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Original Article

β-elemene suppresses the epithelial-mesenchymal transition of non-small-cell lung cancer via the wnt/β-catenin pathway

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Abstract: Objective: Lung cancer is the leading cause of cancer-associated deaths worldwide. Of the various types of lung cancer, non-small-cell lung cancer (NSCLC) has the highest incidence. The epithelial-to-mesenchymal transition (EMT) in the tumor microenvironment plays an significant role in NSCLC invasion and tumor metastasis. The Wnt/β-catenin pathway has been shown to involve the EMT in NSCLC. In our study, we profiled β-elemene expression and its related functions on the proliferation, apoptosis, invasion, and EMT in two different types of NSCLC cells. Method: During this study, we used A549 and H1299 cells. The cell proliferation was assessed with the MTT experiments. The apoptotic cell death was assessed using an Annexin V/PI detection kit. The cell invasion ability was assessed through Transwell assays. The Wnt/β-catenin pathway target gene expression and the related EMT protein was determined through Western blotting. Results: We demonstrated that β-elemene downregulates the A549 and H1299 proliferation abilities and induces cell apoptosis in a concentration-based way. We found that the pretreatment of cells with β-elemene for 24 h decreases invasion. We also demonstrated that β-elemene upregulates the level of the EMT-related protein E-cadherin and downregulates the vimentin, β-catenin, and N-cadherin levels. The triggering of Wnt/β-catenin signaling by LiCl reverses the influence of β-elemene on NSCLC cell invasion and the expression of the EMT-related transcription factors. In addition, LiCl also reverses the influence of β-elemene on NSCLC cell proliferation and the apoptotic process. Conclusion: Our findings show that β-elemene suppresses the EMT and the invasion of NSCLC cells via the Wnt/β-catenin pathway for the first time, suggesting that β-elemene may prevent NSCLC metastasis.

Keywords: β-elemene, non-small-cell lung cancer, epithelial-mesenchymal transition, Wnt/β-catenin pathway

Introduction

Although efforts have been made to increase its survival duration, lung carcinoma remains the principal cause of tumor-related deaths. Non-small-cell lung cancer (NSCLC) accounts for almost 85% of all lung carcinoma cases [1]. As a result of distant and local metastasis, less than 25% of patients with NSCLC receive surgical therapy [2]. Targeted therapies have been developed to treat advanced or metastatic NSCLC [3]. However, tumor cells generally develop resistance to these therapies within a few months. Hence, the development of new effective therapies is urgently needed to improve the outcomes of NSCLC.

Metastasis is an intricate and multi-stage activity including cell migration, invasion, and extravasation. The epithelial-mesenchymal transition (EMT) is critical for tumor dissemination. During the EMT, epithelial cells lose the junction between cells and undertake the remodeling of the cytoskeleton. Concurrently, they acquire the characteristics of mesenchymal cells and stem cells, facilitating metastasis [4]. The EMT is responsible for the progression, drug resistance, and stemness of NSCLC [4]. Therefore, targeting the EMT could prevent NSCLC metastasis. Several developmental pathways involved in the EMT are associated with the progression of NSCLC [4]. Among these, the

Wnt/β-catenin pathway has attracted attention for its potential as a therapeutic target [5].

β-elemene is the main component of elemene, which is extracted from *Rhizoma zeodaria* and has antitumor, anti-inflammatory, and antifibrotic activities [6, 7]. The antitumor activity of β-elemene has been demonstrated in lung cancer, glioblastoma, prostate cancer, and colorectal cancer [8, 9]. Importantly, β-elemene suppresses invasion and the EMT in breast cancer and melanoma [10, 11]. However, whether β-elemene inhibits the EMT in NSCLC, and the underlying mechanism, are unclear.

Our study focuses on the effects of β-elemene on the biological function of NSCLC and its potential molecular mechanism. Our research also suggests that β-elemene may inhibit the metastasis of NSCLC by suppressing the EMT of NSCLC via the Wnt/β-catenin pathway. Our findings will likely promote the clinical application of β-elemene.

Materials and methods

Cell lines

The NSCLC cell lines H1299 and A549 were obtained from the Shanghai Institute for Biological Sciences, CAS (Shanghai, China).

Drugs and reagents

β-elemene was purchased from the CSPC-Yuanda Pharmaceutical Co. (Dalian, Liaoning, China) and was prepared to 50 μg/mL (0.244 μmol/mL), 100 μg/mL (0.489 μmol/mL), 150 μg/mL (0.734 μmol/mL), and 200 μg/mL (0.979 μmol/mL) with complete medium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Shanghai EKEAR Bio@Tech Co. (Shanghai, China). Dimethyl sulfoxide (DMSO) was obtained from BioFROXX (Shanghai, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining kit was provided by BD Science (San Diego, CA). Then, the rest of the antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the EMT-related factors, and the Wnt/β-catenin were provided by Cell Signaling Technology (Beverly, MA, US). LiCl was obtained from Amresco (Solon, Ohio, US).

MTT assay

A549 or NCI-H1299 cells (3×10^3) were seeded in 96-well plates. The cells were treated with β-elemene (50, 100, and 150 μg/mL) for 48 h, then 15 μL MTT was dripped into the wells and the cells were cultured at 37°C for 4 h. The medium was subsequently poured off, and 200 μL DMSO was put in. The optical density (OD) at 492 nm was calculated using a SpectraMax® i3 plate reader (Molecular Devices, US).

Apoptosis assay

Cells cultured with 50, 100, and 150 μg/mL β-elemene for 1 d were obtained and then resuspended in a binding buffer. Annexin V-FITC/PI staining was carried out next following the manufacturer's instructions. The FACSCanto II Flow Cytometer (BD, San Diego, CA) was employed to analyze the apoptosis of the NSCLC cells.

Western blotting

We lysed the cells in a radioimmunoprecipitation assay buffer mixed with phenylmethylsulfonyl fluoride on ice for 2 h. After that, we centrifuged the lysate at a speed of 12,000 r/min (10 min, 4°C). The total protein was extracted using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and put onto polyvinylidene difluoride membranes (Millipore, US). The membranes were blocked with 5% nonfat milk and probed with the primary antibodies overnight (4°C). Next, he membranes were washed five times and the secondary antibodies were cultured in an environment of 37°C for 60 min. The bound proteins were imaged using enhanced chemiluminescence (Amersham Biosciences, Sweden). The densities of the protein bands were normalized to that of GAPDH using Image J software (NIH, Bethesda, MD). The antibodies used in this study included: vimentin (1:1000, 5741T, Cell Signaling), E-cadherin (1:1000, 3195T, Cell Signaling), N-cadherin (1:1000, 13116T, Cell Signaling), GAPDH (1:10000, 181602, Abcam), β-catenin (1:5000, ab32572, Abcam), p-GSK-3β (1:10000, ab75814, Abcam), and GSK-3β (1:5000, ab32391, Abcam).

Transwell assay

The cell invasive performance was evaluated by applying 24-well plates equipped with Ma-

trigel-coated chamber inserts (8-μm; Corning, US). Cells (5×10^4) resuspended in medium with no serum (100 μL, supplemented with 50-150 μg/mL β-elemene, 25 mM LiCl, or DMSO) were added into the upper chamber; 600 μL complete DMEM with 10% FBS was transferred into the lower chamber. After incubating for 12 h (at 37°C), the cells were fixed with 100% methanol for 0.5 h and stained with 0.1% crystal violet for 10 min. Finally, we observed and calculated the number of cells in 16 random fields under a microscope.

qRT-PCR

An RNeasy Extract Kit (Solarbio, Beijing, China) was employed to extract total RNA according to the manufacturer's instructions. Using total RNA and the HiScript II first-strand synthesis system (Vazyme Biotech, Nanjing, China), cDNA was prepared using reverse transcription following the manufacturer's instructions. The cDNA samples were then stored at -20°C before we performed the quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR amplification was done using a SYBR Premix Ex Taq kit (Takara) in a 20 μL reaction containing 0.4 μL of each primer, 0.4 μL SYBR green dye and 2 μL of cDNA. The primer sequences were as follows: MMP-7, TGGCCTACCTATAACTGG, reverse primer TAAAGCCTTTGACACTAATC; Cyclin D1, forward primer GCGAGGAACAGAAGTGCG, reverse primer TGGAGTTGTCGGTGTAGATGC; C-Myc, reverse primer CTGCGACGAGGAGGAGAA, reverse primer CCGAAGGGAGAAGGGTGT; β-actin, forward primer AGCGAGCATCCCCCAAAGTT, forward primer GGGCACGAAGGCTCATCATT. The RT-PCR was carried out on a Roche Light Cycler 480II with the steps below: 95°C for 30 sec followed by 95°C for 5 sec (40 cycles) and 58°C for 34 sec. The relative gene expression levels were measured using the 2-ΔΔCt method employing β-actin as the internal control.

TUNEL assay

The density of the cells was regulated to 10^5 cells/mL and the cells were then added into 48-well plates. Then 50 μg/mL β-elemene and 25 mM LiCl were added and the solution was cultured for 24 h at 37°C. Then we cleaned the solution with ice-cold PBS twice and then we examined the solution using TUNEL reagents. The cells were then treated with proteinase K for 10 min. Then the reaction solution was added and cleaned with PBS 3 times. The apoptotic cells were counted using a fluorescence microscope.

Statistical analysis

The values are the means ± standard deviations. SPSS software (v. 22.0, US) was used for the Statistical analysis. The comparisons between two groups were performed using Student's *t*-tests. The variance analyses for multiple comparisons were conducted by applying the Dunnett test after one-way ANOVA. Each experiment was repeated three times. P values < 0.05 indicated statistical significance.

Results

β-elemene suppresses the proliferation of NSCLC cells

To further investigate the antitumor activity of the NSCLC cells and to eventually identify the suitable concentrations for subsequent experiments, the A549 and H1299 cells were processed as described above. The proliferation was not suppressed by culturing with 50, 100, or 150 μg/mL β-elemene for 24 h (**Figure 1A**), but the treatment for a longer period or with a higher concentration significantly inhibited cell proliferation (**Figure 1B**). Thus, the β-elemene suppressed the proliferation of NSCLC cells. The nontoxic treatments with concentrations below 150 μg/mL β-elemene were performed in the subsequent experiments.

β-elemene promoted NSCLC cell apoptosis

The impact of β-elemene on the apoptosis of the A549 and H1299 cells was investigated using flow cytometry. After treatment with 50, 100, and 150 μg/mL β-elemene for 24 h, the apoptotic rates were 20.9%, 28.31%, and 36.77%, respectively, in the A549 cells and 19.58%, 26.86%, and 31.25%, respectively, in the H1299 cells; all the rates were significantly higher than the rates of the untreated cells (A549 cells: 5.24%, H1299 cells: 6.67%; **Figure 2A-D**). Furthermore, the apoptotic rates of both cells in the early (lower-right quadrant) and late (top-right quadrant) phases were notably higher than those in the untreated controls

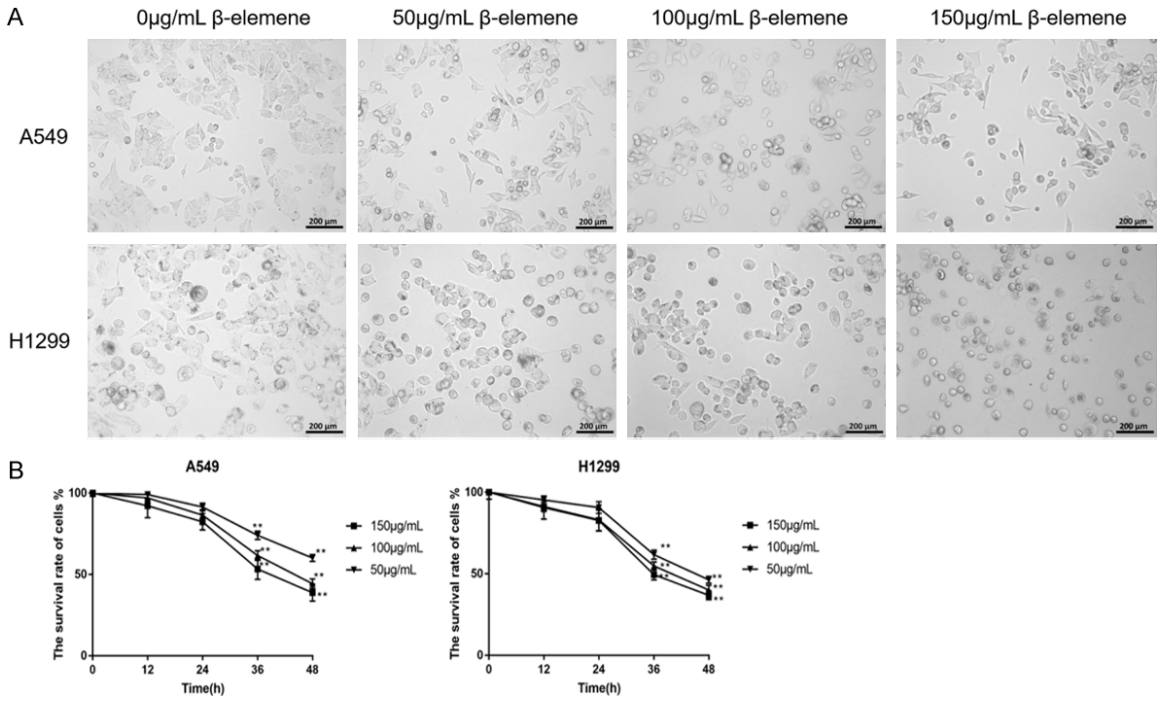

Figure 1. β-elemene inhibited the proliferation of A549 and H1299 cells. A. Optical micrograph of the cell morphology after 24 h treatment with 50, 100, and 150 μg/mL β-elemene. Scale bar, 200 μm. B. The survival rates of the NSCLC cells after various concentrations of β-elemene treatment at 12, 24, 36, and 48 h. *P < 0.05, **P < 0.01 when compared with the control group.

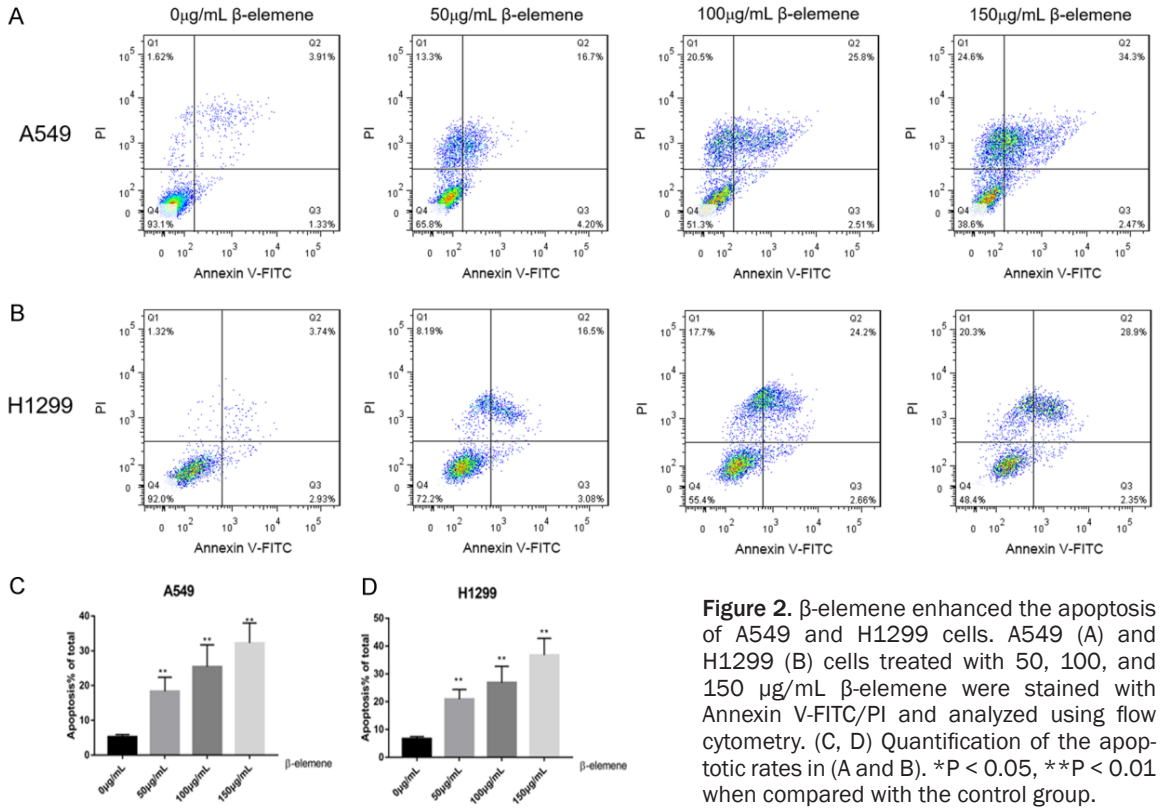

Figure 2. β-elemene enhanced the apoptosis of A549 and H1299 cells. A549 (A) and H1299 (B) cells treated with 50, 100, and 150 μg/mL β-elemene were stained with Annexin V-FITC/PI and analyzed using flow cytometry. (C, D) Quantification of the apoptotic rates in (A and B). *P < 0.05, **P < 0.01 when compared with the control group.

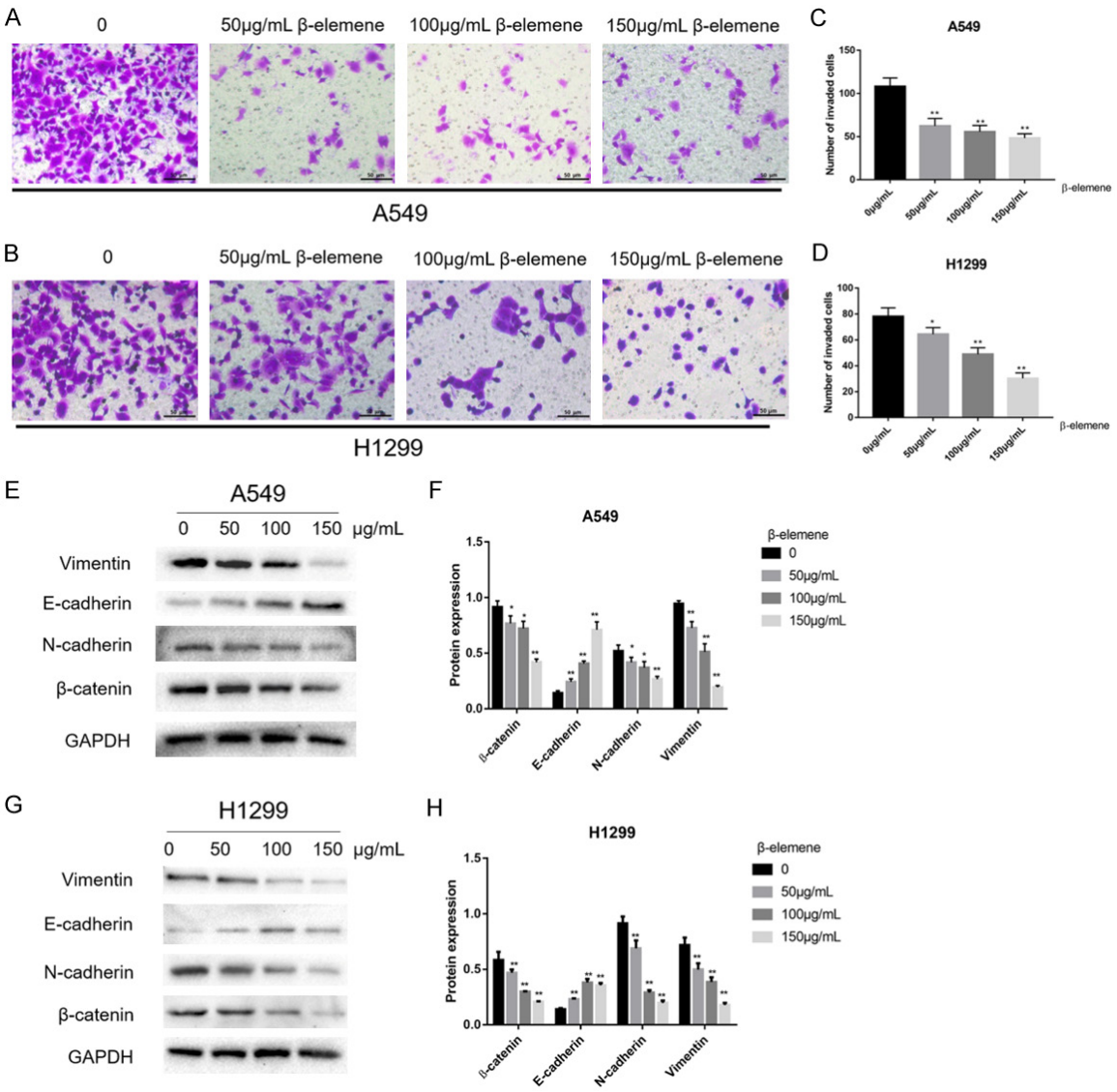

Figure 3. β-elemene suppressed the invasion and EMT of A549 and H1299 cells. A549 (A) and H1299 (B) cells cultured with 50, 100, and 150 μg/mL β-elemene were subjected to a Transwell assay. Scale bar, 50 μm. (C, D) Numbers of invaded cells in (A and B). (E, G) The A549 and H1299 cells were treated with 50, 100, and 150 μg/mL β-elemene for 24 h and subjected to western blotting for vimentin, E-cadherin, β-catenin, and N-cadherin; GAPDH was the internal control. (F, H) Protein levels in (E and G). *P < 0.05, **P < 0.01 when compared with the control group.

(**Figure 2A**, **2B**). In addition, the proportion of apoptotic cells increased with the β-elemene concentration (**Figure 2C**, **2D**). Thus, the apoptosis of the A549 and H1299 cells was enhanced by β-elemene in a concentration-dependent manner.

β-elemene inhibits NSCLC cell invasion and reverses the EMT

To assess the effect of β-elemene on the NSCLC cell invasion, a Transwell assay was performed. A549 and H1299 cells were incubated for 24 h. The numbers of invading cells were notably decreased in the β-elemene groups, particularly in the 150 μg/mL group (**Figure 3A-D**).

The EMT plays an important role in cancer dissemination and is characterized by an increase in N-cadherin expression and a downregulation of the E-cadherin expression. Thus, the levels of the EMT-related proteins were analyzed to confirm whether β-elemene could inhibit the

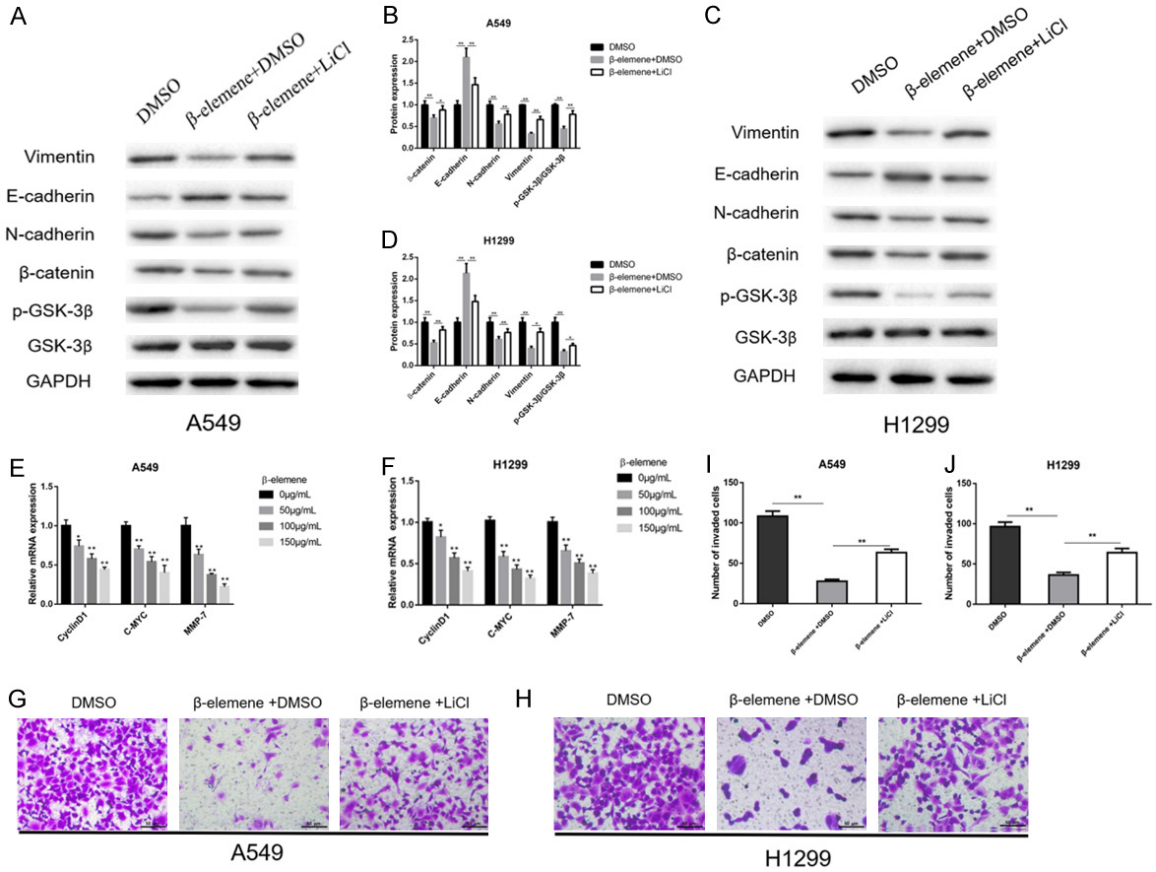

Figure 4. β-elemene inhibited the invasion and EMT of A549 and H1299 cells through the Wnt/β-catenin pathway. The vimentin, E-cadherin, N-cadherin, GSK3β, p-GSK3β, and β-catenin levels in the A549 (A) and H1299 (C) cells treated with DMSO, 50 μg/mL β-elemene + DMSO, or 50 μg/mL β-elemene + 25 mM LiCl were determined using western blotting. (B, D) Protein levels in (A and C). (E, F) The mRNA expressions of the Wnt target genes in the A549 and H1299 cells after the β-elemene treatment. The Transwell system was used to evaluate the influence of LiCl on the invasion of the A549 (G) and H1299 (H) cells, and the numbers of invaded cells were counted (I, J). Scale bar, 50 μm. *P < 0.05, **P < 0.01 when compared with the control group.

EMT. A549 and H1299 cells were cultured with β-elemene (50, 100, and 150 μg/mL) for 24 h. Western blotting showed that, relative to the untreated group, β-elemene increased the level of E-cadherin and decreased vimentin, N-cadherin, and β-catenin levels in a concentration-dependent manner in both cell lines (**Figure 3E-H**). Thus, β-elemene inhibited the invasion and suppressed the EMT of the NSCLC cells.

β-elemene attenuated the EMT and invasion of NSCLC by inhibiting the Wnt/β-catenin signaling pathway

We found that the β-catenin levels were decreased in A549 and H1299 cells, so next we investigated the involvement of the Wnt/β-catenin pathway in the inhibition of the EMT by β-elemene. H1299 and A549 cells were incubated for 1 d. The Wnt/β-catenin pathway was triggered by 25 mM LiCl; the activation was confirmed by the upregulation of p-GSK3β and β-catenin. LiCl elevated the vimentin, β-catenin, and N-cadherin levels, and reduced the E-cadherin level (**Figure 4A-D**). To further verify the inhibitive effect of β-elemene on the Wnt signaling, the Wnt target gene expression was examined using qRT-PCR, and the results showed that the mRNA levels of Cyclin D1, c-Myc, and MMP7 in the NSCLC cells were significantly decreased after the β-elemene treatment (**Figure 4E**, **4F**). The Transwell assay showed that the NSCLC cell invasion was also reversed by LiCl (**Figure 4G-J**). Thus, activating the Wnt/β-catenin pathway could block β-elemene's effect on the invasion and EMT in human NSCLC cells.

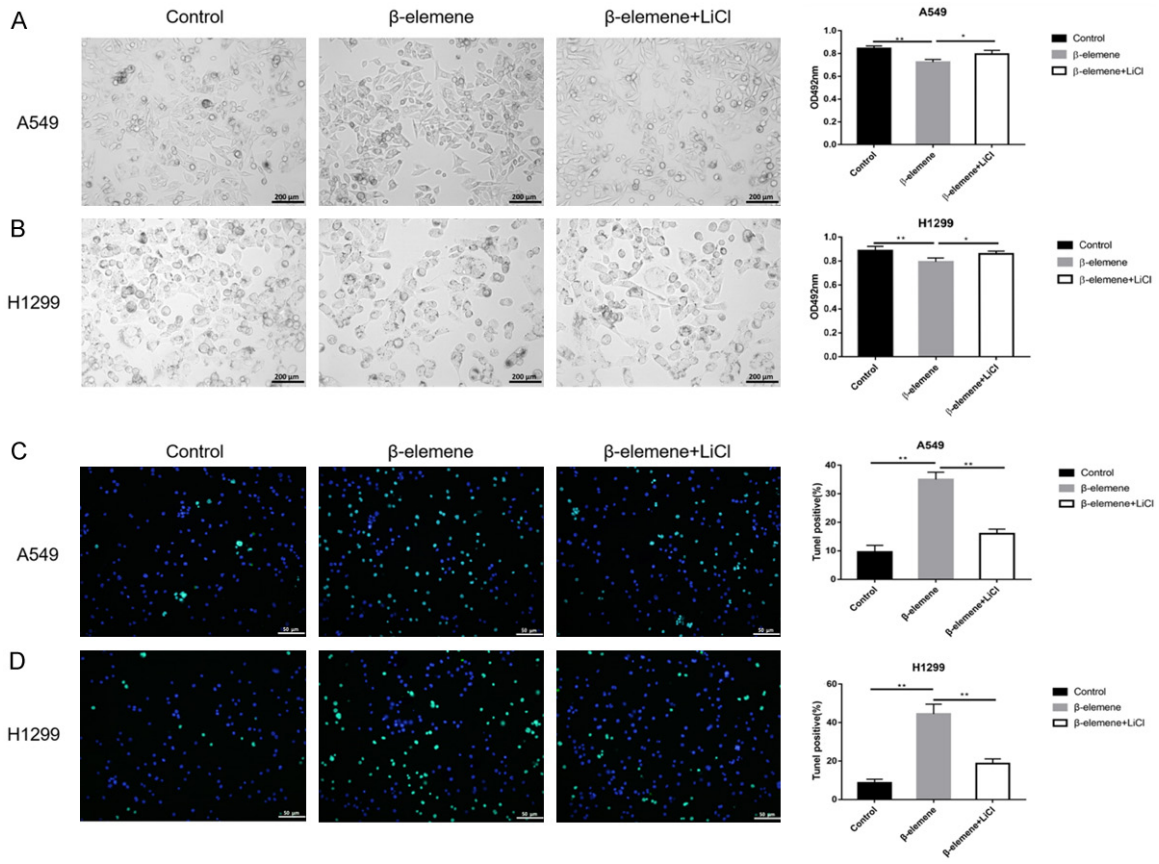

Figure 5. β-elemene inhibited the proliferation and promoted the apoptosis of A549 and H1299 cells through the Wnt/β-catenin pathway. A, B. Optical micrograph of NSCLC cell morphology and optical density at 492 nm after 24 h treatment with 50 μg/mL β-elemene or 50 μg/mL β-elemene + 25 mM LiCl. Scale bar, 200 μm. C, D. The TUNEL assay of the NSCLC cells after 24 h treatment with 50 μg/mL β-elemene or 50 μg/mL β-elemene + 25 mM LiCl. Scale bar, 50 μm. **P < 0.01 when compared with the control group.

Additionally, we investigated whether β-elemene inhibited the proliferation and promoted the apoptosis of NSCLC cells via the Wnt pathway. The MTT study demonstrated that the ability of proliferation can be restrained by β-elemene in NSCLC cells, but LiCl could reverse this suppression (**Figure 5A**, **5B**). The TUNEL assay showed that LiCl also inhibited the apoptosis promoted by β-elemene (**Figure 5C**, **5D**). Collectively, our results demonstrated that β-elemene inhibited multiple biological activities of NSCLC through the Wnt/β-catenin pathway.

Discussion

The EMT increases the migration, invasion, and metastasis of cancer cells. Cancer cells acquire stem cell-like features and drug resistance during the EMT [12]. Hence, drugs that inhibit the EMT may suppress the metastasis of tumors, including NSCLC. β-elemene is the main component of elemene, which is extracted from the herbal medicine *Rhizoma zeodaria* and is used clinically to treat multiple types of cancer (*e.g.*, breast cancer, liver cancer, glioblastoma, and leukemia) [8, 9]. β-elemene reportedly blocks the EMT of breast cancer and glioblastoma cells [10, 13]. We found that β-elemene suppresses the EMT of two human NSCLC cell lines via the Wnt/β-catenin pathway.

In this study, β-elemene inhibited the proliferation and promoted the apoptosis of A549 and H1299 cells. Our findings are consistent with the effects of β-elemene on other cancer cells [14, 15]. For example, β-elemene inhibited the proliferation of U251 glioblastoma cells and rat GBM cells by activating the p38-MAPK pathway [16]. This antitumor activity of β-elemene was attributed to the inhibition of the PI3K/Akt/mTOR and MAPK/ERK signaling pathways. The

suppression by β-elemene of the NSCLC cell proliferation might be caused by the triggering of cell-cycle arrest with the level of cyclin A protein and CDK2 phosphorylation on Thr-160 downregulated. Also, β-elemene enhanced the apoptosis of H460 cells in a mitochondrial cytochrome c release-dependent manner [17].

The EMT is an evolutionarily-conserved developmental process during which epithelial tumor cells undergo morphological and molecular changes, leading to the formation of mesenchymal cells with aggressive capabilities. In accordance with a previous report [4], we found that the loss of epithelial-cell factors (*e.g.*, E-cadherin) and the gain of mesenchymal-cell factors (*e.g.*, N-cadherin, and vimentin) in NSCLC cells undergoing the EMT. E-cadherin downregulation results in adherence junction breakdown and is accountable for enhanced invasiveness and anoikis resistance [18]. Zhang *et al*. [10] found that β-elemene inhibits breast cancer cell migration and invasion by upregulating E-cadherin expression via the ERα/MTA3/Snail signaling pathway. We found that β-elemene inhibits EMT and invasion by suppressing the Wnt/β-catenin pathway. The reduction of E-cadherin is accompanied by the cytoplasmic accumulation of β-catenin, an important component of adherens junctions. β-Catenin in cooperation with Wnt-controlled signaling plays an important role in cancer stemness and EMT [19, 20]. The Wnt/β-catenin signaling pathway reportedly affects the maintenance of cancer stem cells [20, 21] and participates in lung carcinogenesis [21]. Patients with a high Wnt1 expression and an altered β-catenin expression have a poorer prognosis [22]. Wnt2 enhances the proliferation of NSCLC cells via the Wnt/β-catenin pathway. Thus, the suppression of the Wnt/β-catenin signaling pathway shows therapeutic potential for NSCLC. β-elemene also influences the Wnt/β-catenin pathway in the treatment of cervical cancer and glioblastoma [13, 23].

In this study, β-elemene reversed the EMT and decreased the level of β-catenin. The activation of the Wnt/β-catenin pathway by LiCl enhances cell invasion and rescues the effect of β-elemene on the EMT, confirming that the anti-EMT effect of β-elemene is mediated via the Wnt/β-catenin pathway. The decreased level of Wnt target genes further confirms the inhibitive effect of β-elemene on the Wnt pathway.

In conclusion, β-elemene suppresses the proliferation ability and induces the apoptosis of NSCLC cells. β-elemene also inhibits the invasion and reverses the EMT via the Wnt/β-catenin signaling pathway. Thus, β-elemene shows promise for the treatment of NSCLC and other types of cancer.

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Disclosure of conflict of interest

None.

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