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ORIGINAL RESEARCH

β-Caryophyllene from Chilli Pepper Inhibits the Proliferation of Non-Small Cell Lung Cancer Cells by Affecting miR-659-3p-Targeted Sphingosine Kinase 1 (SphK1)

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Background: β-Caryophyllene is the main ingredient of chilli pepper and used for the prevention of various cancers, while the molecular mechanism for its effects on non-small cell lung cancer (NSCLC) remains unclear.

Methods: NSCLC cell lines A549 and NCI-H1299 were treated with β-Caryophyllene and miR-659-3p (a potential tumor suppressor) mimic or siRNA. The levels of miR-659-3p, sphingosine kinase 1 (SphK1), apoptotic factors and oxidative stress factors were investigated.

Results: β-Caryophyllene inhibited NSCLC growth, promoted their apoptotic rate, increased the level of miR-659-3p, apoptotic factors (cleaved caspase-3 and BAX), antioxidant factors (SOD, CAT and GPx) and reduced the level of oxidative stress (ROS and NO) and SphK1. miR-659-3p mimic and siRNA affected NSCLC growth, their apoptosis, and biochemical indices.

Conclusion: β-Caryophyllene of chilli pepper exerts inhibitory activity in NSCLC cells possibly by affecting miR-659-3p-targeted SphK1.

Keywords: β-caryophyllene, chilli pepper, non-small cell lung cancer, miR-659-3p, sphingosine kinase 1

Introduction

Lung cancer is the malignant tumor with the highest morbidity and mortality among all tumors.[1] The widespread and global incidence of lung cancer requires more advanced treatment methods. In the global survey and analysis of lung cancer, the incidence of non-small cell lung cancer (NSCLC) is as high as 85%, which is significantly higher than that of small cell lung cancer.[2] NSCLC is highly malignant, easy to relapse and metastasize. The first-line and traditional treatments for NSCLC include chemotherapy,[3,4] radiotherapy,[5,6] and surgery.[7,8] In the past ten years, targeted therapy and tumor immunotherapy have become new treatment methods for NSCLC, and they have developed rapidly. The availability of new drugs has also produced more differences and side effects.[9] It is necessary to explore new nature potential drug with few side effects.

Chrysanthemum boreale (Asteraceae) is a wild flowering plant in East Asia and its main ingredient β-caryophyllene exerts anti-proliferative activities in lung cancer cells A549 and NCI-H358 cells by inducing G 1 phase cell cycle arrest in human lung cancer cells.[10] However, the related molecular mechanism remains widely unknown.

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Comparatively, chilli pepper is a common vegetable with rich β-caryophyllene and can be easily available.[11] Chili pepper (*Capsicum annuum L.*) belongs to Solanaceae Capsicum, is often used as a spice, flavour enhancer, vegetable and ingredients in traditional Chinese medicine and its many phytochemicals (alkaloids, anvils, flavonoids, phenols, unsaturated fatty acids) exert health-promoting properties, including anticancer,[12] anti-inflammatory,[13] antioxidant,[14] and the metabolism of glucose and cholesterol.[15] β-Caryophyllene is often reported nature product with anti-cancer properties and widely existed in the volatile compounds of pepper and chilli pepper.[16,17] However, the impact of β-Caryophyllene on NSCLS and related molecular mechanisms remain unclear.

MicroRNA (miRNA) is a small non-coding RNA of 18–25 nucleotides, which inhibits target gene expression by directly binding to the 3'-untranslated region (3'-UTR) of mRNA. It is estimated that more than 60% of human protein-coding genes are regulated by miRNAs.[18] miRNAs play a role in many physiological and pathophysiological processes, such as embryonic development, stem cell differentiation, inflammation and tumors. Much more evidence shows the key regulatory role of miRNA in various types of malignant tumors, including NSCLC.[19,20] For example, miR-205 connects TMPRSS4 (a membrane-anchored protease involved in cell migration and invasion in NSCLC) to accelerate the cell cycle progression of NSCLC cells.[21] In addition, previous studies have shown that the expression of miR-126 decreases in A549 cells, and the up-regulation of miR-126 inhibits the proliferation, migration and invasion of A549 cells by targeting phosphatidylinositol-3-kinase regulatory subunit 2 (PIK3R2).[22] miRNA also plays an important role in the chemical and radio-sensitivity of NSCLC cells by regulating DNA repair genes.[23] Therefore, miRNA is a potential biomarker for the diagnosis, prognosis, prevention and treatment of NSCLC. With the progress of research, many natural Chinese medicine ingredients have been found to have the effect of regulating microRNA.

Sphingosine kinase 1(SphK1) is a key kinase for intracellular sphingomyelin metabolism. It can catalyze the production of sphingosine (Sph) to sphingosine-1-phosphate (S1P) and participate in the regulation of multiple intracellular sphingosine kinases. The transmission of important signal transduction pathways plays an important role in maintaining cell homeostasis, regulating cell proliferation, differentiation and apoptosis. Recent studies have shown that SphK1 is a carcinogenic enzyme, which is closely related to the proliferation and transformation of cells and the survival, invasion and migration of tumor cells. SphK1 gene itself has many characteristics of oncogene. The expression level of SphK1 is significantly increased in a variety of tumor tissues and tumor cells cultured in vitro, including breast cancer, colorectal cancer, gastric cancer, liver cancer, bladder cancer, endometrial cancer, etc.

miR-659-3p, a tumor suppressor, exerts its function via downregulating SphK1 by binding to its 3'-UTR and increasing the apoptosis of cancer cells.[24] β-Caryophyllene controls various diseases by regulating miRNA.[25] β-Caryophyllene has been used for exploring the prevention of various cancers, while its effects on NSCLC and the related molecular mechanism remain unclear. In this study, we investigated the inhibitory potential of β-caryophyllene on NSCLC cells and explored the underlying molecular mechanisms associated with the antioxidant properties and antitumor activities.

Materials and Methods

Materials

The following agents were purchased from Sigma, including 2-Methyl-1-tetralone, Catalogue Number: 1590-08-5; β-Caryophyllene, Catalogue Number: 87-44-5; β-Elemene, Catalogue Number: 515-13-9; Hexanal, Catalogue Number: 66-25-1; Furfural, Catalogue Number: 98-01-1; Nonanal, Catalogue Number: 124-19-6 and 2-methyldecane, Catalogue Number: 6975-98-0 were purchased from Zhengzhou JACS Chem Co., Ltd (Zhengzhou, China).

Integrated Bioinformatics Analysis of SphK1 in Lung Cancer and Interaction with Potential miRNA

SphK1 expression in lung cancer was analyzed by using the data from GEPIA2 database, including the median expression of SphK1 in tumor and normal samples via bodymap. GEPIA2 database was used to obtain the SphK1 expression levels in lung cancer and normal tissues, such as LUAD, lung adenocarcinoma and LUSC, lung squamous cell carcinoma. SphK1 expression was investigated at different stages of lung cancer. Kaplan–Meier analysis was used to analyze the overall survival of lung cancer patients based on SphK1 expression in GEPIA2 database. miRDB is an online database for miRNA target prediction and functional annotations.[26,27] SphK1 is predicted to be targeted by potential miRNAs via

miRDB online tools. Finally, the target site was also analyzed.

GC/MS Analysis of Volatile Compounds from Chilli Pepper

About 1.5 kg of dried chili pepper was rinsed with clean water, dried, and cut into small pieces less than 1cm in length. Five hundred grams was taken into a bag, and marked as A, B, and C, and homogenized with liquid nitrogen, and stored in a freezer at −20°C for later use. About 5g of homogenized sample was added to 5 mL saturated NaCl solution, vortexed for 3 min, placed in a 15mL headspace bottle, and heated on a magnetic stirrer at 65°C for 15 min. About 65 μm PDMS/DVB was inserted into the headspace bottle, and headspace solid-phase micro extraction (HS-SPME) was performed at 250°C for 45 min. The powder was located about 1 cm above the liquid surface in the headspace bottle. The stirring speed was adjusted to 1000r/min and heated to 80°C. After 30 min, the extraction tool was inserted into the inlet of the gas chromatograph at 250°C for 5 min. GC-MS instrument was operated, and data acquisition and analysis were performed.

The instrument includes Agilent HP-5MS chromatographic column (30 m × 250 μm × 0.25 μm). The carrier gas was helium, the linear flow rate was set at 1 mL/min, the inlet temperature was 250°C, the desorption temperature was 250°C, and the desorption time was 5 min. The sample was injected in split mode, and the injection volume was 1 μL. The heating program was set to an initial temperature of 40°C, maintained for 5 min, heated to 160°C at 4°C/min, maintained for 5 min, and then heated to 250°C at 6°C/min, maintained for 5 min. Mass spectrometry condition was proved as follows: ion source type EI, 70 eV; ion source temperature and interface temperature were both 230 °C; quadrupole temperature 150 °C. It adopted full scan mode, and the mass scan range was 35~450m/z.

After the data were collected using the Agilent 7890B/597A gas chromatography-mass spectrometer system, the data were analyzed by using the Mass Hunter GC/MS Acquisition Software (B.07.00) and MSDChemStat 1.0. According to the automatic search and matching of the structural formula of each peak in the data spectrum, with the aid of manual analysis, the mass spectrum of each compound was compared with the standard spectrum, and the retention index was combined with the literature for qualitative analysis; the peak area normalization method was used for quantification analysis. Finally, volatile compounds were identified by matching retention times and mass spectra with authentic standards.

Cell Culture

NSCLC cell lines A549 and NCI-H1299 were purchased from Shanghai Institute for Biological Sciences (Shanghai, China), and the cell line HCC827 and PC9 from Chengdu Yuanquan and cultured in DMEM media with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 u/mL), and streptomycin sulfate (100 μg/mL). Cells were cultured at 37 °C and 5% CO_2.

Cell Transfection

The following sequences were synthesized from TaKaRa Biotechnology Company, miR-659-3p siRNA, 5'-GAAC CAAGGAAGAGTCACAGT-3'; siRNA scrambled, 5'-GGCAAACCAGGGAAATACGTA-3'; miR-659 mimic, 5'-CTTGGTTCAGGGAGGGTCCCCA-3 and miR-659-3p mimic scrambled, 5'-GGTGCTAGGCGCTCGC TCATGA-3'. Lipofectamine 2000 transfection reagent was used to transfect miR-659-3p, siRNA and scrambled samples according to the manufacturer's instructions. Six-well plates were used for cell culture, and 5×10^4 cells were inoculated in each well of the six-well plate. Liposome-nucleic acid complex was prepared, and 250 μl Opti-MEM was added to dilute Lipofectamine 2000, mixed well at room temperature and incubated for 5 min. About 7.5 μl miRNA mimics/siRNA was mixed with 250μl Opti-MEM and incubated at room temperature for 20 min. The mixture was transferred to the six-well cell plate, placed in a cell incubator, and cultured at 37 °C and 5% CO_2.

MTT Assay

Cell growth rate was measured by using MTT assay. Cells were harvested and seeded in 96-a well plate at 1×10^5/mL in 200 μL/well. The cells were treated with 10, 20 and 40 μM of the individual volatiles compound standard. After 24-, 48- and 72-h incubation, 50 μL of MTT was added to each well for 4 h. The supernatants were discarded, and MTT crystals were solubilized with 100 μL DMSO for individual well and absorbing values were measured at 540 nm. Inhibitory rates were calculated, 100% x (the values of control cells − the values of treated cells)/the values of control cells. All cells were assigned into the following groups, CG, control group; EG, β-Elemene (40 μM) treated group; IG, miR-659-3p siRNA group; ING, miR-659-3p siRNA scrambled group;

EIG, β-Elemene treated miR-659-3p siRNA group; MG, miR-659-3p mimic group; MNG, miR-659-3p mimic scrambled group and EMG, β-Elemene treated miR-659-3p mimic group. Inhibitory rates were calculated and compared among different groups using the above method.

Apoptosis Analysis

After treatment with different concentrations β-caryophyllene or miR-659-3p siRNA or mimics for different time times, the cells were collected with trypsin and rinsed with ice-cold PBS twice. The pellets were resuspended and fixed in 70% EtOH at 4 °C overnight. Before being detected by flow cytometry, the cells were washed twice with PBS and resuspended in a solution containing PI (1 μg/mL) and RNase A (10 μg/mL) for 30 min. Cells were digested with 0.25% trypsin for 3 min. PBS was added to make cell suspension, and cells were mixed evenly and observed under an inverted microscope. The cell suspension was transferred into a 10 mL centrifuge tube and centrifuge at 4°C, 1000 rpm for 5 min. The supernatant was removed, and precipitation was washed twice with PBS, and centrifuged at 1000 rpm for 5 min, and collected. Five hundred-microliter binding buffer was added, and the cells were resuspended. Five-microliter Annexin V-EGFP was added, and then 5 μL PI was added, mixed well. The mixture was centrifuged at 1000 rpm for 5 min, and the supernatant was removed. One hundred and ninety microliters of Annexin V-FITC binding solution was added, and 10μL of PI staining solution was added and mixed well on the ice bath for 15 min. Flow cytometry detection was performed at excitation wavelength of 488 nm and emission wavelength of 638 nm.

qRT-PCR Analysis

RNA was isolated from cells following the instructions of RNA extraction kit (Invitrogen, USA). Reverse transcription of mRNA was synthesized by using cDNA synthesis kit (TaKaRa, Japan). The primer for caspase-3 and BAX was used from the reported reports.[28] The primers for miR-659-3p, SphK1, U6 and GAPDH were used from the previous report[29] and synthesized from TaKaRa.

qRT-PCR was performed on Biosystems Step One Plus™ Real-Time PCR System (Life Technologies TM, USA). First, perform the pre-denaturation at 95°C for 10 min, and then alternately cycle 40 times at 95°C for 15 s and 60°C for 60 s. Three parallel samples were set in parallel for each sample. The PCR system software was used for data collection and analysis. The 2-ΔΔCT threshold cycle method was used for quantitative data analysis. GAPDH was used as the control.

ELISA Analysis

Collected cells were disrupted by using lysis buffer (1:10, w/v)[30] and centrifuged at 12,000 × g for 10 min and the supernatant was collected. The concentration of SphK1, cleaved caspase-3 and BAX was analyzed by using the ELISA kits from Beyontime Co. (Beyotime, China).

Measurement of ROS and NO

The DCFH-DA (2,7-dichlorofluorescein diacetate) probe was used to determine the ROS in the cells. The single-cell suspension was incubated with 10 μM DCFH-DA at 37°C for about 30 min and then centrifuged at 1000 g for 10 min. The fluorescently labelled cells were collected and resuspended in 9× volume of PBS. The fluorescence intensity of DCF (dichlorofluorescein) in the resuspension of single cells was measured with an automatic multifunctional microplate reader (Thermo). The maximum excitation spectrum was 495 nm, and the maximum emission spectrum was 529 nm. The nitrate reductase method was used to determine the production of nitric oxide (NO). According to the operating instructions provided by the manufacturer of the nitric oxide kit (Nanjing Jiancheng Institute of Biological Engineering). About 0.5 mL of the supernatant and 0.3 mL of color developer were mixed and measured using a spectrophotometer (Shimadzu, Japan) at 550nm wavelength.

Measurement of Antioxidants

Glutathione peroxidase (GPx) (EC 1.11.1.9) is a selenium-containing antioxidant that effectively reduces H_2O_2 and lipid peroxides to water and lipid alcohols. The Sod, Cat and GPx enzyme activities were measured using Sod assay kit (WST-1 method), Cat assay kit (visible light method) and GPx assay kit (colorimetric method), respectively, and the relevant operations were in accordance with the instructions provided by the manufacturer. All kits were purchased from Nanjing Jiancheng Biological Engineering Company. The absorbance is measured using a microplate reader (Thermo) or an ultraviolet spectrophotometer (Shimadzu, Japan).

Statistical Analysis

All data were expressed as mean ± standard deviation (Mean ± SD). SPSS software (IBM Statistics SPSS 22 for Windows, USA) was used to test the data for normal

distribution and homogeneity of variance. Analysis of Variance (ANOVA) was performed under the conditions of satisfying the two tests. Differences between groups were analyzed by two-way analysis of variance (two-way ANOVA). An independent sample *t*-test was used for the difference between the two groups of means. $P < 0.05$ was used as the criterion of statistical difference.

Results

Volatile Compounds of Chilli Pepper

There are 7 main volatile compounds (2-Methyl-1-tetralone, β-Elemene, β-Caryophyllene, Hexanal, Furfural, Nonanal, and 2-methyldecane, Supporting Information Figure S1) with more 15% β-Caryophyllene in the chilli pepper (Figure 1A) according to standards (Figure 1B).

SphK1 Expression in Lung Cancer from GEPIA2 Database and Interaction with miRNA

Bodymap shows that the median expression of SphK1 increased in many tumor tissues and reduces in the normal samples (Figure 2A, $P < 0.05$). SphK1 expression levels increased in lung cancer tissues and reduced in normal tissues (Figure 2B, $P < 0.05$). SphK1 expression increased with lung cancer development and reduced at stage IV (Figure 2C, $P < 0.05$). The overall survival time of lung cancer patients based on high-level SphK1 expression was shorter than the low-level expression (Figure 2D, $P < 0.05$). miRDB analysis shows that SphK1 was predicted to be targeted by 12 miRNAs and miR-659-3p with the highest target scores (Figure 2E, $P < 0.05$). The target site sequence (GAACCAA) at 3'- UTR of SphK1 could be recognized by the sequence of miR-659-3p (CUUGGUU) via complementary binding ability (Figure 2F).

β-Caryophyllene and miR-659-3p Intervention Inhibited NSCLC Growth

MTT analysis showed that β-Caryophyllene had significant inhibitory functions on NSCLC cell lines A549 (Figure 3A, $P < 0.05$), NCI-H1299 (Figure 3B, $P < 0.05$), HCC827 (Figure 3C, $P < 0.05$), and PC9 (Figure 3D, $P < 0.05$) after 24-, 48- and 72-h culture. β-Caryophyllene treatment and miR-659-3p mimic inhibited the growth of NSCLC cell lines A549 (Figure 3E, $P < 0.05$), NCI-H1299 (Figure 3F, $P < 0.05$), HCC827 (Figure 3G, $P < 0.05$), and PC9 (Figure 3H, $P < 0.05$) after 24-, 48- and 72-h culture. In contrast, miR-659-3p siRNA promoted the growth of NSCLC cell lines NSCLC cell lines A549 (Figure 3E, $P < 0.05$), NCI-H1299 (Figure 3F, $P < 0.05$), HCC827 (Figure 3G, $P < 0.05$), and PC9 (Figure 3H, $P < 0.05$) after 24-, 48- and 72-h culture. The results suggest that β-Caryophyllene and miR-659-3p intervention inhibits NSCLC growth. The cells were treated with 10, 20 and 40 μM of the individual volatiles compound standard. About 40 μM of β-Caryophyllene showed the highest inhibitory effects on NSCLC growth and used in the subsequent experiment.

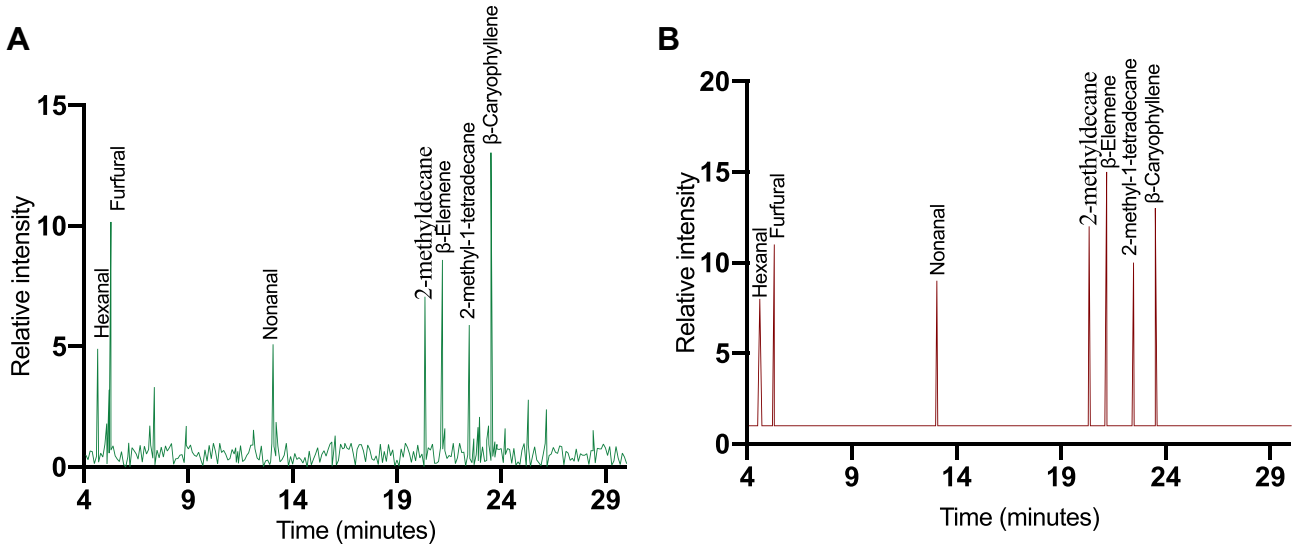

Figure 1 GC-MS analysis of volatile compounds of chilli pepper and their standards. (**A**) The main volatile compounds of chilli pepper. (**B**) The standards of the main compounds (2-Methyl-1-tetralone, β-Caryophyllene, β-Elemene, Hexanal, Furfural, Nonanal, and 2-methyldecane).

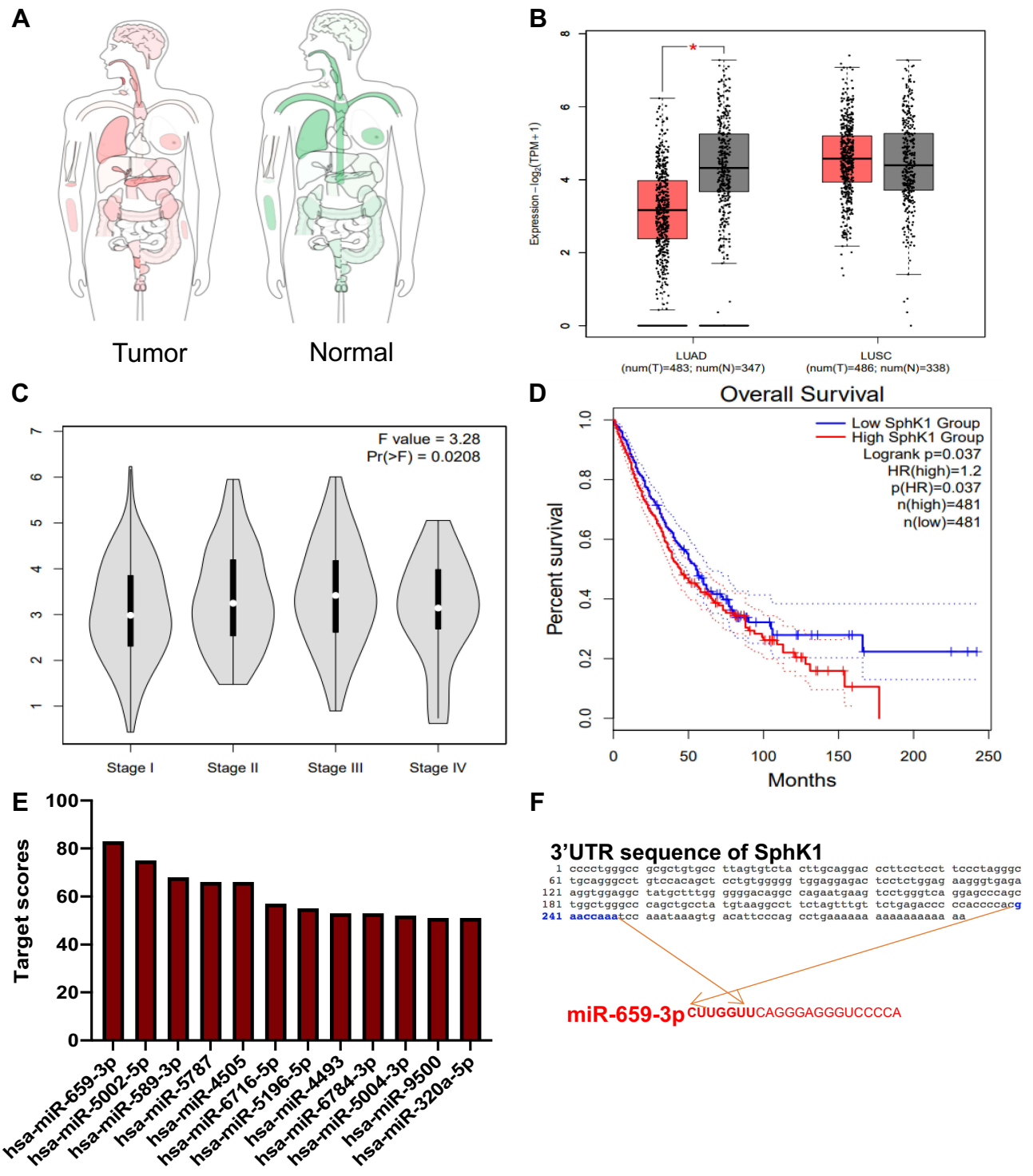

Figure 2 SphK1 expression in lung cancer from GEPIA2 database and interaction with miRNA. (**A**) The median expression of SphK1 tumor and normal samples in bodymap. Red represents upregulation and green represents down-regulation. (**B**) GEPIA2 database was used to obtain the SphK1 expression levels in lung cancer and normal tissues. (**C**) SphK1 expression at different stage of lung cancer. (**D**) Kaplan-Meier analysis of the overall survival of lung cancer patients based on SphK1 expression in GEPIA2 database. (**E**) SphK1 is predicted to be targeted by 12 miRNAs in miRDB. (**F**) Target site analysis between SphK1 and miR-659-3p.
Abbreviations: LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

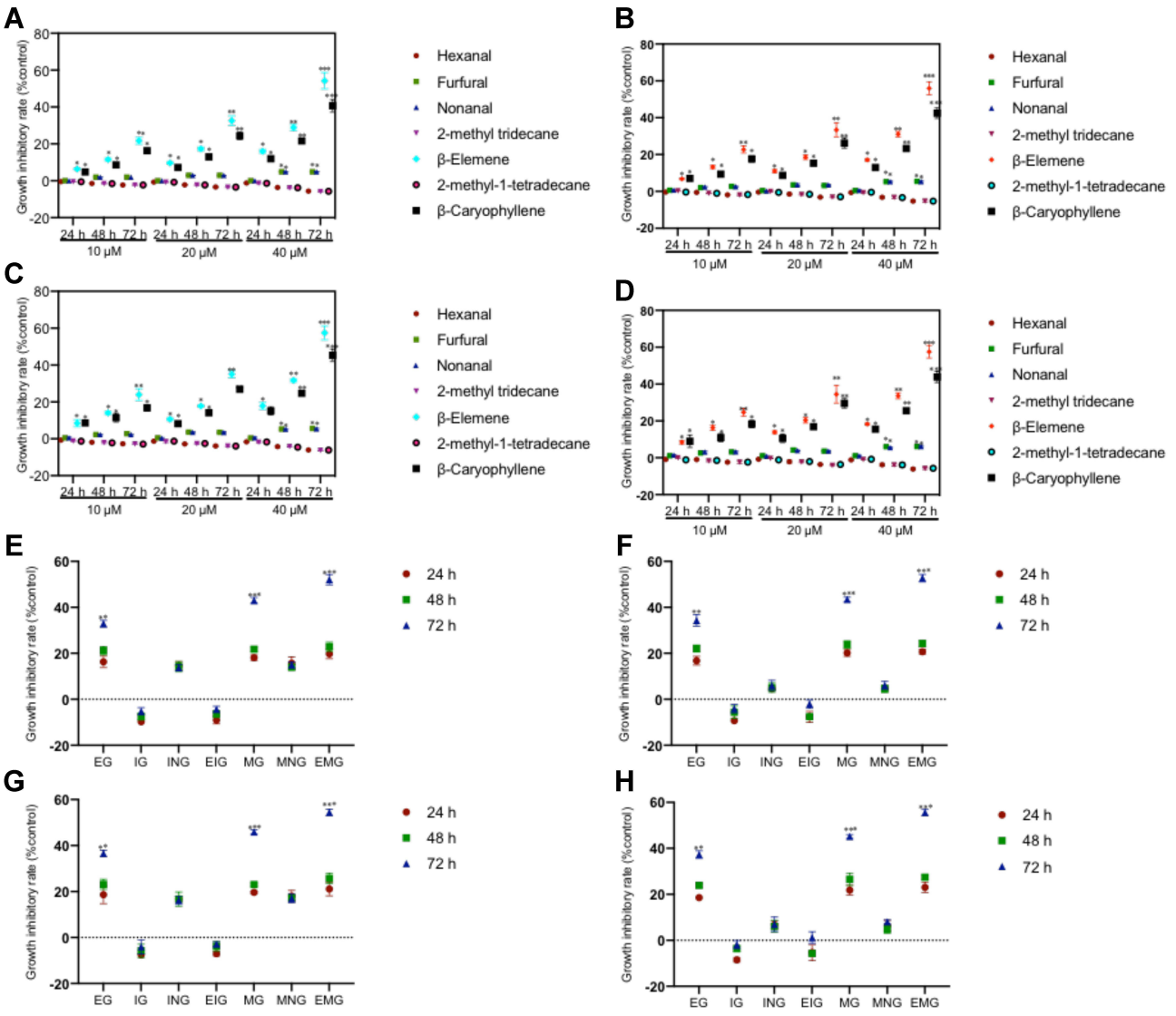

Figure 3 MTT analysis of the effects of β-Caryophyllene and miR-659-3p on the growth of lung cancer cells. (**A**) The effects of β-Caryophyllene on the growth of non-small cell lung cancer (NSCLC) cell lines A549. (**B**) The effects of β-Caryophyllene on the growth of NSCLC cell lines NCI-H1299. (**C**) The effects of miR-659-3p on the growth of NSCLC cell lines A549. (**D**) The effects of miR-659-3p on the growth of NSCLC cell lines NCI-H1299. (**E**) The effects of miR-659-3p mimic or siRNA on the growth of NSCLC cell lines A549. (**F**) The effects of miR-659-3p mimic or siRNA on the growth of NSCLC cell lines NCI-H1299. (**G**) The effects of miR-659-3p mimic or siRNA on the growth of NSCLC cell lines HCC827. (**H**) The effects of miR-659-3p mimic or siRNA on the growth of NSCLC cell lines PC9. All experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs the CG group.

Abbreviations: CG, control group; EG, β-Caryophyllene treated group; IG, miR-659-3p siRNA group; ING, miR-659-3p siRNA scrambled group; EIG, β-Caryophyllene treated miR-659-3p siRNA group; MG, miR-659-3p mimic group; MNG, miR-659-3p mimic scrambled group; EMG, β-Caryophyllene treated miR-659-3p mimic group.

β-Caryophyllene Intervention Increased miR-659-3p Level and Apoptotic Factors mRNA Level, and Reduced SphK1 mRNA Level

qRT-PCR analysis showed that β-Caryophyllene had significant effects on miR-659-3p level in NSCLC cell lines A549 (Figure 4A, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 4B, $P < 0.05$) among all volatile compounds. Therefore, β-Caryophyllene was selected to explore the possible mechanisms. miR-659-3p siRNA reduced the level of miR-659-3p and the mimic increased the level of miR-659-3p in NSCLC cell lines A549 (Figure 4C, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 4D, $P < 0.05$), suggesting that miR-659-3p was successfully blocked or over-expressed. In contrast, miR-659-3p siRNA increased the mRNA level of SphK1 and the mimic reduced the mRNA level of SphK1 in NSCLC cell lines A549 (Figure 4E, P <

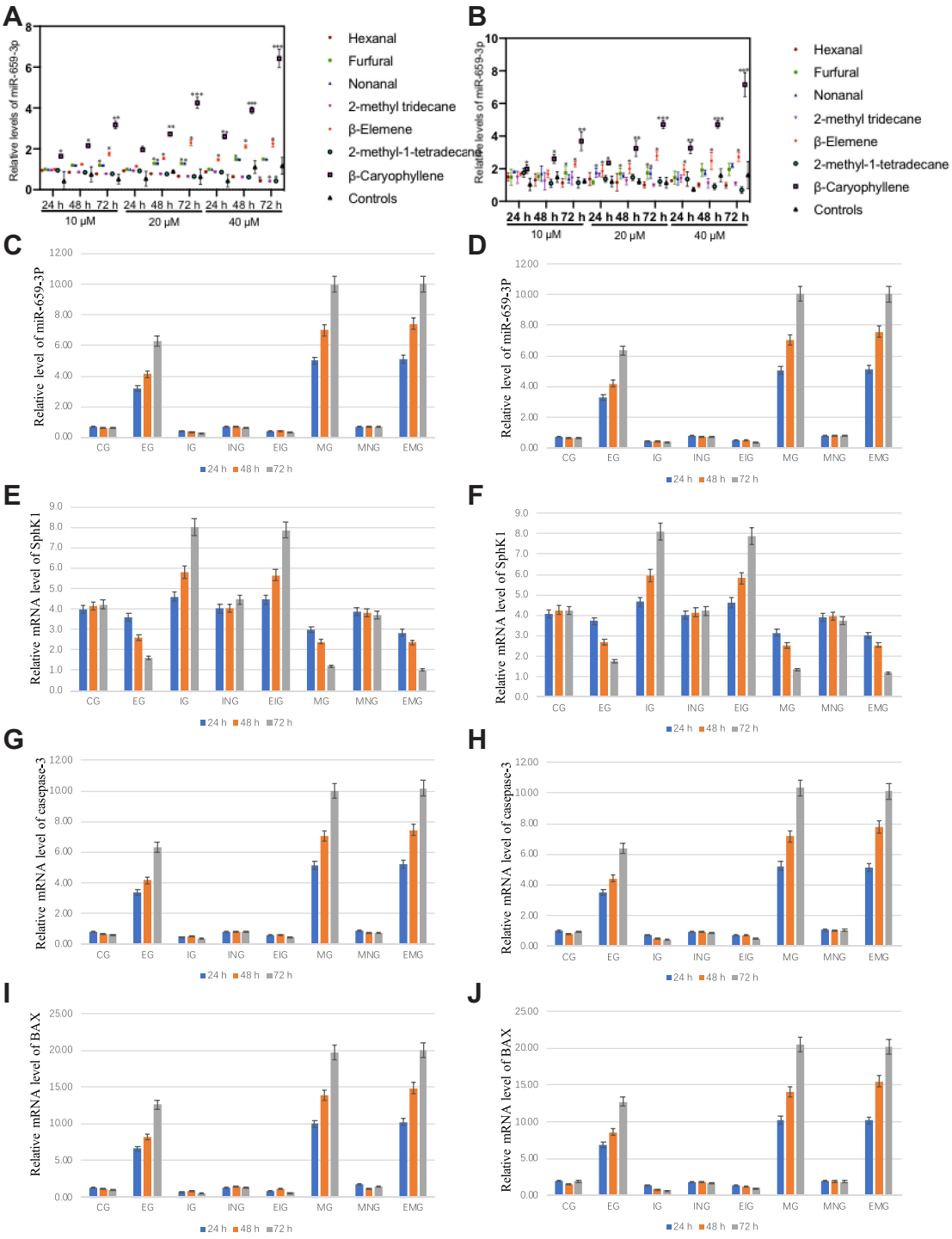

Figure 4 The RNA levels of miR-659-3p/SphK1 loop and apoptotic factors. (**A**) The effects of volatile compounds (Hexanal (**A**), Furfural (**B**), Nonanal (**C**), 2-methyldecane (**D**), β-Elemene (**E**), 2-Methyl-1-tetralone (**F**), β-Caryophyllene (**G**) and controls (**H**)) of chilli pepper on miR-659-3p level in NSCLC cell lines A549. (**B**) The effects of volatile compounds of chilli pepper on miR-659-3p level in NSCLC cell lines NCI-H1299. (**C**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on miR-659-3p level in NSCLC cell lines A549. (**D**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on miR-659-3p level in NSCLC cell lines NCI-H1299. (**E**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on relative mRNA level of SphK1 in NSCLC cell lines A549. (**F**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on relative mRNA level of SphK1 in NSCLC cell lines NCI-H1299. (**G**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on relative mRNA level of caspase-3 in NSCLC cell lines A549. (**H**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on relative mRNA level of caspase-3 in NSCLC cell lines NCI-H1299. (**I**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on relative mRNA level of BAX in NSCLC cell lines A549. (**J**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on relative mRNA level of BAX in NSCLC cell lines NCI-H1299. 40 μM of β-Caryophyllene showed the highest inhibitory effects on NSCLC growth and used in the subsequent experiment. All experiments were performed in triplicate.*P<0.05, **P<0.01 and ***P<0.001 vs the CG group.

0.05) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 4F, $P < 0.05$), suggesting that SphK1 was closely targeted by miR-659-3p. miR-659-3p siRNA reduced the mRNA level of caspase-3 and the mimic increased the mRNA level of caspase-3 in NSCLC cell lines A549 (Figure 4G, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 4H, $P < 0.05$). miR-659-3p siRNA reduced the mRNA level of BAX and the mimic increased the mRNA level of BAX in NSCLC cell lines A549 (Figure 4I, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 4J, $P < 0.05$). All these results suggest that miR-659-3p can affect the mRNA levels of apoptotic factors.

β-Caryophyllene and miR-659-3p Intervention Reduced the Protein Level of SphK1 and Increased Relative Protein Levels of Apoptotic Factors

ELISA analysis showed that β-Caryophyllene had significant inhibitory effects on SphK1 protein concentration in NSCLC cell lines A549 (Figure 5A, $P < 0.05$) and NCI-

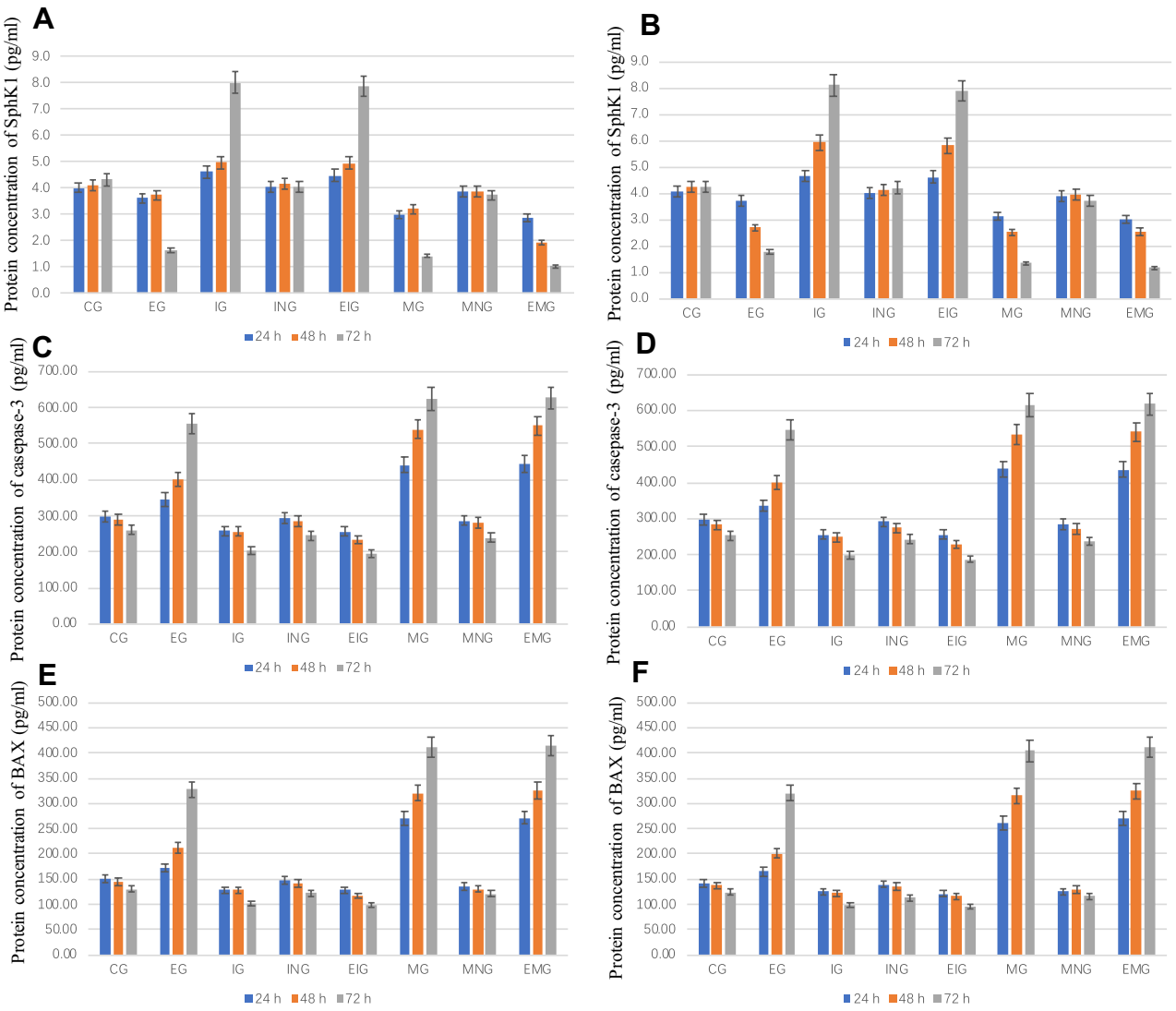

Figure 5 Protein concentration of SphK1 and apoptotic factors. (**A**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on protein concentration of SphK1 in NSCLC cell lines A549. (**B**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on protein concentration of SphK1 in NSCLC cell lines NCI-H1299. (**C**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on protein concentration of cleaved caspase-3 in NSCLC cell lines A549. (**D**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on protein concentration of cleaved caspase-3 in NSCLC cell lines NCI-H1299. (**E**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on protein concentration of BAX in NSCLC cell lines A549. (**F**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on protein concentration of BAX in NSCLC cell lines NCI-H1299. 40 μM of β-Caryophyllene showed the highest inhibitory effects on NSCLC growth and used in the subsequent experiment. All experiments were performed in triplicate.

H1299 after 24-, 48- and 72-h culture (Figure 5B, $P < 0.05$). Similarly, miR-659-3p siRNA increased the protein level of SphK1 and the mimic reduced the protein level of SphK1 in NSCLC cell lines A549 (Figure 5A, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 5B, $P < 0.05$), suggesting that SphK1 was closely targeted by miR-659-3p. β-Caryophyllene had significant promoting effects on cleaved caspase-3 protein concentration in NSCLC cell lines A549 (Figure 5C, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 5D, $P < 0.05$). miR-659-3p siRNA reduced the protein level of cleaved caspase-3 and the mimic increased the protein level of cleaved caspase-3 in NSCLC cell lines A549 (Figure 5C, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 5D, $P < 0.05$). Caryophyllene had significant promoting effects on BAX protein concentration in NSCLC cell lines A549 (Figure 5E, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 5F, $P < 0.05$). miR-659-3p siRNA reduced the protein level of BAX and the mimic increased the protein level of BAX in NSCLC cell lines A549 (Figure 5E, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 5F, $P < 0.05$). β-Caryophyllene and miR-659-3p intervention may promote NSCLC apoptosis via SphK1.

β-Caryophyllene and miR-659-3p Intervention Reduced Oxidative Stress and Increased Antioxidant Level of NSCLC

β-Caryophyllene had significant inhibitory effects on ROS levels in NSCLC cell lines A549 (Figure 6A, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6B, $P < 0.05$). β-Caryophyllene also had significant inhibitory effects on NO level in NSCLC cell lines A549 (Figure 6C, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6D, $P < 0.05$). miR-659-3p siRNA increased the ROS level and the mimic reduced ROS level in NSCLC cell lines A549 (Figure 6A, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6B, $P < 0.05$). miR-659-3p siRNA increased the NO level and the mimic reduced NO level in NSCLC cell lines A549 (Figure 6C, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6D, $P < 0.05$). The results suggest that β-Caryophyllene and miR-659-3p intervention reduce oxidative stress in the NSCLC.

β-Caryophyllene had significant promoting effects on SOD activity in NSCLC cell lines A549 (Figure 6E, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6F, $P < 0.05$). β-Caryophyllene had significant promoting effects on CAT level in NSCLC cell lines A549 (Figure 6G, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6H, $P < 0.05$). β-Caryophyllene also had significant promoting effects on GPx level in NSCLC cell lines A549 (Figure 6I, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6J, $P < 0.05$). miR-659-3p siRNA reduced the SOD activity and the mimic increased SOD activity in NSCLC cell lines A549 (Figure 6E, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6F, $P < 0.05$). miR-659-3p siRNA reduced the CAT activity and the mimic increased CAT activity in NSCLC cell lines A549 (Figure 6G, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6H, $P < 0.05$). miR-659-3p siRNA also reduced the GPx activity and the mimic increased GPx activity in NSCLC cell lines A549 (Figure 6I, $P < 0.05$) and NCI-H1299 after 24-, 48- and 72-h culture (Figure 6J, $P < 0.05$). The results suggest that β-Caryophyllene and miR-659-3p intervention increases antioxidant properties in the NSCLC.

β-Caryophyllene and miR-659-3p Intervention Induced NSCLC Apoptosis

β-Caryophyllene had significant inhibitory effects on the apoptotic level in NSCLC cell lines A549 (Figure 7A and 7B, $P < 0.05$) and NCI-H1299 after 72-h culture (Figure 7C and 7D, $P < 0.05$). miR-659-3p siRNA reduced the apoptotic rate level and the mimic increased apoptotic rate in NSCLC cell lines A549 (Figure 7A and 7B, $P < 0.05$) and NCI-H1299 after 72-h culture (Figure 7C and 7D, $P < 0.05$). All these results suggest that β-Caryophyllene and miR-659-3p intervention promoted NSCLC apoptosis.

Discussion

Natural product intervention is one of the crucial approaches to reduce or delay the occurrence of NSCLC.[31–33] The potential value of this approach has been demonstrated with trials in breast,[34,35] prostate,[36,37] and colon cancer.[36,37] Because of low cytotoxicity to normal cells, minimal side effects, and a wide margin of safety, natural products can be used to develop new pharmaceutical drugs.

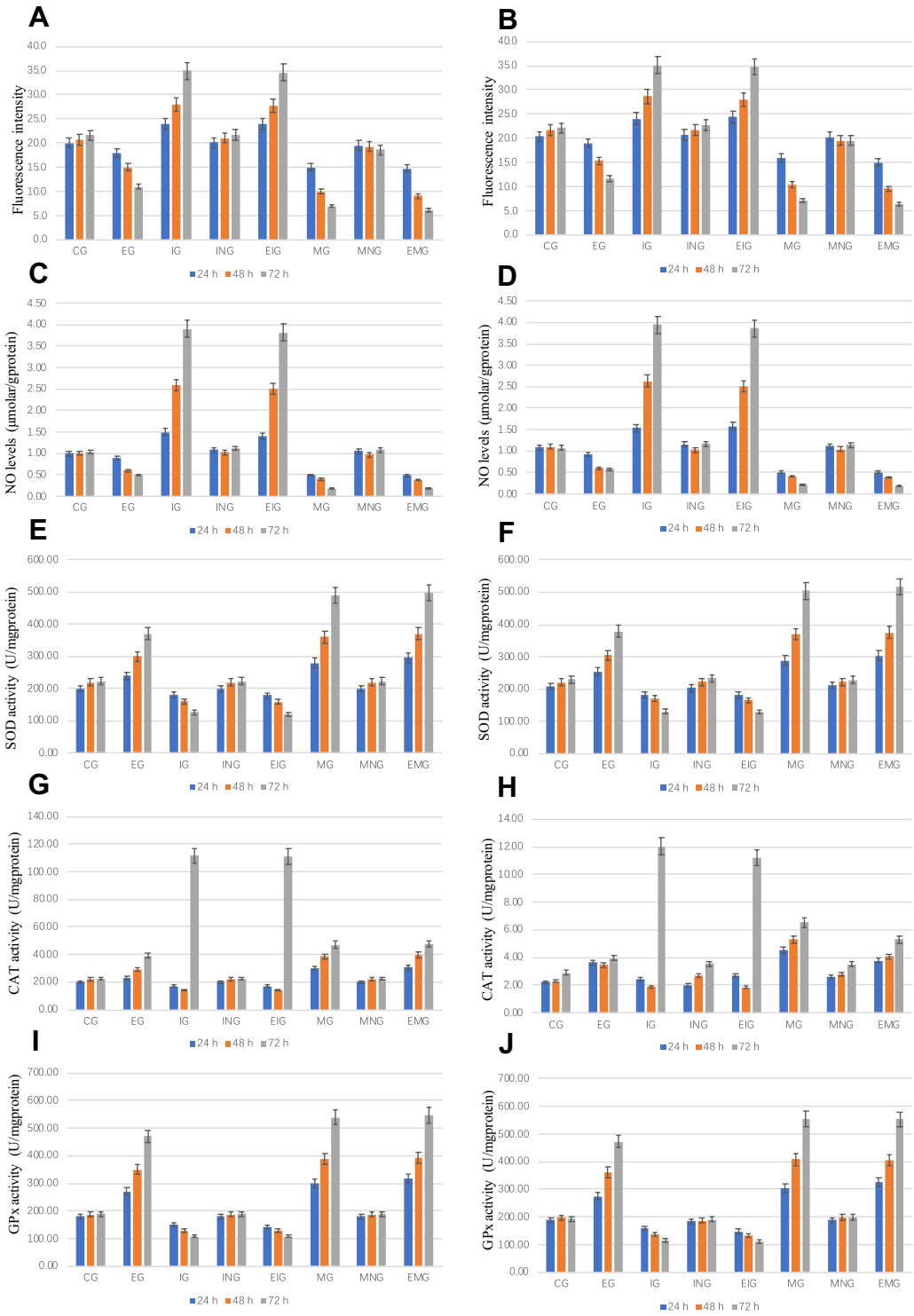

Figure 6 The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on the levels of oxidative stress and antioxidants in NSCLC cells. (**A**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on ROS level in NSCLC cell lines A549. (**B**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on ROS level in NSCLC cell lines NCI-H1299. (**C**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on NO level in NSCLC cell lines A549. (**D**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on NO level in NSCLC cell lines NCI-H1299. (**E**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on SOD activity in NSCLC cell lines A549. (**F**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on SOD activity in NSCLC cell lines NCI-H1299. (**G**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on CAT activity in NSCLC cell lines A549. (**H**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on CAT activity in NSCLC cell lines NCI-H1299. (**I**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on GPx activity in NSCLC cell lines A549. (**J**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on GPx activity in NSCLC cell lines NCI-H1299. 40 μM of β-Caryophyllene showed the highest inhibitory effects on NSCLC growth and used in the subsequent experiment. All experiments were performed in triplicate.

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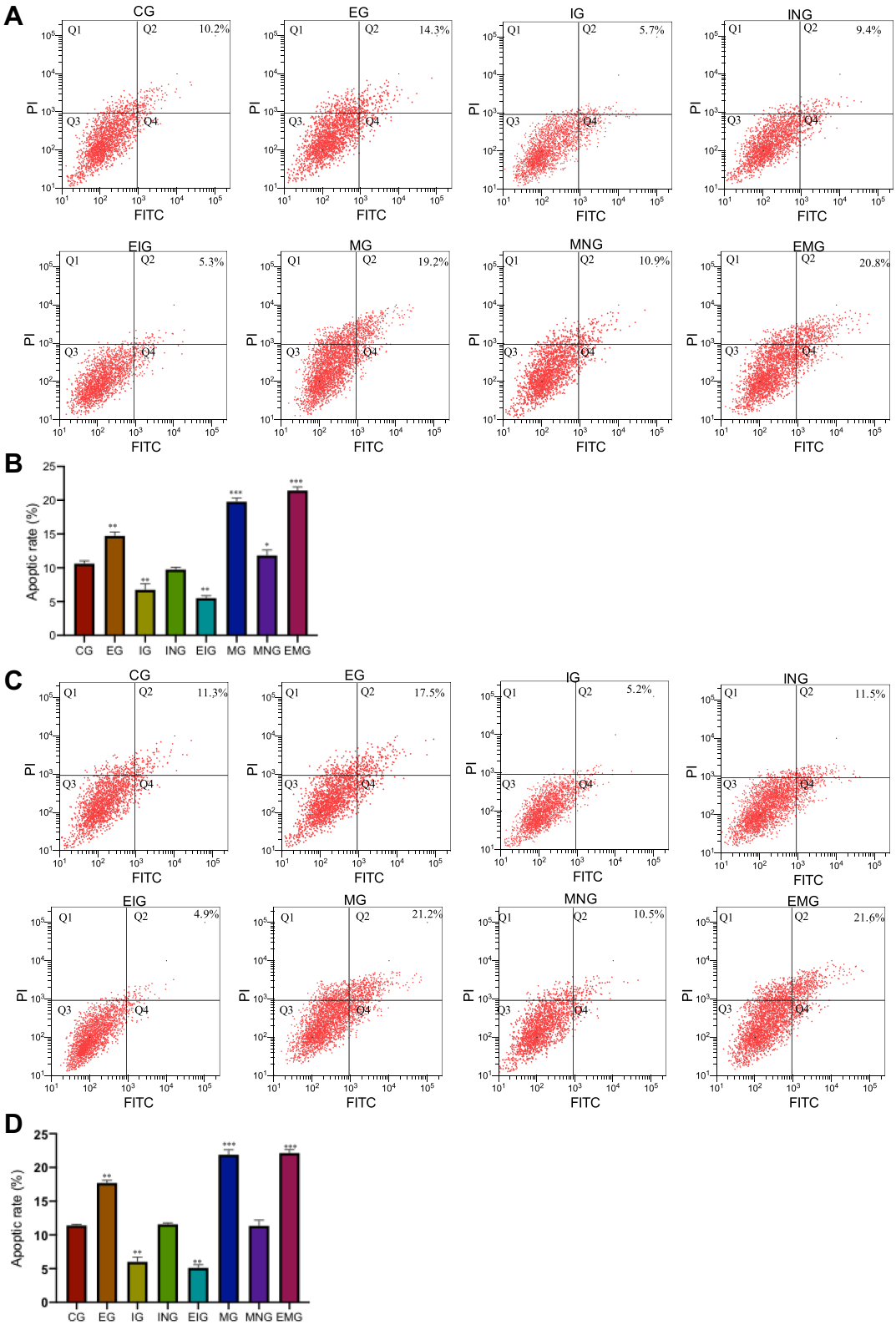

Figure 7 The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on the apoptotic levels in NSCLC cells. (**A**) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on apoptotic levels in NSCLC cell lines A549. (**B**) Different apoptotic levels among different groups in NSCLC cell lines A549. (C) The effects of miR-659-3p siRNA and mimic, and or β-Caryophyllene treatment on apoptotic levels in NSCLC cell lines NCI-H1299. 40 μM of β-Caryophyllene showed the highest inhibitory effects on NSCLC growth and used in the subsequent experiment. (**D**) Different apoptotic levels among different groups in NSCLC cell lines NCI-H1299. All experiments were performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs the CG group.

Abbreviations: CG, control group; EG, β-Caryophyllene treated group; IG, miR-659-3p siRNA group; ING, miR-659-3p siRNA scrambled group; EIG, β-Caryophyllene treated miR-659-3p siRNA group; MG, miR-659-3p mimic group; MNG, miR-659-3p mimic scrambled group; EMG, β-Caryophyllene treated miR-659-3p mimic group.

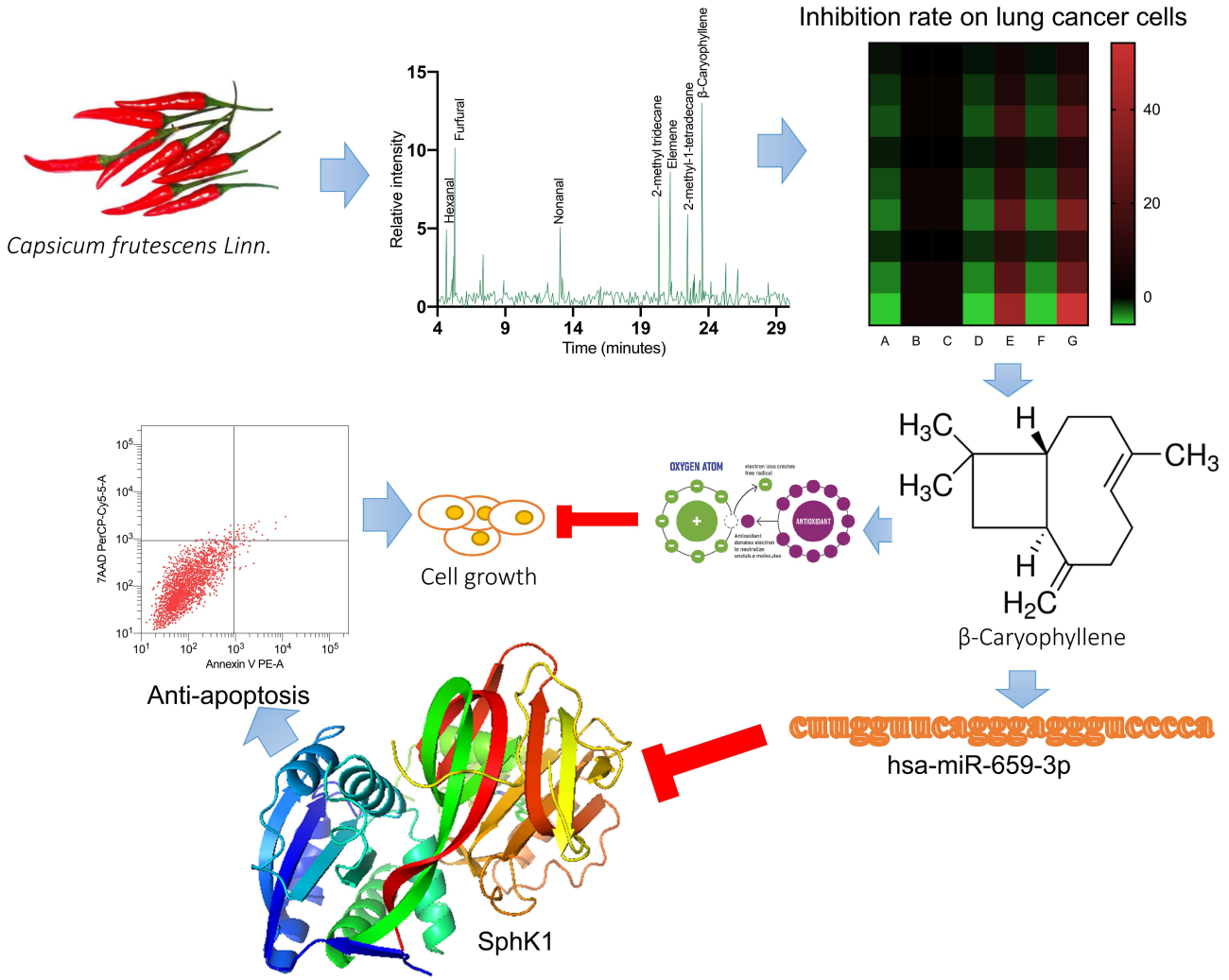

Figure 8 The molecular mechanism for β-Elemene from chilli pepper inhibiting the proliferation of non-small cell lung cancer cells by affecting miR-659-3p-targeted SphK1 and antioxidant properties.

The present findings show that β-Caryophyllene is the main ingredient of volatile compounds of Chilli pepper and had significant inhibitory effects on NSCLC growth (Figure 3). Furthermore, β-Caryophyllene treatment reduces the oxidative stress level and increases antioxidant activities and apoptotic factor level (Figures 4–7). miR-659-3p mimic and siRNA prevented and induced the growth of NSCLC cells, their apoptosis, and these biochemical indices. On the other side, β-Caryophyllene intervention increases the level of miR-659-3p and reduces the level of SphK1. Bioinformatic analysis shows SphK1 may be targeted by miR-659-3p, which is also consistent with the previous report.[38] SphK1 has also been reported to inhibit apoptosis in gastric cancer cells by downregulating Bim via the upregulation of Akt/forkhead box O3a (FoxO3a) signaling.[39] Therefore, β-caryophyllene from volatile compounds of chilli pepper exerts inhibitory activity in NSCLC cells possibly by affecting miR-659-3p-targeted SphK1 and increasing antioxidant properties (Figure 8).

High-level reactive species including ROS are the main products produced as a consequence of high-level metabolic reactions in the mitochondria of cancers.[40] Therefore, the antioxidant may be a potential approach for cancer therapy.[41] The present experiment approves that β-Caryophyllene increases antioxidant properties in NSCLC and reduces oxidative stress, which is also consistent with the reported work.[42] However, antioxidant inhibitors have been reported to be used in cancer therapy,[43] suggesting there are still other mechanisms for the differences.

Our results indicated that β-caryophyllene induces NSCLC apoptosis. β-Caryophyllene oxide has been reported

to prevent tumor growth and induces apoptosis through the suppression of Phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of the rapamycin (mTOR)/S6 Kinase 1 (S6K1) pathways and ROS-mediated mitogen-activated protein kinases (MAPKs) activation.[44] However, the β-caryophyllene on NSCLC apoptosis is still seldom reported. Previous work also suggests that β-caryophyllene may induce cell cycle arrest and lead to apoptosis.[45]

There are some limitations to the present work. We have separated different fractions from chilli pepper. Unfortunately, the purity of some ingredients was less than 90%. To avoid the interference of other ingredients, only the standard samples from a commercial company were used. We have not tried other purified commercial sources either. The present study indicated that β-caryophyllene in the volatile compounds of chilli pepper may display its antiproliferative effect via in miR-659-3p-targeted SphK1 in NSCLC. The present data suggest that β-caryophyllene of chilli pepper should be considered as potential natural product in the prevention of NSCLC. To obtain clear conclusion, further work is needed to be done in the future since the present work is only carried out at cellular level.

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Disclosure

The authors declare no conflict of interest.

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