Knockdown of POLE2 expression suppresses lung adenocarcinoma cell malignant phenotypes *in vitro*

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Abstract. In the present study, we profiled β-elemene-regulated gene expression and investigated the effects of the silencing of the DNA polymerase epsilon 2, accessory subunit (POLE2) in lung cancer cells. Differently expressed genes were profiled in A549 cells incubated in the presence or absence of β-elemene by Affymetrix Human Gene Expression Array. POLE2 shRNA was then constructed to knock down POLE2 expression. Cells were counted and phenotypes were assessed via CCK-8, colony formation and caspase-3/-7 activity assays. PathScan antibody array analysis was used to identify shPOLE2-regulated genes. The cDNA microarray identified a total of 721 differentially expressed genes in the A549 cells. Furthermore, knockdown of POLE2 expression inhibited A549 and NCI-H1299 cell proliferation and apoptosis. The PathScan data indicated that expression levels of p-Akt (phosphorylated-protein kinase B, p-AKT/p-PKB), p-Smad2 (phosphorylated mothers against decapentaplegic homolog 2), p-p38 MAPK (phosphorylated mitogen-activated protein kinases p38), p-SAPK/JNK (phosphorylated c-Jun N-terminal protein kinase/stress activated protein kinase), cleaved caspase-7, IκBα (nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor, α), p-Chk1 (phosphorylated checkpoint kinase 1), p-IκBα, p-eIF2α (phosphorylated eukayotic translational initiation factor 2α), p-TAK1 (phosphorylated TGF-B-activated kinase 1), survivin and α-tubulin were significantly lower in shPOLE2 cells than these levels in the shCtrl cells. The PathScan data indicated that the expression levels of p-p53 (phosphorylated tumor protein 53) were significantly higher in the shPOLE2 cells than these levels in the shCtrl cells. β-elemene can restrain human lung cancer A549 and NCI-H1299 cell proliferation and apoptosis by suppressing POLE2 expression.

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Introduction

An estimated 1.8 million new cases of lung cancer were diagnosed in 2012 which consisted of 13% of all cancers diagnosed that year worldwide. Lung cancer contributed to ~1.6 million cancer-related deaths worldwide (1,2). In China, 4.2 million new cases and >2.8 million cancer-related deaths were estimated to occur in 2015 (2). Histopathologically, lung cancer can be classified into four major types, i.e., squamous cell, adenocarcinoma, large cell and small cell lung cancer. Among them, lung adenocarcinomas account for ~40% of all lung cancers globally (3). To date, surgery is still the most effective treatment option for lung cancer, although recently developed molecular and targeting therapy represents promising treatment strategies for certain patients. Most lung cancers are currently diagnosed at an advanced disease stage, leading to very poor prognoses (4). Novel therapeutic or preventive strategies are urgently needed to better control this deadly disease. In addition, a better understanding of the molecular mechanisms responsible for lung carcinogenesis and tumor progression could provide novel biomarkers for diagnosis, surveillance and novel targets for control of lung cancer.

β-elemene, a member of the class of organic compounds known as elemane sesquiterpenoids, is found in a variety of plants and has been demonstrated to possess antitumor activity in a variety of human cancers (5-15). For example, β-elemene exhibited activity to induce cell cycle arrest and apoptosis of non-small cell lung cancer cells (10), and was found to enhance cisplatin or taxane chemosensitivity or radiation sensitivity of lung cancer cells (6,8,14). A previous clinical trial conducted in China revealed that β-elemene was beneficial for cancer patients (16), but further randomized clinical trials are required to confirm its therapeutic potential. The human genome sequencing project and gene profile analyses have further elucidated the molecular mechanisms underpinning cancer and cancer chemotherapy, and thus may be useful in revealing the potential therapeutic mechanism of β-elemene.

In our previous study, we profiled differentially expressed genes in lung adenocarcinoma A549 cells after β-elemene treatment, and confirmed that POLE2 expression was altered by β-elemene. In the present study, we further investigated the effects of POLE2 knockdown on the regulation of lung cancer cell proliferation, apoptosis and the underlying molecular events. This study illuminated the molecular mechanisms responsible for the antitumor activity of β-elemene, and may aid in the development of novel therapeutic tools and targets for lung cancer treatment or prevention.

Materials and methods

Cell lines and culture. Human lung cancer cell line A549, obtained from the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), was isolated from a patient with lung adenocarcinoma. This cell line is frequently used as an *in vitro* model for lung alveolar basal epithelial cells. Two other lung cancer cell lines, NCI-H1975 and NCI-H1299, were also obtained from the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. A549 cells were cultured in F-12K complete medium (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NCI-H1299 cells were cultured in RPMI-1640 complete medium (Santa Cruz Biotechnology, Inc.) and NCI-H1975 cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM; Corning, Inc., Corning, NY, USA). Complete medium was supplemented with 10% fetal bovine serum (FBS; Vian-Saga Co., Ltd., Shanghai, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldirch; Merck KGaA, Darmstadt, Germany) and cells were cultured in a humidified incubator with 5% CO_2 at 37˚C. Cells in the exponential growth phase were used for our experiments. Furthermore, a human embryonic kidney cell line 293T was obtained from Shangha GeneChem Co., Ltd. (Shanghai, China) and also cultured in complete DMEM.

Profiling of differentially expressed genes in A549 cells. We first profiled differentially expressed genes in A549 cells treated with or without β-elemene (DRUG and NC groups, respectively) using the GeneChip® PrimeView™ Human Gene Expression Array (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA). In brief, total RNA was isolated from A549 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The RNA concentration of samples was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA integrity was assessed using Agilent 2100 Bioanalyzer ($1.7 < A260/A280 < 2.2$ and $RIN \geq 7.0$ and $28S/18S > 0.7$, respectively).

Next, 100 ng of each RNA sample was mixed with a poly(A) RNA to form double-stranded cDNA (complementary RNA, cRNA) and these cDNA samples were used to produce aRNA (amplified RNA, aRNA) by using the *in vitro* transcription (IVT) primers with the GeneChip 3'-IVT Express kit (Affymetrix; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. After that, the aRNA samples were purified and fragmented, and then hybridized to human cDNA microarrays with hybridization reaction mixtures for 16 h at 45˚C in a GeneChip Hybridization Oven 645 (Affymetrix; Thermo Fisher Scientific, Inc.). On the next day, the arrays were washed in the GeneChip Fluidics Station 450 (Affymetrix; Thermo Fisher Scientific, Inc.) with the GeneChip Hybridization Wash and Stain Kit (Affymetrix; Thermo Fisher Scientific, Inc.) and scanned with the GeneChip Scanner 3000 (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

We then utilized the Affymetrix GeneChip Analysis Software v1.3 for data acquisition, the first-level data analysis, and desktop data management for the entire GeneChip System, and the Robust multichip analysis (RMA) to normalize gene expression levels against the level of background variability between different hybridizations.

We then performed differential gene expression analysis in R environment using the Limma (linear models for microarray data) package (http://www.bioconductor.org/packages/release/bioc/html/limma.html). The fold change was calculated relative to baseline controls. Three independent replicate experimental data were used to perform a paired two-sample t-test for each differentially expressed gene. Dysregulated genes were defined as fold change of ≥ 2 or ≤ -2 ($P<0.05$) between the DRUG and NC cells. Such data were then compared to those from lung cancer in large sample data and Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and further analyzed using volcano and scatter plots and cluster diagrams.

Construction of the shPOLE2 lentiviral vector, lentivirus production and cell infection. We first selected and designed a short hairpin RNA (shRNA) to knock down POLE2 expression in A549 and NCI-H1299 cells using the following sequences (5'-GATTGTTCTTGGAATGATA-3'). The negative control (NC) shRNA sequences were 5'-TTCTCCG AACGTGTCACGT-3'. The synthesized DNA oligonucleotides were annealed to form double-stranded DNA and inserted into the *Age*I/*Eco*RI sites of linearized vector GV115 carrying a Green Fuorescent Protein (GFP) gene (Shanghai GeneChem Co., Ltd.). After amplification and DNA sequence confirmation, these lenti-shRNA vectors were co-transfected into 293T cells with two packaging plasmids pHelper 1.0 and pHelper 2.0 (Shanghai GeneChem Co., Ltd.) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h according to the manufacturer's instructions. Lentiviral particles were purified from the culture supernatant. A549 and NCI-H1299 cells were infected in 6-well plates at the density

of $2x10^5$ cells/well at a multiplicity of infection (MOI) of 10 and 5, respectively. Infection efficiency was evaluated under a florescence microscope by detection of GFP-positive cells vs. total cells in the plate. The POLE2 knockdown efficiency was calculated using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; Roche Diagnostics, Inc., Basel, Switzerland) and western blot analysis, respectively.

qRT-PCR. Total RNA was isolated from A549 and NCI-H1299 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturers' instructions. The qPCR was then carried out in the LightCycler 480 II Real-time PCR instruments (Roche Diagnostics) with the SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan). The qPCR conditions were as follows: an initial cycle at 95˚C for 30 sec and 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec with the primers (POLE2, 5'-TGAGAAGC AACCCTTGTCATC-3' and 5'-TCATCAACAGACTGACT GCATTC-3'; and GAPDH, 5'-TGACTTCAACAGCGACA CCCA-3' and 5'-CACCCTGTTGCTGTAGCCAAA-3'). GAPDH was used as an internal control. The qPCR products were 84 and 121 bp, respectively for POLE2 and GAPDH. The level of POLE2 mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (17). The experiment was in duplicate and repeated at least once.

Western blotting. An expression vector carrying POLE2-flag tag fusion cDNA was purchased from Shanghai GeneChem Co., Ltd., and co-transfected into 293T cells with shPOLE2 or shCtrl expression vector for 48 h using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total cellular protein was then extracted using a lysis buffer from Beyotime Institute of Biotechnology (Shanghai, China) according to the manufacturer's protocol. The protein concentration was assessed using BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal amount of protein samples (20 μg for each loading) was separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were then incubated in 5% skim milk-Tris-based saline (TBS) at room temperature for 1 h, and then further incubated with a mouse anti-FLAG antibody (cat. no. F1804; Sigma-Aldrich, Merck KGaA) diluted at 1:2,000 at 4˚C overnight. On the next day, the membranes were washed with TBS-Tween 20 (TBS-T) and then incubated with a secondary antibody (cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) at a dilution of 1:2,000 at room temperature for 2 h. Afterwards, the membrane was washed with TBST three times for 10 min each. Protein expression was developed on X-ray film after brief incubation with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). The experiment was conducted in duplicate and repeated at least twice. The gray analysis was scanned using an Epson Perfection V370 Photo scanner (Epson America Inc., Long Beach, CA, USA) and ImageJ 1.51w software (National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assay. Lentivirus-infected A549 and NCI-H1299 cells in the logarithmic phase were reseeded into 96-well plates (Corning Inc.) at a density of 2,000 cells/well and the plates were incubated for up to 4 days. At the end of each experiment, cells were incubated with 10 μl of the CCK-8 reagent (Sigma-Aldrich; Merck KGaA) at 37˚C for 3 h. The optical density (OD) at 450 nm was measured by a microplate reader (Tecan Infinite; Tecan Austria GmbH, Groedig, Austria) according to the manufacturer's instructions. The data are expressed as % of the control. The experiments were carried out in triplicate and repeated at least twice.

Colony formation assay. Lentivirus-infected A549 and NCI-H1299 cells in the logarithmic phase were reseeded into 6-well plates at a density of 500 cells/well and incubated for 14 days. The cell culture medium was refreshed every three days. At the end of each experiment, the cells were fixed with 4% paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 30 min and then washed with phosphate-buffered saline (PBS) and stained with 500 μl of Giemsa (Shanghai Dingguo Biotechnology Co., Ltd., Shanghai, China) for 10 min. Cell colonies were photographed under an inverted microscope and cell colonies with >50 cells or more were counted. The experiment was in duplicate and repeated at least twice.

Caspase-3/-7 activity assay. The A549 cells were seeded into 96-well plates at a density of $1.5x10^4$ cells/well and NCI-H1299 cells at a density of $1.0x10^4$ cells/well, grown in a 5% CO_2 incubator at 37˚C and infected with shPOLE2 and shCtrl lentiviruses. After 3-5 days, cells were detached using 0.5% trypsin and counted. A549 and NCI-H1299 cell suspensions with $1x10^4$ cells/well were added into a new 96-well plate. Cells were then lysed with a mixture of 1:1 Caspase-Glo3/7 substrate buffer (Promega) and cell growth medium on a shaker (43 x g) for 30 min according to the manufacturer's protocol. The plate was further incubated at room temperature for 2 h. The activity of caspase-3/-7 was then measured using a microplate reader (Tecan Infinite; Tecan Austria GmbH). The experiment was carried out in triplicate and repeated twice.

PathScan stress and apoptosis signaling antibody array. A PathScan® Stress and Apoptosis Signaling Antibody Array kit was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and used to detect changes in vital signaling molecules after knockdown of POLE2 expression in A549 cells. In brief, cells were grown and infected with shPOLE2 and shCtrl lentiviruses for 48 h and then lysed using 1X cell lysis buffer (Cell Signaling Technology, Inc.). The concentration of cell lysate was measured and adjusted to 0.2-1.0 mg/ml and these protein samples were subjected to analysis by a PathScan® Antibody Array kit according to the manufacturer's protocols. Each experiment was repeated twice.

Statistical analysis. The data are expressed as the mean ± standard deviation (SD) and all statistical analyses were performed using SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA). The statistical significance of differences between the two groups was determined by a two-sided Student's t-test, and one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple comparisons

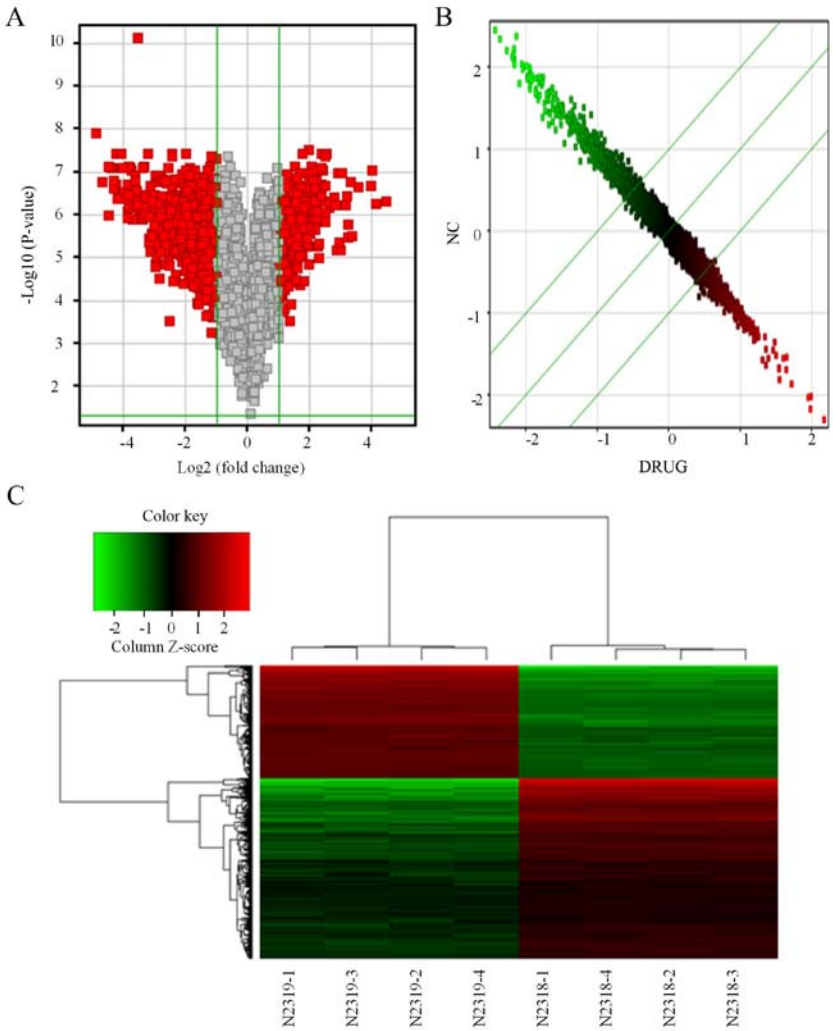

Figure 1. Illustration of the Volcano plot, Scatter plot and Cluster diagram data on β-elemene-induced differentially expressed genes in lung cancer cells. (A) Each dot represents the average value (log2 fold change between β-elemene-treated and control cells). Negative values indicate a decrease in gene expression after β-elemene treatment, whereas the positive values show an increase in gene expression. (B) Red region, P<0.05; fold change, 2. The values of the x-coordinate and y-coordinate in the Scatter plot are the averaged normalized signal values. The green lines in B are fold change lines and the regions above and below the green line denote that it formed >2-fold change of genes between two groups. (C) Due to the clustering Figure stratified cluster of 721 differential genes, including 273 upregulated genes and 448 downregulated genes (fold change ≥2, P<0.05).

test was used for comparison between multiple groups. P<0.05 was considered to indicate a statistically significant result.

Results

Identification of differentially expressed genes in lung adenocarcinoma cells after β-elemene treatment. We compiled data from a large lung cancer sample data set in the GEO database with our Affymetrix whole-profile gene expression chip data. We performed gene expression profiling analysis to identify differentially expressed genes in lung adenocarcinoma cells after incubation with or without β-elemene. Specifically, we used the Limma package in R to identify genes expressed in A549 cells at levels differing by 2-fold between β-elemene and NC groups with a false discovery rate (FDR) of P-value <0.05. We identified 721 differentially expressed genes in lung cancer cells after treatment with β-elemene, of which 273 were upregulated and 448 were downregulated. The Volcano plot showed that the distribution of the differential genes between the treatment and NC groups (Fig. 1A). The scatter plot data showed the distribution of signal values between the treatment and NC groups on the plane of the rectangular coordinate system (Fig. 1B). The Cluster diagram analysis shows the aggregation of all samples and differentially expressed genes (Fig. 1C).

Knockdown of POLE2 expression inhibits A549 and NCI-H1299 cell proliferation and colony formation. We then assessed the effects of POLE2 knockdown on lung cancer cell proliferation. The fluorescence microscopy data showed that lentiviruses carrying shCtrl and shPOLE2 at MOI of 10 and 5, respectively, to infect A549 and NCI-H1299 cells had an 80% transfection efficacy at 72 h (Fig. 2A). qRT-PCR data showed that the level of POLE2 mRNA was 75.6% lower in the shPOLE2-infected A549 cells than that noted in the shCtrl cells 5 days after infection, and the level of POLE2 mRNA was 74.3% lower in the shPOLE2-infected NCI-H1299 cells than that noted in the shCtrl cells 5 days after infection (Fig. 2B). The level of POLE2 expression in the NCI-H1299 and A549 cells was higher than that in the NCI-H1975 cells (Fig. 3A).

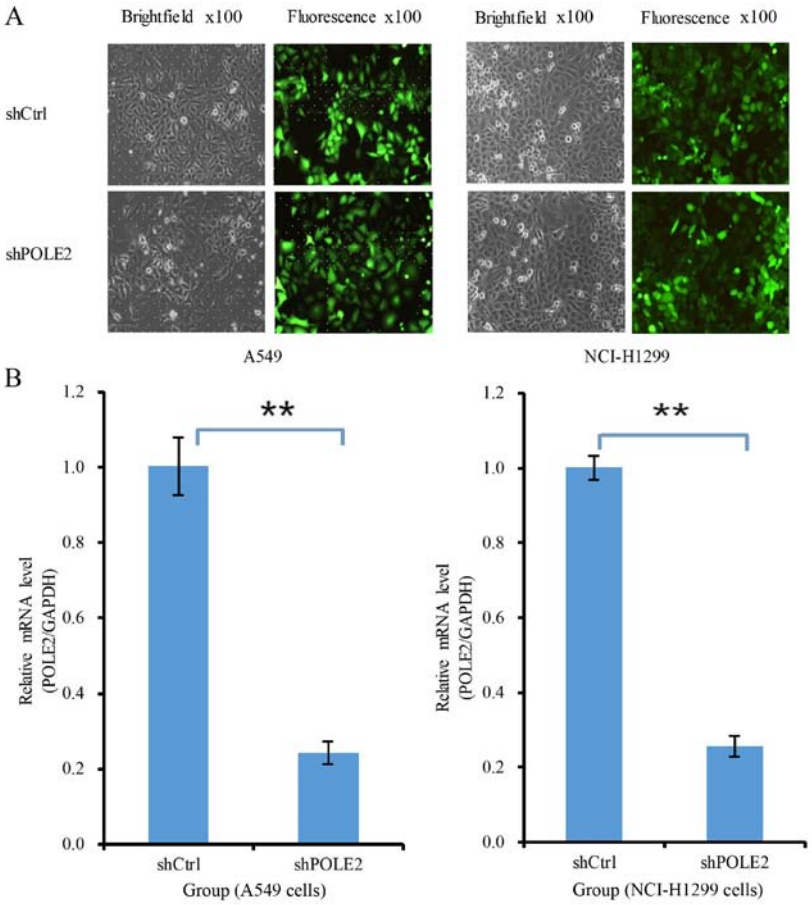

Figure 2. shPOLE2 knockdown of POLE2 expression in lung cancer A549 and NCI-H1299 cells. (A) Fluorescence microscopy. A549 and NCI-H1299 cells were grown and infected with shPOLE2 or shCtrl lentivirus for 48 h and subjected to fluorescence microscopy. (B) qRT-PCR. A549 and NCI-H1299 cells were grown and infected with shPOLE2 or shCtrl lentivirus for 48 h and subjected to qRT-PCR. The level of POLE2 mRNA was significantly downregulated in shPOLE2-infected A549 and NCI-H1299 cells compared with the shCtrl group ($^{**}P<0.05$).

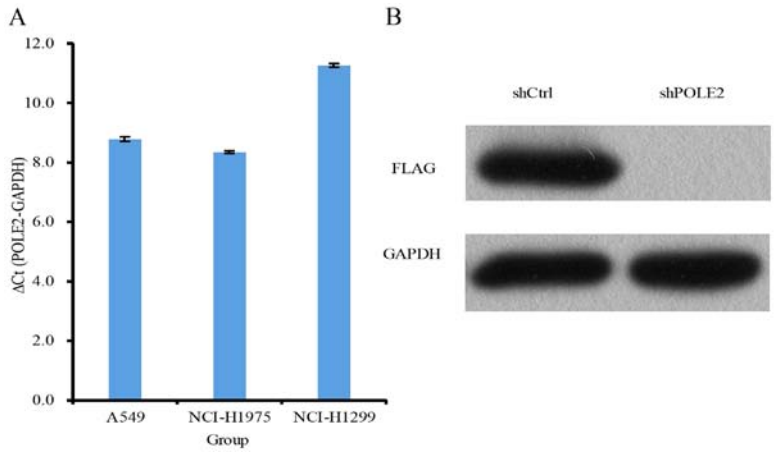

Figure 3. The level of POLE2 expression in human lung adenocarcinoma cell lines and the level of POLE2 protein in shPOLE2 lentivirus-infected A549 cells. (A) qRT-PCR. Human lung adenocarcinoma cell lines A549, NCI-H1975 and NCI-H1299 were subjected to qRT-PCR analysis of POLE2 expression. (B) Western blot analysis. A549 cells were grown and infected with the shPOLE2 or shCtrl lentivirus for 48 h and subjected to western blot analysis.

We further assessed the level of POLE2 protein using western blotting and found significantly lower levels of protein in the shPOLE2 lentivirus-infected A549 cells (Fig. 3B).

We then assessed proliferation of these infected A549 and NCI-H1299 cells using the Celigo (Nexcelom Bioscience, Lawrence, MA, USA) cell count method and found that shCtrl-infected A549 cells proliferated normally and had an increase in number by 6.07- and 9.53-fold by day 4 and 5, respectively (Fig. 4A). The shCtrl-infected NCI-H1299 cells proliferated normally and had an increase in number by 6.72- and 10.92-fold by day 4 and 5, respectively (Fig. 5A). In contrast, proliferation of shPOLE2-infected A549 cells

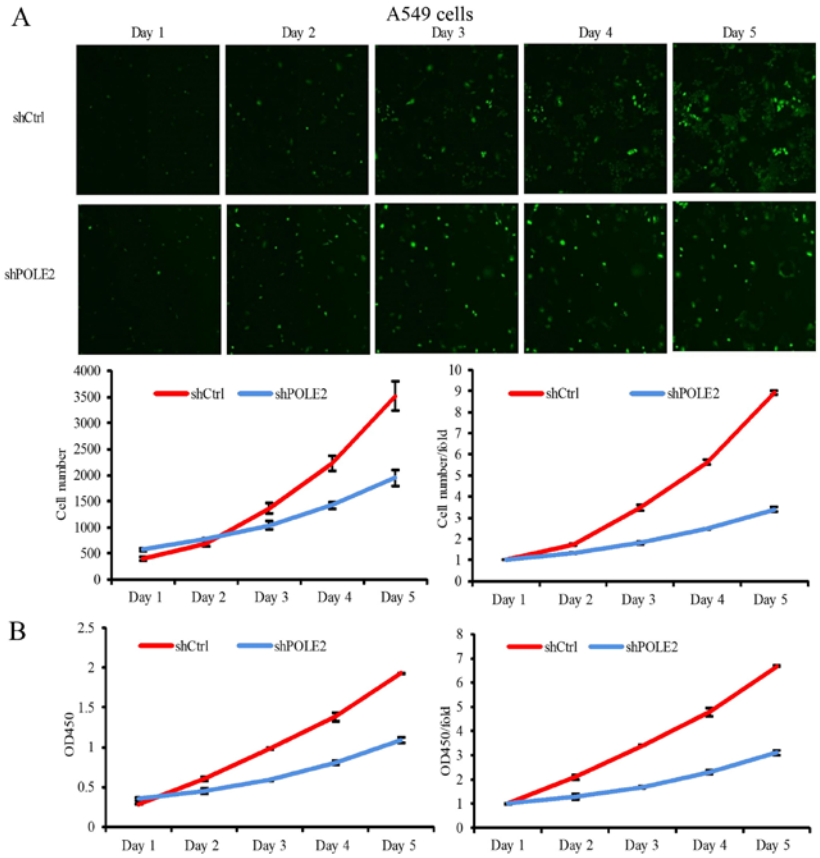

Figure 4. Effect of POLE2 knockdown on A549 cell proliferation. (A) Cell counting assay. The high content screening (HCS) was used to observe A549 cell growth for 5 consecutive days after transfection with shPOLE2 or shCtrl. (B) CCK-8 assay. A549 cells were grown and infected with the shPOLE2 or shCtrl lentivirus for 48 h and subjected to CCK-8 assay.

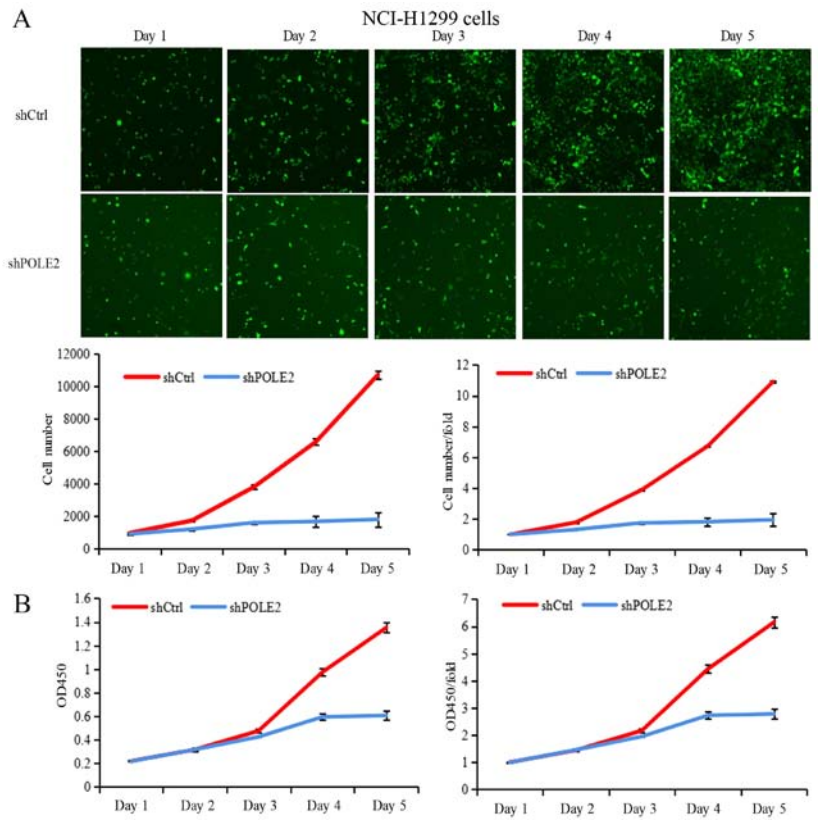

Figure 5. Effect of POLE2 knockdown on NCI-H1299 cell proliferation. (A) Cell counting assay. The high content screening (HCS) was used to observe NCI-H1299 cell growth for 5 consecutive days after transfection with shPOLE2 or shCtrl. (B) CCK-8 assay. NCI-H1299 cells were grown and infected with shPOLE2 or shCtrl lentivirus for 48 h and subjected to CCK-8 assay.

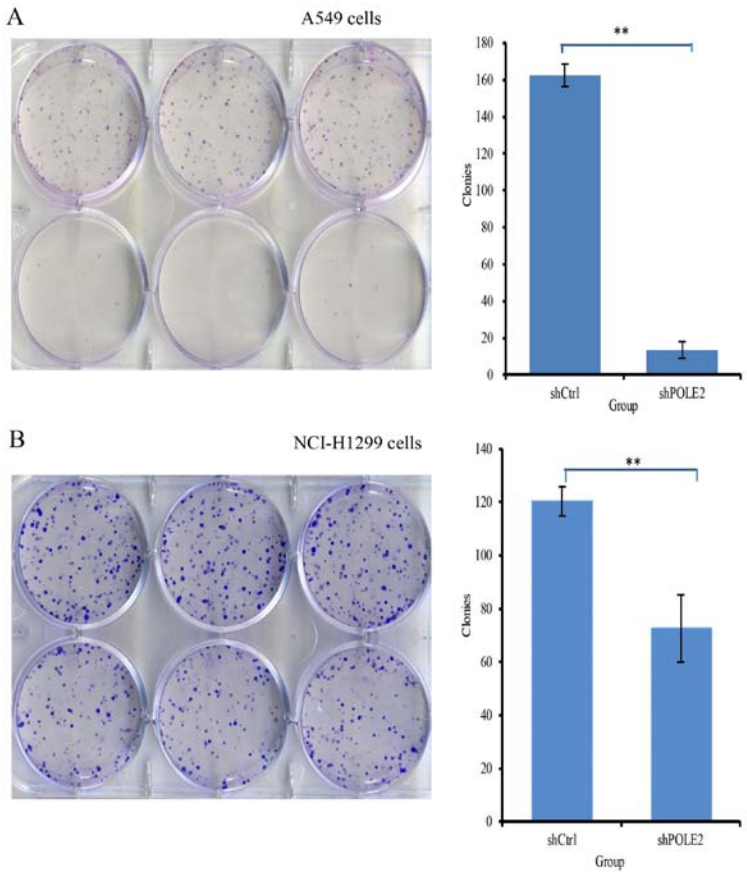

Figure 6. Effect of POLE2 knockdown on A549 and NCI-H1299 cell colony formation capacity. (A) A549 cells were grown and infected with the shPOLE2 or shCtrl lentivirus for 48 h and subjected to colony formation assay. The graph is the summarized data of the colony formation assay (**P<0.05 vs. shCtrl cells). (B) NCI-H1299 cells were grown and infected with the shPOLE2 or shCtrl lentivirus for 48 h and subjected to colony formation assay. The graph is the summarized data of the colony formation assay (**P<0.05 vs. shCtrl cells).

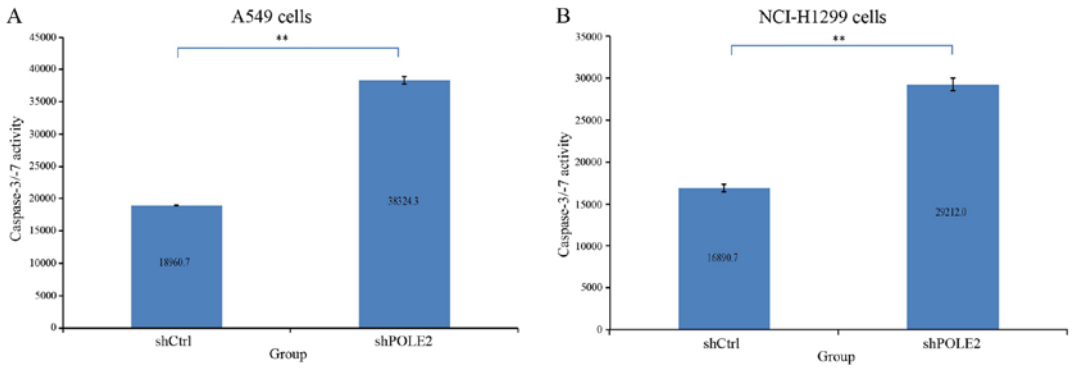

Figure 7. Effect of POLE2 knockdown on induction of caspase-3/-7 activity. (A) A549 cells were grown and infected with the shPOLE2 or shCtrl lentivirus for 48 h and subjected to colony formation assay. The graph shows the summarized data of the caspase-3/-7 activity assay (**P<0.01 vs. shCtrl cells). (B) NCI-H1299 cells were grown and infected with the shPOLE2 or shCtrl lentivirus for 48 h and subjected to colony formation assay. The graph shows the summarized data of the caspase-3/-7 activity assay (**P<0.05 vs. shCtrl cells). Data are expressed as the mean ± SD.

was significantly slower, increasing in number by only 4.01- and 5.86-fold by day 4 and 5, and proliferation of shPOLE2-infected NCI-H1299 cells was also significantly slower, increasing in number by only 1.82- and 1.95-fold by day 4 and 5, respectively (Figs. 4A and 5A). Furthermore, the CCK-8 assay showed that the proliferation of shPOLE2 A549 and NCI-H1299 cells were also significantly reduced on day 4 (P<0.001; Figs. 4B and 5B).

Colony formation measures cell tumorigenic ability *in vitro*. After shPOLE2 and shCtrl infection in A549 cells, the colony forming capacity was 163±6 and 13±5 (P<0.05; Fig. 6A). In NCI-H1299 cells, the colony forming capacity was 120±6 and 73±13, respectively (P<0.05; Fig. 6B).

Impact of POLE2 knockdown on A549 and NCI-H1299 cell apoptosis. We further assessed the effect of shPOLE2 on A549

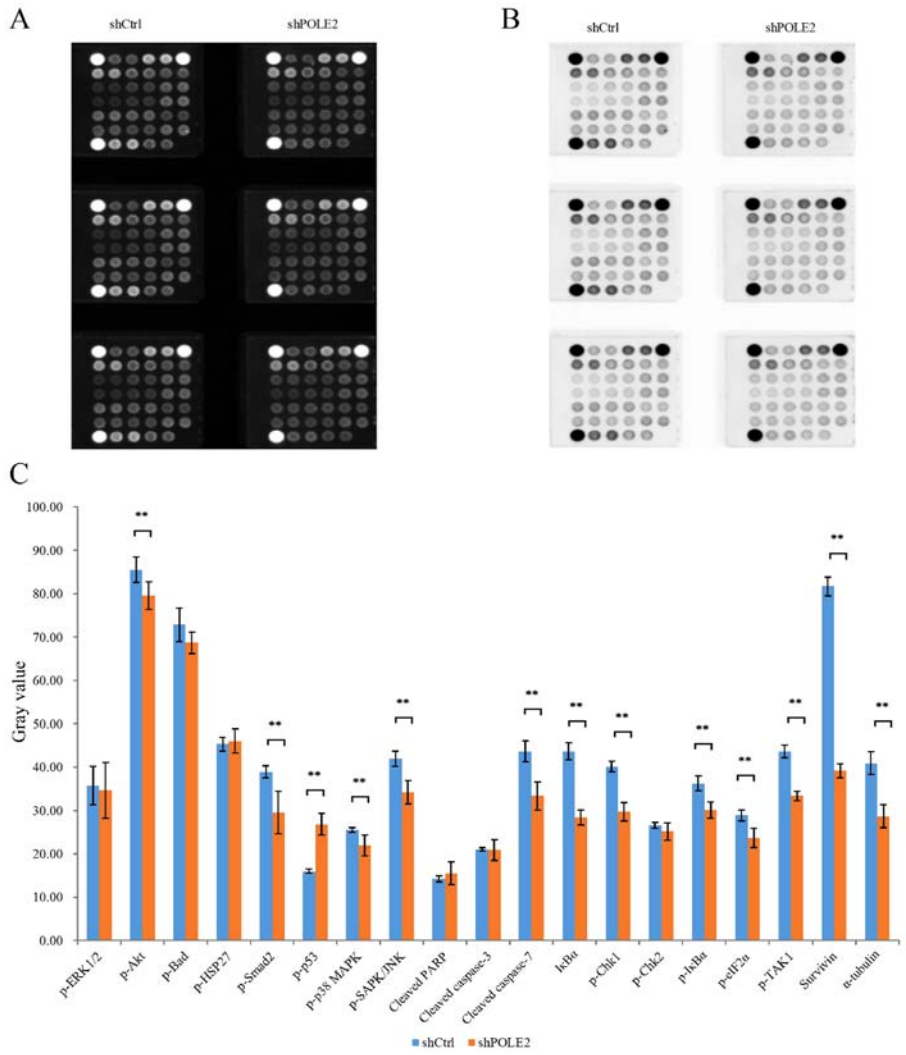

Figure 8. Identification of shPOLE2 knocked down-related gene expression. (A and B) A Stress and Apoptosis Signaling Antibody Array kit was used to generate the chemiluminescence and gray scale results. (C) Statistical analysis of the gray values revealed that expression levels of p-Akt, p-Smad2, p-p38 MAPK, p-SAPK/JNK, cleaved caspase-7, IκBα, p-Chk1, p-IκBα, p-eIF2α, p-TAK1, survivin and α-tubulin were significantly decreased in the shPOLE2 compared to shCtrl cells and p-p53 was significantly increased in the shPOLE2 compared to shCtrl cells (**P<0.05).

and NCI-H1299 cell apoptosis. Forty-eight hours after lentivirus infection, the activity of caspase-3/-7 was significantly higher in the shPOLE2-infected A549 and NCI-H1299 cells than the activity noted in the control cells (P<0.01; Fig. 7). These results indicate that downregulated POLE2 suppressed A549 and NCI-H1299 apoptosis.

Identification of genes mediating POLE2 activity in lung cancer cells. To investigate the molecular events that may be responsible for the observed impact of POLE2, we performed PathScan stress and apoptosis signaling antibody array analysis in A549 cells infected with shPOLE2 or shCtrl lentiviruses. The chemiluminescence and gray scale data (Fig. 8A and B) indicated that shPOLE2 altered expression of 19 cell stress and apoptosis-related signaling molecules. Statistical analysis of the gray values showed that expression levels of p-Akt, p-Smad2, p-p38 MAPK, p-SAPK/JNK, cleaved caspase-7, IκBα, p-Chk1, p-IκBα, p-eIF2α, p-TAK1, survivin and α-tubulin were significantly lower in shPOLE2-transfected cells and p-p53 was significantly higher in the shPOLE2-transfected cells when compared with these levels in the shCtrl-infected cells (P<0.05; Fig. 8C). These data further indicate that downregulated POLE2 may suppress lung tumor cell apoptosis.

Discussion

To better understand the antitumor activity of β-elemene, we profiled differentially expressed genes in A549 cells after treatment with or without β-elemene. POLE2 was identified as one of the genes most significantly downregulated by β-elemene. We then investigated the effects of POLE2 knockdown on lung cancer cell proliferation and apoptosis. We found that knockdown of POLE2 expression significantly inhibited A549 and NCI-H1299 cell proliferation and colony formation, but induced tumor cell apoptosis. Statistical analysis of the gray values revealed that expression of p-Akt, p-Smad2, p-p38 MAPK, p-SAPK/JNK, cleaved caspase-7, IkBα, p-Chk1, p-IκBα, p-eIF2α, p-TAK1, survivin and α-tubulin were significantly lower in the shPOLE2-transfected and p-p53 was signficantly higher in the shPOLE2-transfected cells than that noted in the shCtrl-transfected A549 cells (P<0.05). Further study will be required to explore whether

the strategy of targeting POLE2 expression could control lung adenocarcinoma clinically.

β-elemene is the active ingredient in Curcuma wenyujin and is found in other plants (18). β-elemene is one of the second-class of novel non-cytotoxic broad-spectrum antitumor agents developed in China as it possesses wide-spectrum antitumor activity and mild toxicity in various types of human cancers (5-15). To date, β-elemene has been assessed as a potential complementary, replacement or alternative medicine for human cancer, including lung cancer. β-elemene was found to restrain tumor cell synthesis of DNA, RNA and protein (19) and mitosis, therefore inhibiting tumor proliferation by arresting tumor cell cycle and inducing apoptosis (10). Moreover, β-elemene also reversed resistance of cancer cells to chemotherapy and increased chemosensitivity (11). β-elemene was also found to suppress expression of Bcl-2 and Bcl-xL (18,20). In the present study we revealed that POLE2 was downregulated in lung cancer cells treated with β-elemene.

The human genome contains at least 15 DNA polymerases for genome replication and DNA repair (21). The eukaryotic DNA polymerase epsilon was first isolated from Saccharomyces in 1970, and belongs to the DNA polymerase B family, which contains four subunits. The largest subunit is POLE (subunit A), the second largest subunit is POLE2 (subunit B) with a molecular weight of 59 kDa, and the two smaller subunits POLE3 and POLE4 have molecular weights of 17 and 12 kDa (22), respectively. POLE2 was previously reported to be expressed in breast, colorectal cancer, mantle cell lymphoma, cervical and bladder cancer (21,23-26), yet to date, alteration of POLE2 expression has not been reported in lung adenocarcinoma. In the present study, we used Affymetrix gene expression profile chip and GEO database search to identify a total of 721 differentially expressed genes, including 273 upregulated and 448 downregulated genes in lung adenocarcinoma A549 cells after incubation with β-elemene.

Our current data show that POLE2 is highly expressed in three different lung cancer cell lines and we selected A549 and NCI-H1299 cells as the model in which to knock down POLE2 expression to assess the role of POLE2 in lung cancer. We found that POLE2 knockdown significantly reduced A549 and NCI-H1299 cell proliferation and colony formation, but induced tumor cell apoptosis. These observations are consistent with reports of the antitumor activity of β-elemene on human cancer cells (9,10). To date, studies of POLE2 are rare, but a small number of genomic studies have shown mutations in POLE2 in human diseases such as colorectal cancer (27,28). Thus, further study is needed to evaluate and identify the role of POLE2 in lung cancer development and progression. Furthermore, we also observed that knockdown of POLE2 expression reduced expression of p-Akt, p-Smad2, p-p38 MAPK, p-SAPK/JNK, cleaved caspase-7, IκBα, p-Chk1, p-IκBα, p-eIF2α, p-TAK1, survivin and α-tubulin proteins and the knockdown of POLE2 expression increased the expression of p-p53. These proteins are involved in cell stress responses, proliferation, and apoptosis, which further confirmed the tumor-promoting effects of POLE2 in lung cancer cells. In the present study, caspase-3/-7 activity assay showed a significant increase in caspase-3/-7 activity after POLE2 knockdown, but our Stress and Apoptosis Signaling Antibody Array experiment did not show a significant change in the levels of cleaved caspase-3, although the change in cleaved caspase-7 was significant. This discrepancy could be due to the sensitivity of these two assays, i.e., activity assay is much more sensitive than that of western blotting. However, the role of POLE2 in apoptosis needs further investigation.

The present study is simply a proof-of-principle one and does have some limitations. For example, only two repeats were performed for certain experiments; cell proliferation was assessed by using Celigo Cell Counting, CCK-8 and colony formation assays, while apoptosis was only measured by caspase-3/-7 activity and Stress and Apoptosis Signaling Antibody Array. In the future, we will further utilize more apoptotic assays and precisely characterize the role of POLE2 in lung cancer. In conclusion, this study for the first time reports that POLE2 is downregulated by β-elemene. We assessed the tumor-promoting effects of POLE2 in lung cancer. These data indicate that POLE2 may represent a useful therapeutic target for the treatment of lung adenocarcinoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contribution

JL and YW conceived and designed the experiments; JL, JY and YZ have been involved in drafting the manuscript or revising it critically for important intellectual content; JW performed the Affymetrix Human Gene Expression Array of the A549 cells; YD performed the experiments of gene knockdown and PathScan antibody array; YF performed the experiments of CCK-8, colony formation and caspase-3/-7 activity assays. NL and YZ obtained data, and analyzed and interpreted the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable as human or animal tissues were not utilized in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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