Am J Transl Res 2016;8(11):4857-4868
www.ajtr.org /ISSN:1943-8141/AJTR0035289

Original Article

Fisetin, a dietary phytochemical, overcomes Erlotinib-resistance of lung adenocarcinoma cells through inhibition of MAPK and AKT pathways

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Received July 7, 2016; Accepted November 13, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Erlotinib (Tarceva) is a selective epidermal growth factor receptor tyrosine kinase inhibitor for treatment of non-small cell lung cancer (NSCLC). However, its efficacy is usually reduced by the occurrence of drug resistance. Our recent study showed that a flavonoid found in many plants, Fisetin, might have a potential to reverse the acquired Cisplatin-resistance of lung adenocarcinoma. In the present study, we aimed to test whether Fisetin could have the ability to reverse Erlotinib-resistance of lung cancer cells. Erlotinib-resistant lung adenocarcinoma cells, HCC827-ER, were cultured from the cell line HCC827, and the effects of Fisetin and Erlotinib on the cell viability and apoptosis were evaluated. The possible signaling pathways in this process were also detected. As expected, the results showed that Fisetin effectively increased sensitivity of Erlotinib-resistant lung cancer cells to Erlotinib, possibly by inhibiting aberrant activation of MAPK and AKT signaling pathways resulted from AXL suppression. In conclusion, Fisetin was a potential agent for reversing acquired Erlotinib-resistance of lung adenocarcinoma. Inactivation of AXL, MAPK and AKT pathways might play a partial role in this process.

Keywords: Lung adenocarcinoma, Erlotinib-resistance, Fisetin, signaling pathways, reversion

Introduction

Lung cancer is the most common invasive cancer in the world, and specifically, 80% of which is non-small cell lung cancer (NSCLC) [1]. Currently, the most effective therapeutic method for early-stage NSCLC patients (stage I-II) is surgical resection, which results in 30%-60% of five-year survival [2]. Nevertheless, because most patients were diagnosed at advanced stage when the cancer was unresectable, the five-year survival rate decreased to about 10-15%. Though chemotherapy has led to a modest improvement in outcomes of patients with advanced-stage NSCLC, the treatment often results in severe toxicity.

It has been reported that a portion of NSCLC patients harbor specific epidermal growth factor receptor (EGFR) tyrosine kinase mutation that causes EGFR signaling addiction for malignant proliferation [3]. Thus, two EGFR tyrosine kinase inhibitors (TKIs), including Gefitinib and Erlotinib, have been developed. Reports showed that these two agents displayed significant clinically effect on NSCLC with activating EGFR mutations [4], and thus, they have been clinically used as standard first-line agents for treatment of mutant EGFR NSCLC [5]. However, unfortunately, most of the responding patients eventually develop drug resistance that markedly decreased the drug efficacy with the duration of therapy [6]. Once the patients developed TKI-resistance, no optimal therapy has yet been established up to date.

Molecular mechanisms underlying acquired Erlotinib-resistance are still unclear. Several reports have shown that an EGFR T790M gatekeeper mutation is related to about 50% of acquired Erlotinib-resistance [7, 8]. Other mechanisms include aberrant expression and/or activation of a series of receptors and signaling molecules, such as MET [9], PTEN [10], HGF

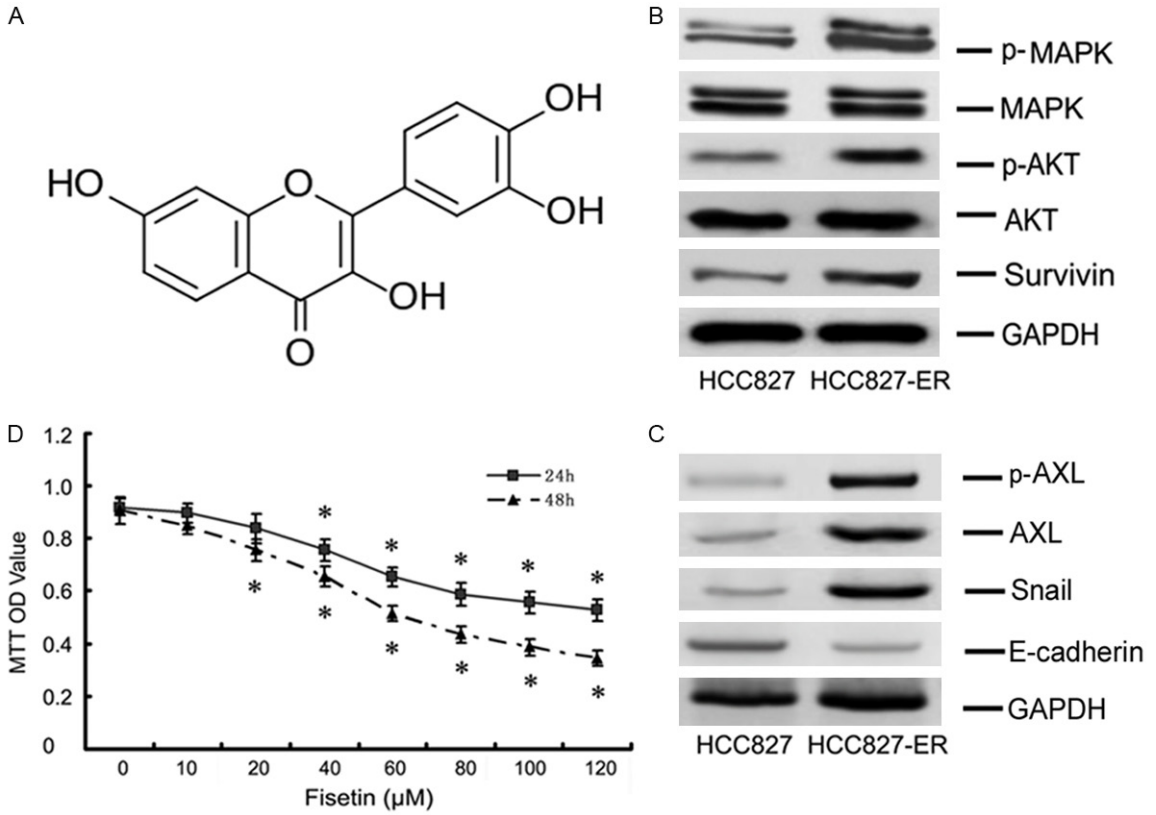

Figure 1. A: The chemical structure of fisetin. B: Characterization of HCC827 and HCC827-ER cells. Western blot analysis showed p-MAPK, p-Akt and Survivin were up-regulated in HCC827-ER compared to HCC827cells ($P<0.05$). C: Western blot analysis showed p-AXL and Snail were up-regulated while E-cadherin was down-regulated in HCC827-ER compared to HCC827cells. D: Effects of Fisetin on the viability of HCC827-ER cells. Cells were treated with Fisetin (0-120 μM) for 24 and 48 h. Viability was determined by MTT assay. The data is presented as mean ± SD of three separate experiments. *$P<0.05$, vs control group (0 μM).

[11], FGF, PI3K/Akt and ERK/MAPK [12, 13]. Moreover, recent evidence showed that epithelial-mesenchymal transition (EMT) [14] and histological changes [15] also confer cell resistance to Erlotinib. However, in approximately 30% of cases, the mechanisms of the acquired resistance are unclear [16].

Recently, abundant therapeutic approaches trying to reverse acquired Erlotinib-resistance have been reported. Nevertheless, the therapeutic effects of these approaches are not satisfactory due to the unclear pathogenesis. We have previously used connectivity mapping analysis to screen the agent that may reverse Erlotinib-resistance, and found that Valproic acid has a potential to reverse the resistance to any extent [17].

Recently, much attention has been focused on the roles of natural agent for cancer therapy. Fisetin (**Figure 1A**), a structurally distinct chemical substance that belongs to the flavonoid group of polyphenols, can be found in many plants, fruits and vegetables, such as parrot tree, honey locust, strawberries, apples, grapes and onions [18]. Fisetin has a wide variety of biological activities, such as anti-aging, anti-inflammatory [19], anti-carcinogenic [20], anti-oxidation, and anti-viral effects [21]. Previous studies have demonstrated that Fisetin had extensively anti-tumorigenic ability in a variety of cancers, such as melanoma [22], bladder cancer [23] and prostate cancer [24]. Our recent experiment showed that Fisetin can overcome the acquired cisplatin-resistance of lung adenocarcinoma [25].

To our knowledge, whether Fisetin could overcome the Erlotinib-resistance of lung cancer has not been published to date. Thus, in the present study, we aimed to address the possible roles of Fisetin in reversion of the lung cancer Erlotinib-resistance, and further investigate the precise mechanisms if necessary.

Materials and methods

Establishment of Erlotinib-resistant lung adenocarcinoma cells

Two types of lung cancer cell lines, HCC827 and its Erlotinib-resistant type, HCC827-ER, were obtained and cultured as described previously [17].

HCC827, the human EGFR mutant NSCLC cell lines that was sensitive to Erlotinib, was obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM medium (Hyclone, Logan) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) in a humidified atmosphere with 5% CO_2. An acquired Erlotinib-resistant subclone cell line of HCC827 was established in continuous culture with gradually increasing Erlotinib (Genentech, San Francisco, CA) [26]. In brief, cells were initially maintained in Erlotinib at a concentration of 0.02 μM (IC50) and the dose was increased step by step during a period of 18 weeks until the final concentration of Erlotinib was 15 μM. Then, to select resistant cells, single-cell cloning techniques were used, and consequently, the cells were established and named HCC827-ER. Then, these Erlotinib-resistant cells were maintained in DMEM including 10% FBS and 15 μM Erlotinib.

Cell viability analysis

MTT assay was used to assess the cell viability. In brief, the cells (1×10^4) were plated in 96-well cell culture plates in RPMI containing 10% FBS in a final volume of 0.2 mL. If the cells reached 50% confluence, Agents were added to appropriate concentrations and incubation continued for an additional 72-96 h. Next, MTT reagent was added to 400 μg/ml and incorporated for 4 hours. After that, the MTT medium mixture was removed and 200 μl of dimethyl sulfoxide was added to each well. Absorbance was measured at 490 nm by a multi-well spectrophotometer (Thermo Electron, Andover, USA).

Chou-Talalay median-effect analysis

The combined effects of Fisetin and Erlotinib on the viability of HCC827-ER cells were analyzed with the CalcuSyn software program (Biosoft, Ferguson, MO) in accordance with the method described by Chou and Talalay [27]. Firstly, the data from the cell viability assay (MTT) were converted to Fraction affected (Fa; range 0-1), where Fa=0 represents 100% viability and Fa=1 represents 0% viability) [28]. Then, the combination index (CI) was calculated according to the following equation: CI=(D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination, and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone [29]. CI values indicate the ways of interaction between two drugs (CI<1 indicates synergism; CI=1 indicates an additive effect; and CI>1 indicates antagonism).

Cell apoptosis analysis

Apoptotic cells were evaluated by using an annexin V-FITC kit (Beyotime, China). The cells were scraped and stained with annexin V-FITC and propidium iodide according to the manufacturer's protocol. In brief, the cells were washed with PBS. After 195 μl of the binding buffer was added, 5 μl of FITC-labeled annexin V was added and incubated for 10 min at 25°C. The cells were then incubated with 10 μl propidium iodide for 10 min in an ice bath in the dark and the apoptotic cells were determined by flow cytometry (FACS) analysis.

Western blot

The cells were harvested, pelleted by centrifugation, washed with ice-cold PBS, and lysed with RIPA buffer [150 mM NaCl, 50 mM Tris base (pH 8.0), 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM DTT, 1 mM PMSF, and 1 mM Na_3VO_4] that was supplemented with a protease inhibitor. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Life Technologies, Gaithersburg, MD). The blots were then incubated in a fresh blocking solution with an appropriate dilution of the primary antibody at 4°C for 24 h.

The sources of antibodies were as follows: GAPDH mouse polyclonal antibody (Santa Cruz); p-MAPK (Thr202/Tyr204), MAPK, p-AKT (Ser473) and AKT rabbit monoclonal antibody (Cell Signaling); Survivin and Cytochrome C mouse monoclonal antibody (Santa Cruz), Snail, E-cadherin, Caspase-3 and Caspase-8 rabbit

polyclonal antibody (Santa Cruz). R428 was purchased from Selleck Chemicals (Houston, Texas). Antibodies for Axl and phospho-Axl were purchased from R&D systems (Minneapolis, MN).

After the blots were extensively washed, the membranes were incubated with horseradish peroxidase-coupled secondary antibody (1: 2000, Zhongshan Biotech Company, China) at 25°C for 1 h. The bands were visualized and quantified using the Image-Pro Plus 5.0 software (Media Cybernetics). p-AXL, p-MAPK and p-AKT band intensities were normalized to AXL, MAPK and AKT band intensities, respectively. Other factors were adjusted by the GAPDH band intensities.

Statistical analysis

Data were expressed as mean value ± SD. Differences between groups were analyzed using an ANOVA or a *t*-test. These analyses were performed on SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL). A *P*-value of <0.05 was considered statistically significant.

Results

Development and characterization of Erlotinib-resistant HCC827-ER and HCC827 cells

HCC827 and HCC827-ER were obtained as described previously [17]. In brief, TKI-resistant HCC827-ER was obtained by gradual increase of Erlotinib in cell culture. The IC50 of Erlotinib was 19.6 nM (0.02 μM) for HCC827 and 98.3 μM for HCC827-ER cells, respectively, which reflected that higher Erlotinib concentrations were required for growth inhibition of HCC827-ER cells. Conclusively, since HCC827 cells were highly sensitive to Erlotinib, these data demonstrated that HCC827-ER cells were highly resistant to it, indicating the establishment of the Erlotinib-resistant lung adenocarcinoma cells.

Evidence indicates that activation of pro-survival PI3K/AKT pathway and/or mitogenic Ras/Raf/MAPK pathway might generate high anti-apoptosis ability of Erlotinib-resistant cancer cells [30]. We next investigated these molecular pathways in HCC827 and HCC827-ER cells by immunoblotting analysis. As illustrated in **Figure 1B**, expression of p-AKT and p-MAPK proteins were higher in HCC827-ER than those in HCC827 cells, suggesting that activation of both AKT and MAPK may play a crucial role in this Erlotinib-resistant cancer cell model.

Accordingly, Survivin, an important anti-apoptotic signaling protein [31], was markedly up-regulated in HCC827-ER compared with HCC827 cells, which might be responsible for the acquired resistance to Erlotinib. Additionally, previous research has described that activation of AXL kinase participated in resistance of EGFR-targeted therapy in lung cancer through induction of epithelial-to-mesenchymal transition (EMT) [26]. Therefore, in this study, AXL, Snail, and E-cadherin were detected in HCC827 and HCC827-ER cells, respectively. The immunoblotting analysis showed that expression of AXL and Snail proteins were up-regulated while E-cadherin expression was down-regulated in HCC827-ER compared with those in HCC827 cells (**Figure 1C**). The results indicated that AXL might also play important roles in Erlotinib-resistance of HCC827-ER cells, but its concrete mechanisms deserve further research.

Effects of Fisetin on HCC827-ER cell growth

To evaluate the effects of Fisetin on HCC827-ER cell growth, cells were incubated with various concentrations (0, 10, 20, 40, 60, 80, 100, 120 μM) of Fisetin (Sigma, St Louis, MO) for 24 h and 48 h respectively. Then, the cell viability was tested by MTT assay. As illustrated in **Figure 1D**, the cell viability was inhibited by Fisetin, and the inhibitory effect was strengthened with an increase in Fisetin concentration or the time length of incubation. Fisetin alone significantly suppressed cell viability and induced apoptosis of HCC827-ER when the dose was equal to or greater than 40 μM. The estimated IC10, IC25 and IC50 of the HCC827-ER cells treated with Fisetin were 26.02 μM, 49.71 μM, and 180.84 μM for 24 h, and 15.42 μM, 32.90 μM and 76.44 μM for 48 h, respectively, suggesting that Fisetin could inhibit the cell viability of HCC827-ER in a time- and dose-dependent manner.

Effects of Fisetin and Erlotinib on HCC827-ER cancer cells and the possible signaling pathways

To learn whether Fisetin could reverse Erlotinib-resistance of HCC827-ER to any extent, we conducted further experiments. Cells were divided

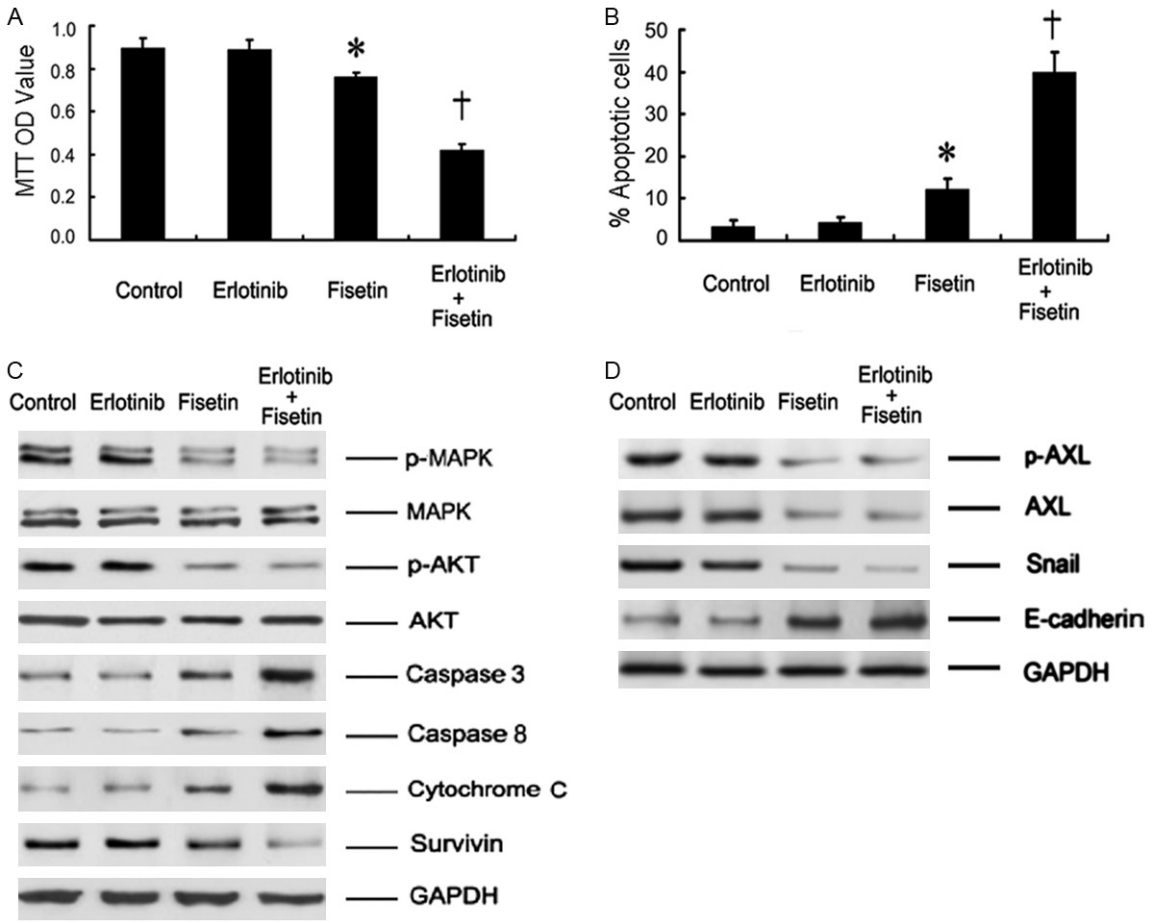

Figure 2. Cells were divided into four subgroups and treated with 40 μM Fisetin, 15 μM Erlotinib, 40 μM Fisetin + 15 μM Erlotinib, and DMEM as a control for 24 h, respectively. Then, cell viability and apoptosis were tested. Cell viability (A) and apoptosis (B) in HCC827-ER assessed by MTT and apoptosis assay. (*P<0.05 vs Control. †P<0.05 vs Control) Expression of the MAPK, AKT, Survivin and apoptosis pathway-related proteins (C) and AXL, Snail and E-cadherin proteins (D) assessed by immunoblotting.

into four subgroups and treated with 40 μM Fisetin, 15 μM Erlotinib, 40 μM Fisetin + 15 μM Erlotinib, and DMEM as a control for 24 h, respectively. Then, cell viability and apoptosis were tested.

As shown in **Figure 2A**, **2B**, Single use of Erlotinib (15 μM) had no evident influence on the cell viability and apoptosis of HCC827-ER cells, confirming that the HCC827-ER cells were markedly resistant to Erlotinib. However, the combinational treatment of Fisetin (40 μM) with Erlotinib (15 μM) resulted in a marked suppression of cell viability and induction of cell apoptosis as compared with the cells treated with Fisetin or Erlotinib alone, respectively, indicating that Fisetin made Erlotinib-resistant lung cancer cells vulnerable to the cytotoxicity of Erlotinib.

To explore the status of signaling pathways, we further detected the signaling proteins by western blot analysis. As shown in **Figure 2C**, Erlotinib alone at a dose of 15 μM could not affect the protein expression of phosphorylation levels of MAPK and AKT, as well as Survivin and apoptotic pathway-related molecules including Caspase-3, Caspase-8 and Cytochrome C. Treatment of Fisetin alone, or combined with Erlotinib, might lead to a decrease in the expression of p-MAPK, p-AKT and Survivin protein. Accordingly, an increase in the expression of Caspase-3, Caspase-8 and Cytochrome C was also observed in these two groups, respectively, indicating that Fisetin might reverse Erlotinib-resistance of cancer cells via inactivation of MAPK and AKT pathways and repression of Survivin expression. Thus, the apoptotic signaling might be initiated in HCC827-ER cells.

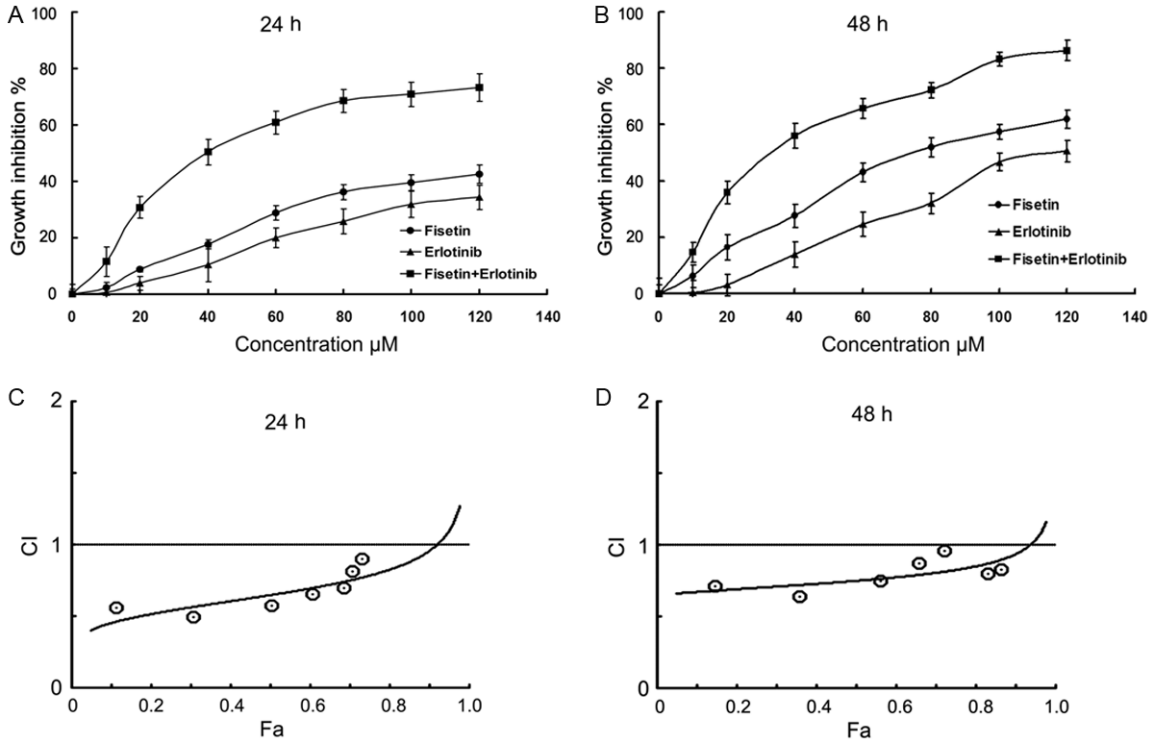

Figure 3. Effects of Fisetin and Erlotinib on the growth inhibition of HCC827-ER. Cell growth inhibition curves of HCC827-ER cells treated with Fisetin, Erlotinib or the combination of Fisetin and Erlotinib for 24 h (A) or 48 h (B). Analysis of combined effects of Fisetin and Erlotinib on HCC827-ER cells for 24 h (C) or 48 h (D) treatment. CI values were determined according to the median-effect method by Chou and Talalay, with less than 1 at all given concentrations indicating highly synergistic effects. The molar ratios of Fisetin and Erlotinib were fixed at 1:1 with increasing doses (from 10 μM to 120 μM), respectively. Data were presented as means ± SD, n=3.

Next, we further determined whether Fisetin treatment has an effect on AXL expression and EMT-related proteins including Snail and E-cadherin. As a result, treatment of Erlotinib alone could hardly influence the expression of these proteins, whereas treatment of Fisetin alone, or a combination of Erlotinib and Fisetin could markedly suppress the expression of p-AXL and Snail. Accordingly, E-cadherin expression was elevated (**Figure 2D**), implying that AXL pathway might be involved in the Erlotinib-resistance and EMT reversion caused by Fisetin. Nevertheless, the exact mechanisms in this process deserve further investigation.

Fisetin acts synergistically with Erlotinib to inhibit the growth of HCC827-ER cells

The combinational effect of Fisetin with Erlotinib on HCC827-ER cells was quantified using the median-drug effect analysis according to Chou and Talalay [27] as mentioned above. The Fisetin and Erlotinib combinations were evaluated at a fixed molar ratios of 1:1 (Fisetin: Erlotinib) with increasing dose (from 10 μM to 120 μM), respectively. As shown in **Figure 3**, the significant synergistic effect of Fisetin plus Erlotinib was observed in HCC827-ER cells for 24 h (**Figure 3A** and **3C**) and 48 h (**Figure 3B** and **3D**), respectively, with CI values less than 1 at all given concentrations of Fisetin combined with Erlotinib, especially at a low concentration. For example, the CI value was 0.54, 0.65, and 0.79 at inhibition of 25% (Fa=0.25), 50% (Fa=0.50), and 75% (Fa=0.75) for cell proliferation, respectively, when the cells were treated with Fisetin plus Erlotinib for 24 h. Moreover, the CI value was 0.70, 0.75, and 0.83 at inhibition of 25%, 50%, and 75% for cell proliferation, respectively, when the cells were treated with the drugs for 48 h. The data demonstrated that the combined treatment of Fisetin and Erlotinib yielded strong growth inhibition compared to Fisetin or Erlotinib alone.

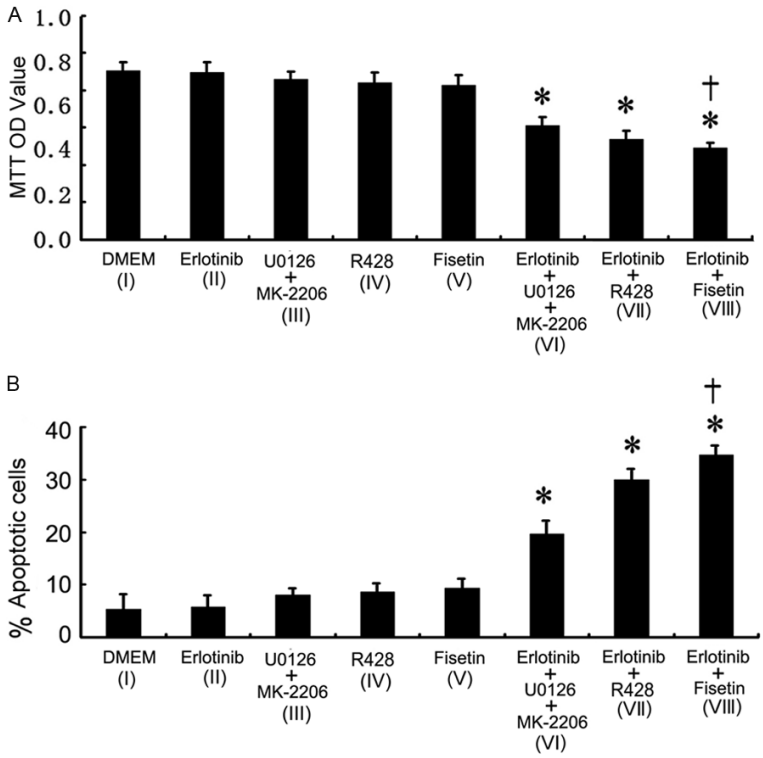

Figure 4. HCC827-ER cells were divided into eight subgroups as I to VIII, respectively. They were treated with different reagent for 24 h as follows: DMEM (I), Erlotinib (II), U0126 + MK-2206 (III), R428 (IV), Fisetin (V), Erlotinib + U0126 + MK-2206 (VI), Erlotinib + R428 (VII), and Erlotinib + Fisetin (VIII). Cell viability (A) and apoptosis (B) of the cells were assessed by MTT and apoptosis assays, respectively. (*P<0.05 vs I or II; †P<0.05 vs VI or VII).

Involvement of AXL, MAPK and AKT pathways in Erlotinib-resistance reversion

To investigate the roles of the singling pathways by which Fisetin reverses Erlotinib-resistance of HCC827-ER cells, we conducted further investigations.

HCC827-ER cells were divided into eight groups as I, II, III, IV, V, VI, VII, and VIII, respectively. Group I was treated with DMEM for 24 h as a control. Group II to V were treated with Erlotinib (15 μM; Group II), a combination of 10 μM U0126 (a specific MAPK inhibitor, Cell signaling) and 10 μM MK-2206 (a specific AKT inhibitor, Cell signaling; Group III), 1 μM R428 (Group IV) and 20 μM Fisetin (Group V) for 24 h, respectively. For group VI and VIII, cells were treated with Erlotinib (15 μM) for 24 h after a pre-incubation of a combination of 10 μM U0126 and 10 μM MK-2206 (VI) or 1 μM R428 (VII) for 2 h. For group VIII, cells were treated with a combination of 15 μM Erlotinib and 20 μM Fisetin for 24 h. Cell viability and apoptosis were assessed by MTT and apoptosis assays, respectively.

When the cells were treated with any single reagent, we found that treatment of Erlotinib (II), signaling inhibitors (III or IV), or Fisetin at a low concentration (V) could hardly suppress cell viability and induce cell apoptosis relative to those in the control group (I), while treatment with Erlotinib + inhibitors (VI or VII) exhibited marked inhibition of cell viability and induction of apoptosis compared with the controls. However, as shown in **Figure 4**, in Group VIII (Erlotinib + Fisetin), Evident effects on cell viability suppression and apoptosis induction were observed compared with those in Group VI or VII (Erlotinib + inhibitors), respectively, suggesting that although Fisetin at a low concentration (For example, 20 μM) exerts little effect on the cell viabilities, it could evidently overcome the Erlotinib-resistance of HCC827-ER cells. Moreover, inhibition of AXL, MAPK and AKT pathways might play a role in the mechanisms by which Fisetin reverses Erlotinib-resistance. However, in addition to this, other unknown mechanisms might be involved in this process. In other words, activation of AXL, MAPK and AKT pathways might play partial roles in the development of acquired Erlotinib-resistance.

Inhibition of AXL pathway might lead to inactivation of MAPK and AKT pathways

The above results showed that Fisetin could inhibit expression of AXL, MAPK and AKT, with an increase in apoptosis of HCC827-ER in response to Erlotinib. The data indicated an involvement of these pathways in the Erlotinib-resistance reversion of HCC827-ER caused by Fisetin. Nevertheless, the relationship among the pathways has not been evaluated. Evidence suggests that MAPK and AKT pathways were

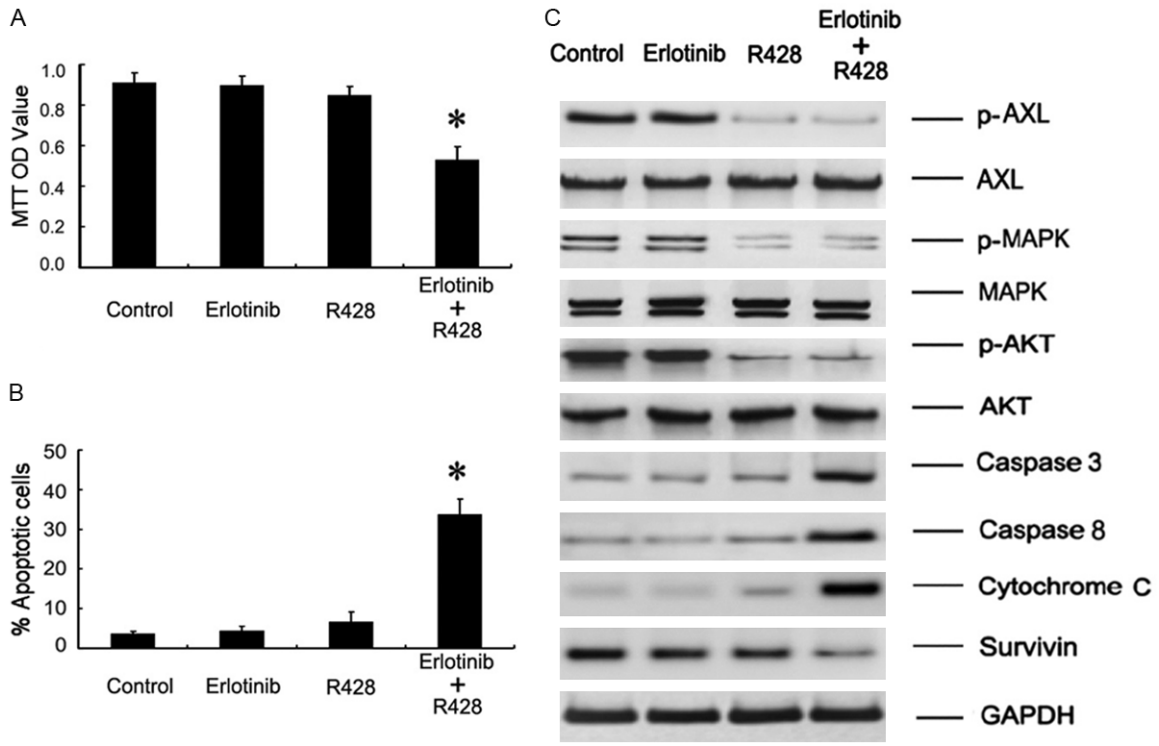

Figure 5. HCC827-ER cells were divided into four subgroups and treated with 15 μM Erlotinib, 1 μM R428 (a specific AXL inhibitor), 1 μM R428 + 15 μM Erlotinib, and DMEM as a control for 24 h, respectively. Cell viability (A) and apoptosis (B) in HCC827-ER were assessed by MTT and apoptosis assays, respectively. (*P<0.05 vs Control). Expression of the AXL, MAPK, AKT, Survivin and apoptosis pathway-related proteins were assessed by immunoblotting.

mediated by AXL pathways [32, 33]. Likewise, in this section, we aimed to explore whether inhibition of AXL leads to MAPK or AKT inactivation.

HCC827-ER cells were divided into four subgroups and treated with 15 μM Erlotinib, 1 μM R428 (a specific AXL inhibitor), 1 μM R428 + 15 μM Erlotinib, and DMEM as a control for 24 h, respectively. Then, cell viability and apoptosis as well as the expressions of relevant proteins were tested. Interestingly, as shown in **Figure 5**, single use of R428 could obviously suppress p-AXL expression, resulting in down-regulation of both p-MAPK and p-AKT expression, and nevertheless, expressions of Survivin and the apoptosis-related proteins such as Caspase-3 were unchanged. In this subgroup (single use of R428), the cell apoptosis was slightly changed without significance compared with those in the control group or the single Erlotinib administration group. However, combined use of Erlotinib and R428 could down-regulate Survivin expression other than p-MAPK and p-AKT, and up-regulate the apoptosis-related proteins such as Caspase-3. Accordingly, a marked increase in cell apoptosis was observed in this group (Erlotinib + R428) compared with those in the other three groups (DMEM; R428; or Erlotinib), respectively. The results indicated that inhibition of AXL pathway might lead to MAPK and AKT inactivation, and therefore make the HCC827-ER cells vulnerable to the cytotoxicity of Erlotinib.

Discussion

In this present study, we found that Fisetin, a flavonoid widely found in fruits and vegetables, had a potential to reverse acquired Erlotinib-resistance of lung adenocarcinoma cells. Fisetin significantly decreased proliferation of Erlotinib-resistant cells treated with Erlotinib. The combination of Fisetin and Erlotinib markedly down-regulated the expressions of p-AXL, p-MAPK, p-AKT, and Survivin, caused the activation of Caspase-3, -8 and Cytochrome C, induced apoptosis, and inhibited cell viability of Erlotinib-resistant NSCLC cells in vitro, demonstrating that Fisetin might overcome Erlotinib-

resistance and providing an alternative therapeutic strategy in patients with acquired resistance to EGFR-TKIs. To our knowledge, we for the first time report the potential role of Fisetin as an anticancer drug that might reverse Erlotinib-resistance in NSCLC cells.

Fisetin induces cell apoptosis through various mechanisms. Reports showed that Fisetin could induce p53 expression and suppress mTOR and p70S6K pathways [22, 23]. Moreover, Fisetin could suppress cell migration and invasion by inhibiting matrix metalloproteinases [34], and reverse chemoresistance of cancer cells through inhibition of MAPK and NF-kappaB pathways [35, 36]. In the present study, the data showed that Fisetin alone could repress cell viability and induce apoptosis of HCC827-ER cells in a time- and dose-dependent manner. Then, treatment of a combination of Fisetin and Erlotinib could exhibit strong inhibition effects on HCC827-ER cells compared with Fisetin or Erlotinib alone. These results demonstrated that Fisetin could sensitize Erlotinib-resistant NSCLC cells to Erlotinib and enhance Erlotinib-induced apoptosis through a synergistic action, and indicated that Fisetin might act as a potential agent for reversing Erlotinib-resistance in treating NSCLC patients. However, the mechanisms underlying this process remain unclear. Thus, we further explored the possible signaling pathways involved in this issue.

Ras/Raf/MAPK and PI3K/AKT/mTOR pathways have been indicated to play a role in multiple cellular processes, such as cell proliferation, apoptosis, transcription, and cell migration. Evidence shows that hyperactive Akt pathway and MAPKs pathway have been associated with resistance to EGFR-TKIs in NSCLC [37, 38]. The results of the present study showed that single use of Erlotinib could hardly affect the phosphorylation of AKT and MAPK expression, with the cell apoptosis unchanged, whereas Fisetin alone could slightly decrease the levels of p-AKT and p-MAPK in HCC827-ER cells, with an increase in cell apoptosis. However, combination of Fisetin with Erlotinib could markedly induce cell apoptosis, with inactivation of both AKT and MAPKs pathways, implying that Fisetin might sensitize the Erlotinib-resistant cells to Erlotinib through inhibition of MAPK and AKT pathways. The activation of MAPK and AKT signaling pathways provided a survival signal for the Erlotinib-resistant cells and the inhibition of both pathways released an apoptotic signal. Nevertheless, we found that co-treatment of Fisetin and Erlotinib presented strong inhibition effects on cells compared with other groups, indicating that activation of these two pathways might only play partial roles in acquired Erlotinib-resistance of HCC827-ER cells. In addition to these two pathways, other signaling pathways might be involved in this process.

AXL is a member of receptor tyrosine kinases (RTKs), which has been widely detected in a variety of cancers and thus been thought to be associated with cell proliferation, migration, EMT, and cancer progression [39]. Recently, AXL has been suggested to play a role in drug-resistance of cancers [40, 41], particularly resistance of EGFR-TKI for lung cancer [26, 42, 43]. In the present study, over-expression of p-AXL was detected in Erlotinib-resistant lung cancer cells. Administration of Fisetin could inactivate AXL pathway and reverse EMT in the HCC827-ER cells. Interestingly, inhibiting of AXL might result in suppression of MAPK and AKT, indicating that AXL might mediate the activation of these two pathways in this process.

Over-expression of Survivin has been widely detected in various types of cancer and suggested to correlate with tumor progression and drug resistance [31]. Previous reports have shown that EGFR signaling-related activation of Ras/Raf/MAPK and PI3K/AKT/mTOR pathways may have a correlation with up-regulated Survivin expression [44, 45]. Persistent Survivin expression contributes to acquired Erlotinib-resistance of NSCLC cells. Down-regulation of Survivin is associated with Erlotinib-induced apoptosis in NSCLC cells with a TKI-sensitive EGFR mutation [46]. Our results demonstrated that Erlotinib could scarcely reduce Survivin expression and fail to cause apoptosis in HCC827-ER cells, while Fisetin significantly down-regulated Survivin expression and induced apoptosis in these cells. Furthermore, co-treatment of Fisetin and Erlotinib presented strong inhibition of Survivin expression and induction of cell apoptosis compared with Fisetin alone. Therefore, co-treatment of Fisetin and Erlotinib led to remarkably increased cleavage of Caspase-3, -8 and release of Cytochrome C. The results suggested that down-regulation

of Survivin might be involved in the mechanisms by which Fisetin reverses Erlotinib-resistance of lung cancer cells.

Several limitations might be involved in the present study. First, only one cell line, HCC827, was used in this experiment. Future studies using other EGFR mutant cell lines, such as H3255, might strengthen the significance of the results. Second, only a small proportion of underlying mechanisms by which Fisetin overcomes the Erlotinib-resistance of lung cancer cells were evaluated in this study. Other pathways that might play crucial roles in this process need to be deeply determined in further investigations.

In conclusion, we found that Fisetin, a natural product, might be a potential agent that can reverse Erlotinib-resistance of lung adenocarcinoma cells. Fisetin might reverse acquired Erlotinib-resistance of lung adenocarcinoma cells, by partly inactivating AXL, AKT and MAPKs pathways as well as suppressing Survivin expression. Future studies are needed to confirm the results.

Acknowledgements

This work was partly supported by the special foundation for the 1130 Project of Xinqiao Hospital of Third Military Medical University (2012).

Disclosure of conflict of interest

None.

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