



# Erlotinib enhanced chemoradiotherapy sensitivity via inhibiting DNA damage repair in nasopharyngeal carcinoma CNE2 cells

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**Background:** Nasopharyngeal carcinoma (NPC), arising from nasopharynx epithelium, is a rare type of malignant carcinoma that has a specific geographical distribution and a high risk of distant metastases. For most of the diagnosed NPC patients, the total survival rate decreased significantly due to the high local recurrence rate and metastasis rate. Concurrent chemoradiotherapy (CCRT), as routine therapy strategy of NPC, usually accompanies with high-dosage cytotoxic agents and serious toxic side reaction. Therefore, there is an urgent need for update the existing therapy strategies. In this study, we sought to investigate the effects of a combined therapy strategy, erlotinib combined with cisplatin and radiotherapy, on biological characteristics of NPC CNE2 cells and the potential reasons.

**Methods:** CNE2 cells in logarithmic phase seeding in 96-well plates received concentration gradients of erlotinib (at 0, 10, 20, 40, 80, 160, 320 mmol/L) or cisplatin (at 0, 0.25, 0.5, 1, 2, 4, 8 mg/L), in order to obtain the optimal working concentration of erlotinib and cisplatin via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Then, cells were divided into control group and four treatment groups (Group I–IV). All treatment groups received irradiation of 4 Gy, moreover, Group II–IV respectively received erlotinib, cisplatin and erlotinib plus cisplatin at optimal working concentration. After 24 and 48 h of irradiation, growth inhibition rate was determined; invasion ability and migration ability was respectively detected by Boyden's chamber assay and cell scratch test; flow cytometry was performed for determining apoptosis rate and cell cycle distribution; the expression levels of epidermal growth factor receptor (EGFR) signal pathway proteins were semi-quantitatively analyzed by Western-blot.

**Results:** Compared with Group I, all the other treatment groups showed better inhibition effect on cell viability, invasion and migration ability and higher apoptosis rate, while Group IV showed the strongest growth inhibition effect and highest apoptosis rate. In addition, EGFR signal pathway proteins of Group IV showed the lowest expression level.

**Conclusions:** In combined therapy with radiotherapy/chemotherapy, erlotinib could enhance radiotherapy/chemotherapy sensitivity, probably because it could suppress DNA damage repair after radiotherapy/chemotherapy, thus weakening radiotherapy/chemotherapy resistance of tumor cells.

**Keywords:** Erlotinib; chemoradiotherapy; nasopharyngeal carcinoma; CNE2 cell; DNA damage repair

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## Introduction

Southern China is one of the high-incidence areas of nasopharyngeal carcinoma (NPC) in the worldwide. Currently, the effective treatment strategy of NPC is radiotherapy, and the 5-year survival rate was 71.7–89.2%, 50.2–75.4%, 53.1–60.3% and 30.1–37.7% for patients at stage I, stage II, stage III and stage IV, respectively (1–5). For mid and late stage's patients, the total survival rate decreased significantly due to the high local recurrence rate and metastasis rate by radiotherapy alone. Concurrent chemoradiotherapy (CCRT), as routine therapy strategy of NPC (6,7), accompanying with improved therapeutic effect, high-dosage cytotoxic agents usually lead to serious toxic side reaction. Therapy resistance, especially cisplatin (DDP) resistance, is the main cause of disease failure (8). Hence, a new potent systemic management that leads to the clinically desirable to develop treatments with fewer side effects (9) is urgent for this cancer.

Epidermal growth factor receptor (EGFR), an important member of the ErbB family of receptors, its overexpression is a common characteristic of head and neck squamous cell carcinomas (HNSCC), and it usually predicts a poor clinical outcome. By binding to the ligand, EGFR is phosphorylated and activated, which leads to activation of the downstream signaling cascades, facilitating carcinoma cells proliferation, migration and invasion and inhibiting apoptosis. The best-known involved pathways include the rat sarcoma (Ras)/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) signaling pathways, whose roles in promoting tumor growth, survival, and progression are well characterized (10). Besides, EGFR modulates DNA double-strand break (DSB) repair by regulating non-homologous end-joining via MAPK signaling (11).

Erlotinib is a small, reversible tyrosine kinase inhibitor that competes with adenosine triphosphate for binding to the intracellular catalytic domain of EGFR, inhibiting phosphorylation. In the field of lung cancer and other cancers, there are many reports demonstrate the antitumor efficacy of EGFR inhibitors in combination treatment, especially in the treatment of non-small cell lung cancer and squamous-cell lung cancer (12–14). In a study of an anti-angiogenesis drug combination of erlotinib and a ruthenium-based compound, Berndsen *et al.* (15) validated the drug combination results in strong synergistic inhibition of cell viability in human endothelial and human ovarian

carcinoma cells and also confirmed effective anti-angiogenic and anti-proliferative activity *in vivo*. Yoo *et al.* (16) and Gilbert *et al.* (17) also demonstrated that erlotinib combined with radiotherapy and chemotherapy improved the effective rate of advanced HNSCC. Anisuzzaman and colleagues examined the combined effects of erlotinib and BKM120 on cell growth suppression, apoptosis and signaling pathway, and they demonstrated that cotargeting of EGFR and PI3K is synergistic and induces apoptosis of HNSCC cell lines by inhibiting both axes of the AKT-mTOR pathway and translational regulation of antiapoptotic Bcl-2 proteins (18). But because the biological characteristics of NPC are quite different from the other HNSCCs, it is not clear whether erlotinib enhances the efficacy of chemoradiotherapy on NPC.

The aim of this study was to evaluate the combined effects of erlotinib and chemoradiotherapy on biological characteristics of NPC cells and explore the possible reasons.

## Methods

### Reagents and cell culture

Erlotinib was obtained from Roche (Basel, Switzerland), Dulbecco's modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco (Glasgow, UK). Rabbit anti-human monoclonal antibodies and horseradish peroxidase (HRP) labeled anti-rabbit secondary antibody were purchased from Cell Signaling Technology Com (Danvers, US). Human poorly differentiated NPC cell line CNE2 (purchased from Cell Bank of Chinese Academy of Science) was cultured in DMEM at 37 °C humidified atmosphere containing 5% CO<sub>2</sub>. The medium was supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were exposed to X-ray of 4 Gy using an X-ray irradiation system.

### MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay

CNE2 cells in logarithmic growth phase were seeded into 96-well plates (5,000 cells/well), erlotinib (at 0, 10, 20, 40, 80, 160, 320 nmol/L) or DDP (at 0, 0.25, 0.5, 1, 2, 4, 8 mg/L) was added into the medium in advance. Then, cells were continued to be cultured for 24, 48 and 72 h. Following the addition of 20 µL MTT to each well, cells were incubated for 2 h. The absorbance was read at a wavelength of 490 nm. The experiment was performed

in triplicate and repeated at least three times. The half maximal inhibitory concentration (IC<sub>50</sub>) values, IC<sub>10</sub> (10% inhibitory concentration values) and IC<sub>75</sub> (75% inhibitory concentration values) were calculated using SPSS 16.0 software. Based on the results, we chose the optimal working concentrations to continue the experiments.

Next, cells were divided into control group and four treatment groups (Group I–IV). Control group was set as blank group without any treatment, the four treatment groups all received irradiation of 4 Gy X-ray. Besides, at the optimal working concentration, erlotinib, DDP and erlotinib combined with DDP was given Group II, Group III and Group IV, respectively. Irradiation (dose rate of 1 Gy/min) was followed after 12 h of administration. At 24 and 48 h post-irradiation, cell growth-inhibition rate was calculated as follows:

$$\text{Growth-inhibition rate (\%)} = (1 - \text{OD}_{\text{treatment}} / \text{OD}_{\text{blank}}) \times 100\%.$$

#### ***Boyden's chamber assay and cell scratch test***

The chambers were put into 24-well plates and coated with Matrigel (100  $\mu\text{L}$ /well) overnight at 37 °C. CNE2 cells were grouped and treated according to MTT assay, and continued to be cultured for 24 h after completion of irradiation. Then they were made into cell suspension ( $5 \times 10^5$  cells/mL) and seeded into the upper chamber (200  $\mu\text{L}$ /well). Concurrently, the lower chambers were filled with 500  $\mu\text{L}$  medium containing 10% FBS. After 24 h, cells were fixed and stained with crystal violet for 10 min. Cells number was observed microscopically.

For cell scratch test, CNE2 cells in logarithmic growth phase were seeded into 6-well plates ( $5 \times 10^5$  cells/well) and treated as mentioned above. Cells number of migration was detected using a digital camera system and imaged at 0 and 24 h postirradiation.

#### ***Flow cytometry***

CNE2 cells seeding in 6-well plates ( $5 \times 10^5$  cells/well) were treated as mentioned above and fixed in 95% ethanol at 24 h postirradiation. Following rehydration in PBS for 30 min at 4 °C, cells were treated with 1% RNAase for 30 min at 37 °C and stained with propidium iodide for 5 min. Then cells were analyzed using a flow cytometer.

#### ***Western blot***

Proteins of whole cell extracts were detected by Western

blot according to standard protocols. Primary antibodies (anti-EGFR (Cat. # 4267, RRID: AB\_2246311, Cell Signaling Technology), anti-ERK1/2 (Cat. # 9102, RRID: AB\_330744, Cell Signaling Technology), anti-AKT (Cat. # 9272, RRID: AB\_329827, Cell Signaling Technology), anti-p-EGFR (Cat. # 3777, RRID: AB\_2096270, Cell Signaling Technology), anti-p-ERK1/2 (Cat. # 9101, RRID: AB\_331646, Cell Signaling Technology) and anti-p-AKT (Cat. # 9271, RRID: AB\_329825, Cell Signaling Technology)) and HRP labeled anti-rabbit IgG secondary antibody (Cat. # 7074, RRID: AB\_2099233, Cell Signaling Technology) were used for determining target proteins levels. Signals were determined via chemiluminescence.

#### ***Statistical analysis***

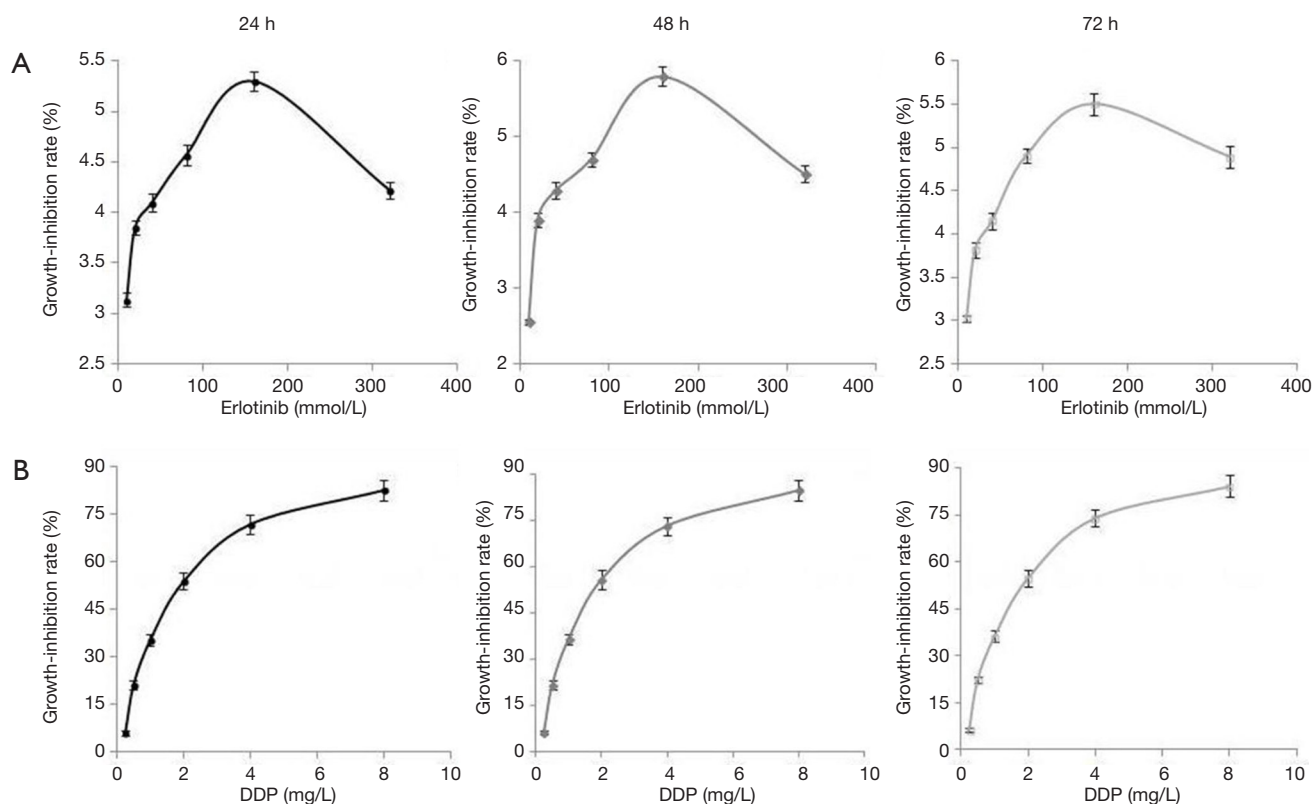
SPSS (RRID: SCR\_002865) version 16.0 statistical software was used for statistical analysis. Measurement data were presented as means  $\pm$  standard deviation (SD). Comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA). Data complying with normal distribution between groups were compared utilizing *t*-test. Statistical significance was reported if the *P* value was  $<0.05$ .

### **Results**

#### ***Erlotinib combined with DDP and irradiation inhibited cell viability***

As erlotinib concentrations rose, its inhibitory effect on cell viability enhanced gradually and peaked at 160 mmol/L. However, this inhibitory effect was not dose-dependent because the maximum inhibitory effect was no more than 6% (*Figure 1*). In contrast, *Table 1* showed the inhibitory effect of DDP was dose-dependent, IC<sub>10</sub> and IC<sub>50</sub> values after 24, 48, 72 h of treatment were 0.264, 0.264, 0.260 mg/L and 1.798, 1.702, 1.691 mg/L, respectively. In order to achieve as low agent cytotoxicity as possible, the optimal working concentration of erlotinib (160 mmol/L) and DDP (1 mg/L) was determined.

The results of *Table 2* and *Figure 2* demonstrated erlotinib combined with irradiation could obviously inhibit cell viability, compared with irradiation alone ( $P < 0.05$ ); when cells were treated with erlobinib combined DDP and irradiation, the inhibitory effect was the most significant ( $P < 0.05$ ).



**Figure 1** Inhibitory effect of erlotinib and DDP on CNE2 cells. Cells were exposed for 24, 48 and 72 h to increasing concentrations of erlotinib or DDP. (A) Cells were treated with erlotinib; (B) cells were treated with DDP. The data was presented as the mean  $\pm$  SD from three different experiments performed in triplicate.

**Table 1** The inhibitory concentration values of DDP in CNE2 cells at different time points after treatment

Time point	IC10 (95% CI)	IC50 (95% CI)	IC75 (95% CI)
24 h post-administration	0.264 (0.177–0.357)	1.798 (1.520–2.139)	4.689 (3.769–6.192)
48 h post-administration	0.264 (0.179–0.354)	1.702 (1.444–2.014)	4.320 (3.504–5.628)
72 h post-administration	0.260 (0.176–0.349)	1.691 (1.434–2.003)	4.312 (3.495–5.624)

IC10, 10% inhibitory concentration values; IC50, half maximal inhibitory concentration values; IC75, 75% inhibitory concentration values.

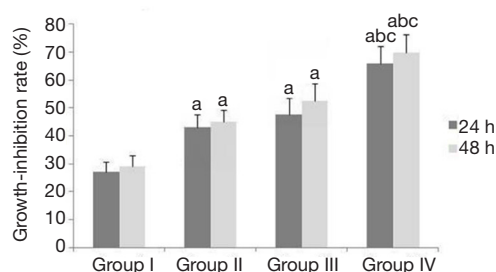
**Table 2** Cell viability was determined at 24 and 48 h post-irradiation at 490 nm

Time point	Control group	Group I	Group II	Group III	Group IV
24 h post-irradiation	0.69 $\pm$ 0.02	0.49 $\pm$ 0.03	0.39 $\pm$ 0.05 <sup>a</sup>	0.37 $\pm$ 0.06 <sup>a</sup>	0.27 $\pm$ 0.04 <sup>abc</sup>
48 h post-irradiation	0.87 $\pm$ 0.02	0.61 $\pm$ 0.04	0.50 $\pm$ 0.05 <sup>a</sup>	0.46 $\pm$ 0.05 <sup>a</sup>	0.26 $\pm$ 0.04 <sup>abc</sup>

<sup>a</sup>, vs. Group I,  $P < 0.05$ ; <sup>b</sup>, vs. Group II,  $P < 0.05$ ; <sup>c</sup>, vs. Group III,  $P < 0.05$ .

### Effects of erlotinib combined with DDP and irradiation on the abilities of invasion and migration, apoptosis and cell cycle distribution of CNE2 cells

After treatment, the results of Table 3, Figure 3 and Figure 4 showed that compared with Group I, the invasion/



**Figure 2** CNE2 cells growth inhibition rate was determined at 24 and 48 h post-irradiation by MTT assay. <sup>a</sup>, vs. Group I,  $P < 0.05$ ; <sup>b</sup>, vs. Group II,  $P < 0.05$ ; <sup>c</sup>, vs. Group III,  $P < 0.05$ .

migration ability of the other three treatment groups all showed significant reduction ( $P < 0.05$ ), and this reduction of Group IV was the most significant ( $P < 0.05$ ). Concerning the apoptosis rate, Group II–IV were all higher than Group I, and Group IV had the highest ( $P < 0.05$ , Figure 5).

From the perspective of cell cycle distribution, compared with Group I, Group II–IV all showed more obvious cell cycle arrest with blocking to G2/M phase, especially Group IV. These results indicated erlotinib disturbed cell cycle distribution significantly.

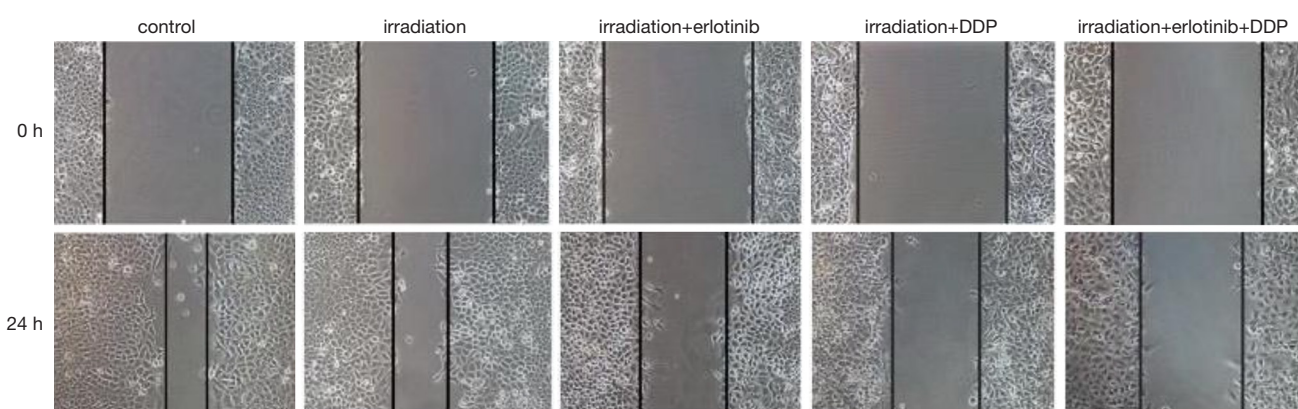
### The combined effects of erlotinib, DDP and irradiation on EGFR downstream signaling pathways

Because EGFR binding to its ligand was strongly related to DNA damage repair of tumor cells after radiotherapy/chemotherapy (11), we speculated erlotinib intervention made it hard for EGFR to bind to ligand, leading to the failure of DNA damage repair. Meanwhile, erlotinib could

**Table 3** Effects of different treatments on CNE2 cells' biological characteristics

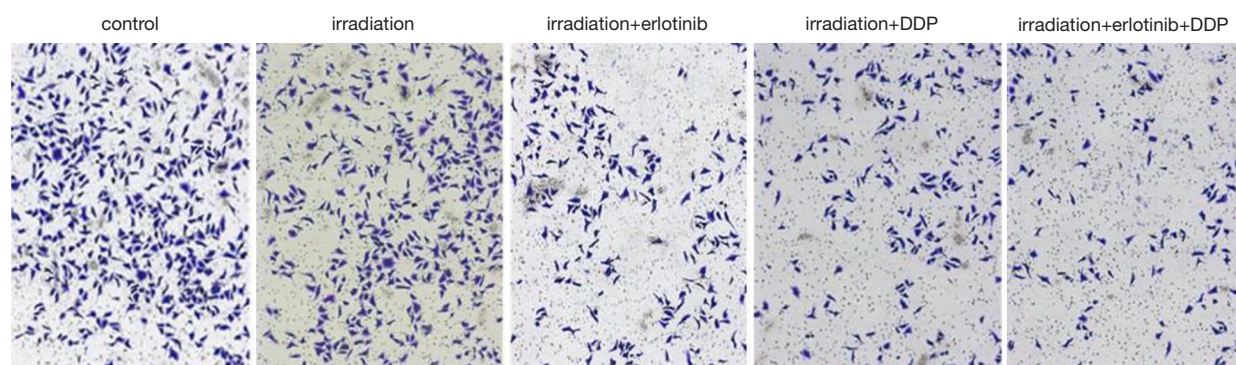
Cell biological characteristics	Control group	Group I	Group II	Group III	Group IV
Boyden's chamber assay	104±5.5	64±6.5	46±6.3 <sup>a</sup>	41±6.0 <sup>a</sup>	23±4.4 <sup>abc</sup>
Cell scratch test	521±20.4	286±17.5	192±13.8 <sup>a</sup>	177±13.5 <sup>a</sup>	89±10.2 <sup>abc</sup>
Apoptosis rate (%)	4.5±0.8	12.1±1.4	13.9±1.2 <sup>a</sup>	25.5±2.6 <sup>ab</sup>	33.9±3.3 <sup>abc</sup>
Cell cycle distributions					
G0/G1 (%)	58.6±2.2	25.1±1.8	15.9±1.7 <sup>a</sup>	17.2±1.6 <sup>a</sup>	11.2±2.1 <sup>ac</sup>
G2/M (%)	13.0±1.4	50.1±2.9	63.9±3.8 <sup>a</sup>	60.3±2.8 <sup>ab</sup>	78.5±4.9 <sup>abc</sup>
S (%)	29.4±1.7	24.9±1.6	21.0±1.3 <sup>a</sup>	23.6±1.7 <sup>b</sup>	15.1±1.5 <sup>abc</sup>

<sup>a</sup>, vs. Group I,  $P < 0.05$ ; <sup>b</sup>, vs. Group II,  $P < 0.05$ ; <sup>c</sup>, vs. Group III,  $P < 0.05$ .

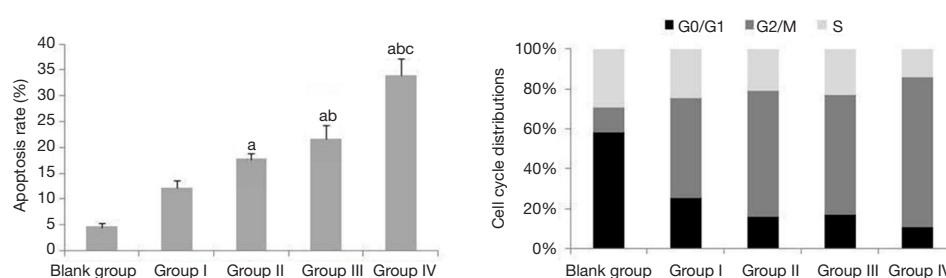


**Figure 3** CNE2 cells' migration ability was determined through scratch test at 0 and 24 h after treatment by microscopy (100×).





**Figure 4** CNE2 cells' invasion ability was determined by Boyden's chamber assay at 24 h after treatment by microscopy (100×).



**Figure 5** Apoptosis rate and cell cycle distributions after exposing to treatment at optimal working concentration or/and irradiation. <sup>a</sup>, vs. Group I,  $P < 0.05$ ; <sup>b</sup>, vs. Group II,  $P < 0.05$ ; <sup>c</sup>, vs. Group III,  $P < 0.05$ .

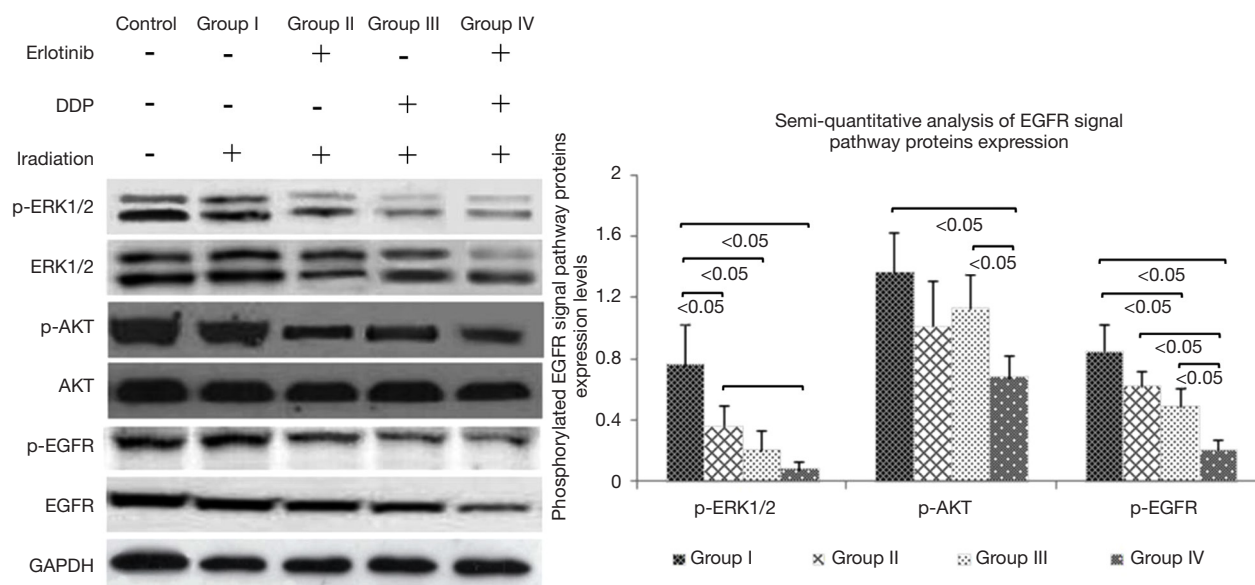
disturb cell cycle distribution and eventually intensified apoptosis induced by DDP or/and irradiation. *Figure 6* showed when erlotinib combined with DDP and irradiation, the reductions of EGFR signal pathways proteins (p-ERK1/2, p-AKT and p-EGFR) expression levels were the most significant ( $P < 0.05$ ), which indicated erlotinib's inhibitory effect on EGFR signal pathway activation was one of the main mechanisms in combination treatment.

## Discussion

Erlotinib can inhibit proliferation, invasion, metastasis, and angiogenesis in tumor cells (19,20). When used as monotherapy, the response rate of erlotinib was not satisfactory. However, the promising disease control rate, time-to-progression and overall survival rate in many trials encouraged researchers to explore the use of erlotinib in conjunction with other anticancer therapies (21-23). In the present study, erlotinib combined with DDP and irradiation showed the most significant reduction in cell viability, invasion and migration ability and the highest increase in apoptosis, when compared to other treatment

strategies. These results indicated that erlotinib enhanced the sensitivity of cells to irradiation or/and DDP (24-26).

In order to explore the mechanisms involved, the expression and activation of EGFR downstream signaling pathways proteins in NPC cells were analyzed. Our results demonstrated these proteins expression and activation were inhibited obviously, when cells received erlotinib combined with irradiation and DDP. These results indicated erlotinib prevented activation of EGFR downstream signaling pathways, thus suppressing DNA damage repair in tumor cells (27,28). Though cell cycle G2/M phase arrest was enhanced by combined treatment strategy, this arrest could not be the buffer time for repairing DNA damage induced by irradiation/cytotoxic agent because of erlotinib intervention. Instead, the cell cycle arrest (at G2/M phase) intensified DNA damage induced by DDP or/and irradiation. As a result, erlotinib intensified apoptosis rate, because it suppressed DNA damage repair, tumor cells' sensitivity to chemoradiotherapy enhanced. Besides, anti-EGFR therapy could reduce the percentage of cells in S phase (because elevated percentage of cells are in G2/M phase, sensitive phase to radiotherapy) (29).



**Figure 6** Effects of combination treatment with erlotinib, DDP and irradiation on expressions level of EGFR, EGFR/AKT and EGFR/MAPK-ERK1/2. The control group cells were blank cells without any treatment. GAPDH was used as a loading control. EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase.

Nevertheless, some studies reported erlotinib monotherapy barely killed tumor cells (30,31). When we designed the optimal working concentration of erlotinib, we found the inhibitory effect on tumor cells growth was very weak (the highest inhibitory effect was less than 6%) and not dose-dependent. This from a side, confirmed erlotinib monotherapy had little effect on CNE2 cells. Zhang *et al.* also confirmed that treatment of NPC cells with erlotinib alone had no significant effect on tumor cell proliferation (32). Given this, it can be assumed that erlotinib takes effect perhaps depending on DNA damage induced by irradiation or cytotoxic agents.

In conclusion, in combination treatment with radiotherapy and cytotoxic agents, erlotinib enhanced tumor cells' sensitivity to chemoradiotherapy, whose reason can be attributed to its inhibitory effect on DNA damage repair, and then reducing the chemoresistance and radiotherapy resistance of tumor cells.

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## Footnote

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-19-466>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors of this study are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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