ORIGINAL RESEARCH

In vitro and in vivo efficacy of afatinib as a single agent or in combination with gemcitabine for the treatment of nasopharyngeal carcinoma

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Purpose: Epidermal growth factor receptor (EGFR) is usually overexpressed in nasopharyngeal carcinoma (NPC). We tested the antitumor effects of irreversible ErbB family inhibitor afatinib on human NPC using in vitro and in vivo models.

Materials and methods: The effect of afatinib on NPC cells was evaluated using the Cell Counting Kit 8 (CCK8) assay, flow cytometry, and Western blot analyses. The effect of afatinib, as either a single agent or in combination with gemcitabine (GEM), on tumor growth was determined using NPC tumor xenografts in mice.

Results: Afatinib inhibited cell growth in all three NPC cell lines tested in a dose-dependent manner. Afatinib promoted cell cycle arrest at the S and G2/M phases, and it significantly inhibited epidermal growth factor (EGF)-induced activation of EGFR and its downstream signaling factors. Co-treatment with afatinib and GEM more effectively inhibited tumor growth than either drug alone but was associated with increased toxicity.

Conclusion: Afatinib induced cell cycle arrest and inhibited the proliferation of NPC cell lines. Afatinib in combination with GEM demonstrated significant antitumor effect in an NPC xenograft model. The administration of afatinib with GEM in NPC needs to be modified in order to be effective and tolerable.

Keywords: nasopharyngeal carcinoma, EGFR, afatinib, gemcitabine, preclinical

Introduction

Nasopharyngeal carcinoma (NPC) has a distinct geographic distribution, with low morbidity in most areas worldwide (generally <1/100,000).[1] Eighty percent of all NPC cases occur in the People's Republic of China, with the highest incidence rates in South China.[2] NPC-related morbidity in the northern region of the People's Republic of China is comparable to that observed in the remaining parts of the world. The incidence of NPC in South China is the highest in the world (~20–30/100,000 for nearly 30 years).[3]

NPC is very sensitive to radiotherapy, and radiotherapy is the treatment of choice for early nonmetastatic NPC.[4] With advances in imaging and radiation therapy, the 5-year local control rate of nonmetastatic NPC has risen to 94.9%, and the 5-year relapse-free survival rate has reached 76.7%. The main cause of treatment failure of NPC is distant metastases, whereby ~20% of patients with locally advanced disease present with remote metastasis.[5]

More than 90% of NPCs are undifferentiated carcinomas, which show relatively good reactivity to chemotherapy. Many cytotoxic chemotherapeutic agents have been tested for NPC, and currently, platinum-based treatment is the basic regimen for

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palliative care. For salvage therapy after failure of platinum regimen, yew, gemcitabine (GEM), and others are effective.[6,7] Despite the relatively high efficacy of these therapies and the eventual availability of various new drugs, the survival of patients with late NPC has not significantly improved, with progression-free survival (PFS) at about 5.6–10.6 months and overall survival (OS) at about 7.6–19.6 months.[6,8–10] Identification of novel drugs, especially targeted therapies, is needed to improve the mortality rate in patients with advanced NPC.

Epidermal growth factor receptor (EGFR) is overexpressed in 73%–89% of NPC patients, contributing to an increased risk of metastasis and decreased OS.[11–14] However, EGFR mutations are rarely detected in NPC,[15] suggesting that EGFR mutations might not be a critical factor in NP carcinogenesis. The first-generation EGFR–tyrosine kinase inhibitors (TKIs) were effective in patients with EGFR mutations, which may explain why previous studies found that EGFR-TKIs only stabilized the disease in most patients with refractory metastatic NPC.[16,17]

Compared to the first-generation reversible EGFR-TKIs gefitinib and erlotinib, afatinib is a potent irreversible ErbB inhibitor that blocks the activity of all kinases related to ErbB family members, including wild-type and mutated EGFR (eg, T790M) and human epidermal growth factor receptor 2 (HER2).[18] Owing to its efficacy in the EGFR mutation-associated non-small-cell lung cancer (NSCLC), afatinib was approved as a first-line therapy for advanced NSCLC with mutant EGFR.[19]

Recent studies have focused on whether afatinib can be applied to EGFR wild-type tumors, and some preclinical and clinical data have shown that afatinib does suppress the growth of cancer cells with wild-type EGFR.[19,20] There are ongoing clinical studies investigating the antitumor efficacy of afatinib in multiple solid tumors with ErbB deregulations.[21–23] Similar to NPC, overexpression of EGFR is commonly found in squamous cell cancer of the head and neck (SCCHN). Young et al[24] demonstrated that the efficacy of afatinib was comparable to that of cetuximab in SCCHN cell lines and xenograft models. A Phase II study[25] comparing the efficacy of cetuximab with afatinib in refractory recurrent SCCHN patients showed similar response and survival rates. Taken together, these data provided the rationale for investigating the use of afatinib for NPC. A Phase III clinical trial compared afatinib with methotrexate (MTX) as a second-line treatment for patients with refractory metastatic head and neck cancer after failure of platinum treatment and found that afatinib significantly prolonged PFS relative to MTX (2.6 months vs 1.7 months; P=0.03). In addition, afatinib improved the objective response rate (ORR: 10.2% vs 5.6%; P=0.10) and the disease control rate (49.1% vs

38.5%; P=0.04), with tolerable side effects,[26] relative to MTX. These results showed that afatinib as a second-line therapeutic regimen had a better curative effect than MTX in refractory head and neck cancer and that its application in head and neck cancer required further exploration.

In this study, we explored whether afatinib has curative effects in preclinical studies of NPC, which will shed light on its use for other applications. We selected GEM for combination with afatinib because GEM is a novel and effective agent for the treatment of NPC and is currently being investigated as a first-line therapy in metastatic NPC.[6,8–10,27] Moreover, former in vitro studies showed that combination treatment with afatinib and GEM had synergistic effects.[23] We hypothesized that combination therapy with afatinib and GEM may have enhanced the antitumor activity relative to the individual administration of these two agents.

Materials and methods
Cell lines
All cell line experiments were approved by the Ethical Committee of Sun Yat-sen University Cancer Center. Three poorly differentiated human NPC cell lines were studied: HNE-1, CNE-2, and SUNE-1. HNE-1 was kindly provided by Professor Kaitai Yao (Southern Medical University, Guangzhou, People's Republic of China). CNE-2 and SUNE-1 were kindly provided by Professor Musheng Zeng (Sun Yat-Sen University Cancer Center, Guangzhou, People's Republic of China). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Logarithmically growing cells were used in the experiments.

Reagents and drug preparation
Afatinib was kindly provided by Boehringer Ingelheim Pharmaceuticals (Ingelheim am Rhein, Germany). GEM (Gemzar; Eli-lilly Pharmaceuticals, Indiana, IN, USA) was obtained as a commercial product from our hospital pharmacy.

Afatinib was dissolved in dimethylsulfoxide at a stock concentration of 10 mM and stored at −20°C. For in vivo studies, afatinib was dissolved in plain water at a concentration of 1.25 mg/mL. GEM was dissolved in plain water at a stock concentration of 100 mM and stored at −20°C.

Proliferation assay
Tumor cells were cultured in 96-well plates at an appropriate density per well. Varying concentrations of afatinib, GEM, and the combination were added to the cells 24 hours after plating and incubated for 72 hours, followed by Cell Counting Kit 8

(CCK8; Dojindo, Tokyo, Japan) assay. Afatinib and GEM were added at the same concentration when in the combination assay (eg, both 0.625 µM). The optical density was measured at 450 nm on an enzyme-linked immunosorbent assay reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). The half-maximal inhibitory concentration (IC_{50}) value was defined as the concentration resulting in 50% of cell growth inhibition relative to untreated control cells. The assay was performed in triplicate in more than three independent experiments. The combination index (CI) was calculated by CalcuSyn (Biosoft, Cambridge, UK). CI <1 indicates synergism; CI =1 indicates additive effects; CI >1 indicates antagonism.[28]

Cell cycle analysis

Cells were seeded into six-well plates containing RPMI 1640/10% FBS with or without various concentrations of afatinib. Following 48-hour incubation, cells were harvested and fixed with 70% ethanol and stored at −20°C. Tumor cells were incubated with propidium iodide/RNase buffer (Becton Dickinson, Bedford, MA, USA) for 30 minutes at room temperature in the dark. Analysis was performed using the FACScan flow cytometer (Beckman Coulter Inc., Brea, CA, USA) and CellQuest Pro software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Western blotting

Cells were treated with afatinib for 24 hours and then stimulated with 10 nM epidermal growth factor (EGF) (Cell Signaling Technology, Beverley, MA, USA) for 30 minutes before harvest. Cells were harvested and lysed in cell lysis buffer (Cell Signaling Technology). Proteins were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Hoffman-La Roche Ltd., Basel, Switzerland). The membranes were incubated overnight at 4°C with primary antibodies. After being incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, bands were detected using an enhanced chemiluminescence system (Cell Signaling Technology). The expression of phosphorylated-EGFR (pEGFR), pAKT, and p-extracellular regulated kinase (pERK) was analyzed to detect the activation of EGFR and its downstream pathways (all antibodies were purchased from Cell Signaling Technology). The quantitative analysis was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Tumor xenograft studies

Six- to 8-week-old male BALB/c nude mice weighing ~16–18 g were maintained under specific pathogen-free conditions. All animal experiments were conducted in accordance with the United Kingdom Co-ordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia. All animal experiments were approved by the Experimental Animal Ethical Committee of Sun Yat-sen University. HNE-1 cells (1×10^7 cells resuspended in 0.2 mL of 0.9% NaCl solution) were inoculated subcutaneously into the right flank of nude mice. When tumors reached ~150 mm³, animals were randomly assigned to one of four groups (n=8 per group): afatinib group, GEM group, combination group, and control group. Afatinib (12.5 mg/kg) was administered by oral gavage for 5 days a week. Intraperitoneal injection of GEM (100 mg/kg) was dosed with 0.9% NaCl every week. NaCl (0.9%, 10 mL/kg) was administered by oral gavage for 5 days a week, and intraperitoneal injection of 0.9% NaCl was administered every week as control treatment. Mice body weight and tumor size were measured and recorded every 3 days. Tumor volume was calculated using the following equation: volume (mm³) = length × width² ×0.5. After 4 weeks of treatment, mice were sacrificed, and tumors were harvested, fixed in 10% buffered formalin, and embedded in paraffin.

Statistics

Statistical analyses were performed using SPSS Version 16.0 software (SSPS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. Comparisons in the in vivo studies were analyzed by the unpaired *t*-test with Welch correction. Two-sided $P<0.05$ was considered statistically significant.

Results

Afatinib inhibited proliferation in NPC cell lines and showed synergistic effect with GEM

Three human NPC cell lines – CNE-2, HNE-1, and SUNE-1 – were treated with afatinib (0–10 µM) for 72 hours. Afatinib demonstrated a steep dose–response relationship, with dose-dependent growth inhibition in all cell lines (Figure 1). The IC_{50} values for afatinib in HNE-1, CNE-2, and SUNE-1 cell lines were 4.41±0.73 µM, 2.81±0.35 µM, and 6.93±0.54 µM, respectively. When combined with GEM, the CI values were separately 0.293, 0.435, and 0.654 when the concentrations were 0.625 µM, 1.25 µM, and 2.5 µM for both drugs, which indicates that synergistic effect exists when afatinib is combined with GEM.

Afatinib arrested the cell cycle at S and G2/M phases

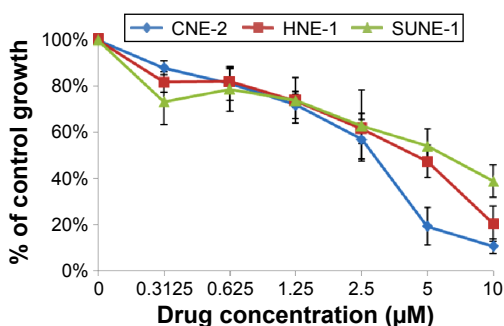The effects of afatinib on the cell cycle of NPC cells were determined by flow cytometry. HNE-1 cells were treated

Figure 1 Inhibitory effect of afatinib on growth of NPC cell lines.
Notes: Cancer cell proliferation was calculated as a percentage of growth of the control cells. Each point represents the mean of triplicate values ± standard deviation.
Abbreviations: CNE, HNE, SUNE: three human NPC cell lines; NPC, nasopharyngeal carcinoma.

with various concentrations (0 µM, 0.625 µM, 1.25 µM, 2.5 µM, and 5 µM) of afatinib for 48 hours, resulting in a dose-dependent increase in the percentage of cells in the S and G2/M phases and a concomitant decrease in the proportion of cells in the G0/G1 phase (Figure 2).

Afatinib inhibited the EGFR signaling pathway

We examined the activity of EGFR and its downstream targets AKT and ERK in HNE-1 cells treated with afatinib. As shown in Figure 3, after exposure to afatinib for 24 hours,

EGF-induced phosphorylation of EGFR, AKT, and ERK was suppressed by afatinib. However, afatinib treatment did not affect the levels of p-EGFR, p-AKT, and p-ERK in the absence of EGF stimulation. The quantitative analysis is provided in Figure S1.

Antitumor activity of afatinib in vivo

We evaluated the efficacy of afatinib in HNE-1 xenograft mouse model, both as a single agent and in combination with GEM. As shown in Figure 4A, afatinib modestly inhibited NPC tumor growth in the HNE-1 xenograft model. In the human NPC cell line xenograft model, combination treatment with afatinib and GEM significantly inhibited tumor growth more than that obtained by the two treatments alone. However, toxicity was more severe, as evidenced by a greater decrease in body weight, in the combination group than in the other treatment groups (Figure 4B).

Discussion

This preclinical study is the first to investigate the efficacy of afatinib in NPC, and our findings suggest that afatinib is a promising agent for the treatment of NPC. The IC_{50} values of afatinib were found to be around 3–7 µM in three poorly differentiated human NPC cell lines, suggesting that these cell lines are marginally sensitive to afatinib monotherapy.
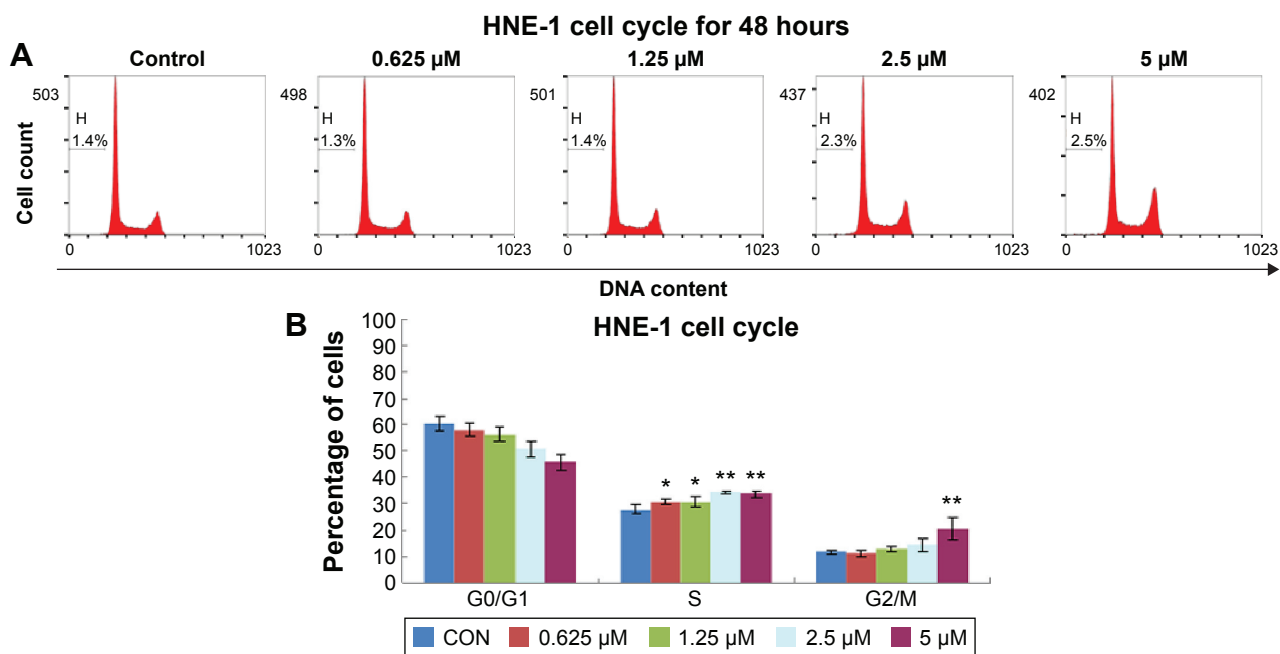

Figure 2 Afatinib induced cell cycle arrest at G1 phase in HNE-1 cell line.
Notes: (**A**) Cell cycles were assessed by flow cytometry and one representative experiment is shown. (**B**) Statistical results are shown in the graph. Values are presented as mean of triplicate ± SD. *Represents $P < 0.05$, **represents $P < 0.01$.
Abbreviations: CON, control; H, percentage of sub-G1 phase; HNE-1, human NPC cell line; NPC, nasopharyngeal carcinoma; SD, standard deviation.
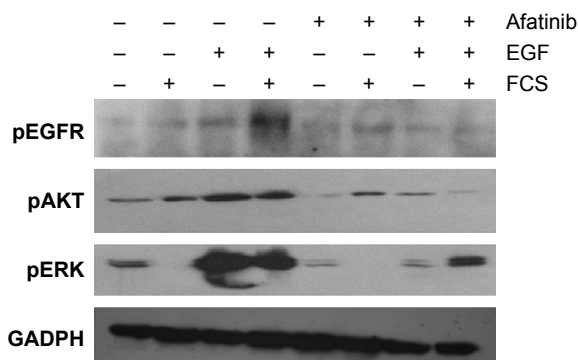
Figure 3 Effect of afatinib on levels of phosphorylated forms of EGFR, AKT, and ERK in the HNE-1 cell line.
Notes: Some cells were incubated with 10 ng/mL EGF for 30 minutes. Afatinib (5 µM) was administered as described in the "Materials and methods" section.
Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular regulated protein kinases; FCS, fetal calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HNE-1, human NPC cell line; NPC, nasopharyngeal carcinoma; p, phosphorylated.

In the Phase I trials of afatinib monotherapy for advanced solid tumors, different doses of afatinib lower than the maximum tolerated dose achieved an average steady-state maximum concentration ($C_{max,ss}$) of 63.4 ng/mL (~0.13 µM). We observed that the growth inhibition rate of this dose of afatinib was only about 10%–20%. The IC_{50} of all three cell lines after conversion was 1.37–3.38 µg/mL (the molecular weight of afatinib is ~486 g/mol), and this drug dosage is much higher than the human tolerated blood drug concentration of afatinib. Western blot data also showed that afatinib

did not inhibit the EGFR signaling pathway when EGFR was not overactive. In tumors not driven by EGFR mutations, such as gastric cancer, pancreatic cancer, and SCCHN, the combination of afatinib and other agents or radiotherapy might have better response than afatinib alone.[21–23] Therefore, we believe that the currently used dose of afatinib may fail to produce satisfactory curative effects as monotherapy in patients with advanced NPC, whereas its use in combination with other drugs (targeted drugs or chemotherapy drug) may be ideal.

Previously, preclinical studies[22,23] reported that afatinib mainly inhibited the growth of tumor cell lines by causing retardation of the cell's G_1 phase. Because chemotherapy drugs mainly act on cells in the proliferating and division phase, there is no appropriate theoretical basis to support the existence of synergic efficiency during concurrent application of small molecule inhibitors and chemotherapy drugs. In addition, both in vitro and in vivo experiments have shown no positive results.[29] In our study, cell cycle analysis demonstrated that afatinib induced cell arrest in the G2/M and S phases. This is a very interesting finding, as it provides some support for the use of combination treatments with afatinib and chemotherapeutic agents for NPC patients. Because GEM is an S phase-specific cytotoxic agent, we predicted that this combination might increase efficacy. Our in vivo experiments using combination treatment with afatinib and GEM confirmed this hypothesis.
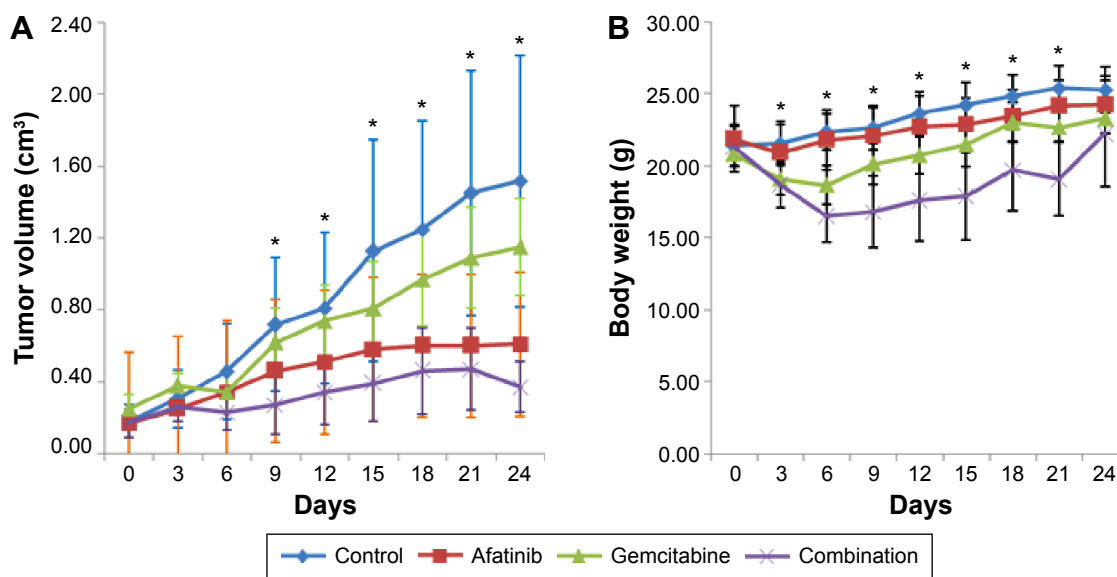

Figure 4 Effect of afatinib, gemcitabine, or combination on the growth of HNE-1 xenografts.
Notes: (**A**) Tumor volume of HNE-1 xenografts. Mean tumor volume is shown. (**B**) Body weight of HNE-1 xenografts. Mean body weight is shown. Data points represent the mean value from six to nine mice; Bars represent the standard deviation. *$P<0.05$ versus control.
Abbreviations: HNE-1, human NPC cell line; NPC, nasopharyngeal carcinoma.

The human NPC xenograft model demonstrated good sensitivity toward afatinib monotherapy treatment at 12.5 mg/kg. Afatinib in combination with GEM resulted in significant inhibition of tumor growth. However, more weight loss was observed in the combination group, showing that toxicity might limit the clinical applicability of concurrent therapy. To achieve safe and effective results in subsequent clinical studies, the dosages of both afatinib and GEM need to be further explored.

Another option for including afatinib in treatment regimens for NPC is sequential therapy. Previous studies have shown that most EGFR-TKIs induce G0/G1 cell cycle arrest, which might protect cells from the cytotoxic effects of cell cycle phase-dependent chemotherapy agents.[29] Sequential administration of EGFR-TKIs following chemotherapy may provide greater efficacy than concurrent administration in NSCLC.[30,31] In other preclinical models,[32] sequential administrations of two potent agents could delay disease progression. In our preclinical study, afatinib and GEM, both active drugs against NPC, were administered concomitantly. The combination treatment, although effective, induced more toxicity than either agent alone did. On the basis of these observations, we suggest modifying the method of administration. Further studies are needed to evaluate the full potential of this combination.

In conclusion, afatinib effectively arrested the growth of NPC cell lines in vitro. Data from the NPC xenograft model suggested that concurrent administration of afatinib with GEM might lead to significant efficacy but at the cost of enhanced toxicity. Combination therapy of GEM along with afatinib might be an effective treatment option for NPC once alternative dosage or methods of administration (schedules) are optimized in model systems, resulting in a larger therapeutic window for this combination.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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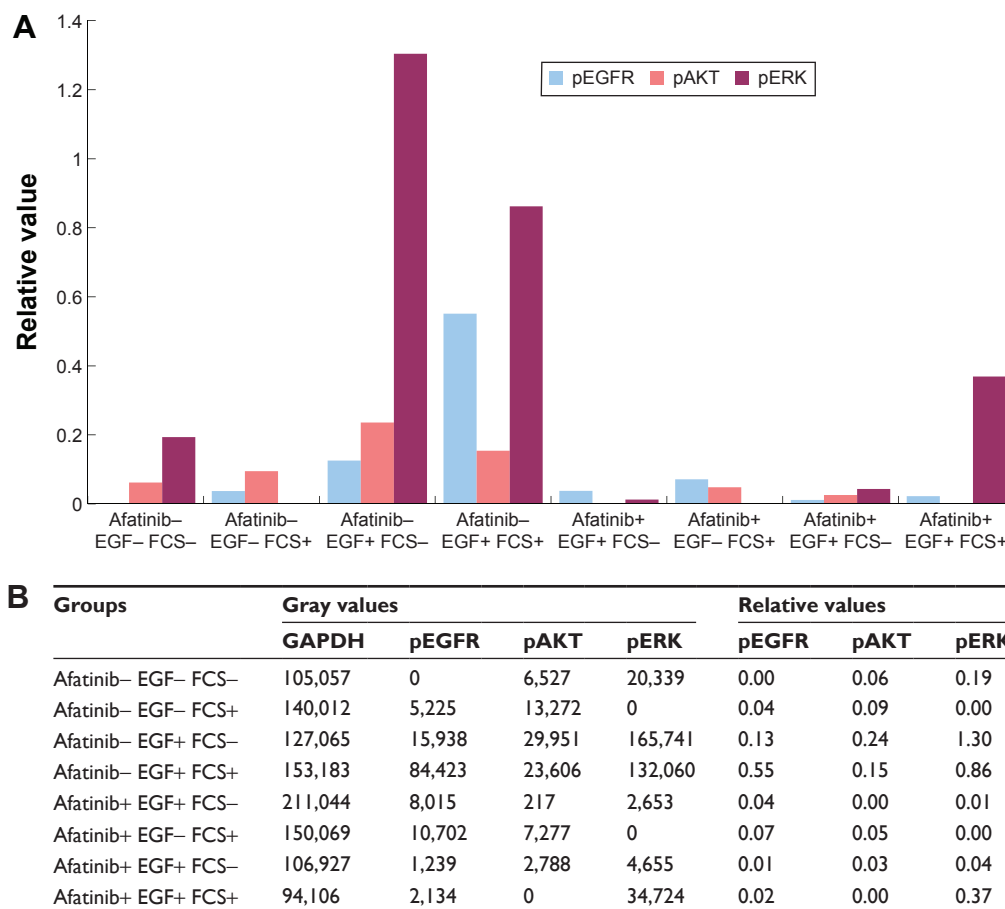
Supplementary material

A

B

Groups	Gray values				Relative values		
	GAPDH	**pEGFR**	**pAKT**	**pERK**	**pEGFR**	**pAKT**	**pERK**
Afatinib− EGF− FCS−	105,057	0	6,527	20,339	0.00	0.06	0.19
Afatinib− EGF− FCS+	140,012	5,225	13,272	0	0.04	0.09	0.00
Afatinib− EGF+ FCS−	127,065	15,938	29,951	165,741	0.13	0.24	1.30
Afatinib− EGF+ FCS+	153,183	84,423	23,606	132,060	0.55	0.15	0.86
Afatinib+ EGF+ FCS−	211,044	8,015	217	2,653	0.04	0.00	0.01
Afatinib+ EGF− FCS+	150,069	10,702	7,277	0	0.07	0.05	0.00
Afatinib+ EGF+ FCS−	106,927	1,239	2,788	4,655	0.01	0.03	0.04
Afatinib+ EGF+ FCS+	94,106	2,134	0	34,724	0.02	0.00	0.37

Figure S1 The relative quantity of phosphorylated EGFR, AKT, and ERK in HNE-1 cell line.
Notes: (**A**) Statistical graph of the relative quantity. (**B**) The gray values and the relative values.
Abbreviations: EGF, epidermal growth factor; ERK, extracellular regulated protein kinase; FCS, fetal calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HNE-1, human NPC cell line; pEGFR, phosphorylated epidermal growth factor receptor; p, phosphorylated.