

Original Article

DHA increases the anti-tumor effect of gefitinib on non-small cell lung cancer with EGFR mutations *in vitro*

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Abstract: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are approved as first-line therapy for patients with non-small cell lung cancer (NSCLC) harboring *EGFR* activating mutations. Docosahexaenoic acid (DHA) exerts anti-neoplastic activity in human lung cancer cells. In this study, we investigated whether DHA increases the anti-tumor effects of gefitinib on NSCLC cells with *EGFR* mutations and the related mechanisms of action. We determined the effects of DHA and gefitinib on the proliferation, apoptosis, cell cycle, and signaling pathways of NSCLC cells with *EGFR* activating mutations (PC9 cells) and TKI resistance (A549 cells). DHA had an obvious inhibitory effect on both cell lines, and enhanced the anti-tumor effects of gefitinib on the cells *in vitro*. Combined gefitinib and DHA therapy had a synergistic effect, inducing apoptosis, causing G0/G1 arrest in the PC9 cells and affecting EGFR and ERK1/2 signaling. These results suggest that DHA can act as a sensitizer of gefitinib in NSCLC cells with *EGFR* mutations. Nutritional intervention with DHA is a promising approach to enhancing the therapeutic effect of gefitinib.

Keywords: DHA, gefitinib, non-small cell lung cancer, EGFR mutation

Introduction

Lung cancer continues to be the leading cause of cancer mortality worldwide [1]. The high mortality is due to the fact that lung cancer is often detected at an advanced stage, and the opportunity for radical surgery is lost. Chemotherapy plays an important role in the management of advanced lung cancer; however, the adverse effects of chemotherapy are usually difficult to tolerate and the effect of chemotherapy is disappointing [2, 3]. In addition to radiation and chemotherapy, molecular targeted therapy has become a novel approach for treating advanced non-small cell lung cancer (NSCLC). In the last decade, targeted drugs such as tyrosine kinase inhibitors (TKIs) have greatly changed the management of patients with advanced epidermal growth factor receptor (EGFR)-mutated NSCLC [4, 5]. Gefitinib, a representative EGFR TKI, was approved by the US Food and Drug Administration for treating advanced NSCLC (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2003/021399_iressa.cfm) [6].

Docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid (n-3 PUFA) long approved as a dietary supplement, confers a broad range of health benefits [7]. DHA plays an important role in preventing and treating several chronic diseases, including cardiovascular, inflammatory, and neurodegenerative diseases. Epidemiological studies have also suggested that DHA-rich diets are inversely correlated with the development of cancer [8]. Recent *in vivo* and *in vitro* experimental studies have shown that n-3 PUFAs have significant anti-tumor action [7, 9-11]. Some researchers have also proved that n-3 PUFAs may enhance the effectiveness of some chemotherapy drugs [12].

The present study addresses the influence of DHA on gefitinib-induced cytotoxic action on the human lung adenocarcinoma cell lines PC9 and A549. We evaluated the effect of DHA, a representative n-3 PUFA, and gefitinib on cell growth and cell cycle progression in the two cell lines. We designed this study to determine whether DHA can be used as an adjuvant of gefitinib.

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This research also sheds some light on the mechanism of action involved, and it contributes to our understanding of molecular targeted therapies.

Materials and methods

Materials

Gefitinib was purchased from Selleckchem (Houston, TX, USA). Antibodies were from Abcam (Cambridge, MA, USA). DHA was purchased from Sigma (St. Louis, MO, USA). All other culture medium and additives were purchased from Sigma-Aldrich (Bangalore, India).

Cell culture conditions

The *EGFR*-mutated PC9 (*EGFR* exon 19del E746-A750) and wild-type *EGFR* A549 cell lines were purchased from Shanghai Gefan Biotechnology (Shanghai, China).

The cells were supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and were incubated in a humidified incubator in 5% CO₂ at 37°C.

MTT assay for the inhibition of cell growth

Cells (6×10^3 /well) were seeded in 96-well plates and incubated for 24 h. A series of concentrations of DHA (50, 75, 100 and 125 µg/ml) or GEF (PC9 cells: 20, 40, 60 and 80 nmol/l; A549 cells: 5, 10, 15, 20 µmol/l) were added to the wells for 24, 48 and 72 h. Meanwhile, as for the effect of DHA on gefitinib-induced cytotoxicity in NSCLC cells, gefitinib (PC9 cells: 60 nmol/l, A549 cells: 15 µmol/l) and a suboptimal dose of DHA (100 µg/ml) were added for 24, 48 and 72 h. At the end of each treatment period, viable cell numbers were measured using tetrazolium dye (MTT) assay. Briefly, MTT (5 g/l, 20 µl/well) was added to each well and incubated at 37°C for 4 h. Next, dimethyl sulfoxide (150 µl/well) was added to each well to dissolve any crystals, and the plates were agitated for 10 min. Absorbance values at 570 nm (A570) were detected using a microplate reader (Infinite M200; Tecan, Geneva, Switzerland). The rate of cell growth was expressed as the percentage of cell growth as compared with the blank control in the same treatment group.

Cell growth inhibition was calculated using the following formula: Cell growth inhibition rate (%) = $[1 - A570 \text{ (experimental group)} / A570 \text{ (control group)}] \times 100$. Each experiment was repeated three times.

The same doses of gefitinib and DHA were used to examine whether DHA had additive effects on gefitinib, as described in the following sections.

Colony forming assay

PC9 and A549 cells were plated into the 6cm plates at a density of 500 cells/dish and maintained in DMEM containing 10% FBS. After being incubated for 8 days, the cells were fixed with formaldehyde, and then were incubated in room temperature for one hour. Cells were stained with giemsa for 30 min before being photographed and counted. The colony numbers were counted using ImageJ software. All experiments were repeated three times.

Flow cytometry detection of apoptosis

Cells (2×10^5 /well) were seeded in 6-well plates and incubated with DHA and/or gefitinib for 24 h, and then collected by trypsinization and washed with phosphate-buffered saline (PBS). Following annexin V-phycoerythrin and 7-amino-actinomycin D staining, apoptosis was immediately detected using flow cytometry (Millipore, Billerica, MA, USA).

Examination of nuclear morphology

Cells (5×10^4 /well) were incubated with DHA and/or gefitinib for 24 h. Then, cells present in the monolayer were fixed in methanol, stained with the DNA-specific fluorochrome DAPI [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide], and their nuclear morphology was observed under a fluorescent microscope (Dialux, Lietz, Germany).

Analysis of cell cycle distribution

Cells (2×10^5 /well) were seeded in 6-well plates and treated with gefitinib and/or DHA for 48 h. Then, the cells were collected and washed with ice-cold PBS, fixed in 75% ethanol at 4°C for 12 h, and then washed twice with PBS. The cells were stained at 4°C for 30 min with propidium iodide (50 µg/ml in PBS), and 2×10^4 cells were analyzed using a flow cytometer.

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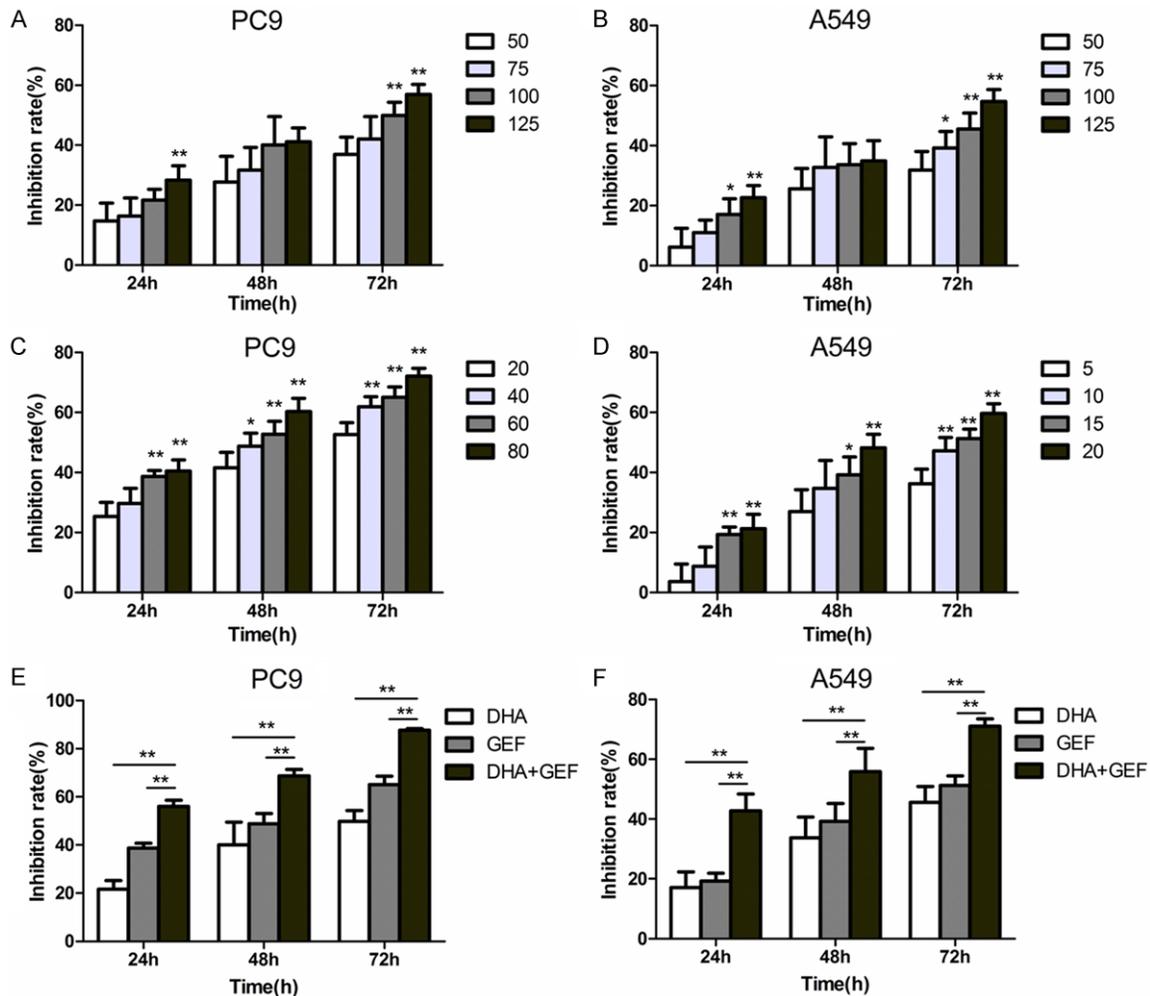


Figure 1. DHA and gefitinib (GEF) inhibit PC9 and A549 cell proliferation in a dose- and time-dependent manner. Cell proliferation was detected by MTT assay. PC9 (A, C) and A549 (B, D) cells were treated with DHA (50, 75, 100, 125 $\mu\text{g/ml}$) and GEF [20, 40, 60, 80 nmol/L (C); 5, 10, 15, 20 $\mu\text{mol/L}$ (D)] for 24 h, 48 h, and 72 h. In the combined treatment, 100 $\mu\text{g/ml}$ DHA and 60 nmol/L GEF were administered to the PC9 cells (E), and 100 $\mu\text{g/ml}$ DHA and 15 $\mu\text{mol/L}$ GEF were administered to the A549 cells (F). Bars represent the mean \pm SD of three separate experiments. Each test group was compared with the corresponding control group; * $P < 0.05$, ** $P < 0.01$.

Western blot analysis

After 1-h gefitinib and/or DHA treatment, the cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride and 1% phosphatase inhibitors. The cell lysates were analyzed using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (PerkinElmer, Waltham, MA, USA). The membrane was blocked in blocking buffer (5% bovine serum albumin in Tris-buffered

saline with 0.1% Tween 20 [TBST]) for 1 h at room temperature. Primary antibodies against EGFR, phosphorylated EGFR (p-EGFR), mitogen-activated protein kinase (ERK) 1/2, p-ERK1/2, and tubulin were diluted in blocking buffer and added to the membrane, which was gently shaken overnight at 4°C. After washing three times with TBST, the membrane was immersed in horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer for 1 h at room temperature, and then washed with TBST. The washed membranes were visualized using a chemiluminescence kit (PerkinElmer). The band intensity was measured by densitometry.

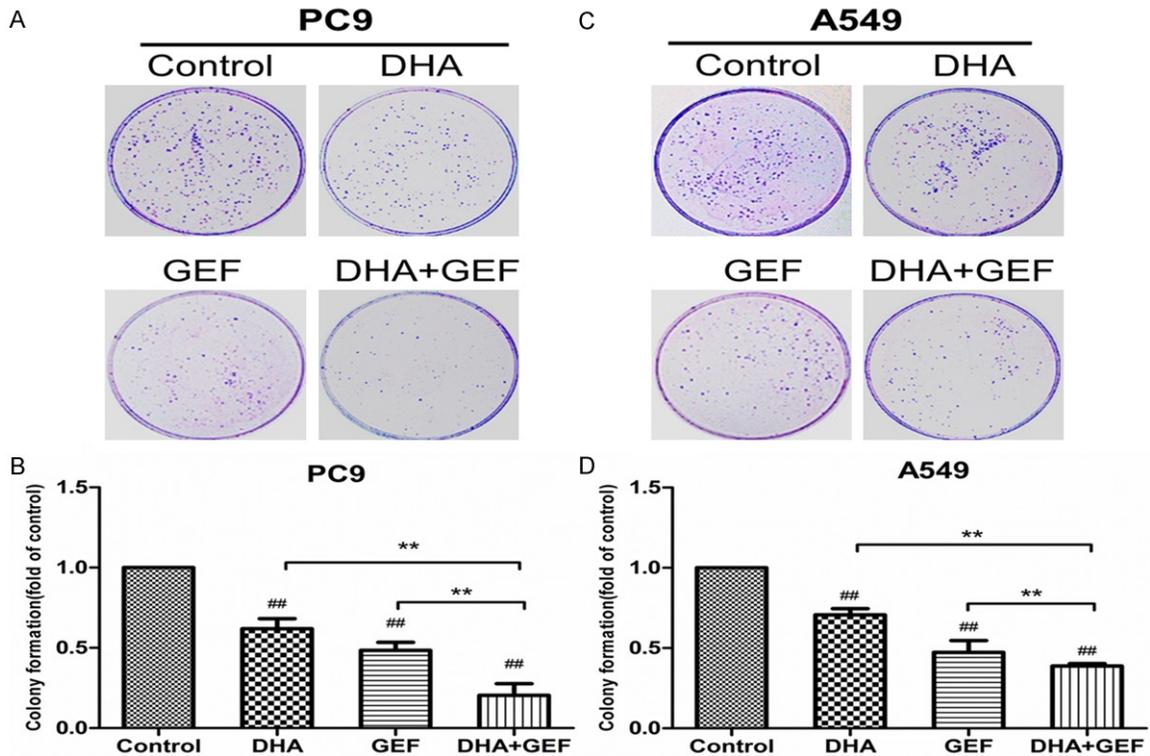


Figure 2. DHA and gefitinib (GEF) inhibit colony formation of PC9 and A549 cell lines. And the combination treatment led to significant suppression of colony forming capacity. The two cell lines were divided into four treatment groups, and treated with DHA and/or GEF for 24 h. Both cell lines were treated with 100 $\mu\text{g/ml}$ DHA; the PC9 and A549 cells were treated with 60 nmol/l and 15 $\mu\text{mol/l}$ GEF, respectively. (A and C) Are representative sets of images from 3 reproducible independent experiments. Bars represent the mean \pm SD of three separate experiments (B, D). ** $P < 0.01$, ## $P < 0.01$.

Statistical analysis

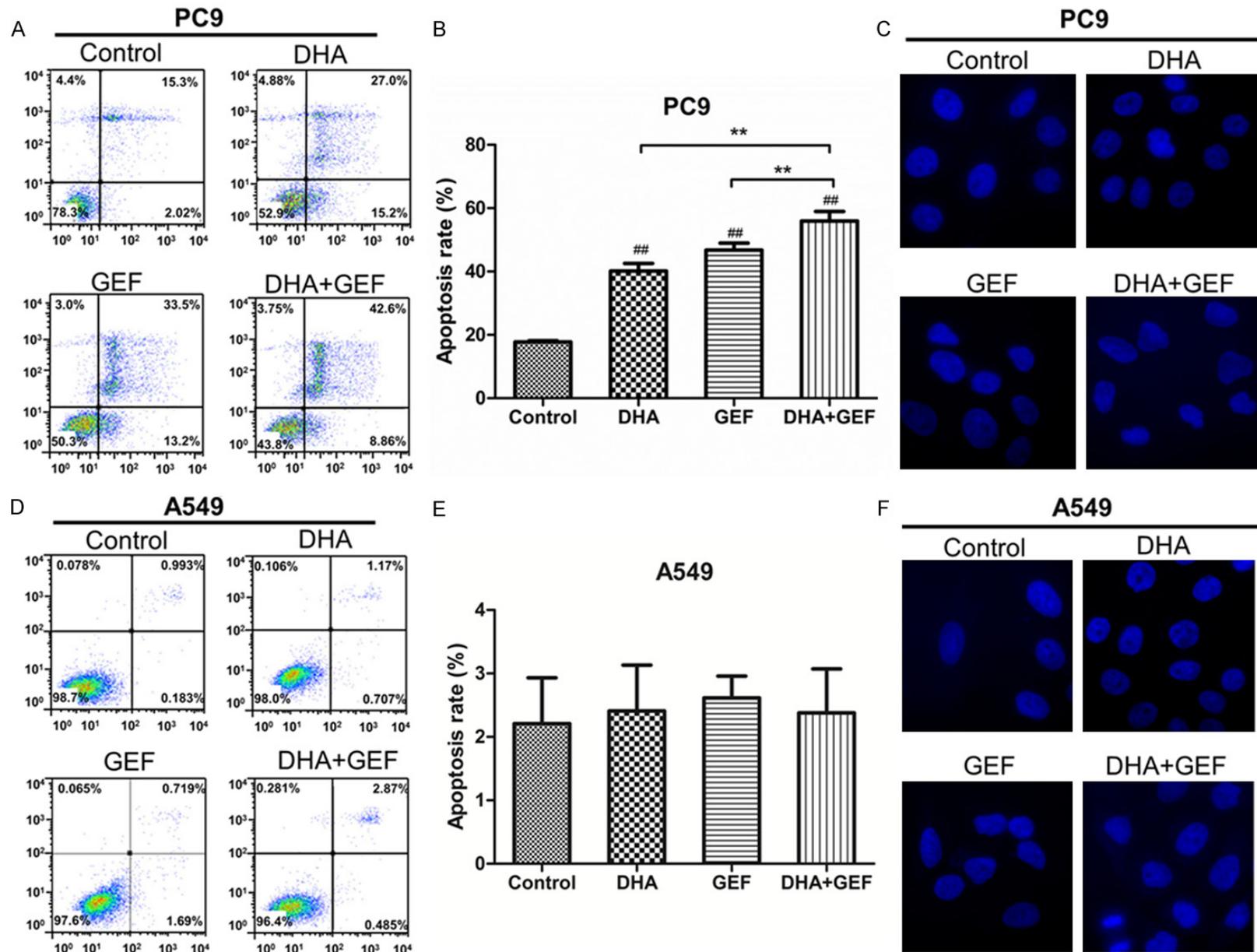
One-way analysis of variance (ANOVA) and following Newman-Keuls Multiple Comparison test were applied to analyze difference between multiple groups of cell proliferation, cell apoptosis, cell cycle progression and EGFR and ERK1/2 phosphorylation of cell lines. The data of at least three independent experiments are expressed as the mean \pm standard deviation (SD). Statistical significance ($P < 0.05$, $P < 0.01$) was indicated in the graphs by appropriate signal or double symbols, respectively. GraphPad Prism 5.0 software was used to analyze the results.

Results

DHA enhanced the gefitinib-induced inhibition of NSCLC cell growth

An MTT assay was used to examine the viability inhibition effect of DHA/Gefitinib on PC9/A549

cells. DHA suppressed PC9 and A549 cell viability in a concentration- and time-dependent manner (Figure 1A and 1B). Following 72-h treatment with 125 $\mu\text{g/ml}$ DHA, the rates of PC9 and A549 cell growth inhibition were $56.9 \pm 3.3\%$ and $54.8 \pm 3.9\%$, respectively. Gefitinib significantly suppressed PC9 and A549 cell viability in a concentration- and time-dependent manner (Figure 1C and 1D), and had a much stronger inhibitory effect on the PC9 cells. Following 72-h treatment with high concentrations of gefitinib (PC9 cells: 80 nmol/l ; A549 cells: 20 $\mu\text{mol/l}$), the rates of PC9 and A549 cell growth inhibition were $72.1 \pm 2.6\%$ and $59.7 \pm 3.2\%$, respectively. The combined gefitinib (60 nmol/l) and DHA (100 $\mu\text{g/ml}$) treatment had more potent inhibitory effects on the cell index as compared to gefitinib or DHA monotherapy. The combined therapy was superior to monotherapy in inhibiting the growth of both PC9 and A549 cells ($P < 0.01$) (Figure 1E and 1F). Following 72-h gefitinib plus DHA treat-



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Figure 3. DHA and gefitinib (GEF) induce apoptosis in PC9 and A549 cell lines. The two cell lines were divided into four treatment groups, and treated with DHA and/or GEF for 24 h. The percentage of apoptosis was determined by flow cytometry (A, D). Both cell lines were treated with 100 µg/ml DHA; the PC9 and A549 cells were treated with 60 nmol/l and 15 µmol/l GEF, respectively. Bars represent the mean ± SD of three separate experiments (B, E). Morphological changes in PC9 and A549 cells, including nuclear condensation and fragmentation, were observed following DAPI staining under a fluorescence microscope (C, F). **P < 0.01, ##P < 0.01.

ment, the rate of inhibition of PC9 cell growth was $87.6 \pm 0.7\%$.

DHA and Gefitinib inhibited NSCLC cell colony formation

A colony forming assay was used to examine the growth inhibition effect of DHA/Gefitinib on PC9/A549 cells. As is shown in **Figure 2A** and **2B**, DHA or Gefitinib alone significantly suppressed the colony forming capacity of PC9 cells compared with control group ($P < 0.01$). Combined gefitinib and DHA treatment significantly decreased the colony formation number of PC9 cells as compared with gefitinib or DHA monotherapy ($P < 0.01$). Meanwhile, as depicted in **Figure 2C** and **2D**, consistent results were gotten in A549 cell lines ($P < 0.01$). DHA combined with gefitinib significantly reduced A549 cell colony formation, which was also further than the cell line was treated with gefitinib or DHA alone. The results confirmed that DHA increased the gefitinib-induced inhibition of colony forming of NSCLC cells.

DHA enhanced gefitinib-induced apoptosis in PC9 cells

The effects of gefitinib and/or DHA on the induction of apoptosis in PC9 and A549 cells were detected by flow cytometry. Both gefitinib and DHA had slight apoptosis induction effects on the A549 cells, where the apoptosis index of the cells was increased, but without statistical significance as compared with the control group ($P > 0.05$) (**Figure 3D** and **3E**). Gefitinib or DHA significantly induced apoptosis in the PC9 cells ($P < 0.01$). Combined gefitinib and DHA treatment significantly increased the percentage of apoptotic cells as compared with gefitinib or DHA monotherapy ($P < 0.01$) (**Figure 3A** and **3B**). Therefore, DHA increases the gefitinib-induced apoptosis induced in NSCLC cells with *EGFR* activating mutation.

Figure 3C and **3F** depict the change in the nuclear morphology of NSCLC cells treated with DHA and/or gefitinib. The results con-

firmed that DHA increased the gefitinib-induced apoptosis in NSCLC cells, especially in NSCLC cells with *EGFR* activating mutation.

DHA enhanced gefitinib-induced G0/G1 accumulation in PC9 cells and S-phase decrease in A549 cells

DHA increased the anti-tumor effect of gefitinib on NSCLC cells *in vitro*. Next, we detected the effects of DHA and/or gefitinib on the cell cycle. In the PC9 and A549 cells (**Figure 4A** and **4D**), 48-h treatment with gefitinib and DHA monotherapy both significantly increased the percentage of G0/G1 cells and decreased the percentage of S-phase cells ($P < 0.05$) as is shown in **Figure 4B** and **4C**. Gefitinib and DHA induced G2/M accumulation in PC9 cells, but had no effect on the G2/M phase in A549 cells. Compared with monotherapy, combined gefitinib and DHA treatment increased the percentage of G0/G1 PC9 cells and decreased the percentage of S-phase A549 cells. The combined treatment increased the percentage of G2/M PC9 cells as compared to the control group, and decreased the percentage of G2/M PC9 cells as compared with monotherapy.

DHA enhanced gefitinib-induced inhibition of ERK1/2 phosphorylation in PC9 cells

In the PC9 cells (**Figure 5A**), gefitinib inhibited *EGFR* and ERK1/2 phosphorylation in an obvious manner. Gefitinib also inhibited p-*EGFR* and p-ERK1/2 expression in the A549 cells (**Figure 5B**), decreasing expression partially relative to the decrease in the PC9 cells. In the two cell lines, DHA upregulated *EGFR* phosphorylation and downregulated p-ERK1/2 expression.

In the PC9 and A549 cells, the combined treatment markedly reduced the level of *EGFR* phosphorylation as compared to the control group ($P < 0.01$), while *EGFR* phosphorylation was relatively higher than that following gefitinib monotherapy ($P > 0.05$). In the PC9 cells, the level of ERK1/2 phosphorylation following combined

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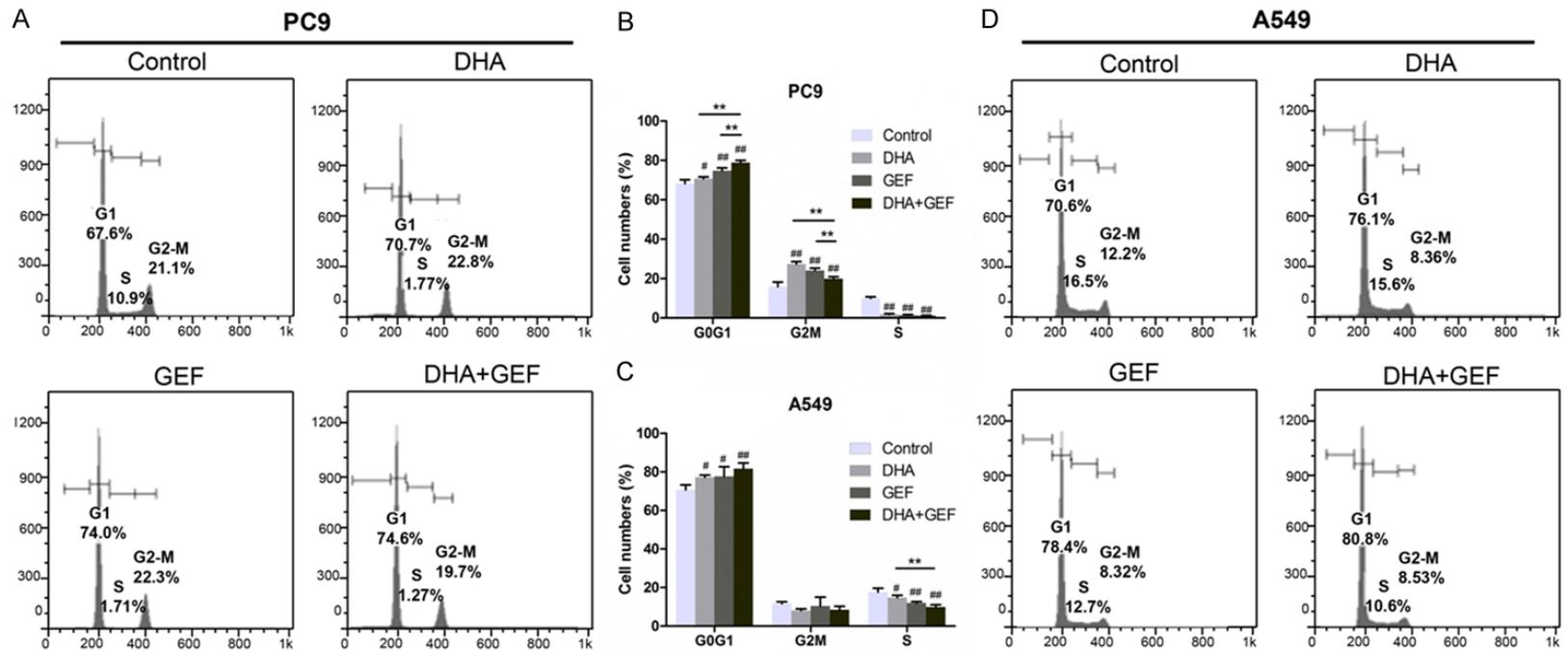


Figure 4. DHA and gefitinib (GEF) affected the PC9 and A549 cell cycles. The DNA content of PC9 (A) and A549 (D) cells was analyzed by flow cytometry; G0/G1, S, and G2/M are the different cell cycle phases. Histograms of three separate experiments are shown (B, C). #P < 0.05, ##P < 0.01 for test group compared with control, *P < 0.05, **P < 0.01 for DHA+GEF group compared with DHA or GEF monotherapy group.

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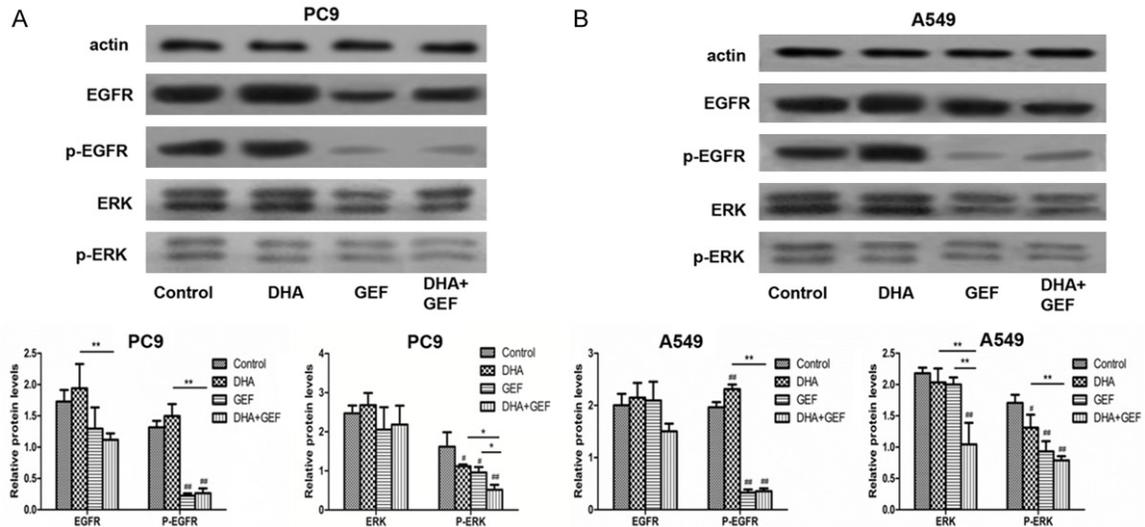


Figure 5. Western blot detection of the effect of DHA and gefitinib (GEF) on the protein expression of the EGFR and ERK signaling molecules in PC9 and A549 cells. (A) PC9 and (B) A549 cells were treated with gefitinib and/or DHA, and the data are expressed as the mean and SD of three separate experiments. #*P* < 0.05, ##*P* < 0.01 for test group compared with control, **P* < 0.05, ***P* < 0.01 for DHA+GEF group compared with DHA or GEF monotherapy group.

therapy was lower than that following monotherapy (*P* < 0.05).

Discussion

As an important factor in the development of cancer, DHA has anti-tumor effects on many tumor types but has few adverse effects on most normal cells [13-15]. The epidemiological and laboratory data suggest that DHA also has anti-tumor effects on lung cancer cells [10, 12, 16]. In the present study, DHA inhibited PC9 and A549 cell proliferation in a dose- and time-dependent manner. In addition, DHA enhances the effectiveness of some chemical agents: When combined with many chemotherapeutic drugs, including paclitaxel, celecoxib, or 5-fluorouracil, DHA enhances colon cancer cell responsiveness to the drugs [17, 18]. Polavarapu et al. found that DHA augments the growth inhibitory action of bleomycin on human neuroblastoma cells [19]. Moreover, DHA increases the anti-tumor effects of targeted therapy. In 2015, Zou et al. reported that DHA enhanced the effectiveness of the human EGFR 2 (HER2)-targeting drug trastuzumab by inhibiting the HER2 pathway [20]. In the present study, we aimed to find the influence of DHA on gefitinib-induced cytotoxic action on human lung adenocarcinoma cells.

The MTT assay results confirmed that both DHA and gefitinib suppress PC9 and A549 cell proliferation in a concentration- and time-dependent manner. The combined DHA and gefitinib treatment showed much greater inhibitory effects as compared with monotherapy in the NSCLC cells.

DHA induces apoptosis in lung cancer cells. The potential mechanism of action includes the proapoptotic effects caused by DHA, regulation of the expression of the dual phosphatase mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1), and the resulting modifications in the phosphorylation state of MAPKs, especially ERK1/2 and p38 [21]. In the present study, flow cytometry showed that DHA enhanced gefitinib-induced apoptosis in PC9 cells (*P* < 0.01), but increased apoptosis only slightly in the A549 cells (*P* > 0.05).

In both the TKI-resistant A549 and TKI-sensitive PC9 cell lines, 48-h gefitinib treatment significantly increased the percentage of G0/G1 cells and decreased the percentage of S-phase cells; both cell lines followed the same trend after DHA treatment. The combined therapy had more significant effects on the cell cycle than monotherapy did. These synergistic effects contributed to the inhibition of NSCLC cell proliferation.

Gefitinib, an EGFR TKI, is one of the most representative targeting therapeutic drugs. Numerous studies have shown that gefitinib inhibits *EGFR*-mutated NSCLC mainly through EGFR signaling [6, 22, 23]. Gefitinib blocks the adenosine triphosphate (ATP) binding pocket of EGFR, inhibiting autophosphorylation and the subsequent activation of the phosphatidylinositol 3-kinase (PI3K)/AKT, MAPK/ERK, and Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) downstream signaling that is essential for tumor migration, proliferation, differentiation, survival, and apoptosis [24, 25]. To investigate the molecular mechanisms responsible for the effects induced by the combined therapy along the EGFR signaling pathway, the expression of both the inactive (unphosphorylated) and activated (phosphorylated) forms of EGFR and its downstream effector ERK1/2 were investigated using western blotting. In the PC9 cells, gefitinib markedly reduced p-EGFR and p-ERK1/2 expression. In the A549 cells, which were used as a comparative cell line because they harbor wild-type *EGFR*, gefitinib downregulated p-EGFR and p-ERK1/2 expression partially as compared with that in the PC9 cells. In both cell lines, DHA upregulated EGFR phosphorylation while downregulating p-ERK1/2 expression. The results are consistent with that of previous studies [21, 26]. When the cells were treated with DHA, there were inconsistent changes in the EGFR and ERK1/2 phosphorylation levels. DHA induces membrane microdomain alterations, resulting in the exclusion of EGFR from caveolin-rich lipid raft fractions [26]. Lipid raft microdomains play an important role, functioning as platforms that aggregate specific proteins, including EGFR, to facilitate cell signaling. Although it upregulated EGFR phosphorylation, we believe that DHA disrupted EGFR signaling and did not activate downstream signaling, as the EGFR protein was transferred out of the cell signal activity area. In 2004, Calviello et al. showed that n-3 PUFAs, including DHA, induced apoptosis by inhibiting ERK1 and ERK2 phosphorylation in human colon cancer cells [27]. Serini et al. also found that DHA downregulated the phosphorylation state of MAPKs, especially ERK1/2, in lung cancer cells [21]. These results are in agreement with ours.

The level of ERK1/2 phosphorylation following combined therapy was lower than that of mono-

therapy, especially in the PC9 cells ($P < 0.05$), which suggests that the combined treatment affects the ERK1/2 pathway in NSCLC cells with *EGFR* mutation more effectively than DHA or gefitinib monotherapy. This result may be attributed to synergy between DHA and gefitinib. Nevertheless, we detected the combined effects of DHA and gefitinib only *in vitro*, therefore further experiments *in vivo* are needed.

In summary, we demonstrate that DHA enhances the anti-tumor effects of gefitinib in NSCLC cells with *EGFR* activating mutation and TKI resistance by inducing apoptosis, regulating the tumor cell cycle, and inhibiting ERK1/2 protein activation. Nutritional intervention with DHA is a promising approach for enhancing the therapeutic effect of gefitinib, or potentially reducing the drug dose. More research into the potential of using DHA supplementation is of critical importance.

Disclosure of conflict of interest

None.

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