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Original Article

Preclinical evaluation of afatinib (BIBW2992) in esophageal squamous cell carcinoma (ESCC)

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Abstract: Esophageal squamous cell carcinoma (ESCC) is the eighth most common cancer worldwide. Epidermal growth factor receptors (EGFR) are often overexpressed in esophageal cancers, thus anti-EGFR inhibitors have been evaluated in ESCC. Afatinib was an irreversible inhibitor of these ErbB family receptors. This study characterized the preclinical activity of afatinib in five ESCC cell lines: HKESC-1, HKESC-2, KYSE510, SLMT-1 and EC-1. ESCC cell lines were sensitive to afatinib with IC_{50} concentrations at lower micro-molar range (at 72 hour incubation: HKESC-1 = 0.002 μM, HKESC-2 = 0.002 μM, KYSE510 = 1.090 μM, SLMT-1 = 1.161 μM and EC-1 = 0.109 μM) with a maximum growth inhibition over 95%. Afatinib can strongly induce G_0/G_1 cell cycle arrest in HKESC-2 and EC-1 in a dose- and time-dependent manner. The phosphorylation of ErbB family downstream effectors such as pAKT, pS6 and pMAPK were significantly inhibited in HKESC-2 and EC-1. Apoptosis was observed in both cell lines at 24 hours after exposure to afatinib, as determined by the presence of cleaved PARP. Afatinib could effectively inhibit HKESC-2 tumor growth in mice without obvious toxicity. Afatinib alone has shown excellent growth inhibitory effect on ESCC in both *in vitro* and *in vivo* models, however, no synergistic effect was observed when it was combined with chemotherapeutic agents such as 5-fluorouracil (5-FU) and cisplatin. In summary, afatinib can inhibit cell proliferation effectively by arresting the cells in G_0/G_1 phase, as well as inducing apoptosis in ESCC. These findings warrant further studies of afatinib as therapeutic agent in treating ESCC.

Keywords: Afatinib, ESCC, EGFR, HER2, AKT

Introduction

Esophageal squamous cell carcinoma (ESCC) is the eighth most common cancer worldwide [1] and is endemic in Northern China, Japan, central Asia, parts of South America, South and East Africa. Genetic and environmental factors (e.g. dietary intake of nitrosamine, hot beverages) have been implicated in its pathogenesis. ESCC is the commonest histologic type, although Barrett's esophagus-associated esophageal adenocarcinoma is more prevalent in Western countries. ESCC is the sixth most common cause of death from cancer [1] with 386,000 deaths (5.7% of the total) worldwide. Patients usually present at an advanced stage, therefore the 5-year overall survival rate is only between 10-15% in developed countries like USA and Europe. In Hong Kong, the treatment of ESCC depends on the clinical stage, in which surgery is indicated for operable, localized disease, while radical concurrent chemoradiotherapy is reserved for patients with locoregionally advanced or inoperable disease operable [2]. Palliative chemotherapy with platinum-based regimen either with taxane, or 5-fluorouracil, (5-FU), is generally indicated for patients with recurrent or metastatic ESCC. Response rate to these agents is modest and to date, no systemic agent has significantly improved the survival of patients with ESCC.

The epidermal growth factor receptor (EGFR) and its ligands such as transforming growth factor alpha (TGF-α) and epidermal growth factor (EGF) are often overexpressed in esophageal cancers, which may be activated by autocrine mechanisms. These EGFR ligands are mitogenic in ESCC cell lines, while activation of EGFR may increase the expression of matrix metalloproteases (MMPs) or integrins, therefore promoting tumor invasion and metastasis

[3]. Anti-EGFR inhibitors such as cetuximab and gefitinib have been evaluated in randomized studies of ESCC, either in combination with radiotherapy or chemotherapy. In a randomized phase II study of advanced ESCC, the addition of cetuximab led to a modest increase in response rate from 13% to 19%, and with progression-free survival from 5.7 to 9.5 months [4]. In a multicenter phase III study of patients with advanced esophageal squamous cancer or adenocarcinoma who failed prior chemotherapy, the use of gefitinib was associated with a modest improvement in progression-free survival and quality of life, but not overall survival [5]. Therefore, EGFR inhibitors have shown promising clinical activity, but none of the randomized studies to date have met the primary study endpoints.

Unlike other tyrosine kinase inhibitors such as gefitinib, afatinib (BIBW2992) is an irreversible inhibitor of ErbB family (EGFR and HER2), which has activity against lung cancer and other cancers. In phase I/II trials, afatinib was effective in patients with solid tumors, including those with esophageal cancer, or lung cancer that contained activating EGFR mutations. Afatinib was generally well tolerated with the main adverse effects being gastrointestinal or cutaneous toxicities. It is now approved for the treatment of lung cancer [6, 7] and is being evaluated in phase II trials for breast and prostate cancers, head and neck carcinoma, as well as glioma [8]. Recently, afatinib is found to be active against cetuximab-resistant cancer cell lines that express increased phosphorylation of a c-terminal fragment of HER2 (611-CTF)-when combined with cetuximab [9]. It also downregulates the expression of thymidylate synthase in lung cancer cells and has synergistic effect on cell growth inhibition when combined with 5-FU or pemetrexed [10]. Co-expression of EGFR, HER2 and HER3 may be associated with response to afatinib in colorectal cancer cells, while treatment with afatinib resulted in an increase in G_0/G_1 cycle arrest [11]. In this study, we hypothesized that afatinib may inhibit ESCC cell growth by abrogating EGFR signaling.

Materials and methods

Drugs, chemicals, and antibodies

Afatinib (BIBW2992) was kindly provided by Boehringer Ingelheim GmbH (Germany). Cisplatin was manufactured by Pharmacheime B.V. (Harrlem, The Netherlands). 5-fluorouracil was manufactured by APP Pharmaceuticals, Inc. (Schaumburg, IL). RPMI-1640 medium and fetal bovine serum (FBS), sodium pyruvate (10 mM), penicillin (50 IU/ml) and streptomycin (50 ug/ml) were from Hyclone, Thermo Fisher Scientific (Logan, Utah, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Amersco (Solon, OH). Amersham ECL Western blotting detection reagents were purchased from GE Healthcare Biosciences (Pittsburgh, PA). The following antibodies were bought from Cell Signaling Technology (Danvers, MA): antibodies recognizing EGFR (#4267), HER2 (#4290), HER3 (#12708), Caspase 3 (#9665), PARP (#9542), cleaved PARP (#9541), p-AKT(S473) (#4060), AKT(pan) (#4691), p-S6 (#2215), S6 (#2217), p-p44/42 MAPK (#9101), p44/42 MAPK (#9102), GAPDH (#2118). Phospho-EGFR pTry1086 (#36-9700) was bought from Life Technologies while Actin (#CP01) was purchased from Calbiochem.

Drug preparation

Afatinib was dissolved in DMSO at 100 mM and stored in aliquot at -80°C as recommended by the manufacturer. Aliquots were diluted in corresponding medium just before addition to cell cultures. Cisplatin was prepared as a 1 mg/ml stock solution in 0.9% NaCl. 5-FU was diluted from 50 mg/ml stock solution (Vehicle Control: 9 mg/ml NaCl + 1 mg/ml mannitol; Molecular Weight: 300.05; 1 mg/ml = 3.33 mM).

Cell culture

Human ESCC cell lines, EC-1 (human well-differentiated ESCC), HKESC1 and HKESC2 (moderately differentiated ESCC from Chinese patient) [12, 13], SLMT1 [14], KYSE510 (ESCC from Japanese patient) [15] were generous gifts from Prof. Qian Tao. All cell lines were cultured in RPMI with 10% fetal bovine serum (FBS).

Assay of cytotoxicity

Cytotoxicity was assessed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Tumour cells were cultured in 48-well plates (3000-8000 cells per well) in respective culture medium. Afatinib in complete medium was added at 24 hr after cell plating and incubated at 37°C with 5% CO_2 for 48 and 72 hr. Cell growth inhibition

was expressed as the percentage of absorbance of control cultures measured at 570 nm with a microplate reader (PerkinElmer 1420 Multilabel Counter VICTOR3, Waltham, Massachusetts, USA) and 50% of the maximum growth inhibition (IC_{50}) was calculated by GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA). In each experiment, triplicate wells were performed for each drug concentration (n = 3), and assay was repeated in three independent experiments.

Western blot analysis

Cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail for 10 minutes at 4°C and the lysate was then centrifuged at 4°C, 12,000 rpm for 10 minutes. Supernatant was collected for protein quantitation with Bicinchoninic Acid (BCA) Assay (Pierce Protein Biology) and bovine serum albumin of known concentration as the standard. Twenty-five micrograms of total protein was resolved on SDS-PAGE gel and transferred onto the Trans-Blot nitrocellulose membrane using wet transfer machine (BioRad Laboratories, Hercules, CA). After protein transfer, the membrane was blocked with 5% non-fat dry milk, 0.2% Tween 20 in Tris-buffered saline (TBST) for 2 hours at room temperature. The membrane was incubated with corresponding primary antibody at 4°C overnight and washed thrice with TBST for 15 minutes each. The membrane was then incubated with corresponding secondary antibody for 1 hour at room temperature and washed thrice for 15 minutes. The blot was developed with GE Amersham ECL chemiluminescent substrate by autoradiography.

Cell death detection by enzyme-linked immunosorbent assay (ELISA)

The mechanism of cell death was quantitatively determined using the cell death detection ELISA^{PLUS} assay (Roche, Germany). Briefly, HKESC-2 and EC-1 (0.1×10^4 cells/well) were plated in 96-well flat-bottomed tissue culture plates for 24 hours at 37°C in a humidified incubator before afatinib treatment. The plate was centrifuged at 600 g at 4°C for 10 minutes. The cell pellet containing the apoptotic bodies was resuspended in lysis buffer and incubated for 30 minutes at room temperature. The plate was centrifuged and cell lysate (20 µl) was transferred into streptavidin-coated microplate in triplicate wells, followed by the immune-reagent containing a mixture of anti-histone-biotin and anti-DNA-POD for nucleosome capture (80 µl). The plate was covered with an adhesive cover foil and incubated for 2 hours at 25°C in a microplate shaker at 300 rpm. The wells were washed with incubation buffer to remove unbound antibodies. Substrate (ABTS) was added to the wells for color development and the reaction was stopped by ABTS stop solution. The amount of nucleosomes retained by the POD in the immune-complex was quantitatively determined using microplate reader at a wavelength 405 nm and reference wavelength of 490 nm.

Cell cycle analysis

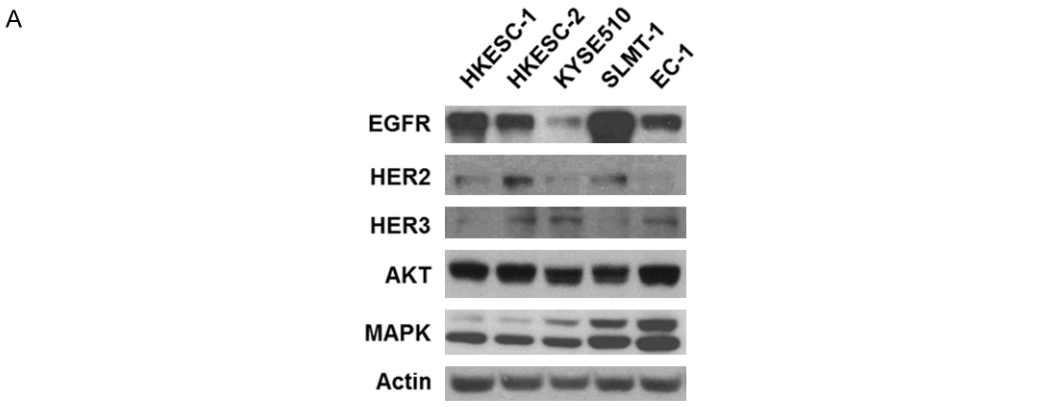
1-2.2 × 10^5 cells were plated in 50-mm^2 Petri dishes, treated with various drug doses according to the individual study. Cells were collected at 16, 24 and 48 hours by trypsinization, fixed with 70% cold ethanol and stored at -20°C. DNA staining was performed with a solution containing RNase (5 µg/ml) and propidium iodide (0.02 µg/ml). Analysis was performed using a BD Bioscience FACSCalibur flow cytometer while data of cell cycle were processed and analyzed with CELLQuest software (Becton Dickinson) and ModFit 4.0 (Verity Software House) respectively. Assay was repeated in three independent experiments.

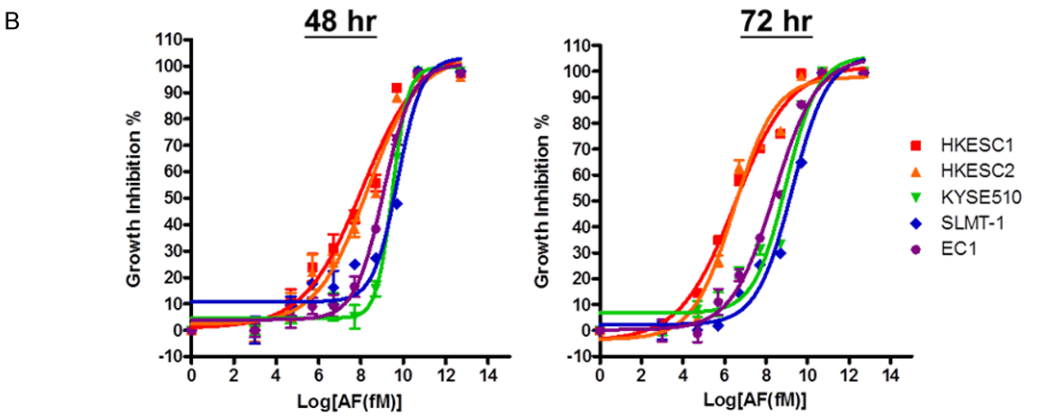
Live cell imaging

HKESC-2 and EC-1 cells (1-1.5 × 10^4) were plated in 24-well plate and cultured in RPMI-1640 medium in Axio Observer Z1 (Carl Zeiss, Germany) with live cell chamber (37°C, 5% CO_2 and humidity control). Afatinib (100 nM and 1 µM for HKESC-2, 1 µM and 10 µM for EC-1) was added to the well right before the start of time-lapse imaging. Cell proliferation and cell division were monitored by time-lapse imaging using ZEN imaging software. Images were captured at 3 hours interval for 72 hours.

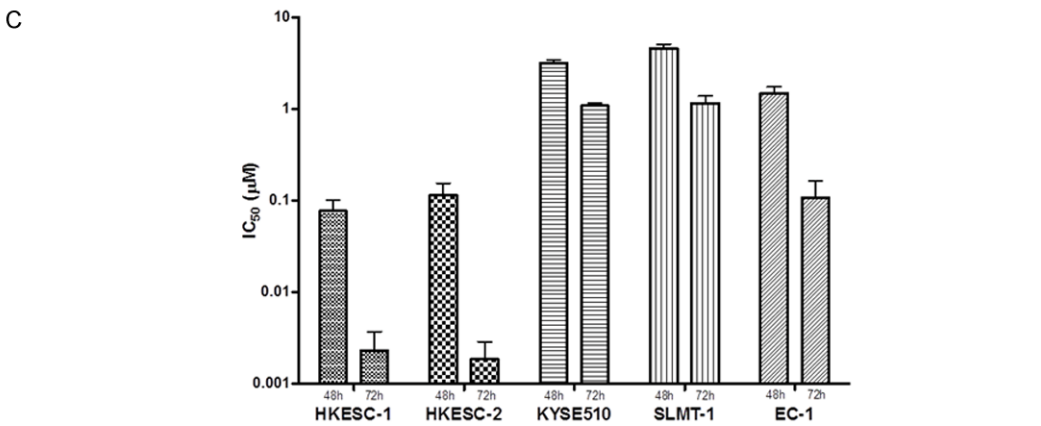
In vivo study of tumor xenograft

Six weeks old female athymic nude mice (nu/nu) weighing about 16-20 gram were housed by Laboratory Animal Services Centre of The Chinese University of Hong Kong. The experiment was conducted by researchers under license from the Hong Kong Government Department of Health and according to approval





Max Inhibition (%)	HKESC-1	HKESC-2	KYSE510	SLMT-1	EC-1
48hr	97.45±0.28	96.23±0.68	97.73±0.53	95.79±1.64	98.03±0.61
72hr	99.67±0.29	98.87±0.34	99.55±0.24	98.58±0.50	99.30±0.13



IC_{50} (μM)	HKESC-1	HKESC-2	KYSE510	SLMT-1	EC-1
48hr	0.078±0.024	0.115±0.041	3.182±0.258	4.625±0.488	1.489±0.269
72hr	0.002±0.001	0.002±0.001	1.090±0.071	1.161±0.224	0.109±0.055

Figure 1. A. Basal protein expressions of ErbB family and signaling components in all ESCC cell lines. B. Representative dose response curve on HKESC-1, HKESC-2, KYSE510, SLMT-1 and EC-1 and more than 95% of maximum inhibition were observed at 48 and 72 hours of afatinib treatment. C. Corresponding IC_{50} for HKESC-1, HKESC-2, KYSE510, SLMT-1 and EC-1 at 48 and 72 hours were in lower end micro-molar concentrations.

given by Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

ESCC xenografts were established by inoculating HKESC-2 (0.6×10^5 cells re-suspended in

50 μl of HBSS-buffer) subcutaneously into both flanks of the nude mice. When tumor size reached to 4-6 mm diameter, they were randomized in either treatment (15 mg/kg) or vehicle control group. Afatinib for treatment was prepared by dissolving in 0.5% methylcellulose before administration. Either drug or vehicle was administered to mouse by oral gavage in a schedule of 5 days on plus 2 days off for two weeks. Drug efficacy was evaluated by monitoring the change in tumor size with caliper. Tumor volume was calculated with the formula Tumor Volume = (width^2 × length)/2.

Statistical analysis

Statistical analyses were performed using PRISM4 Software (GraphPa, La Jolla, CA) and unpaired T-test with Welch Correction. Findings were considered as statistically significant when *P* value is less than 0.05.

Results

Effect of afatinib on cell viability in ESCC cell lines

EGFR was strongly expressed in HKESC-1, SLMT-1 and EC-1 cell lines, and HER2 was strongly detected in HKESC-2 and SLMT-1 cell lines as basal condition. AKT was strongly expressed in all of ESCC cell lines and MAPK was differentially expressed among these cells (**Figure 1A**). All 5 ESCC cell lines were sensitive to afatinib, in which over 95% of growth inhibition was observed after 48 and 72 hours of treatment (**Figure 1B**). The respective IC_{50} concentrations at 48 hours (HKESC-1 = 0.078 μM, HKESC-2 = 0.115 μM, KYSE510 = 3.182 μM, SLMT-1 = 4.625 μM and EC-1 = 1.489 μM) and 72 hours (HKESC-1 = 0.002 μM, HKESC-2 = 0.002 μM, KYSE510 = 1.090 μM, SLMT-1 = 1.161 μM and EC-1 = 0.109 μM) were all in lower micro-molar range (**Figure 1C**). HKESC-2 (with highest HER2, very sensitive to afatinib) and EC-1 cells (with lowest HER2, mildly sensitive to afatinib) were chosen for further experiments.

Afatinib induced cycle arrest in ESCC cell lines

The effect of afatinib on cell cycle arrest in HKESC-2 cells was observed dose-dependently since 16 hours of treatment, where the percentage of cells in G_0/G_1 phase was increased from 38.2% to 68.1% at 0.01 μM of afatinib and to 74.7% at 0.1 μM of afatinib. The G_0/G_1 arrest induced by afatinib in HKESC-2 cells also showed a time-dependent effect, in which more cells were arrested with longer exposure of afatinib, from 24 hours (82.4% G_0/G_1 arrest at 0.01 μM and 86.2% at 0.1 μM) to 48 hours (from 74.7% to 88.2% for 0.01 μM and 91.0% for 0.1 μM) (**Figure 2A**).

In EC-1 cells, afatinib in EC-1 could induce G_0/G_1 arrest at concentrations below and near the IC_{50} concentrations (0.1 μM, 1 μM) following 16 hours of exposure. A time-dependent effect was observed at concentration below the IC_{50} (0.1 μM), in which the percentage of cells arrested in the G_0/G_1 phase was increased from 59.1% to 66.6% and 72.2% at 24 and 48 hours respectively. However, the time dependent effect was not observed at the near-IC_{50} concentration of afatinib (1 μM) (**Figure 2B**). Live cell imaging on ESCC cell growth for 72 hours showed that cell proliferation and division were more significantly reduced in cell lines treated with afatinib than the untreated controls. Cell death of HKESC-2 was clearly found in higher concentration of Afatinib but not in EC-1. (**Figure 2C**).

Effects of afatinib on AKT signaling in ESCC cell lines

Afatinib reduced the phosphorylation of EGFR and the endogenous expression level of HER2 receptors in ESCC cells. In HKESC-2 and EC-1 cell lines, afatinib could suppress AKT phosphorylation in a dose and time dependent manner, in which the phosphorylation was almost completely abrogated when the cells were treated with concentrations around their corresponding IC_{50} (HKESC-2: 0.1 μM, EC-1: 1 μM) for 24 hours (**Figure 3**). Afatinib could also significantly reduce the phosphorylation level of the downstream effectors of the AKT-mTOR axis especially in HKESC-2 cells. The phosphorylation of MAPK was abolished in all treated samples, suggesting that afatinib could inhibit the two major downstream pathways of the ErbB/HER axis, namely, AKT and MAPK pathways in ESCC cell lines.

Effects of afatinib on apoptosis in ESCC cell lines

Apoptosis was detected in both HKESC-2 and EC-1 cell lines following treatment with afatinib

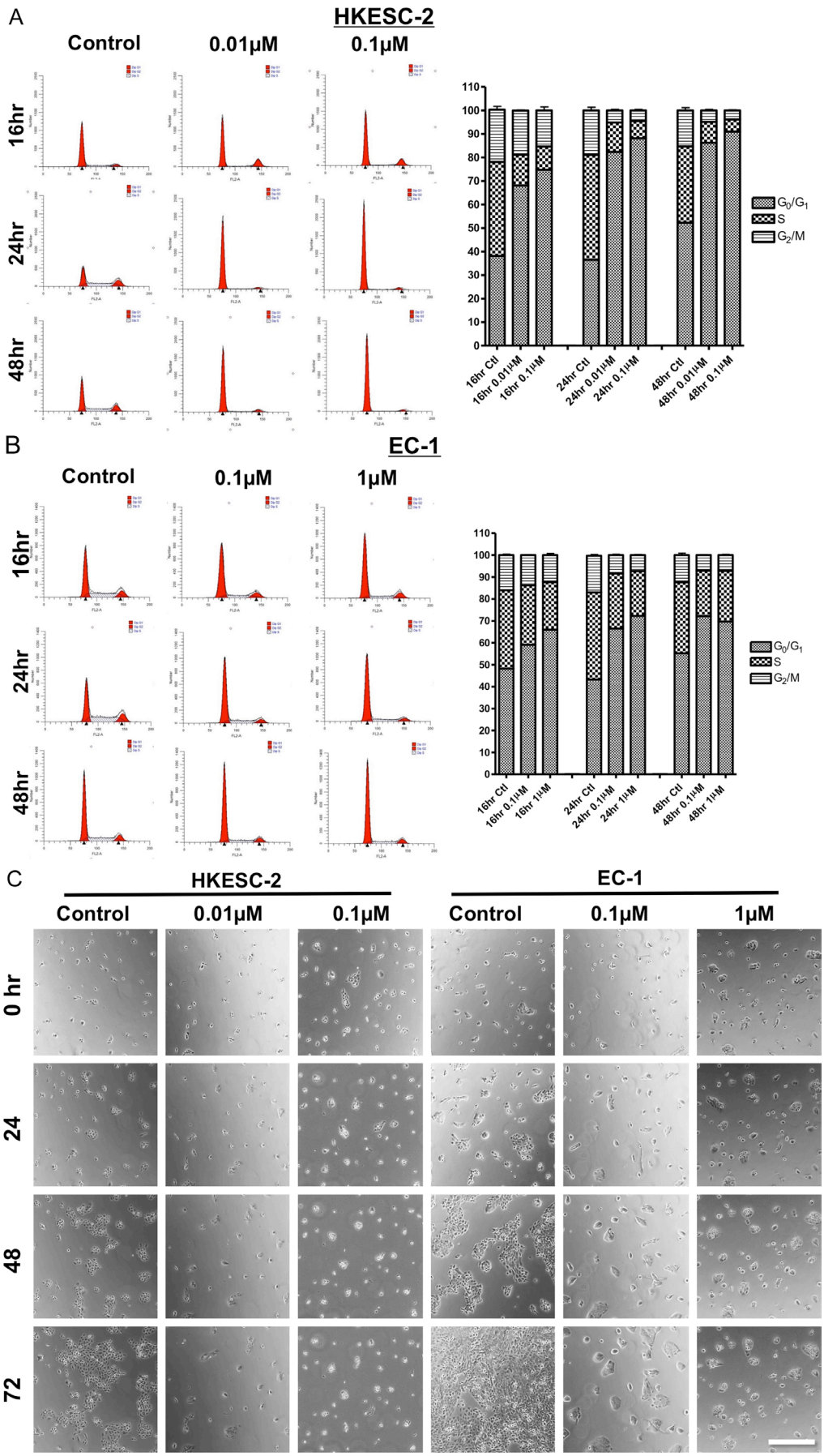

A
HKESC-2
Control
0.01μM
0.1μM
16hr
24hr
48hr
110
100
90
80
70
60
50
40
30
20
10
0
G0/G1
S
G2/M
16hr Ctl
16hr 0.01μM
16hr 0.1μM
24hr Ctl
24hr 0.01μM
24hr 0.1μM
48hr Ctl
48hr 0.01μM
48hr 0.1μM
B
EC-1
Control
0.1μM
1μM
16hr
24hr
48hr
110
100
90
80
70
60
50
40
30
20
10
0
G0/G1
S
G2/M
16hr Ctl
16hr 0.1μM
16hr 1μM
24hr Ctl
24hr 0.1μM
24hr 1μM
48hr Ctl
48hr 0.1μM
48hr 1μM
C
HKESC-2
EC-1
Control
0.01μM
0.1μM
Control
0.1μM
1μM
0 hr
24
48
72

Figure 2. Effect of afatinib on cell cycle distribution in (A) HKESC-2 and (B) EC-1 cell lines at 16, 24 and 48 hours treatment. Afatinib induced G1 cell cycle arrest in both tested ESCC cell lines in a time and dose dependent manner. (C) Live cell imaging revealed that afatinib could effectively suppress HKESC-2 and EC-1 cell proliferation and division compared with the untreated control (scale bar = 500 μm).

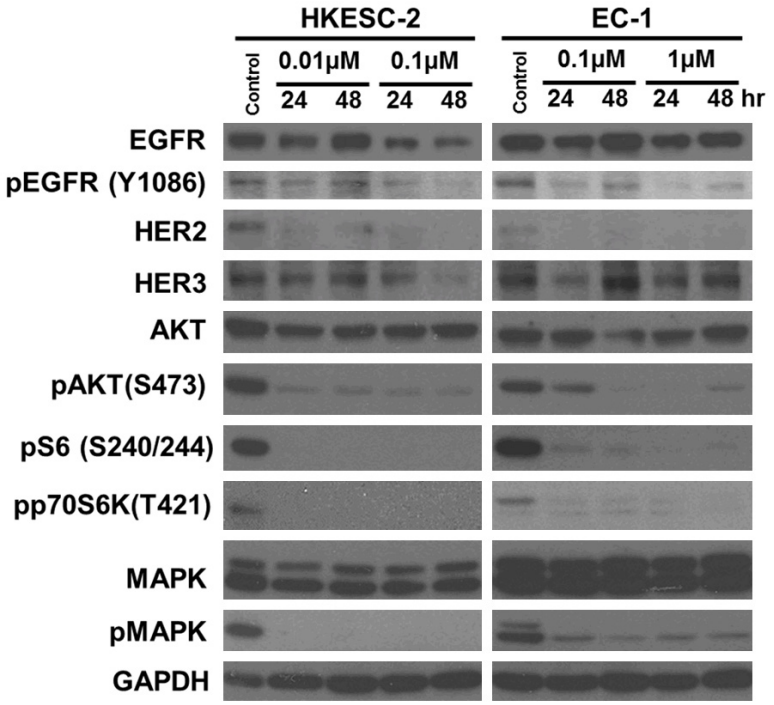


Figure 3. Effect of afatinib on AKT signaling pathway of HKESC-1 and EC-1 cell lines. Protein expression was detected by immunoblotting after 24 and 48 hours of treatment. Phosphorylation of AKT was inhibited effectively with the treatment of afatinib at a concentration that close to IC_{50} (at 48 hours) and its one-tenth (ADP-ribose) polymerase concentration. The phosphorylation of downstream effectors of the AKT-mTOR axis, p70S6K and S6, were also abolished.

for 24 and 48 hours in a dose dependent manner, as indicated by the presence of cleaved PARP. The apoptotic effect was more prominent at 48 hours as shown by a stronger expression level of cleaved Poly (ADP-ribose) polymerase (PARP) in these cell lines (**Figure 4A**). Cell death ELISA assay revealed that cleaved nucleosomes were detected at concentrations below, at and above the IC_{50} for afatinib (at 0.5 × IC_{50}, at IC_{50} and at 2 × IC_{50} concentrations) in both cell lines. These results indicated that afatinib could effectively induce cell death by triggering apoptotic mechanisms in ESCC cell lines.

In vitro activity of afatinib in combination with 5-FU and cisplatin

Two concentrations of afatinib were used in order to study the combination effect with two cytotoxic agents which are commonly used in clinical practice for ESCC, namely, 5-FU and cisplatin. HKESC-2 and EC-1 cells were treated with different concentrations of afatinib alone (0.058 μM and 0.115 μM for HKESC-2 cells, 0.75 μM and 1.5 μM for EC-1 cells), 5-FU alone (at concentrations of 5 μM and 50 μM), cisplatin alone (at concentrations of 0.3 μM and 1.7 μM for HKESC-2, 1.7 μM and 8.3 μM for EC-1), or in combination (afatinib and chemotherapy) for 48 and 72 hours. Afatinib could only slightly enhance the growth inhibitory effect with 5-FU and cisplatin (**Figure 5A**, **5B**). No synergism was observed between afatinib and 5-FU or cisplatin in all concentration and time points. Although the combinations evaluated were not antagonistic, our results suggested that afatinib should be further evaluated in the clinical setting as monotherapy rather than in combination with cisplatin or 5-FU in ESCC.

Inhibition of in vivo tumor growth in xenograft model

Drug administration started at 17 days post inoculation with either 15 mg/kg afatinib or vehicle. Mice in vehicle group were sacrificed at 24 days post inoculation due to rapid growth of tumor that might soon ulcerated while two week schedule was completed for treatment group. Tumor measurement continued until five days post afatinib treatment (**Figure 6A**, **6B**). The data showed that afatinib could strongly inhibit the growth of HKESC-2 tumor once the treatment began. Average tumor sizes of vehicle and treatment at end point were 348±24 mm^3 and 108±36 mm^3 respectively, showing

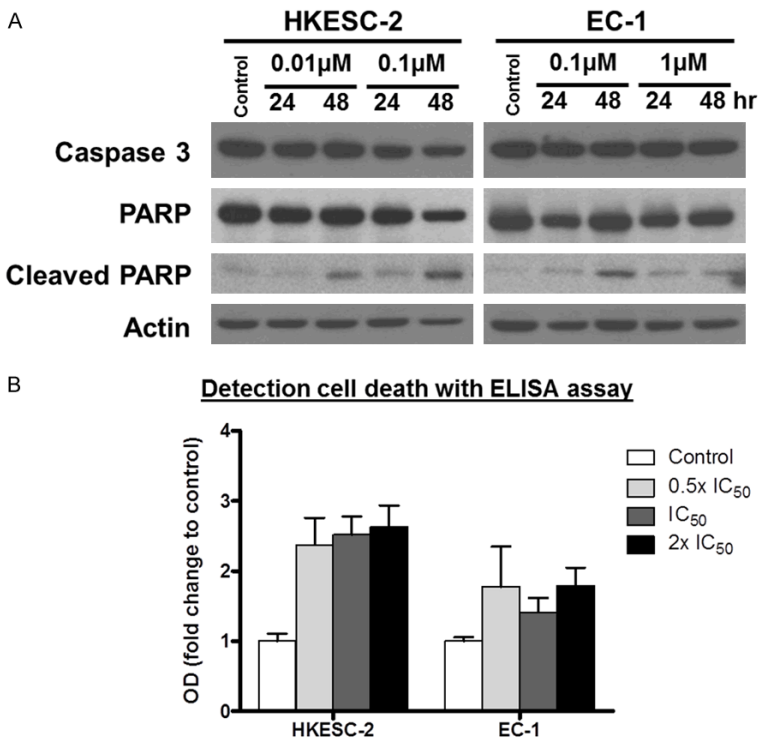


Figure 4. Afatinib effectively induced apoptotic cell death in HKESC-2 and EC-1. A. Cleaved caspase 3 and PARP were detected by immunoblotting after 24 and 48 hours treatment at a concentration that close to IC_{50} (at 48 hours) and its one-tenth concentration, indicating the activation of caspase 3 associated apoptotic mechanism. B. Nucleosomes were detected after treatment of afatinib which indicated the presence of DNA fragmentation upon apoptosis.

significantly difference between them. And apparently tumor size did not bounce back in a short period of time after the end of afatinib administration. Without rapid change of body weight throughout the treatment showed that the toxicity of afatinib was minimal and this drug is well tolerated (**Figure 6C**).

Discussion

The results of clinical studies on EGFR inhibitors have been disappointing. HER-2 overexpression can be found in around 23% (range 0-52%) of ESCC. In ESCC, HER2 overexpression has been correlated with extramural invasion and poor response to neoadjuvant chemotherapy [16]. A recent study found that HER2 amplification can be found in 16.6% of ESCC, and both HER2 protein overexpression and gene amplification may be poor prognostic factors in ESCC [17]. In this study, afatinib was effective in suppressing the growth of ESCC cell lines at lower end of micro-molar concentrations probably by inducing cell cycle arrest and apoptosis. Furthermore, it could significantly suppress AKT phosphorylation as well as the downstream targets of the AKT-mTOR axis, such as p70S6K and S6. It also reduced the endogenous expression of pMAPK in both ESCC cell lines. This inhibitory effect of afatinib on AKT signaling should theoretically drive cells into the cells G_1 cell cycle arrest effect which was observed in the ESCC cells tested in this study. However, at higher concentrations of afatinib, EC-1 cells underwent G_2/M instead of G_1 arrest (Data not shown). In animal study, afatinib was shown to effectively inhibit ESCC tumor while without showing obvious toxicity. Bounce back of tumor size was not observed after treatment may due to the effect of irreversible ErbB inhibition from afatinib, indicating it's a potential candidate for ESCC cancer treatment. However, in this study, no additive effect or a very weak enhancement of the growth inhibition was observed when ESCC cells were treated with afatinib and 5-FU or cisplatin. It has been previously reported that the combination of afatinib with 5-FU or pemetrexed was synergistic in inhibiting the proliferation of gefitinib-resistant NSCLC cell lines [10]. However in another study, the combination of afatinib with 5-FU was antagonistic in 3 out of 5 colorectal cancer cell lines tested, but with additive or synergistic effects in the other two cell lines [11]. The reason of such different outcomes in combined treatment is still unknown. From our study, the combined effect with 5-FU or cisplatin was neither antagonistic nor synergistic, but a very minor enhancement of cell growth inhibition was observed. One possible explanation is that afatinib could potentially reduce cellular sensitivity to cycle-dependent cytotoxic chemotherapy (e.g. antimetabolites) by arresting cancer cells in the G_0/G_1 phase. This is exemplified by clinical experience in non-small cell lung cancer

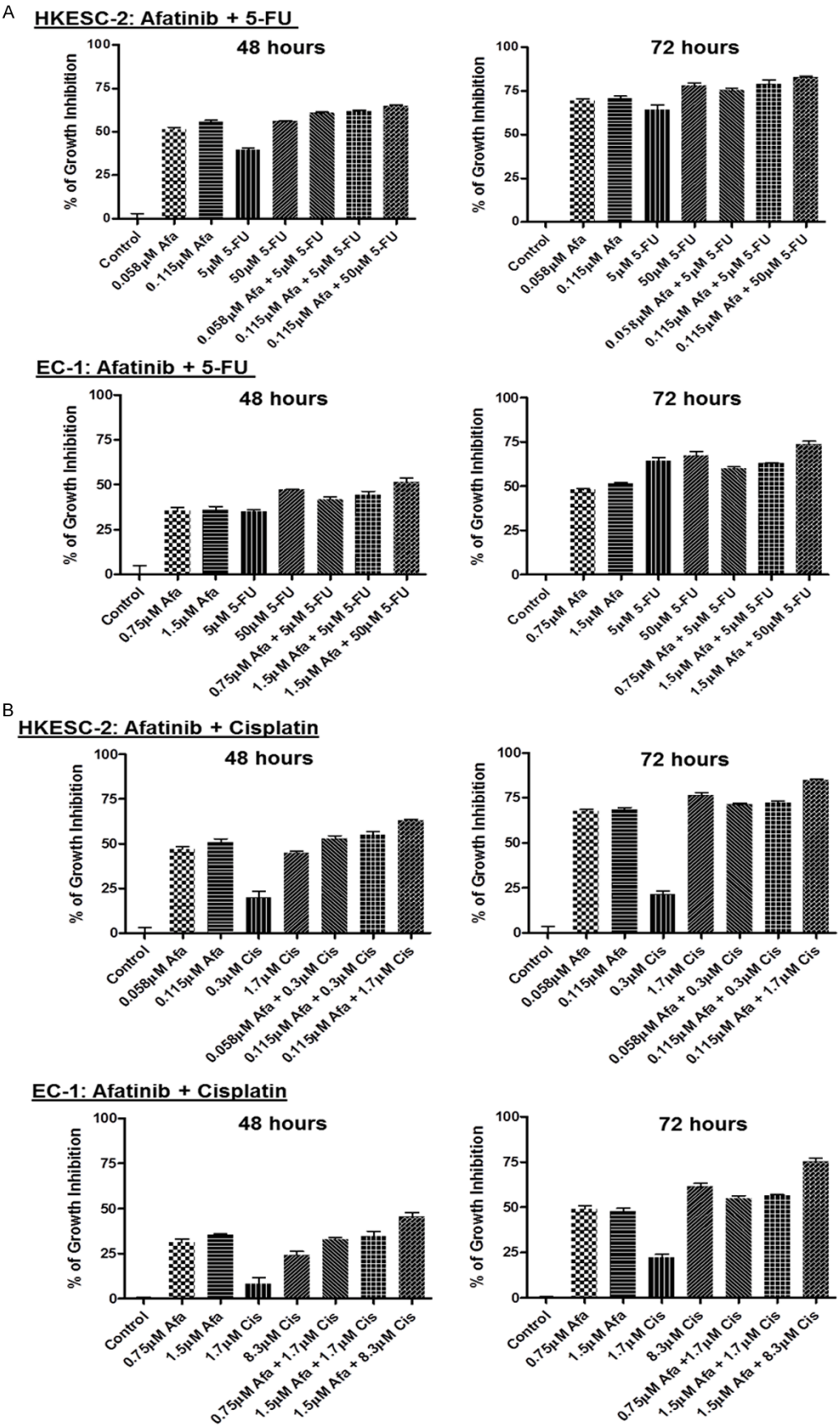

A
HKESC-2: Afatinib + 5-FU
48 hours
72 hours
% of Growth Inhibition
Control
0.058μM Afa
0.115μM Afa
5μM 5-FU
50μM 5-FU
0.058μM Afa + 5μM 5-FU
0.115μM Afa + 5μM 5-FU
0.115μM Afa + 50μM 5-FU
EC-1: Afatinib + 5-FU
48 hours
72 hours
Control
0.75μM Afa
1.5μM Afa
5μM 5-FU
50μM 5-FU
0.75μM Afa + 5μM 5-FU
1.5μM Afa + 5μM 5-FU
1.5μM Afa + 50μM 5-FU
B
HKESC-2: Afatinib + Cisplatin
48 hours
72 hours
Control
0.058μM Afa
0.115μM Afa
0.3μM Cis
1.7μM Cis
0.058μM Afa + 0.3μM Cis
0.115μM Afa + 0.3μM Cis
0.115μM Afa + 1.7μM Cis
EC-1: Afatinib + Cisplatin
48 hours
72 hours
Control
0.75μM Afa
1.5μM Afa
1.7μM Cis
8.3μM Cis
0.75μM Afa + 1.7μM Cis
1.5μM Afa + 1.7μM Cis
1.5μM Afa + 8.3μM Cis

Figure 5. A. Synergistic study of afatinib (0.057 μM or 0.115 μM) and 5-FU (5 μM or 50 μM) in HKESC-2 and EC-1 cell lines at 48 and 72 hours. B. Synergistic study of afatinib (0.058 μM or 0.115 μM) and cisplatin (0.3 μM or 1.7 μM for HKESC-2, 1.7 μM or 8.3 μM for EC-1) in HKESC-2 and EC-1 cell lines at 48 and 72 hours. A very slight enhancement of the growth inhibition was shown, but neither synergism nor antagonism was observed when afatinib was used with chemotherapeutic drugs in treating ESCC cell lines.

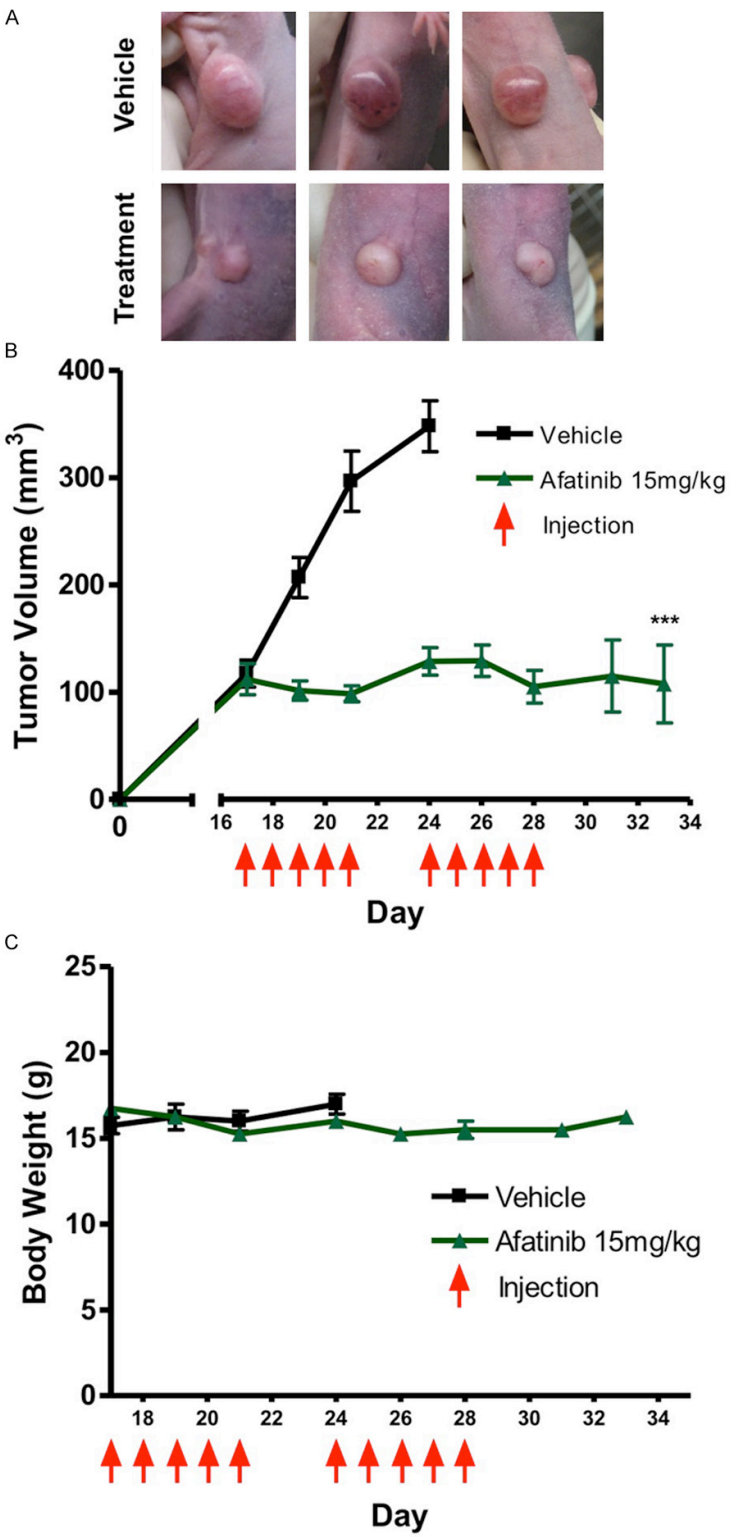


Figure 6. Effect of afatinib on HKESC-2 xenograft in nude mice. A. Representative nude mice with HKESC-2 xenograft in treatment/vehicle groups before tumor harvesting. B. Tumor volume versus number of day after inoculation. Afatinib treatment (N = 8) could evidently suppress tumor growth when compared with vehicle control (N = 6) (***P = 0.002 comparison between the end point of tumor size between them). C. Body weight of mice versus number of day after inoculation.

(NSCLC), where the addition of gefitinib to chemotherapy did not improve clinical outcome irrespective of the EGFR mutation status [18]. Our result suggests that afatinib could be evaluated as a single agent in ESCC patients.

In NSCLC, cell lines that harbored EGFR overexpression, *EGFR* mutation or HER2 overexpression were found to be more sensitive to afatinib, whereas cell lines that expressed mutated *KRAS, BRAF* or *NRAS* were less sensitive to afatinib [19, 20]. In this study, the expression levels of EGFR, HER2 or HER3 in ESCC cell lines did not have direct correlation with cellular sensitivity to afatinib. In the literature, *KRAS* and *BRAF* mutations were very uncommon in ESCC [21-23], except in a study which had reported a 12% prevalence of *KRAS* mutation in ESCC tumors [24]. Recently a large scale pyrosequence analysis had further supported that *KRAS* and *BRAF* mutations were rarely seen in ESCC. *KRAS* codon 12 and 13 mutations were only found in 1 out of 203 ESCC paraffin-embedded tis-

sues and *BRAF* exon 15 (V600E) mutation was absent in all samples [25]. This suggested that the KRAS and BRAF mutations are less likely to be important determinants of cellular sensitivity to afatinib in ESCC than in NSCLC.

Conclusion

ESCC cell lines were highly sensitive to afatinib treatment and we had demonstrated that extremely low micro-molar concentration of drug was effective in inducing G_0/G_1 cell cycle arrest and apoptosis, in which over 95% of growth inhibition was observed. In vivo study showed that afatinib was well tolerated and it could inhibit ESCC growth effectively. But the addition of chemotherapeutic drugs did not improve the preclinical efficacy of afatinib in ESCC. Our study supports the clinical evaluation of afatinib as a single agent in ESCC patients.

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Disclosure of conflict of interest

Brigette Ma-advisory board with Boerhinger Ingelheim. The other authors have no conflict of interests in this study.

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