±2,383.4, 2,610.2±997.4, 1,138.6±592.3 and 1,051.4±562.2 μm^3. The tumor volumes of G + C with those of the G → C pretreatment groups were significantly reduced compared with the cisplatin monotherapy group (Fig. 5C; P<0.01). The volumes of H1975 tumors were as follows: 3,856.7±3,661.2, 1,321.6±915.8, 1,530.5±977.3 and 1,368.6±963.7 μm^3. No significant difference in tumor volumes between G + C or G → C pretreatment groups and the cisplatin monotherapy groups were observed (Fig. 5D; P>0.05). Collectively, the results indicated that gefitinib could selectively enhance the antitumor effect of cisplatin, which was present in H1299 and A549 cells but not H1975 cells.

Synergistic antiproliferative effect of gefitinib and cisplatin combination on H1299 and A549 xenografts. A xenograft mouse model was used to study the effect on xenograft tumor growth in nude mice *in vivo*. H1299, A549 and H1975 cell suspensions were injected into the right flank of each mouse.

The mean ± SEM of largest diameters of H1299 tumors in the control, gefitinib alone, cisplatin alone and gefitinib + cisplatin groups was: 16.31±3.58, 15.02±1.49, 12.9±1.73 and 9.04±2.64 mm respectively. The mean ± SEM in A549 tumors were: 8.14±1.42, 8.62±0.65, 6.99±1.55 and 5.78±1.86 mm. Furthermore, those in H1975 tumors were: 18.98±0.76, 18.33±1.77, 13.76±1.78 and 13.77±1.62 mm. The volumes and qualities of H1299 and A549 tumors were significantly reduced in the gefitinib + cisplatin group combination compared with gefitinib alone and cisplatin alone groups (Fig. 6A-a and b and B-a and b). However, in H1975 tumors, the combination of gefitinib + cisplatin did not significantly inhibit tumor growth compared with cisplatin alone group (Fig. 6A-c and B-c).

Subsequently, the results of tumor H&E staining indicated that in H1299 and A549 tumors, the tissue necrosis was more serious after a combination of gefitinib + cisplatin treatment compared with cisplatin monotherapy. However, no notable difference was observed in necrotic areas for H1975 tumors (Fig. 6C).

Gefitinib selectively inhibits DNA-PK activity on H1299 and A549 cells. The NSCLC cells were treated with 15 μM cisplatin and 10 μM gefitinib for 72 h to examine the mechanism of interaction between cisplatin and gefitinib. In addition, the activation and expression levels of the key proteins of the

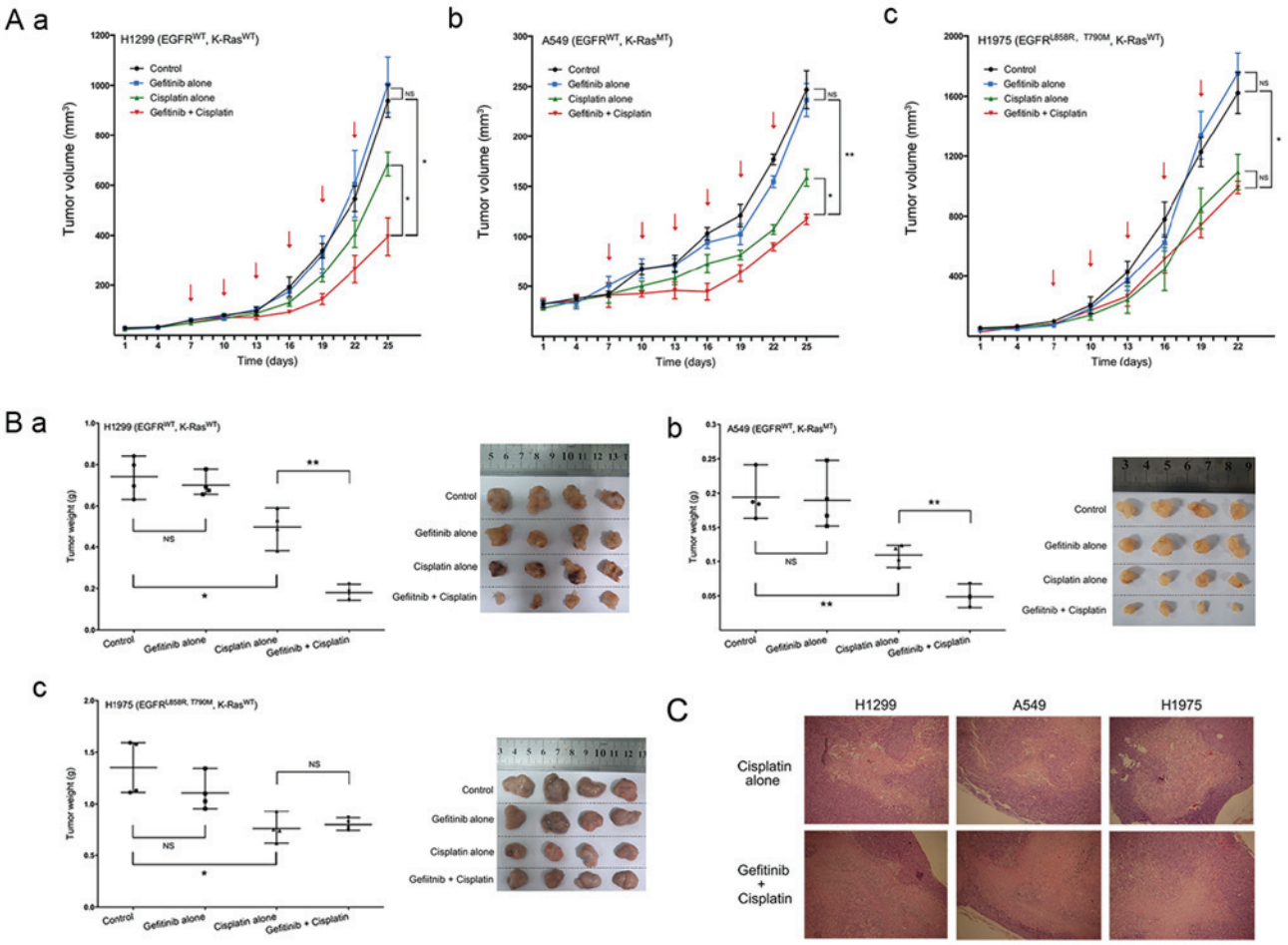

Figure 6. Effect of gefitinib and cisplatin combination on NSCLC xenografts. Tumor volume curves of each treatment group on three NSCLC xenografts, with in (A-a) H1299, (A-b) A549 and (A-c) H1975 cells. Tumor weights and images of three NSCLC xenografts with in (B-a) H1299, (B-b) A549 and (B-c) H1975 cells. (C) Paraffin-embedded tissue sections of the tumors were subjected to hematoxylin and eosin staining. Magnification, x40. The red arrows indicate drug administration time. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. NS, not significant; MT, mutation type; WT, wild-type; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog; NSCLC, non-small-cell lung cancer.

EGFR pathway and platinum-sensitive associated proteins, such as Akt, Erk1/2, PTEN, BRCA1 and ERCC1, were detected via western blot analysis. The results identified that both gefitinib alone and in combination with cisplatin could decrease the expression levels of p-Akt and p-Erk1/2 in H1299, A549 and H1975 cells in comparison with the control group, while cisplatin alone had no effect on the expression levels of p-Akt and p-Erk1/2. Cisplatin monotherapy, gefitinib monotherapy and combination therapy of the two drugs had no effect on the expression of PTEN and ERCC1 proteins in the three cells. Moreover, cisplatin monotherapy and cisplatin + gefitinib could increase the expression of p-BRCA1 in the three cells, but gefitinib monotherapy did not have the same effect. The changes in the activation and expression levels of the above proteins were all consistent in H1299, A549 and H1975 cells (Fig. 7A).

Subsequently, the expression and activation status of EGFR and DNA-PK in the three cell lines were examined. After 15 μM cisplatin treatment alone for 72 h, the expression levels and phosphorylation of EGFR and DNA-PK did not change in these three NSCLC cells. Although the EGFR and DNA-PK expression levels of the three cells did not change significantly after 10 μM gefitinib monotherapy for 72 h, the expression levels of p-EGFR and p-DNA-PK decreased in H1299 and A549 cells. p-EGFR in H1975 cells was also inhibited, but no notable change was observed in DNA-PK phosphorylation. After the combination of 15 μM cisplatin and 10 μM gefitinib, the expression levels of EGFR and DNA-PK in the three NSCLC cells remain unchanged, and the phosphorylation levels of EGFR and DNA-PK of the H1299 and A549 cells were reduced. Furthermore, p-EGFR in H1975 cells was also inhibited, but p-DNA-PK was unchanged (Fig. 7B). Therefore, it was speculated that gefitinib selectively inhibited the pathway of DNA-PK phosphorylation in H1299 and A549 cells, and this inhibition was not associated with EGFR phosphorylation.

Gefitinib selectively inhibits EGFR binding to DNA-PK in H1299 and A549 cells. To further examine whether gefitinib could inhibit DNA-PK activation, NSCLC cells were treated with 20 nM NU7441 (DNA-PK specific inhibitor), 10 μM gefitinib and a combination of different cisplatin concentrations at 37˚C in an incubator for 72 h. In H1299 and A549 cells, the effect of 20 nM NU7441 combined with cisplatin on cell viability had a synergistic effect similar to that of the combination of gefitinib + cisplatin. Moreover, the synergistic

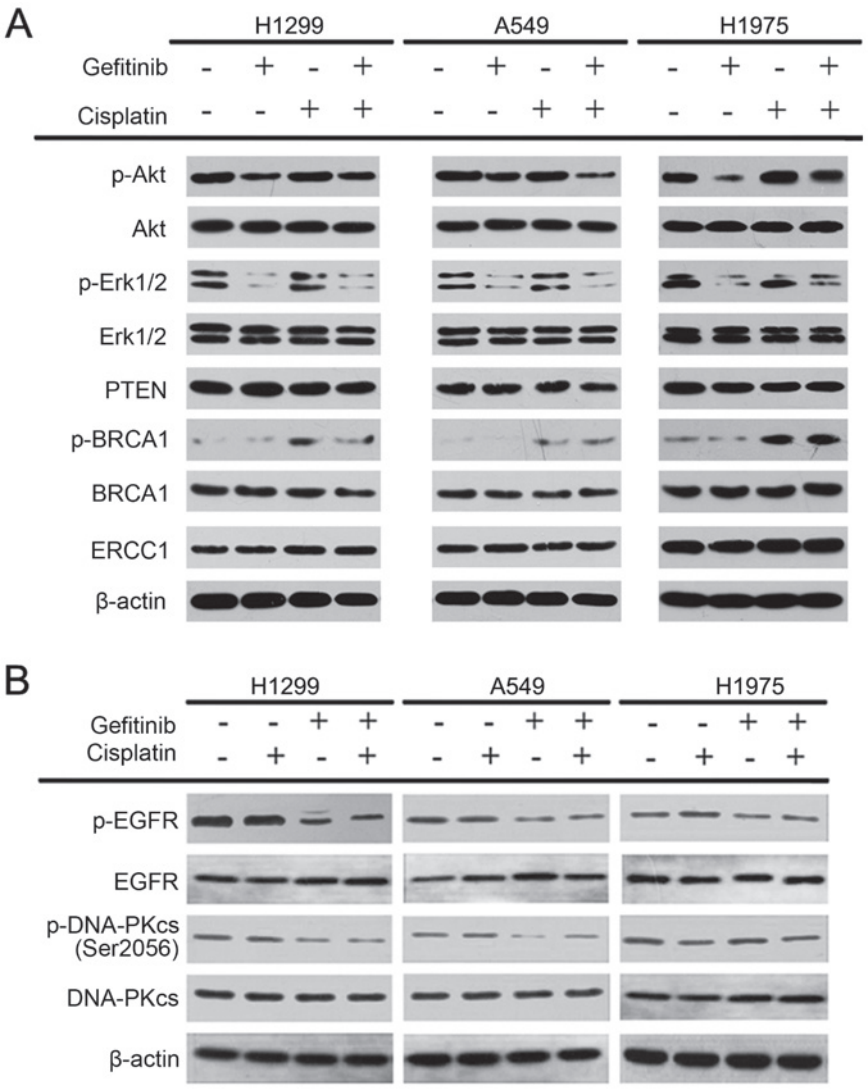

Figure 7. Activation and expression levels of Akt, Erk1/2, BRCA1, PTEN, ERCC1, EGFR and DNA-PKcs on the three non-small-cell lung cancer cells after different treatments. (A) Effects of Akt, Erk1/2, BRCA1, PTEN, and ERCC1 on cisplatin combined with gefitinib. (B) Expression levels and activation of EGFR and DNA-PKcs in the three NSCLC cells after the combination of gefitinib and cisplatin. p-, phosphorylated; BRCA1, BRCA1 DNA repair associated; ERCC1, ERCC excision repair 1, endonuclease non-catalytic subunit; DNA-PK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor.

effect of the combination of NU7441, gefitinib and cisplatin was not stronger than that of the combined NU7441 and cisplatin. However, no synergy in the combination of gefitinib and cisplatin group was observed in the H1975 cells (Fig. 8A). In addition, H1299 and A549 cells pretreated with 20 nM NU7441 were highly sensitive to cisplatin, and this effect was similar to that of the gefitinib pretreatment (10 μM gefitinib at 37˚C for 72 h). However, the effect could not be enhanced by treatment with NU7441 and gefitinib simultaneously (Fig. 8B). Therefore, the data indicated that the synergistic effect of gefitinib combined with cisplatin was achieved via the gefitinib inhibition of the DNA-PK activity (Fig. 8B).

The interaction of EGFR and DNA-PK was further detected via IP after treatment with 15 μM cisplatin and 10 μM gefitinib for 72 h. The results suggested that the IP of EGFR and DNA-PK was positive in all three NSCLC cells without any drug treatment (Fig. 8C). After treatment with cisplatin alone, the IP results of EGFR and DNA-PK of these three cells were not notably different from those of the untreated cells. However, the IP of EGFR and DNA-PK in H1299 and A549 cells was markedly attenuated after gefitinib treatment, but no notable change in H1975 cells was observed (Fig. 8C). Thus, an interaction may occur between EGFR and DNA-PK in the NSCLC cells, and gefitinib could selectively inhibit the interaction between EGFR and DNA-PK in H1299 and A549 cells.

Gefitinib selectively blocks EGFR translocation into the nucleus of H1299 and A549 cells. It was found that DNA-PK was mainly distributed in the nucleus, and cisplatin + gefitinib could not interfere with the DNA-PK distribution. Furthermore, EGFR was evenly distributed in the cytoplasm and nucleus of these cell lines, and EGFR in the nucleus of H1299 and A549 cells was relatively decreased after treatment containing gefitinib (Fig. 9A and B). However, gefitinib did not interfere with EGFR in the nucleus of H1975 cells (Fig. 9C). Western blotting results demonstrated that gefitinib-containing treatment reduced the nuclear EGFR expression of H1299 and A549 cells, and the corresponding cytosolic EGFR expression levels were increased. However, gefitinib had no effect on EGFR in H1975 cells (Fig. 9D). The results indicated that DNA-PK was

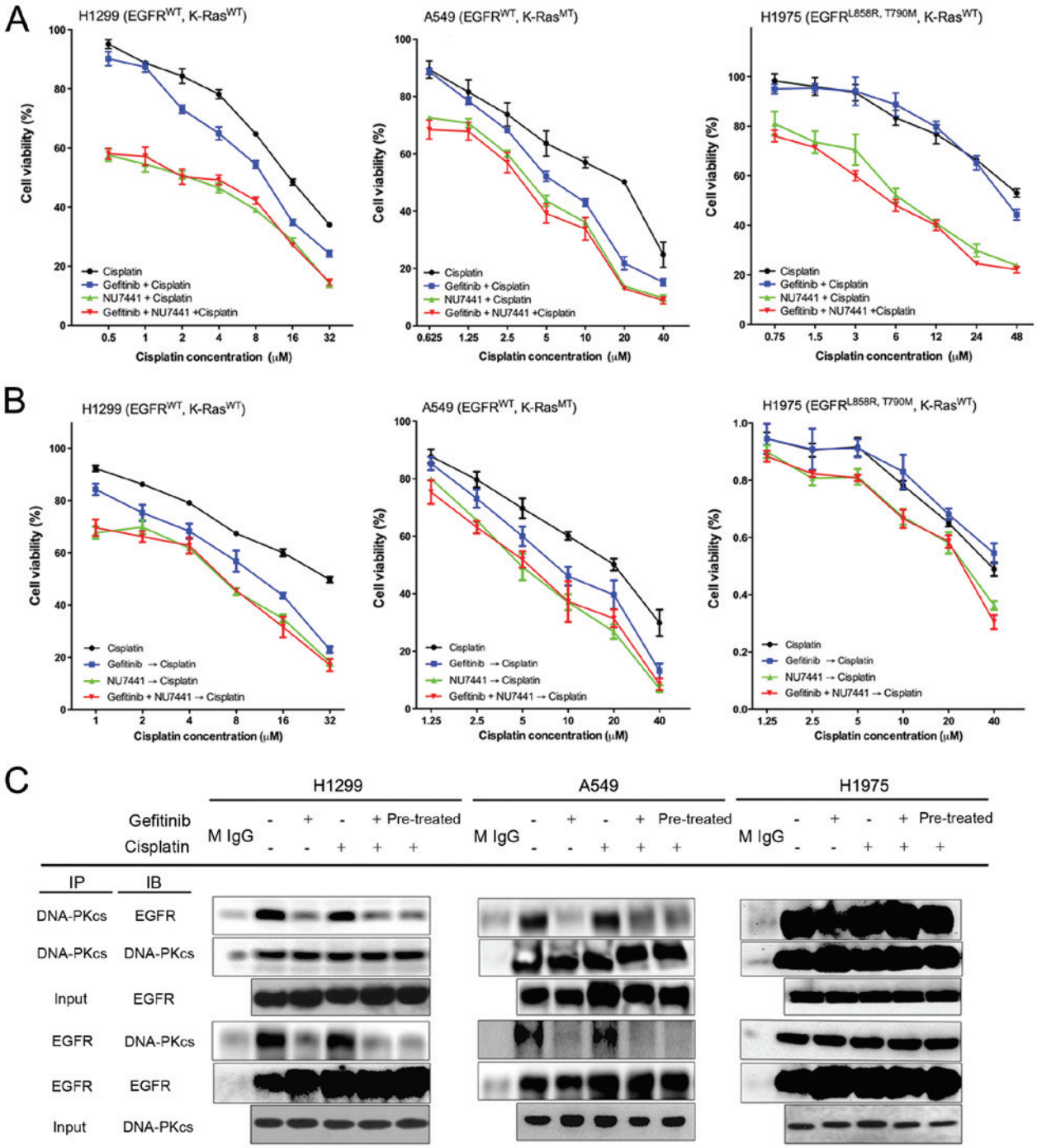

Figure 8. Inhibition of EGFR and DNA-PK by gefitinib in three non-small-cell lung cancer cells. (A) Combination effects of UN7441, gefitinib and cisplatin on the cell viability of the three NSCLC cells. (B) Sequential effects of UN7441, gefitinib and cisplatin on the cell activity of the three cell lines. (C) Interaction of EGFR and DNA-PKcs was detected via immunoprecipitation. DNA-PK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor; MT, mutation type; WT, wild-type; K-ras, Kirsten rat sarcoma 2 viral oncogene homolog.

mainly distributed in the nucleus of NSCLC cells, while EGFR was almost evenly distributed in the cytoplasm and nucleus. Thus, gefitinib could selectively prevent the nuclear enrichment of EGFR in H1299 and A549 cells, but could not prevent translocation of EGFR into the nucleus of the H1975 cells.

Discussion

The sensitivity of NSCLC cells to gefitinib depends on whether EGFR genes harbor sensitive mutations, such as EGFR 19 exon deletion and exon 21 L858R mutation. Furthermore, patients with these mutations are likely to benefit from EGFR-TKIs drugs (5,6). However, Westover *et al* (35) reported that wild-type EGFR, K-ras mutation and secondary EGFR mutation (exon 20 T790M) were associated with resistance to EGFR-TKIs. Zhang *et al* (36) also revealed that the genotypes of EGFR and K-ras in the NSCLC cell line A549 were EGFR wild type and K-ras mutation (exon 2 G12S), which could explain the resistance of A549 to EGFR-TKI (37,38). The genotypes of EGFR-TKI-resistant

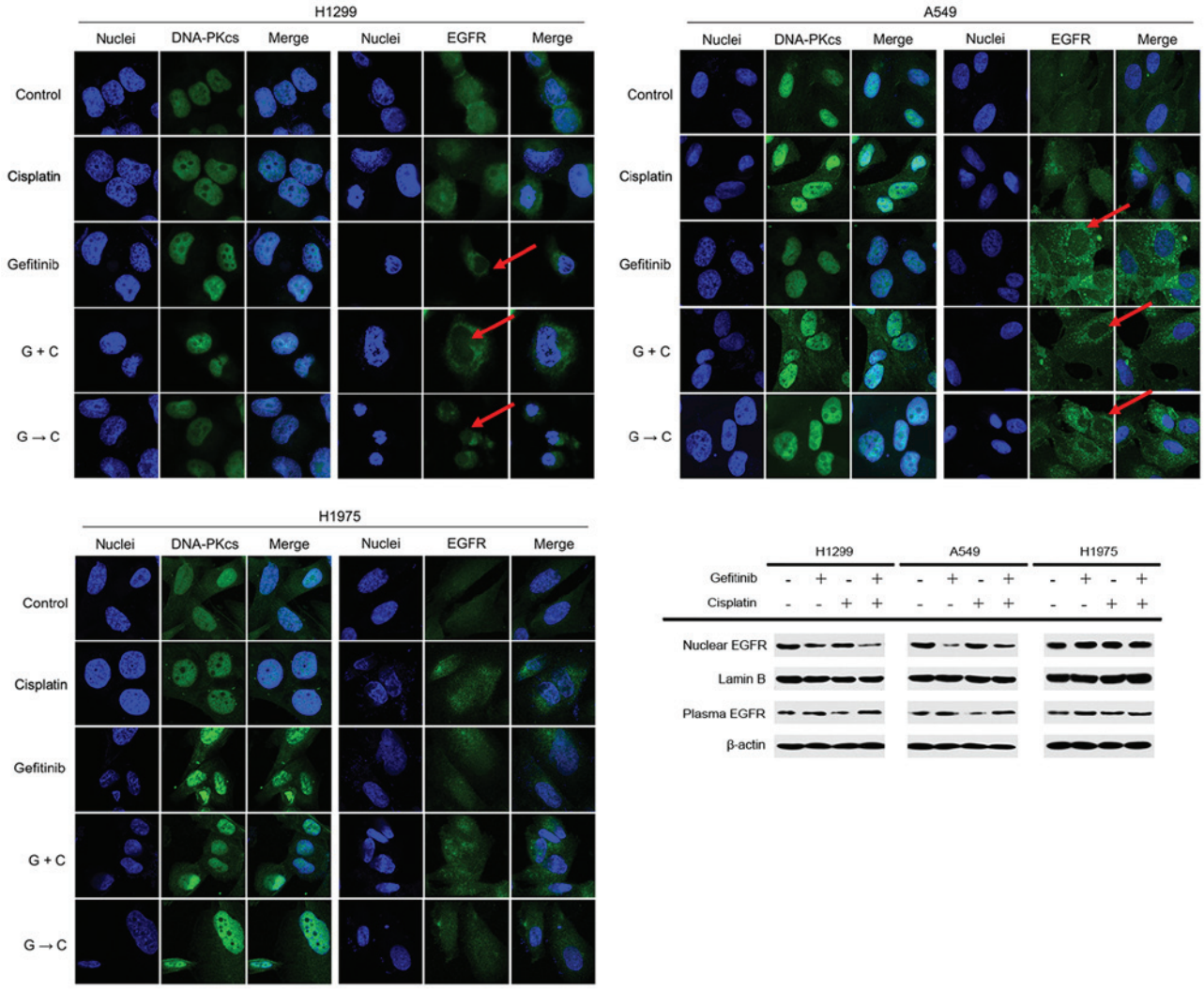

Figure 9. Distribution of EGFR and DNA-PKcs interfered by cisplatin and gefitinib. Cytoplasm and nucleus of cells were shown in each panel. Distributions of EGFR and DNA-PKcs were detected via immunofluorescence. The expression levels of cytoplasm and nuclear EGFR were detected via western blot analysis. The red arrows indicate nucleus without EGFR. DNA-PK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor; G, gefitinib; C, cisplatin.

H1299 cell line have been shown to be wild-type EGFR and wild-type K-ras (39). In addition, the H1975 cell line with secondary EGFR mutation (exon 20 T790M) and exon 21 L858R mutation (36) developed a secondary resistance to EGFR-TKIs (40). All three cell lines have been analyzed and verified with the gefitinib-resistant NSCLC models, and it has been reported that the EGFR and K-ras genotypes of A549, H1299 and H1975 cell lines are different. For example, A549 and H1299 cells with wild-type EGFR are primarily resistant to gefitinib (39,41,42), while H1975 harbors EGFR L858R-sensitive mutation in exon 21 (43) and secondary resistant mutation T790M. The mutation sites of A549, H1299 and H1975 cells in the present study were consistent with the literature (44). It has been reported that the cells resistance to EGFR-TKIs if IC_{50} >2 μM (38,45), but patients with wild-type EGFR may still benefit from EGFR-TKIs in the clinical practice (46). The present results suggested that these three types of NSCLC cell models were all EGFR-TKI resistant, and their IC_{50} values were ~10 μM.

The combined effects of cisplatin and gefitinib were shown to be inconclusive in previous studies (40,47). For instance, Tsai *et al* (47) reported that these two drugs had no synergetic effects on NSCLC cells, but Laurila and Koivunen (48) suggested otherwise. In the present study, it was found that cisplatin + gefitinib combination produced different effects among the three NSCLC cell lines. It was demonstrated that gefitinib monotherapy significantly inhibited the viability of three NSCLC cells *in vitro*. Moreover, cisplatin improved the antiproliferative effect of gefitinib in H1299 and A549 cells *in vivo* and *in vitro*, but such effect was not observed in H1975 cells.

Zhang *et al* (49) reported that some patients with advanced NSCLC with wild-type EGFR could benefit from the combination therapy of EGFR-TKI and platinum-based drugs. The present results suggested that the combination effects of gefitinib and cisplatin in H1299 and A549 varied, despite both cells containing wild-type EGFR. For example, H1299 cells had a consistent synergistic effect *in vitro*, while A549 cells had a complex mixture effect with regards to synergy. Thus, it was speculated that wild-type EGFR could not be used as a single biomarker to predict the combined effect of cisplatin and gefitinib. Hierarchical analyses of

most previous clinical trials are based on EGFR genotype, which may explain the contradictory conclusions from the literature (9,14,15,49). Moreover, the biological difference of tumors may cause this inconsistency. The most obvious differences between the H1299 and A549 cells is that H1299 contains wild-type K-ras, but A549 has the most common mutant of exon 2 G12S. Previous studies have revealed that the K-ras mutation was a mechanism for primary resistance to EGFR-TKI (50), and this could be an indicator for excluding the combination of erlotinib and platinum-based chemotherapy (51). In the present study, gefitinib and cisplatin had extensive synergistic effects in A549 cells with mutant K-ras (exon 2 G12S), but their effects were completely different in H1975 and H1299 cells with wild-type K-ras. Thus, both the present study and previous reports (47,51,52) suggested that the EGFR and K-ras gene status are not reliable indictors to assess the sensitivity of combination treatments.

The Iressa Mutation-Positive Multicenter Treatment Beyond ProgRESsion Study (IMPRESS) trial reported that gefitinib-platinum combination therapy was ineffective in patients with secondary resistance and who were carriers of the EGFR T790M mutation, but the treatment was effective in patients with secondary resistance and who were non-carriers of the T790M mutation (53). Moreover, biomarker analyses suggested that this effect may be driven by the T790M-positive status (53). In the present study, an antagonistic effect was observed in the H1975 cells with the EGFR T790M mutation and this observation was consistent with IMPRESS results, which that may be explained via the synergistic mechanisms between gefitinib and cisplatin that were investigated in the current study. However, no biomarker is available to predict the benefit of the combination therapy.

In the current study, it was indicated that the synergistic effect of cisplatin and gefitinib depended on the increased sensitivity of cisplatin induced by gefitinib. The two drugs demonstrated antagonistic effect in A549 cells when combined at low concentrations, but the effect became synergistic at high concentrations. Therefore, sensitization of gefitinib on cisplatin was dominant at high concentrations. The mechanisms of sensitization could provide novel ideas for investigating new biomarkers for combination therapy.

The present results demonstrated that gefitinib enhanced cisplatin-induced apoptosis in H1299 and A549 cells and activation of caspase-3, but this phenomenon was not observed in H1975 cells. Previous studies have shown that gefitinib could enhance the antitumor ability of cisplatin in clinical practice (54), and some patients with NSCLC with failed platinum-based chemotherapy could resume chemotherapy after treatment of gefitinib for a period of time (55). In the present study, cisplatin inhibited the sensitivity of gefitinib, and this result was different from previous findings (40,56). It was speculated that this difference may be associated with the biological background of NSCLC models. Therefore, the inhibition mechanism of cisplatin on gefitinib requires further examination in future studies.

EGFR has two main downstream signal pathways: The Ras/Raf/Mitogen-activated protein kinase kinase/ERK/ Mitogen-activated protein kinase pathway (57) and the PI3K/Akt/mTOR pathway (58). The core agents of these two pathways are ERK1/2 and Akt proteins, respectively, and EGFR-TKI can inhibit the activation of EGFR downstream pathways via EGFR activation inhibition (45). PTEN is a tumor suppressor protein that blocks the activation of Akt and its related proteins (59), and the deletion or inhibition of PTEN is associated with EGFR-TKI resistance (60-62). Previous studies have shown that the mechanism of the synergistic effects between cisplatin and gefitinib was associated with cisplatin-induced activation of EGFR pathway (40). However, in the current study, this pathway was not observed in all three NSCLC cells.

The present results indicated that DNA-PK phosphorylation could be inhibited by gefitinib in H1299 and A549 cells but not in H1975 cells. Previous studies also revealed that gefitinib inhibited DNA-PK activity in breast cancer cells and decreased DNA-PK phosphorylation (63,64). However, gefitinib had no effect on BRCA1 activation and ERCC1 expression. Moreover, BRCA1 was activated by cisplatin, which may be the stress response of cisplatin. Thus, it was speculated that the synergistic effect between gefitinib and cisplatin in NSCLC cells may be achieved via the inhibition of the DNA-PK pathway.

An interaction between EGFR and DNA-PK was identified in the NSCLC cell nucleus, and the interaction could be selectively inhibited by gefitinib. In addition, the present results suggested that the amount of EGFR in the nucleus could be reduced by gefitinib. Therefore, gefitinib may prevent EGFR from entering the nucleus, thus activating DNA-PK and sensitizing cisplatin. The synergistic effect between gefitinib and cisplatin may due to the enhanced cytotoxicity of cisplatin by gefitinib. Similar mechanisms have also been reported in previous studies, which showed that EGFR could move into the nucleus and bind to DNA-PK to assist in DNA double-strand repair, enhancing tumor cell repair (28,29,63,65). Cetuximab, an EGFR antibody, has also been showed to prevent EGFR from entering the nucleus and inhibiting DNA-PK activity, which increases the sensitivity of chemotherapy (65,66).

Nuclear EGFR can regulate the translation of cell signaling proteins (67). Furthermore, not all types of EGFR can enter the nucleus. It has been revealed that cisplatin and radiation facilitate the wild-type EGFR and EGFR vIII entering the nucleus, but not EGFR with the L858R multination (32). EGFR vIII is an EGFR mutation and it lacks the extracellular ligand-binding region factor (32). The inability of ligand binding inhibits the intracellular activation of EGFR and causes the resistance to EGFR-targeted drugs (68). As H1299 and A549 cells contain wild-type EGFR, and H1975 harbors L858R mutation, it was speculated that wild-type EGFR may be an essential agent for the sensitization of cisplatin by gefitinib.

In conclusion, the present results suggested that gefitinib can selectively inhibit DNA-PK activity and sensitize the cytotoxicity of cisplatin in NSCLC cells by inhibiting nuclear EGFR to active DNA-PK, which is the synergistic mechanism between platinum-based chemotherapy and EGFR-TKI. In addition, wild-type EGFR may be a potential biomarkers for the selection of combination therapy of cisplatin-based chemotherapy and gefitinib; however, further research is required to identify other biomarkers.

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Availability of data and materials

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

Authors' contributions

JJH and SZZ conceived the concept and designed the study. CP, HJD, YNW, CPZ, CHY, YD, DML, CG, DQW, YYW, XHF, JX, ML, YDC, TP and WT performed the experiments. CP and HJD wrote the paper. YDC, TP and WHX reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All of the animal experiments described in this study were approved by the Institutional Animal Care and USE Committee (IACUC) at Zhejiang University. All animals were maintained in accordance with the IACUC guideline.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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