β-elemene inhibits non-small cell lung cancer cell migration and invasion by inactivating the FAK-Src pathway

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Abstract. Despite sustained effort, the prognosis of lung cancer remains poor and the therapeutic responses are limited. Cell movement ability is a prerequisite for lung cancer metastasis, which involves focal adhesion kinase (FAK)-mediated cell migration and invasion via complex formation with Src. Hence, FAK-Src signaling might be an effective target for anti-cancer treatment. β-elemene, the major component of elemene extracted from Curcuma Rhizoma, exhibits broad-spectrum anti-tumor properties. However, the role of β-elemene in lung cancer cell motility and its possible mechanism remain unknown. Herein, the role of β-elemene in the migration and invasion of two non-small cell lung cancer (NSCLC) cell lines was investigated by performing wound-healing and Transwell assays. The mRNA expression levels of genes associated with motility, including *RhoA*, *Rac1*, *Cac42*, matrix metalloprotease (*MMP*)*2* and *MMP9*, were examined by reverse transcription-quantitative polymerase chain reaction. To determine whether β-elemene acts through FAK-Src signaling, western blotting was performed and the levels of phosphorylated FAK and Src were detected. The results indicated that β-elemene inhibited the migration and invasion of A549 and NCI-H1299 (H1299) cells, while the motility-associated genes were de-regulated following exposure to β-elemene. Furthermore, β-elemene decreased the activity of FAK and Src. Overall, these results suggest that β-elemene potentially inhibits NSCLC through FAK-Src signaling.

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Introduction

Lung cancer is one of the most invasive malignancies worldwide. Non-small cell lung cancer (NSCLC) is the major type of lung cancer, with an estimated 5-year survival rate of only 16% in 2014 in the United States (1). While great effort has been made to improve the prognosis of NSCLC, metastasis is still a serious hurdle for its treatment. Metastasis is a complex process; its foundation depends on the activity of cellular movement machinery (2). Therefore, the migration and invasion abilities may be potential targets for preventing metastasis.

Focal adhesions (FAs) act as important connectors between cells and the extracellular matrix (ECM). FA kinase (FAK) is a major kinase with a crucial role in cell motility (3). FAKs are recruited to FAs once integrins bind to ECM proteins in the tumor microenvironment (4). FAK forms a complex with Src when recognized by its SH2 domain. The FAK/Src complex has been demonstrated to promote cell migration and invasion in many types of cancer, including NSCLC (5-7). Inhibition of FAK/Src signaling decreases the migration and metastasis of ovarian and lung cancer cells (8-10). However, current drugs targeting FAK/Src (such as defactinib and dasatinib) showed limited success in the treatment of solid tumors (11).

β-elemene, a major component of Curcuma Rhizoma, has been demonstrated to have anti-tumor effects on different types of cancer, including gastric cancer and breast cancer (12,13). Studies performed on cells and animals have shown that β-elemene induced apoptosis and decreased proliferation in esophageal squamous carcinoma cell, gastric cancer cell and rheumatoid arthritis fibroblast-like synoviocytes (12,14,15). Several experiments have demonstrated the chemotherapeutic effects of β-elemene by decreasing cytotoxicity (16,17). β-elemene has been shown to decrease the migration and invasion of lung cancer cells by suppressing the epithelial-to-mesenchymal transition (EMT) (18). β-elemene was also shown to exert an anti-metastatic effect on breast cancer cells by blocking aerobic glycolysis (13). However, the role of β-elemene in cell motility in lung cancer and its possible underlying mechanism remain poorly defined.

In the present study, it was hypothesized that β-elemene inhibits cell motility in lung cancer cells through the FAK-Src

signaling pathway. The effects of β-elemene on cell migration and invasiveness were investigated in two NSCLC cell lines (A549 and H1299) using wound-healing and Transwell assays. The impact of β-elemene on the expression levels of mRNA and protein associated with FAK-Src signaling was determined. The results suggest that β-elemene could suppress NSCLC cell motility via the inhibition of FAK-Src signaling.

Materials and methods

Reagents. β-elemene was obtained from CSCP Pharmaceutical Group Ltd. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM/H) were purchased from Cytvia. Dimethyl sulfoxide (DMSO) was purchased from BioFROXX and 3-(4,5-dimethylthiazol-2-yl)-2, 5-dephenyltetrazolium bromide (MTT) was obtained from (Shanghai Ica Biotechnology Co., Ltd.; cat. no. MO105-1G). A Matrigel-coated Transwell chamber was purchased from BD Biosciences. Antibodies against phosphorylated (p)-FAKTyr397, total (t)-FAK, p-SrcTyr416, p-SrcTyr527, and t-Src were obtained from Abcam. Crystal violet was obtained from Beyotime Institute of Biotechnology.

Cell culture and treatment. A549 and H1299 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in a 37˚C incubator supplemented with 5% CO_2. β-elemene was diluted to 25, 50, 100 and 200 μg/ml with complete medium. A vehicle control consisting of 0.2% DMSO was used (0 μg/ml β-elemene).

MTT assay. In total, 3,000 cells were plated into each well of a 96-well plate. After incubating overnight, different concentrations of β-elemene (0, 50, 100 and 200 μg/ml) were added with fresh medium to each well. Subsequently, cells were washed with phosphate-buffered saline (PBS) and further incubated with 10 μl MTT for 4 h. Subsequently, the MTT was aspirated and the cells were washed with PBS. DMSO (100 μl) was added to dissolve the formazan crystals. The absorbance at 570 nm was measured with a microplate reader (Molecular Devices SpectraMax i3; Bio-Rad Laboratories, Inc.). Cell numbers were normalized to the 0 μg/ml group.

Migration assay. A549 and H1299 cells were plated into 12-well plates ($2x10^5$ each well). Once cells reached 80-90% confluence, a 200-μl pipette tip was used to generate a scratch. Floating cells were washed with PBS. Then, different concentrations of β-elemene (0 and 50 μg/ml) with fresh serum-free medium were added to each well. The wound gap was imaged with a light microscope (magnification, x4 and x10; XDS-1A; Precision Instruments) after 0, 12 and 24 h of incubation. The migration ability was determined by measuring the width (the shortest measurement horizontally across the gap) of the scratch and was normalized to the 0-μg/ml group.

Invasion assay. The invasion capacity of cells was determined using a Matrigel-coated Transwell chamber (BD Biosciences). After treating with 0 or 50 μg/ml β-elemene with serum-free medium, 5x106 cells were plated on the top chamber of the Transwell insert and stimulated with 500 μl medium with 50% FBS added to the bottom chamber. After 24 h of incubation (5% CO_2 at 37˚C), a cotton swab was used to remove the non-invasive cells on the inside of the upper chamber. The invasive cells on the underside of the upper chamber were fixed using 4% paraformaldehyde (PFA) for 10 min at room temperature and stained with 2% crystal violet (20 min at room temperature). Random fields were imaged (magnification, x100) and measured with a light microscope.

Table I. Primer sequences for PCR amplification.

Names	Sequences, 5' to 3'
RhoA-Human-RT-F	GGAAAGCAGGTAGAGTTGGCT
RhoA-Human-RT-R	GGCTGTCGATGGAAAAACACAT
Rac1-Human-RT-F	ATGTCCGTGCAAAGTGGTATC
Rac1-Human-RT-R	CTCGGATCGCTTCGTCAAACA
Cdc42-Human-RT-F	CCATCGGAATATGTACCGACTG
Cdc42-Human-RT-R	CTCAGCGGTCGTAATCTGTCA
MMP2-Human-RT-F	GATACCCCTTTGACGGTAAGGA
MMP2-Human-RT-R	CCTTCTCCCAAGGTCCATAGC
MMP9-Human-RT-F	GGGACGCAGACATCGTCATC
MMP9-Human-RT-R	TCGTCATCGTCGAAATGGGC
β-actin-Human-RT-F	AGCGAGCATCCCCCAAAGTT
β-actin-Human-RT-R	GGGCACGAAGGCTCATCATT

F, forward; R, reverse; MMP, matrix metalloprotease.

Cell adhesion assay. After treatment with 0 or 50 μg/ml β-elemene, cells were digested and resuspended in complete medium. Then, the cells (2,000 cells/well) were plated in each well of a 96-well plate precoated with 20 μl Matrigel for 1 h. The cells were subsequently gently washed with PBS. The remaining cells were fixed with 4% PFA for 10 min at room temperature and stained with 0.2% crystal violet for 20 min at room temperature. The plates were placed directly under a light microscope (magnification, x100) to measure the adhesive cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from A549 and H1299 cells treated with β-elemene (0 or 50 μg/ml) using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Reverse transcription was performed with a RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) using 100 ng of total RNA (60 min at 42˚C, 5 min at 25˚C followed by 60 min at 42˚C and 5 min at 70˚C). The primers used to amplify (Table I) *RhoA*, *Rac1*, *Cdc42*, matrix metalloproteinase (*MMP*)*2* and *MMP9* were designed with Primer Premier 5.0 software (BBI Life Sciences). The thermocycling parameters for RT-qPCR using SybrGreen qPCR Master mix (cat. no. F-415XL; Thermo Fisher Scientific, Inc.) were: 94˚C for 10 min followed by 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec, and 72˚C for 30 sec. Relative changes in mRNA expression were calculated using the $2^{-\Delta\Delta Cq}$ method with *β-actin* as the internal control (19).

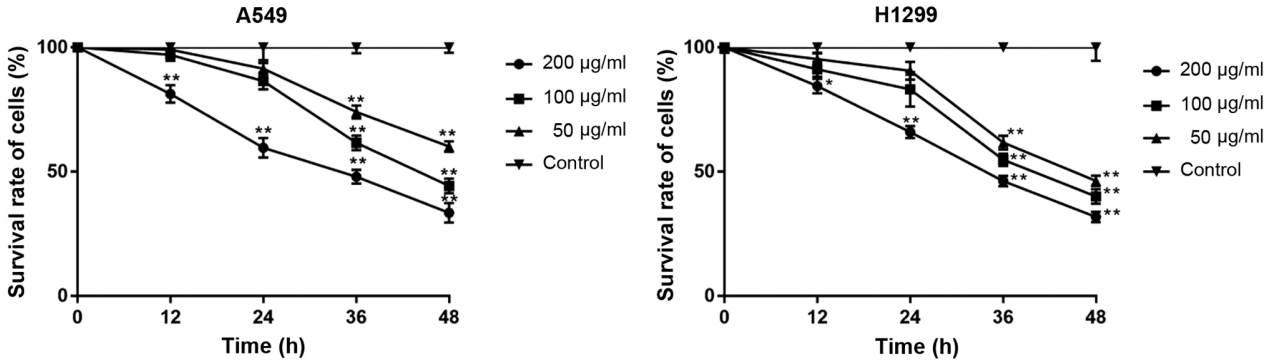

Figure 1. β-elemene decreases the viability of A5469 and H1299 cells. Concentration-dependent decreases in the cell viability of A5469 and H1299 cells, following treatment with different concentrations of β-elemene for 48 h. Cell viability was assessed using the MTT assay. *P<0.05; **P<0.01 vs. 0 μg/ml β-elemene-treated group.

Western blotting. A549 and H1299 cells were treated with 50 μg/ml of β-elemene for 0, 1, 3, 6, 12 and 24 h. Then, radioimmunoprecipitation assay lysis buffer and phenylmethylsulfonyl fluoride were added to the cells, and incubated on ice for 2 h. After centrifuging at 161 x g for 10 min at 4˚C, 30 μg of total protein was loaded on 12% gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was run for 1 h at 120 V. The proteins were transferred to polyvinylidene difluoride membranes and run at 100 V for 1 h. Subsequently, the membranes were blocked with 5% nonfat milk in TBST at room temperature for 1 h. Next, the membranes were incubated with specific primary antibodies in blocking buffer against p-FAK^{Tyr397} (Abcam; cat. no. ab81298; 1:1,000), t-FAK (Abcam; cat. no. ab40794; 1:2,000), p-Src^{Tyr416} (Abcam; cat. no. ab4066; 1:1,000), p-Src^{Tyr527} (Abcam; cat. no. ab32078; 1:5,000), and t-Src (Abcam; cat. no. ab109381; 1:10,000) at 4˚C overnight. The membranes were then washed with TBST three times and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Biosharp Life Sciences; cat. no. BL003A; 1:4,000) in blocking buffer for 1 h at room temperature. The membranes were washed with TBST three times and the bands were visualized using an electrochemiluminescence system (Bio-Rad Laboratories, Inc.) in a dark room according to the manufacturer's instructions. The expression levels of specific proteins in A549 and H1299 cells were normalized to those in cells of the 0 μg/ml group.

Statistical analysis. All data are shown as the mean ± standard error of the mean. More than three independent replicates were performed for each set of experiments. All statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software, Inc.). The differences between treatment groups were compared using the Student's t-test and one-way analysis of variance (ANOVA) with Dunnett's post hoc test. P<0.05 was considered to indicate statistically significant difference.

Results

β-elemene decreases the viability of A549 and H1299 cells. To investigate the effects of β-elemene on cell viability and determine the suitable concentration for subsequent experiments, A549 and H1299 cells were treated with different doses of β-elemene (0-200 μg/ml) for 0-48 h. Cell viability was determined with the MTT assay. A concentration-dependent decrease in viability was observed in both cell lines after exposure to β-elemene (Fig. 1). Treatment with 50 μg/ml β-elemene for 24 h showed no inhibitory effect on either cell line and was selected for subsequent experiments.

β-elemene suppresses the migration of A549 and H1299 cells. Tumor cell migration is a critical step in metastasis. To examine the effects of β-elemene on the migration of A549 and H1299 cells, a scratch wound-healing assay was performed. Cells were inoculated with 0 and 50 μg/ml of β-elemene for 0, 12 and 24 h. As shown in Fig. 2A and B, the scratch width was longer in the β-elemene-treated A549 cells compared with the control group. Similarly, a longer scratch width was observed in the H1299 cells treated with β-elemene (Fig. 2C and D).

β-elemene inhibits the invasion and adhesion of A5469 and H1299 cells. To investigate the effect of β-elemene on NSCLC cell invasion, a Matrigel-coated Transwell assay was performed. A549 and H1299 cells were inoculated with 50 μg/ml of β-elemene for 24 h. As shown in Fig. 3A-C, inoculation with β-elemene significantly decreased invasive cells, represented by crystal violet staining. Next, the anti-adhesion effects of β-elemene on A549 and H1299 cells were assessed using an adhesion assay. Cells were treated with 50 μg/ml of β-elemene for 12, 24 and 48 h. Cells that adhered to the Matrigel were stained with crystal violet. Compared with untreated cells (0 μg/ml), β-elemene exposure resulted in a decrease in the quantity of adherent cells in a time-dependent manner (Fig. 3D-F). Overall, these results indicate that β-elemene inhibits invasion and adhesion in A549 and H1299 cells.

β-elemene decreases the mRNA expression of motility-associated genes in A5469 and H1299 cells. RhoA, Rac1 and Cdc42, which are small GTP-binding proteins in the Rho family, have been reported to regulate the cellular cytoskeleton and cell migration (20). To determine whether β-elemene regulates the expression of genes associated with migration and invasion, the mRNA expression of *RhoA*, *Rac1* and *Cac42* was compared in A549 and H1299 cells by RT-qPCR, following inoculation with 0 and 50 μg/ml of β-elemene for 24 h. β-elemene treatment

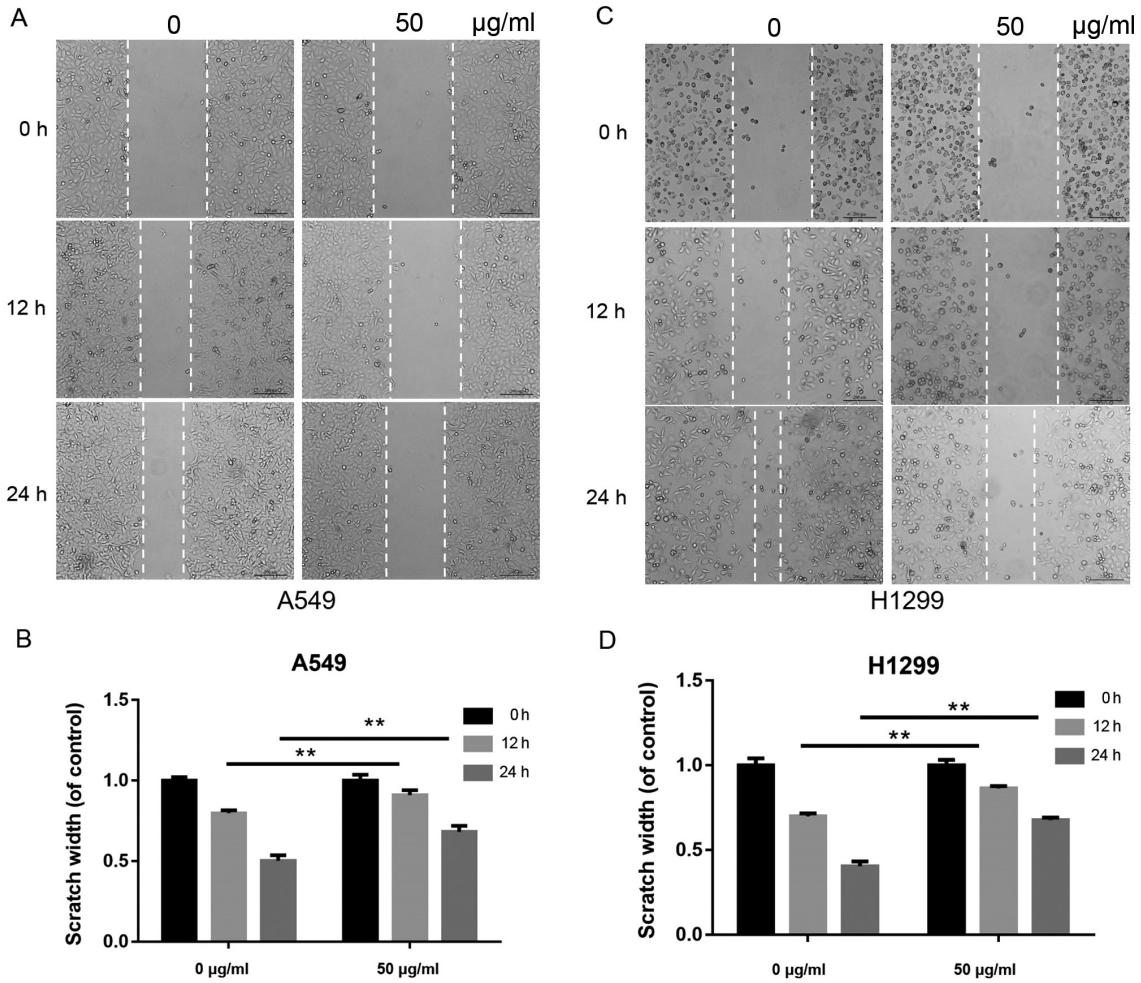

Figure 2. β-elemene inhibits the migration of A549 and H1299 cells. (A and C) A549 and H1299 cells were exposed to β-elemene (0 or 50 μg/ml) for 0, 12 and 24 h. The anti-migration effects of β-elemene were determined using a wound-healing assay. (B and D) The anti-migration effects of β-elemene were quantified by measuring the scratch width. The data shown are representative of more than three independent experiments. **P<0.01 vs. 0 μg/ml or 0 h β-elemene-treated group.

remarkably decreased the mRNA expression levels of *RhoA*, *Rac1* and *Cac42* (Fig. 4A and C). MMP2 and MMP9 are key members of the MMP family, which may also facilitate cell migration and invasion (21,22). As shown in Fig. 4B and D, the mRNA levels of *MMP2* and *MMP9* were decreased after incubation with β-elemene. The data suggest that β-elemene inhibits the expression of genes associated with cell motility.

β-elemene inhibits FAK-Src activation in A5469 and H1299 cells. To elucidate whether FAK-Src signaling was involved in the anti-migration effect of β-elemene on NSCLC cells, the activity of FAK and Src was evaluated by western blotting. As shown in Fig. 5A-D, treatment with 50 μg/ml β-elemene decreased the phosphorylation of FAK (p-FAK) on Y397 and the phosphorylation of Src (p-Src) on Y416 in both A549 and H1299 cells. However, p-Src on Y527 was increased after β-elemene inoculation. Overall, β-elemene may inhibit the activity of the FAK-Src pathway in lung cancer cells.

Discussion

Metastasis is a multi-step process that includes the following: i) Tumor cells detach from the primary tumor by altering cell-cell adhesion; ii) tumor cells infiltrate adjacent tissues; iii) tumor cells migrate into the vasculature; iv) tumor cells survive in the circulation; and v) tumor cells extravasate and proliferate in a new tissue (23). Hence, migration capacity is critical for successful metastasis. In the present study, the role of β-elemene in the motility of NSCLC cells was investigated. The data indicate that β-elemene treatment inhibited the viability, migration, invasion and adhesion of A549 and H1299 cells. Moreover, β-elemene decreased the mRNA expression levels of motility-associated genes, including *RhoA*, *Rac1*, *Cdc42*, *MMP2* and *MMP9*. Finally, it was demonstrated that the anti-migratory and anti-invasive effects of β-elemene might be regulated by the FAK-Src signaling pathway.

β-elemene, an organic compound extracted from Curcuma Rhizoma, has attracted scientific interest due to its good performance in anti-cancer treatment. β-elemene has been reported to inhibit cell growth, induce apoptosis, and block the EMT in different types of cancer cells (12,14,24). Using the MTT assay, it was also demonstrated that β-elemene treatment inhibited the cell viability of NSCLC cells. In A549 and H460 cells, β-elemene induced cell death through G_2-M regulation and apoptosis-modulating proteins, including Cdc2, Bcl-2 and

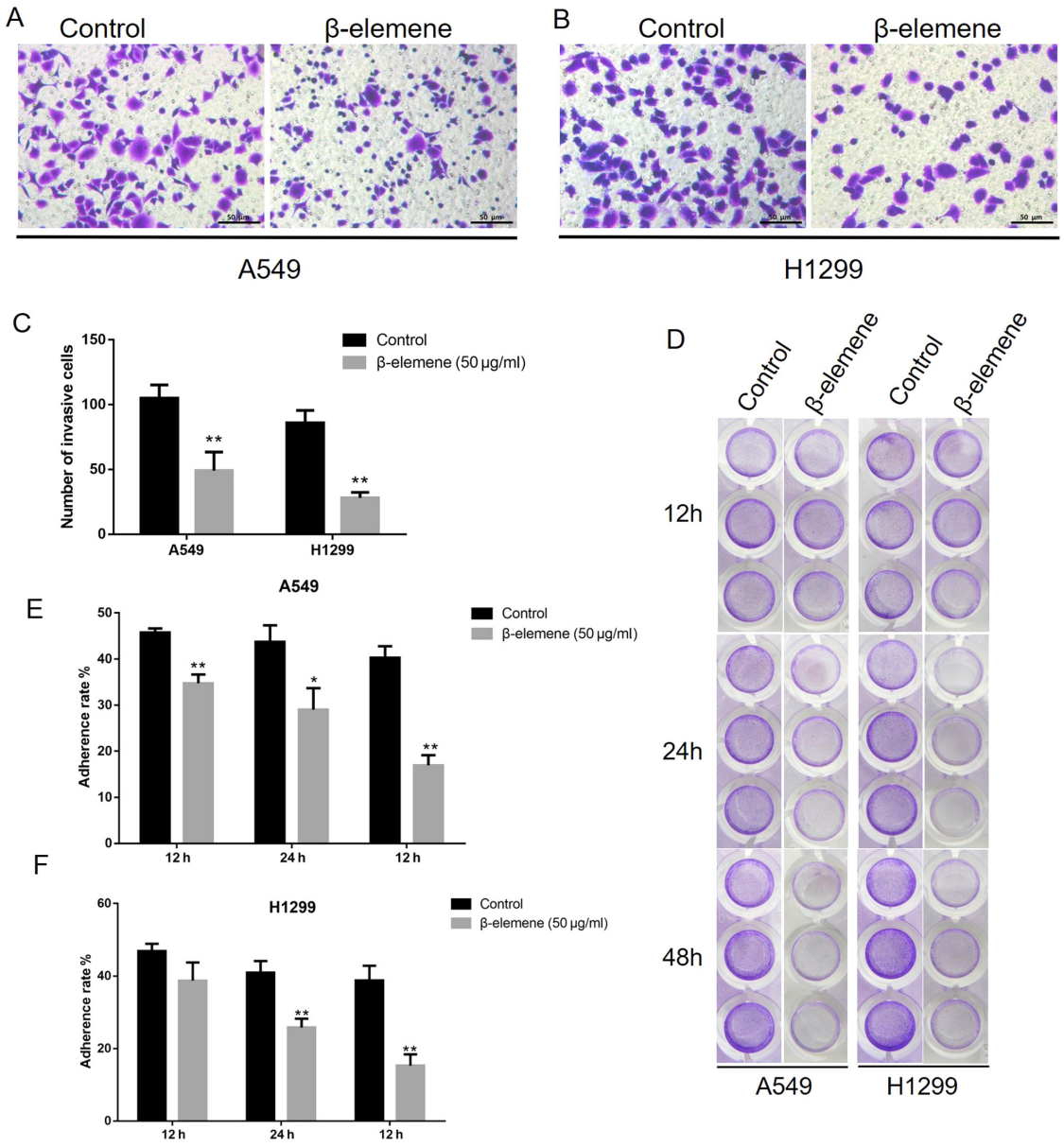

Figure 3. β-elemene inhibits invasion and adhesion of A549 and H1299 cells. (A and B) Cells were treated with 0 or 50 μg/ml of β-elemene for 24 h. Invasion ability was assessed using the Transwell chamber invasion assay. (C) After treatment, cells that translocated to the lower compartment were stained with crystal violet and measured under a light microscope. (D) Cells were seeded on Matrigel-coated plates and treated with 0 or 50 μg/ml of β-elemene for 12, 24 and 48 h. Adherent cells were stained with crystal violet. (E and F) The adherence rate was calculated. The data shown are representative of more than three independent experiments. *P<0.05; **P<0.01 vs. 0 μg/ml or 0 h β-elemene-treated group.

cleaved caspase-9 (25). In H1299 cells, β-elemene inhibited cell growth through the AMPKα- and ERK1/2-regulated inhibition of Sp1 (26). β-elemene also inhibited the migration and the invasive ability of tumor cells in gastric cancer (27). In the present study, it was also found that migration was inhibited in A549 and H460 cells following β-elemene treatment.

FAK and Src are key regulators of integrin-mediated cell adhesion and migration. FAK upregulation and hyperphosphorylation have been demonstrated to increase invasive capability in a variety of human cancer types including gastric cancer and breast cancer (28). The phosphorylation of FAK on Try397, which is the major site of phosphorylation, led to the progression of tumor cells by promoting migration and invasion (29). FAK phosphorylation on Try397 creates a high-affinity binding site for the recognition of the SH2 domain in Src family kinases. Moreover, phosphorylation of FAK on Tyr397 is important for the activation and recruitment of Src via the formation of the FAK-Src complex (30). Numerous studies have demonstrated that FAK-Src signaling is important in the regulation of cell migration (4). Here, we found that β-elemene treatment decreased the phosphorylation of FAK on Tyr397 and of Src on Tyr416 in both A549 and H460 cells, while the phosphorylation of Src on Tyr527 was elevated. The phosphorylation of Src on Tyr527 decreases its recruitment to FAK, while the phosphorylation of Src on Tyr416 activates its recruitment (31). In a previous study, breast cancer cells with FAK-Src inactivation showed decreased metastatic potential (10). The present results suggest that the suppression of FAK-Src signaling by β-elemene may decrease NSCLC metastasis by decreasing cell migration.

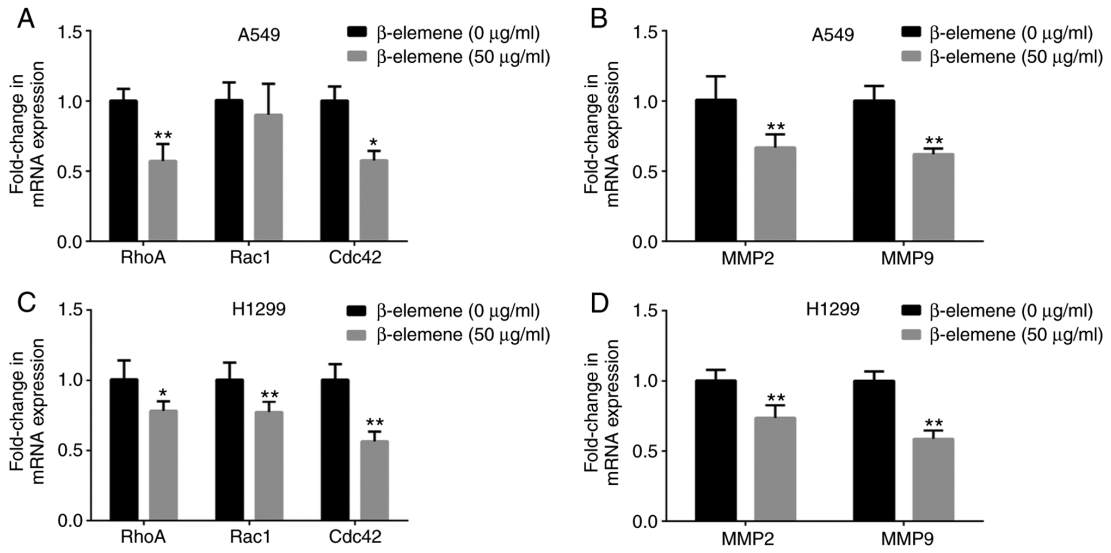

Figure 4. β-elemene inhibits the expression of motility-associated genes in A549 and H1299 cells. (A and B) A549 and (C and D) H1299 cells were inoculated with 0 or 50 μg/m β-elemene for 24 h, and reverse transcription-quantitative PCR was performed to detect changes in *RhoA*, *Rac1*, *Cac42*, *MMP2* and *MMP9* mRNA expression levels. The data shown are representative of more than three independent experiments. *P<0.05; **P<0.01 vs. 0 μg/ml β-elemene-treated group. MMP, matrix metalloprotease.

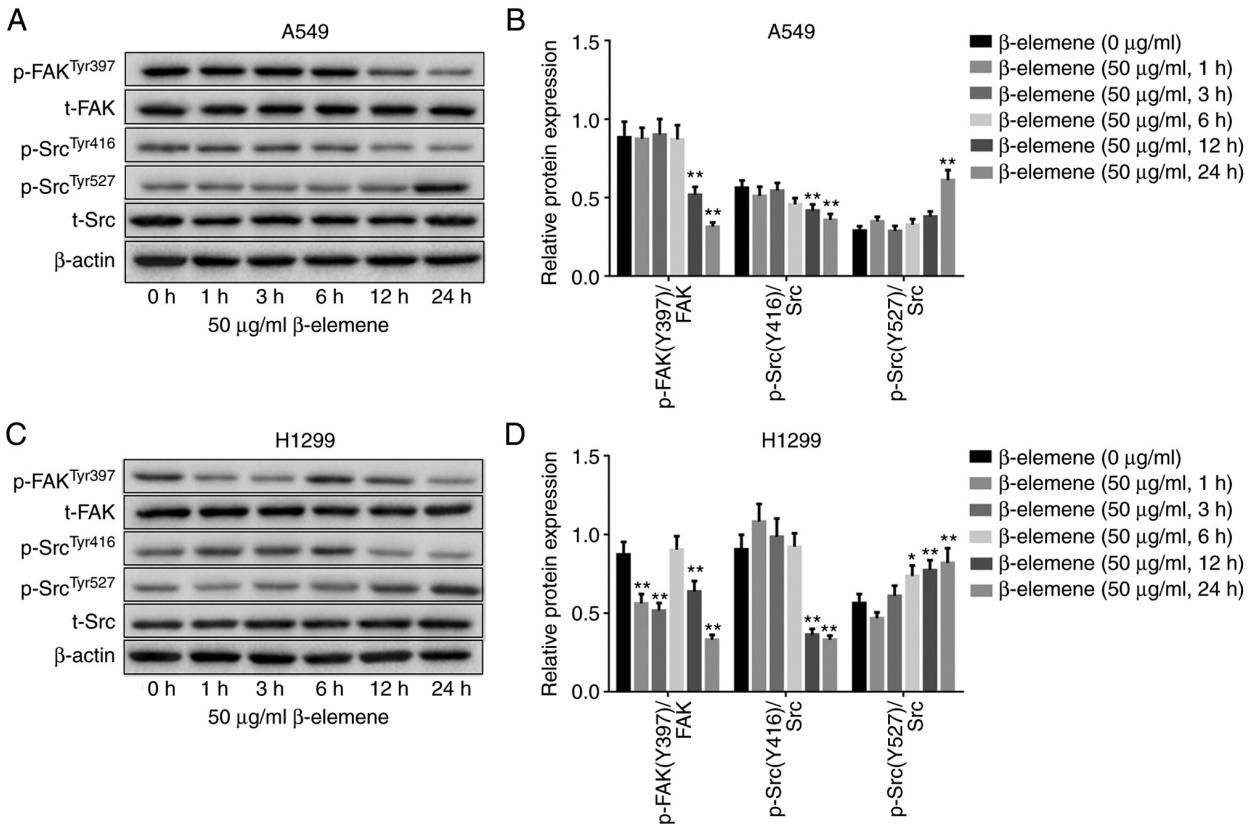

Figure 5. β-elemene suppresses the activity of FAK-Src in A549 and H1299 cells. (A and C) A549 or H1299 cells were exposed to 50 μg/ml β-elemene for 0, 1, 3, 6, 12 and 24 h, and western blotting was performed to analyze the effects of β-elemene on FAK and Src protein levels. (B and D) Relative protein levels were normalized to total protein levels. The data shown are representative of more than three independent experiments. *P<0.05; **P<0.01 vs. 0 μg/ml β-elemene-treated group. FAK, focal adhesion kinase; p-, phosphorylated; t-, total.

The formation and remodeling of FAK-Src-Paxillin contributes to cell migration in a dynamic process, under the regulation of GTPases in the Rho family. Cell movement is dependent on the dynamic organization of the protrusion of filopodia and lamellipodia, whose formation is regulated by Cdc42 and Rac1 (32). RhoA also acts to promote tension in the organization of actin. In addition, the regulation of Rho, Rac, and Cdc42 is coordinated within cells. RhoA is downstream of Rac1, which in turn is downstream of Cdc42. In the present study, treatment with β-elemene led to a remarkable

decrease in the mRNA expression of *RhoA*, *Rac1* and *Cdc42*, suggesting that filopodia formation might be disrupted by β-elemene. MMPs are required for degradation of the ECM, which is required for the dissemination of tumor cells. Among the MMPs, MMP2 and MMP9 are highly associated with cell migration. It has been shown that the inhibition of *MMP2* and *MMP9* decreased angiogenesis and the migration of retinoblastoma cells (33). The present study shows that β-elemene repressed the mRNA expression of MMP2 and MMP9 in A549 and H1299 cells. These results suggest that β-elemene inhibited the expression of cell motility-associated genes.

Although the current study was the first, to the best of our knowledge, to demonstrate that β-elemene inhibited non-small cell lung cancer cell migration and invasion by inactivating the FAK-Src pathway, the exact mechanism for these inhibitory effects is yet to be fully elucidated, such as the molecular target of β-elemene in the FAK-Src signaling pathway. Future studies should therefore elucidate the exact molecular target of β-elemene in non-small cell lung cancer cells and evaluate the therapeutic effects of β-elemene *in vivo*.

Overall, β-elemene exposure inhibits NSCLC cell migration and invasion by suppressing the activity of FAK-Src signaling. Moreover, β-elemene leads to dysregulated expression of motility-associated Rho GTPases and MMPs. The present results suggest that β-elemene holds promise as an anti-metastatic therapy to prevent tumor cell migration.

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Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding authors on reasonable request.

Authors' contributions

The study was conceived and designed by LQ and HZ. HZ and SL conducted most of the experiments with assistance from JB, NG and FH. The manuscript was written by HZ and SL, with contributions from LQ. All authors read and approved the final manuscript. HZ and LQ confirm the authenticity of all raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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