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-IkBα, p-eIF2α (phosphorylated eukaryotic 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.

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 elemene 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-Aldrich; Merck KGaA, Darmstadt, Germany) and cells were cultured in a humidified incubator with 5% CO2 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 Shanghai 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 ≥ 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/l 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'-TTTCCG AACGTGTCACGT-3'. The synthesized DNA oligonucleotides were annealed to form double-stranded DNA and inserted into the AgeI/EcoRI sites of linearized vector GV115 carrying a Green Fluorescent 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'-TGAGAACGAAACCTTGTGATC-3' and 5'-TCATCAACAGACTGACTGCATC-3'; and GAPDH, 5'-GACTTCTACAAGCCGACA CCCA-3' and 5'-CACCCCTTGCTGTTAGCCAAA-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^-ΔΔCT_ 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) and western blot analysis, respectively.

**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 carried out in triplicate and 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.
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).
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.
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
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, IκBα, p-Chk1, p-IκBα, p-eIF2α, p-TAK1, survivin and α-tubulin were significantly lower in the shPOLE2-transfected and p-p53 was significantly 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. 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. 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.
References

The authors state that they have no competing interests.