Eupatilin inhibits EGF-induced JB6 cell transformation by targeting PI3K

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Received April 25, 2016; Accepted June 17, 2016

DOI: 10.3892/ijo.2016.3600

Abstract. Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that play fundamental roles in regulation of multiple signaling pathways, including cell proliferation, survival and cell cycle. Increasing evidence has shown that abnormal activation of PI3K pathway contributes to tumorigenesis and progression of various malignant tumors. Therefore, it is an attractive target of chemoprevention and chemotherapy. Eupatilin, a natural flavone compound extracted from *Artemisia vulgaris*, has antitumor and anti-inflammation efficacy. However, the direct target(s) of eupatilin in cancer chemoprevention are still elusive. In the present study, we reported eupatilin suppressed JB6 cell proliferation and its EGF-induced colony formation. Eupatilin attenuated phosphorylation of PI3K downstream signaling molecules. Downregulation of cyclin D1 expression and arresting in G_1 phase were induced through eupatilin treatment. Furthermore, we found it could bind to the p110α, a catalytic subunit of PI3K, by computational docking methods. Pull down assay outcomes also verified the binding of eupatilin with PI3K. Taken together, our results suggest that epatilin is a potential chemopreventive agent in inhibition of skin cell transformation by targeting PI3K.

Introduction

Cancer development is a multistep and complex process involving initiation, promotion and progression. Cell transformation into cancerous state, is one of the critical steps. Chemoprevention has been considered as the most promising strategy, which describes the use of natural or synthetic chemicals to suppress, delay, or prevent the process of carcinogenesis (1). Phytochemicals are the focus of chemoprevention studies because of their potential human acceptance and low adverse reaction (1,2). Flavonoids, widely present in fruits and vegetables, has gained attention as a phytochemical that could reduce the risk of several types of cancer, including prostate (3), colorectal (4), head and neck cancer (5). Eupatilin is a flavonol isolated from *Artemisia vulgaris*. A previous study showed that eupatilin exerts anti-inflammatory and anti-oxidative activities on gastric mucosal damage, and promotes regeneration of damaged mucosa (6). In recent years, several studies demonstrated that eupatilin could inhibit tumor cell growth and proliferation (7-10). However, the effect of eupatilin on inhibition of cell transformation and the direct molecular target(s) remain unclear.

The phosphatidylinositol 3-kinase (PI3K) pathway is activated following interaction of a growth factor, cytokines, or other environmental cues with a tyrosine kinase receptor (TKR). PI3K plays an important role in regulation of many signaling pathways to control cell proliferation, growth, survival, motility and metabolism (11-14). The abnormal activation of PI3K has been found in numerous cancers (15-18).

Akt is one of downstream kinases of PI3K pathway. PI3K activation catalyzes the PIP2 to generate PIP3, which in turn recruits PDK1 and Akt to bind the plasma membrane at pleckstrin homology domains. Subsequently, Akt is phosphorylated by PDK1 at threonine-308 (T308) or serinine-473 sites through targeting rapamycin complex 2 (mTORC2). Akt activation can lead to phosphorylation of various protein substrates, which are consequently activated or inhibited. GSK3β is an important downstream molecule of Akt, regulated by site-specific phosphorylation and depending on different cell condition.

GSK3β activation and inhibition depend upon the phosphorylation on Tyr216 and Ser9, respectively. Detailed analysis revealed that p-GSK3β^{Ser9} blocked the nuclear export and degradation of cyclin D1, resulting in progressing to S phase from G_0/G_1 (19,20). Hence, PI3K/Akt/GSK-3β pathway regulation is a possible research direction for cancer chemoprevention and PI3K is a potential target of chemoprophylaxis.

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Key words: eupatilin, chemoprevention, phosphatidylinositol 3-kinase, AKT, JB6 cell

In the present study, we found that eupatilin inhibited JB6 cell proliferation and EGF-induced anchorage-independent growth. Treated with eupatilin, downstream kinases of PI3K phosphorylation, including Akt phosphorylation at Ser473 and Thr308, GSK3β phosphorylation at Ser9 were inhibited, causing cell cycle arrest at G_1 phase by suppressing cyclin D1. Furthermore, computer-docking model showed that eupatilin was able to bind at the ATP pocket site of PI3K, which was verified by pull down assay. Hence, this study suggests that eupatilin is a potential chemopreventive agent in inhibition of cell transformation by targeting PI3K.

Materials and methods

Materials. Fetal bovine serum (FBS) was purchased from Gibco-BRL (Gaitherburg, MD, USA). Antibodies were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA) and Cell Signaling Technology (Beverly, MA, USA). Eupatilin was obtained from Chunqiu (Nanjing, China). Epidermal growth factor (EGF) was purchased from BD Biosciences (San Jose, CA, USA). Cell Counting kit-8 (CCK-8) and BeyoECL Plus were purchased from Beyotime Institute of Biotechnology (Shanghai, China). CNBr-Sepharose 4B was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell proliferation. JB6 cells were plated at a density of 10^3 cells/well in 96-well plates and incubated for 6-10 h at 37˚C. The anti-proliferative effect of eupatilin was evaluated in cells cultured with different concentrations of 0, 2.5, 5 or 10 μM and time 24, 48, 72 or 96 h using WST-8. Briefly, 10 μl of the WST-8 solution was added to cell cultures for the designated times. Plates were incubated for 2 h at 37˚C. The absorbance was measured at 450 nm using a microplate reader (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China).

Anchorage-independent cell growth. JB6 cells ($8x10^3$/ml) were cultured in 0.3% Eagle's basal medium-agar containing 10% FBS. Cells were treated with different concentrations of eupatilin (0, 2.5, 5, 10 and 20 μM) along with EGF (10 ng/ml). Plates were incubated for 16 days at 37˚C. The colonies were counted under a microscope with the help of the Image-Pro Plus computer software program.

Western blot assay. In this assay, JB6 cells were cultured in a 10-cm dish at 37˚C. At 90% confluency, cells were starved in MEM containing 0.1% FBS for 16 h to synchronize the cell cycle into G_0 phase (21). Subsequently, the cells were treated with various concentrations of eupatilin (0, 2.5, 5, 10 and 20 μM) for 2 h followed by addition of EGF (final 10 ng/ml concentration). Cells were sonicated by the Ultrusonic cell disrupter system and protein concentration was determined. Total protein (40 μg) from the whole cell lysates was separated by 10% SDS-PAGE and proteins separated in the gel were transferred electrophoretically onto PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 2 h followed by incubated with a 1:1,000 dilution of a specific first antibody [anti-ERK1/2, anti-p-ERK1/2, anti-Akt, anti-p-Akt (Ser473), anti-p-Akt (Thr308), anti-GSK3β, anti-p-GSK3β (Ser9), anti-RSK2, anti-p-RSK2, anti-CREB, anti-p-CREB were from Cell Signaling Technology; anti-cyclin D1, anti-α-tubulin and anti-β-actin were from Santa Cruz Biotechnology] at 4˚C overnight. The next day, the membranes were washed and incubated with their corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 dilution) for 2 h. Protein bands were then developed using BeyoECL Plus on Medical X-ray film.

Cell cycle assay. JB6 cells ($I5x10^5$) were cultured in 6-cm dishes and allow to grow for 24 h at 37˚C. Next, the cells were starved in MEM containing 0.1% FBS for 36 h to synchronize the cells into G_0 phase. Cells were divided into two groups followed by different concentrations of eupatilin (0, 2.5, 5, 10 and 20 μM) and (0 and 20 μM) and PI3K inhibitor LY294002 (final 10 μM concentration). Subsequently, treated with EGF (final 10 ng/ml) and the cells were harvested after 18 h. Later, cells were fixed with 70% ethanol at 4˚C overnight. The following day 500 μl 1X PBS, RNase A (100 μg/ml), propidium iodide (1 mg/ml) were added and incubated at 37˚C in dark for 40 min. Subsequently, cells were analyzed by the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Molecular modeling. Molecular docking was performed in order to understand the detailed binding interactions between eupatilin and PI3K. The initial binding complex structure was constructed from the X-ray crystal structure of human PI3K (PDB ID: 4TV3) (22) and the optimized structure of eupatilin. Initially, the geometry of eupatilin was optimized at the HF/6-31G* level using Gaussian 09 (23). Then, the optimized geometry of eupatilin was used to calculate the electrostatic potential distribution on the molecular surface at the same HF/6-31G* level. The calculated electrostatic potential distribution was used to determine the partial atomic charges by using the standard restrained electrostatic potential (RESP) fitting procedure (24). The determined RESP charges of the eupatilin atoms were used in the following docking studies. Briefly, eupatilin was docked into the possible active site of PI3K by using the AutoDock 4.2 program (25). The atomic charges used for the docking of eupatilin were the restrained electrostatic potential (RESP) charges. During the docking process, a conformational search was performed using the Solis and Wets local search method (26), and the Lamarkian genetic algorithm (LGA) (25) was applied to deal with the PI3K-eupatilin interactions. Among a series of docking parameters, the grid size was set to 60 x 60 x 60 and the grid space was the default value of 0.375 Å. The docked enzyme-ligand complex structures were selected according to the criteria for interacting energy combined with geometric matching quality.

Depicted in Fig. 4A is the constructed structure of PI3K-eupatilin binding complex. Eupatilin fits well in the active site of PI3K and is stabilized by extensive hydrogen bonding, π-π and van der Waals interactions. The benzene ring sandwiched between I800, I848, M772 and D933 establishes extensive van der Waals contacts with PI3K. The two methoxy groups from the benzene ring form hydrogen bonds with K802. The chroman ring forms π-π stacking and van der Waals interactions with the surrounding residues like W780, Y836, M922 and I932. In addition, the hydroxyl group from the chroman

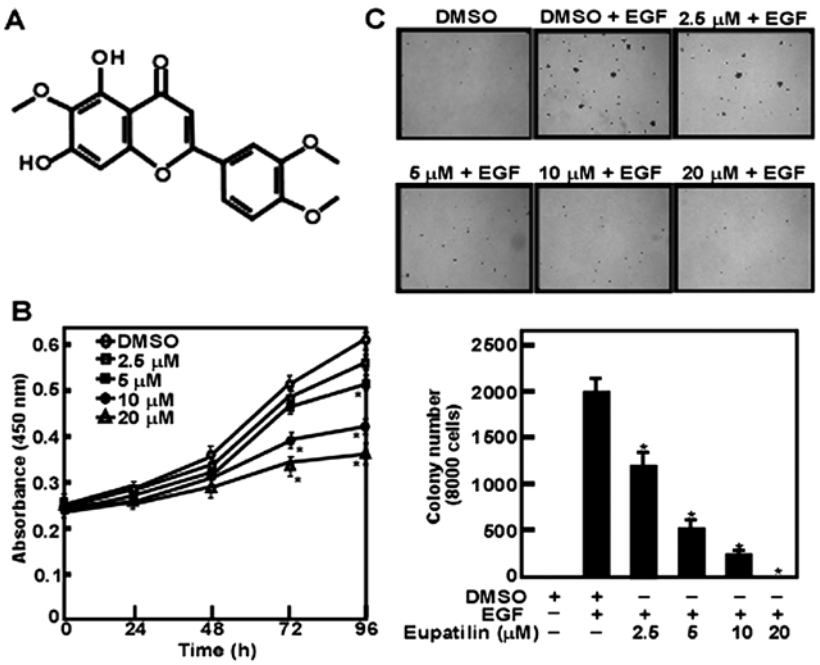

Figure 1. Eupatilin inhibits JB6 cell proliferation and anchorage-independent growth. (A) Chemical structure of eupatilin. (B) Eupatilin inhibits JB6 cell proliferation. JB6 cells (10^3 cells/well in 96 well plate) were treated with the indicated concentrations of eupatilin and proliferation was measured at the indicated time-point as described in Materials and methods. Data are shown as means ± SD from three independent experiments and the $^*P<0.05$ indicates a significant decrease in treated cell proliferation compared with untreated control cells. (C) Eupatilin inhibits JB6 cell anchorage-independent growth. JB6 cells ($8x10^3$) were treated with various concentrations of eupatilin but the same final concentration of EGF except for the control group and grown in soft agar for 14 days. Then the colonies were counted under a microscope with the help of the Image-Pro Plus. Data are shown as means ± SD from three independent experiments and the $^*P<0.05$ indicates a significant decrease in colony formation compared with eupatilin negative cells.

ring forms hydrogen bonds with the backbone of L851. The molecular modeling study enables us to see more clearly the detailed binding interactions of eupatilin with PI3K.

Pull down assay. Preparation of Sepharose 4B beads: the Sepharose 4B beads (0.3 g) were washed with 30 ml of 1 mM HCl 5 min by gentle inversion; repeated 3 times and incubated with 2 mg eupatilin or DMSO as a control in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3) at 4˚C overnight. After washing with coupling buffer (5 ml) the beads were incubated with 0.1 M Tris-HCl (5 ml) (pH 8.0) buffer at 4˚C overnight with rotation. Subsequently, the samples were washed with 0.1 M acetic (pH 4.0), 0.1 M Tris-HCl and 0.5 M NaCl (pH 8.0) and 1 ml PBS was added to resuspension. Cellular supenatant fraction of JB6 cells (500 μg) or active PI3K with eupatilin-Sepharose 4B (or DMSO-Sepharose 4B as a control) beads (100 μl, 50% slurry) were incubated overnight at 4˚C in reaction buffer (150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 50 mM Tris pH 7.5, 1 mM dithiothreitol, 1 μg protease inhibitor mixture, 0.02 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml bovine serum albumin and 0.01% Nonidet P-40) with gentle rotation. The next day, the beads were washed with washing buffer (150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 50 mM Tris pH 7.5, 1 mM dithiothreitol, 0.02 mM phenylmethylsulfonyl fluoride and 0.01% Nonidet P-40) 5 times and the bound PI3K proteins were analyzed by western blotting.

Statistical analysis. All quantitative data are expressed as means ± standard deviation. The one-way analysis of variance and Student's t-test were used for statistical analysis by SPSS 22.0 software (IBM, Amonk, NY, USA). Significant differences are reported at P<0.05.

Results

Eupatilin inhibits EGF induced JB6 cells transformation. Eupatilin is an anticipative natural flavone compound extracted from *Artemisia vulgaris* (Fig. 1A). Our data revealed that JB6 cell proliferation was inhibited by eupatilin in a dose-dependent manner with a maximal concentration at 20 μM (Fig. 1B). Furthermore, we found that JB6 cell anchorage-independent growth was affected by eupatilin. These results showed that eupatilin inhibited EGF-induced cell colony formation dose-dependently (Fig. 1C). However, the effect presented by various concentrations of eupatilin was not caused by the toxicity of eupatilin.

Eupatilin inhibits the transduction of PI3K-mediated downstream signaling pathway. To examine the mechanism of eupatilin inhibition of cell proliferation and anchorage-independent growth, we analyzed the role of eupatilin in activating the EGF-induced Akt and ERK-related signaling pathway by western blotting. We found that EGF-induced phosphorylation of Akt at Ser473 and Thr308 was inhibited dose-dependently by eupatilin. Moreover, GSK3β at Ser9, a downstream molecule of Akt was also downregulated by eupatilin (Fig. 2A). However, the ERK-related signaling pathway (ERK1/2, RSK2 and CREB) was not affected (Fig. 2B).

Eupatilin triggers cell cycle arrest in G_0/G_1 phase by inhibiting the activity of cyclin D1. To reveal the mechanism of eupatilin

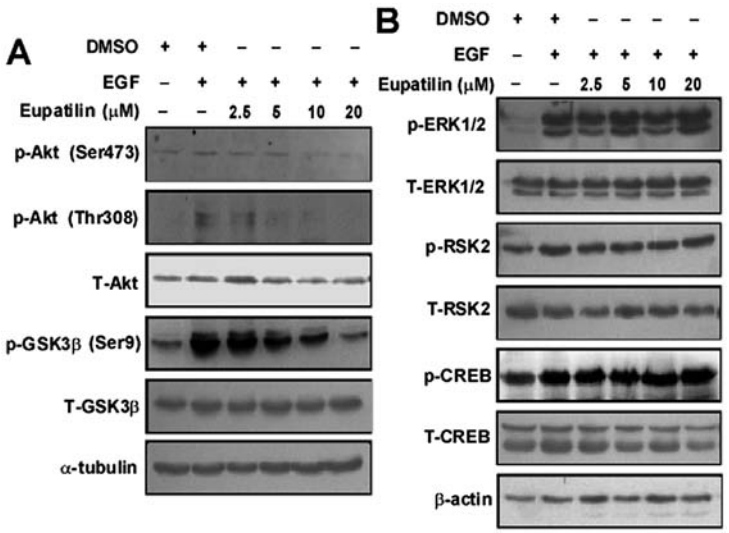

Figure 2. Eupatilin has an inhibitory effect on PI3K/Akt signaling. Eupatilin inhibits PI3K-mediated downstream signaling. (A and B) After the cell cycle synchronized into G_0 phase, JB6 cells were treated with various concentrations of eupatilin for 2 h followed by adding EGF (final 10 ng/ml concentration) for 30 min. The total and phosphorylated protein levels were analyzed by western blotting with specific antibodies. Equal loading of protein was confirmed by α-tubulin or β-actin and the data verified at least three times.

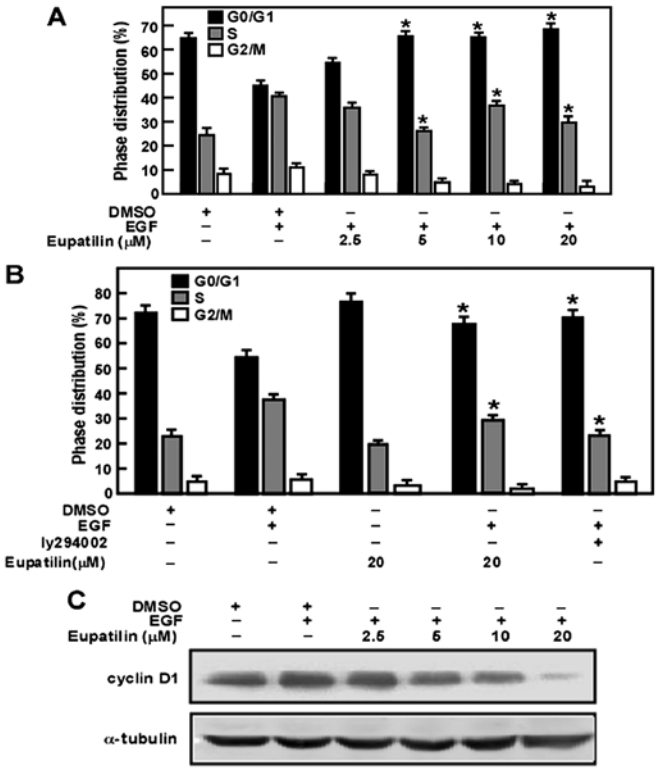

Figure 3. Eupatilin causes cell cycle arrest in G_0/G_1 phase by inhibiting the activity of cyclin D1. JB6 cells synchronized into G_0 phase were treated with various concentrations of eupatilin for 1 h followed by adding EGF (final 10 ng/ml concentration) as a growth stimulator. Subsequently, cells were harvested after 18 h and analyzed by flow cytometry. (A) Data were expressed as the percentage of cells in different phases and showed that eupatilin was the course of cell cycle arrest in G_0/G_1 phase concentration-dependently. Data represent the mean (n=3); bars, ±SD. $^*P<0.05$ vs. EGF stimulated and eupatilin untreated group as determined by Student's t-test. (B) Data revealed that eupatilin has a similar function as the PI3K inhibitor LY294002. Data represent the mean (n=3); bars, ±SD. $^*P<0.05$ vs. EGF stimulated, and not eupatilin or LY294002 treated group as determined by Student's t-test. (C) Data showed that eupatilin inhibits the expression of cyclin D1 in EGF induced JB6 cells dose-dependently. Data represent the mean of at least three repeated independent experiments.

in inhibiting JB6 cell proliferation, we analyzed the effect of eupatilin on the cell cycle. Our results showed that eupatilin arrested cells in G_0/G_1 phase in a dose-dependent manner. Moreover, cyclin D1 is required for the G_1/S transition (27). Thus, these studies indicated that eupatilin might down-regulate the expression of cyclin D1 leading to arrest of the cell cycle in G_0/G_1 phase (Fig. 3A). Furthermore, eupatilin had a similar function as PI3K inhibitor LY294002 (28) (Fig. 3B). Additionally, our results revealed that eupatilin affected downregulation of the expression of cyclin D1 concentration-dependently (Fig. 3C).

Eupatilin specifically binds with PI3K. PI3K plays a decisive role in tumorigenesis (15,16) through Akt phosphorylation. We supposed that PI3K might be a molecular target of eupatilin based on our western blotting data. We tested this idea by constructing a computer docking model, which showed that eupatilin was able to bind at the ATP binding pocket of p110, a catalytic subunit of PI3K (Fig. 4A). Subsequently, we verified the binding of eupatilin with PI3K by pull down assay. We found that eupatilin-Sepharose 4B beads can pull down PI3K, while Sepharose 4B beads could not be bind singly (Fig. 4B). Moreover, we confirmed in cell lysates that eupatilin could bind with endogenous PI3K (Fig. 4C).

Discussion

Cell transformation is a critical characteristic of carcinogenesis. In the present study, we used mouse epidermal JB6 cells, which are ideal in research on the molecular mechanisms of neoplastic transformation (29-32). EGF or TPA was used to make the normal cells to transform into cancer cells (33) through activating some signaling pathways, involved in cell proliferation, survival, motility and metabolism. EGF-induced cells produced moderate size, tumorigenic, anchorage-independent colonies in soft agar assay (34), which is not possible in normal cells. These cancer promoters

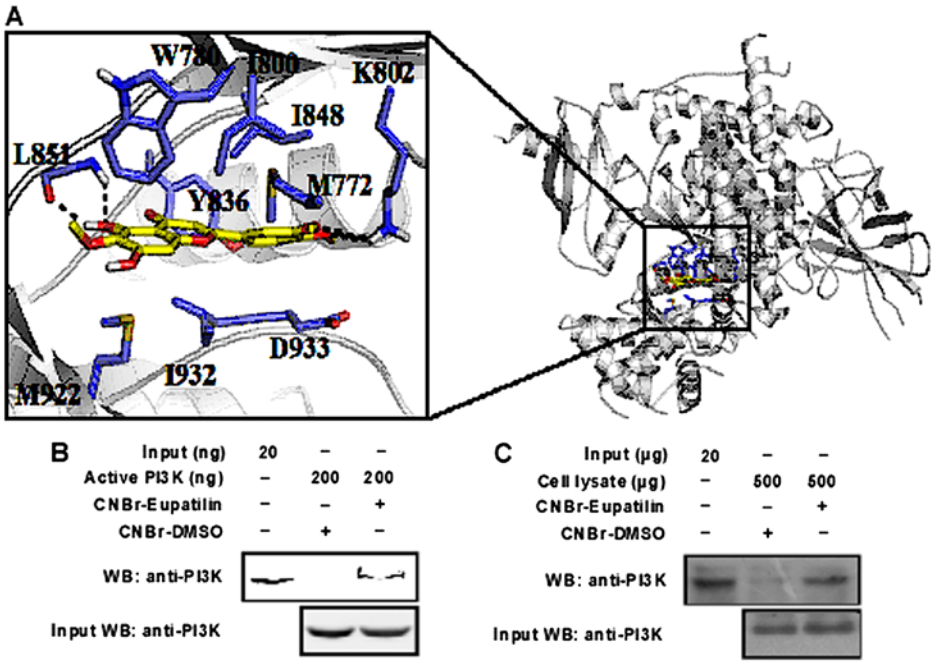

Figure 4. Eupatilin binds PI3K *in vitro* and *ex vivo*. (A) PI3K-eupatilin binding complex structure obtained from docking. The PI3K is represented as cartoon in grey color. Eupatilin is shown as sticks in yellow. The residues interacting with eupatilin are shown as sticks in purple. (B) Eupatilin binds with PI3K *in vitro*. Active PI3K (200 ng) was subjected to a pull down assay with eupatilin conjugated with CNBr-Sepharose 4B beads. Eupatilin binding of PI3K was visualized by western blotting with anti-PI3K. (C) Eupatilin binds with PI3K *ex vivo*. Jb6 cell lysates (500 μg) were used for the pull down assay with CNBr-DMSO or CNBr-eupatilin beads. The pulled-down PI3K was visualized by western blotting with anti-PI3K.

strongly activate PI3K/Akt, and MAPK signaling pathways which have direct role in carcinogenesis (35). Thus, it is an important strategy of cancer chemoprevention to identify a molecular target which activates the signaling pathways in cell transformation phase for novel anticancer molecules.

Flavonoids are well known as promising chemopreventive agents against human cancers. Eupatilin, a dietary flavone compound, has anti-ulcer, anti-inflammatory and cell cycle regulator inhibitory effects (6,8,36). Accumulating evidence shows that eupatilin has antitumor function against the different types of cancer, including gastric and endometrial cancer (36). In the present study, we found that eupatilin could inhibit JB6 cell proliferation and growth in a dose-dependent manner (Fig. 1B). Moreover, anchorage-independent colony formation was decreased with the increase of eupatilin concentration in anchorage-independent cell growth assay (Fig. 1C). These data suggested that eupatilin has cellular targets in EGF-induced JB6 cell transformation.

PI3K/Akt signaling pathway plays a pivotal role (37) in many biological processes such as regulation of cell survival, cell growth (38), apoptosis (39) and cell migration. Missense mutations in PI3K were detected in the colon, brain, breast and stomach cancer, leading to promotion of cell proliferation and tumorigenesis (12,40-42). Overexpression of PI3K/Akt signaling pathways activated the cell cycle dependence protein kinase (CDK) (43) following phosphorylation of GSK-3β at Ser9, leading to inhibition of GSK-3β activity and increasing cyclin D1 expression with the promotion of G_1 period development. Thus, activated PI3K pathway has a role in promoting carcinogenesis (44-47). GSK3β is a complex kinase, acting either as a tumor promoter or suppressor in different types of cancer (48). The different sites of phosphorylation decides the activation of GSK3β. GSK3β phosphorylation at Tyr216 or Ser9 causes activation or inhibition state, respectively. Ma *et al* (49) showed that tumor promoters of EGF and TPA induce strong phosphorylation of GSK3β at Ser9 in JB6 P^+ cells, accompanied by increasing anchorage-independent cell growth. Overexpression of S9A mutant in JB6 cells, leads to inactivation of GSK3β phosphorylation at Ser9 and it becomes less sensitive to EGF induced pGSK3β (Ser9) with the upregulation of cyclin D1. These results indicated that the cells were more resistant to the negative regulation of GK3β (49). In the present study, we showed that EGF induced the activation of PI3K/Akt/GSK-3β signaling pathway in JB6 cells. It also increased the phosphorylation of Akt at Ser473, Thr308 and GSK-3β at Ser9 compared with the control (Fig. 2A). Moreover, EGF treatment promotes the cyclin D1 expression (Fig. 3C) and increases cell percentage of S phase (Fig. 3A). However, we showed that eupatilin effectively reduced phosphorylation of Akt at Ser473 and Thr308 induced by EGF in a dose-dependent manner. Similarly, the phosphorylation of GSK3β (Ser9) was attenuated by eupatilin treatment (Fig. 2A). Eupatilin also decreased the expression level of cyclin D1 (Fig. 3C) and arrest the cell cycle arrest at G_1 phase (Fig. 3A). The results indicated that eupatilin could suppress EGF-induced JB6 cell transformation mediated through the PI3K/Akt/GSK3β pathway. Hence, we speculated that PI3K might be a molecular target of eupatilin. The idea was primarily verified by computer docking models, showing that eupatilin could strongly bind at ATP binding pocket of P110, a catalytic subunit of PI3K (Fig. 4A). Subsequently, we confirmed our hypothesis by pull-down assay with eupatilin-conjugated beads *in vitro* (Fig. 4B) and *ex vivo* (Fig. 4C).

In conclusion, eupatilin significantly contributes to inhibition of EGF-induced JB6 cell transformation through directly

targeting PI3K. Thus, eupatilin is a potential chemopreventive agent which may provide some insights into prevention or therapy for tumorigenesis caused by aberrant PI3K signaling pathway.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (nos. 81372269, 81472324 and 81572812) and the Science Foundation of Henan Education Department (no. 13HASTIT022).

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