Shikonin inhibits the Warburg effect, cell proliferation, invasion and migration by downregulating PFKFB2 expression in lung cancer

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Abstract. Lung cancer is one of the most lethal diseases and therefore poses a significant threat to human health. The Warburg effect, which is the observation that cancer cells predominately produce energy through glycolysis, even under aerobic conditions, is a hallmark of cancer. 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB) is an important regulator of glycolysis. Shikonin is a Traditional Chinese herbal medicine, which has been reported to exert antitumor effects. The present study aimed to investigate the anticancer activity of shikonin in lung cancer. Cell Counting Kit-8 (CCK-8) and colony formation assays were used to analyze proliferation in A549 and H446 cells. Wound healing and Transwell assays were used to measure migration and invasion in A549 and H446 cells. Cell apoptosis was analyzed using flow cytometry. Lactate levels, glucose uptake and cellular ATP levels were measured using their corresponding commercial kits. Western blotting was performed to analyze the protein expression levels of key enzymes involved in aerobic glucose metabolism. Reverse transcription-quantitative PCR was used to analyze the mRNA expression levels of PFKFB2. The results of the present study revealed that PFKFB2 expression levels were significantly upregulated in NSCLC tissues. Shikonin treatment decreased the proliferation, migration, invasion, glucose uptake, lactate levels, ATP levels and PFKFB2 expression levels and increased apoptosis in lung cancer cells in a dose-dependent manner. The overexpression of PFKFB2 increased the proliferation, migration, glucose uptake, lactate levels and ATP levels in lung cancer cells, while the knockdown of PFKFB2 expression exerted the opposite effects. Moreover, there were no significant differences in lung cancer cell migration, apoptosis, glucose uptake, lactate levels and ATP levels between cells with knocked down PFKFB2 expression or treated with shikonin and the knockdown of PFKFB2 in cells treated with shikonin. In conclusion, the results of the present study revealed that shikonin inhibited the Warburg effect and exerted antitumor activity in lung cancer cells, which was associated with the downregulation of PFKFB2 expression.

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Introduction

Lung cancer is one of the most common causes of cancer-related mortality, accounting for 18.4% of cancer-related deaths worldwide (1). In total, ~85% of lung cancer cases are diagnosed as non-small cell lung cancer (NSCLC) and ~15% of lung cancer cases are diagnosed as small cell lung cancer (2). At present, the most common treatment methods for lung cancer include surgical resection, chemotherapy, radiotherapy, immunotherapy and targeted drugs (3). Although targeted therapies, such as EGFR inhibitors, and immunotherapy drugs, such as anti-programmed cell death protein 1/programmed death-ligand 1, have significantly improved the treatment of lung cancer, only a small number of patients are eligible to be treated with targeted therapies, thus the global 5-year survival rate of lung cancer remains at ~20% (4-8). Therefore, identifying specific and sensitive prognostic markers, therapeutic targets and devising novel therapeutic drugs to improve the survival time and quality of life of patients with lung cancer remains a priority.

The Warburg effect is defined by the altered metabolism of glucose, whereby tumor cells produce large amounts of lactate, even in the presence of oxygen and fully functioning mitochondria (9). The intrinsic mechanism of the Warburg effect is complex, and is associated with the activation of numerous oncogenes, inactivation of numerous tumor suppressor genes, the abnormal expression of glycosylase and changes in the tumor microenvironment, amongst other factors (10-12). However, the specific mechanism requires further investigation. It is well established that the cell death program (apoptosis) exerts an anticancer effect (13). The Warburg effect is closely associated with the occurrence and development of cancer, as it provides a growth advantage to tumor cells, helping them to

escape apoptosis and create a suitable environment for tumor metastasis to occur (14-16). The inhibition of the Warburg effect promotes cell apoptosis and inhibits tumor proliferation and metastasis (17). Several key metabolic enzymes [pyruvate kinase M2 (PKM2), pyruvate dehydrogenase kinase 1 (PDK1) and hexokinase 2] have been found to play an important role in the Warburg effect (18-20). The changes in key enzymes can lead to an enhanced glycolytic ability, promote glucose uptake into tumor cells and increase the accumulation of lactic acid, thus further supporting tumor growth and development (21). 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB2) is an enzyme that regulates the synthesis and degradation of fructose-2,6-bisphosphate (Fru-2, 6-P2), which is widely expressed in a variety of cancer cells, such as ovarian, breast and pancreatic cancer cells (22,23). By regulating the expression of Fru-2, 6-P2 (a signaling metabolite that participates in glycolysis), the PFKFB family has been found to regulate intracellular glycolysis in cancer by acting as an oncogene (24,25).

Shikonin is a naphthalene quinone compound extracted from *Lithospermum erythrorhizon* (an herbaceous plant), which has demonstrated anti-inflammatory, antiviral, anti-tumor and wound healing potential, amongst other biological activities (26). The present study aimed to investigate the effects of shikonin on cell proliferation, migration, invasion, apoptosis and aerobic glycolysis in lung cancer cells, and further determined the potential underlying molecular mechanisms of shikonin. The current findings may provide a foundation for the clinical use of shikonin in the treatment of lung cancer.

Materials and methods

Patient studies. A total of 20 NSCLC and adjacent normal tissues were obtained from patients (10 women and 10 men; age range, 50-79 years) who underwent surgical resection at The Affiliated Hospital of Qingdao University (Qingdao, China) between January 2019 and December 2019. Patients who had received chemotherapy or radiotherapy prior to surgery were excluded from the study. Patients who were diagnosed by positron emission tomography/CT scan and were eligible for radical surgery were included in the study. All samples were stored at -80˚C until required for RNA extraction. The study protocols were approved by the Ethics Committee of The Affiliated Hospital of Qingdao University, and written informed consent was obtained from all patients prior to participation.

Cell lines and culture. The Beas-2B lung epithelial cell line and lung cancer cell lines, A549 and H446, were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. All cells were cultured at 37˚C (5% CO_2) in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.).

Cell transfection. Small interfering RNA (siRNA)-negative control (NC) (sense, 5'-UUCUCCGAACGUGUCACG UTT-3' and antisense, 5'-ACGUGACACGUUCGGAGA ATT-3'), siRNA-PFKFB2 (sense, 5'-AUUGUCAUGCCGAAA GAAGUC-3' and antisense, 5'-CUUCUUUCGGCAUGA CAAUGA-3'), pcDNA3.1-PFKFB2 and pcDNA3.1-NC (all Shanghai GenePharma Co., Ltd.) were used to knockdown or overexpress PFKFB2, respectively. Cells were transfected with siRNA (30 nM) or overexpression vectors (1 μg) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. Cells were harvested 48 h post-transfection for use in subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Transfected or untransfected A549 and H446 cells were treated with or without 50 μM shikonin (Shanghai Yuanye Biotechnology Co., Ltd.) for 24 h at 37˚C. Total RNA was extracted from clinical specimens or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using a PrimeScript RT kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. qPCR was subsequently performed on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR® Premix Ex Taq™ kit (Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 3 min; followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. The following primers pairs (Sangon Biotech Co., Ltd.) were used for the qPCR: PFKFB2 forward, 5'-GCTGCTTGG TGGGAGTGATAA-3' and reverse, 5'-TGAGAAGCCAAG TGTCAGGG-3'; and β-actin forward, 5'-GAGGACCCTGGA TGTGACAG-3' and reverse, 5'-AAGACCTGTACGCCAACA CA-3'. Relative mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (27) and normalized to β-actin.

Western blotting. A549 and H446 cells were treated with shikonin [0 (control), 10, 20 or 50 μM] for 24 h at 37˚C, then total protein was extracted from cells using RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) supplemented with protease and phosphatase inhibitor mixture. Total protein was quantified using a BCA assay and 50 μg protein/lane was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes (EMD Millipore) and blocked with 5% skim milk for 2 h at room temperature. The membranes were then incubated with the following primary antibodies at 4˚C overnight: Anti-PFKFB2 (1:1,000; cat. no. ab234865; Abcam), anti-PDK1 (1:2,000; cat. no. ab202468; Abcam), anti-glucose transporter 1 (GLUT1; 1:200; cat. no. ab150299; Abcam), anti-phosphoglycerate kinase 2 (PGK2; 1:1,000; cat. no. ab183031; Abcam), anti-lactate dehydrogenase A (LDHA; 1:1,000; cat. no. ab101562; Abcam), anti-PKM2 (1:1,000; cat. no. ab137852; Abcam), anti-GLUT3 (1:8,000; cat. no. ab41525; Abcam), anti-pyruvate dehydrogenase (PDH; 1:1,000; cat. no. 3205; Cell Signaling Technology, Inc.), phosphorylated (p)-PDH (1:1,000; cat. no. 31866; Cell Signaling Technology, Inc.) and anti-GAPDH (1:2,500; cat. no. ab9485; Abcam). Following the primary antibody incubation, the membranes were incubated with an anti-rabbit HRP-conjugated secondary antibody (1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (EMD Millipore).

Cell proliferation assay. Following transfection, A549 and H446 cells were plated into 96-well plates at a density of $2x10^3$ cells/well and cultured in DMEM supplemented with 10% FBS. For certain experiments, cells were also treated with shikonin [0, 10, 20 or 50 μM] for 24, 48, 72 or 96 h at 37˚C. At each time point, Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc.) was added to each well and incubated for 2 h at 37˚C. The optical density (OD) value was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific). The experiment was repeated in triplicate.

Wound healing assay. A549 and H446 cells were digested using 0.25% trypsin solution (Gibco; Thermo Fisher Scientific, Inc.) and cultured in a 6-well plate at a density of $5x10^5$ cells/well overnight at 37˚C. Upon cells reaching 90% confluence, a single vertical scratch was made in the cell monolayer with a 10-μl micropipette tip. The cells were washed with PBS thrice to remove the detached cells. Subsequently, A549 and H446 cells were cultured in serum-free DMEM with/without shikonin (0, 10, 20 or 50 μM) at 37˚C or transfected A549 cells were cultured in serum-free DMEM with 50 μM shikonin at 37˚C with 5% CO_2 in an incubator. At 0 h and following 24 h of incubation, the wound area was visualized and photographed using a light microscope (magnification, x100) and the migration was analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

Colony formation assay. After transfection for 48 h, A549 and H446 cells were plated into 6-well plates at a density of $0.5\text{-}1x10^3$ cells/well; three wells were plated for each experimental condition. The cells were cultured in complete medium supplemented with 30% FBS in an incubator with 37˚C for 14 days; the medium was changed every 3 days. During the incubation, the cell state and colony size were observed under a light microscope (magnification, x100). After culture, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.2% crystal violet for 5 min at room temperature. The cells were then washed with water, dried, photographed and counted using ImageJ software; >50 cells counted as a colony.

Transwell assay. Transwell assay was used to analyze cell invasion and migration. Transwell chambers (Corning, Inc.) were precoated with (invasion) or without (migration) Matrigel (Becton, Dickinson and Company) at 37˚C for 30 min. Following cell digestion using 0.25% trypsin solution, the culture medium was discarded by centrifugation (300 x g for 5 min at 37˚C) and the cell pellet was washed with PBS 1-2 times. A549 cells were treated with shikonin (0, 10, 20 or 50 μM) for 24 h at 37˚C. Next, cells were incubated in serum-free DMEM at a density of $5x10^5$ cells/ml for 24 h at 37˚C, then 100 μl cell suspension was added to the upper chamber of the Transwell plate. A total of 600 μl medium supplemented with 20% FBS was plated into the lower chambers. Following incubation for 24 h at 37˚C, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature. Finally, the cell numbers were counted under a light microscope (magnification, x200) using ImageJ software.

Flow cytometric analysis of apoptosis. Cell apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) staining kit (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, A549 cells were treated with shikonin (0, 10, 20 or 50 μM) for 24 h at 37˚C or transfected A549 cells were treated with 50 μM shikonin for 24 h at 37˚C and collected by centrifugation (300 x g for 5 min at 37˚C). Cells ($1x10^5$ cells/well) were then washed with cold (4˚C) PBS twice and resuspended in 1X binding buffer (200 μl). Cells were subsequently incubated with 5 μl Annexin V-FITC/PI at room temperature in the dark for 15 min. Apoptotic cells (early and late apoptotic cells) were visualized using a flow cytometer (Accuri™ C6 Plus; BD Biosciences).

Measurement of lactic acid production, glucose uptake and cellular ATP levels. A549 and H446 cells ($1x10^4$ cells/well) were seeded into 6-well plates and cultured in DMEM. A549 and H446 cells were treated with shikonin (0, 10, 20 or 50 μM) for 24 h at 37˚C or transfected A549 cells were treated with 50 μM shikonin for 24 h at 37˚C, then cell culture medium was subsequently collected and centrifuged at 8,000 x g for 5 min at room temperature. The supernatant was used to measure lactate or glucose concentrations, or cellular ATP levels. Extracellular lactate levels were measured using a lactate analysis kit (cat. no. E4341; BioVision, Inc.) according to the manufacturer's protocol. Cell lysates were used to measure glucose levels using a glucose assay kit (cat. no. K686; BioVision, Inc.) according to the manufacturer's protocol. ATP levels were measured using the CellTiter-Glo® luminescent cell activity assay kit (cat. no. G7572; Promega Corporation) according to the manufacturer's protocol. The relative value of lactate, glucose uptake and ATP levels were normalized to the control group (set to 1).

Statistical analysis. Statistical analysis was performed using SPSS 21.0 software (IBM Corp.) and data are expressed as the mean ± SEM of at least three independent experiments. Statistical differences between groups were determined using a one-way ANOVA followed by a Tukey's post hoc test. A paired Student's t-test was used to analyze the mRNA expression levels of PFKFB2 between NSCLC and adjacent normal tissues. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Shikonin inhibits the proliferation, invasion and migration of A549 cells. To investigate the antitumor effects of shikonin, the inhibitory effect of shikonin was determined using cellular functional experiments. The results of the cell proliferation experiment revealed that shikonin (10, 20 or 50 μM) could inhibit the proliferation of A549 cells in a dose-dependent manner compared with the control group (all $P<0.05$; Fig. 1A). The results from the wound healing assay demonstrated that 10, 20 or 50 μM shikonin could significantly inhibit the migration of A549 cells compared with the control group (all $P<0.05$; Fig. 1B). The results of the Transwell assays showed that 10, 20 or 50 μM shikonin could significantly inhibit the migration and invasion of A549 cells in a dose-dependent manner compared with the control group (all $P<0.05$; Fig. 1C). Analysis of cell

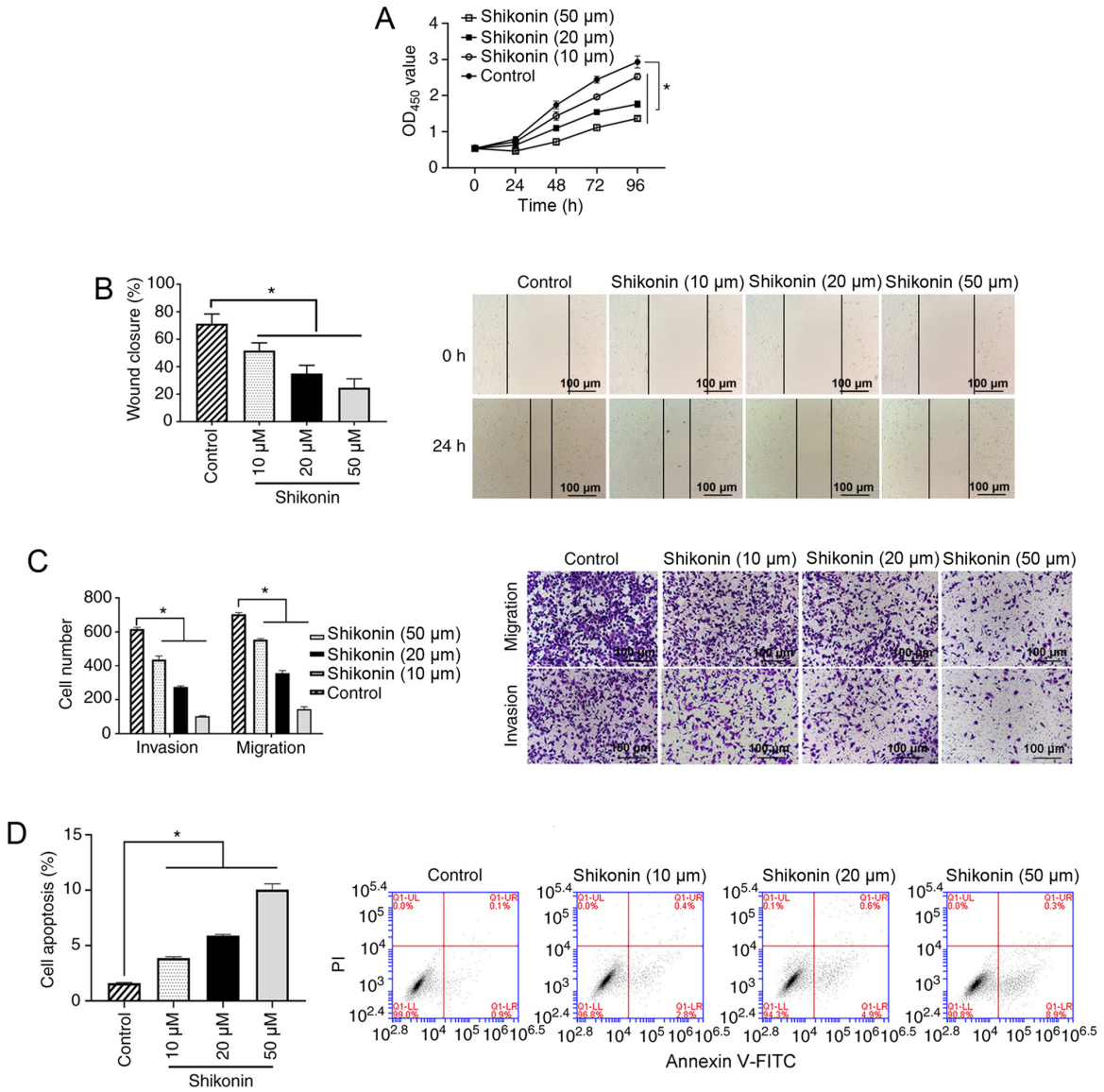

Figure 1. Effect of shikonin on A549 cells. (A) A549 cells were treated with 0, 10, 20 or 50 μM shikonin for 24, 48, 72 or 96 h, and cell proliferation was measured using a Cell Counting Kit-8 assay. *P<0.05. (B) A549 cells were treated with 0, 10, 20 or 50 μM shikonin for 24 h, and the cell migration ability was analyzed using a wound healing assay. Scale bar, 100-μm. (C) A549 cells were treated with 0, 10, 20 or 50 μM shikonin for 24 h, and the cell invasion and migration abilities were measured using Transwell assays. Scale bar, 100-μm. (D) A549 cells were treated with 0, 10, 20 or 50 μM shikonin for 24 h, and cell apoptosis was analyzed using flow cytometry. *P<0.05. OD, optical density; PI, propidium iodide.

apoptosis revealed that 10, 20 or 50 μM shikonin increased the apoptosis of A549 cells compared with the control group (all P<0.05; Fig. 1D).

Shikonin inhibits the Warburg effect in a dose-dependent manner and regulates glucose metabolism in A549 and H446 cells. To further determine the underlying mechanism of the effect of shikonin on lung cancer cells, the effect of shikonin on the Warburg effect and glucose metabolism was investigated in A549 and H446 cells. The results found that 10, 20 or 50 μM shikonin could dose-dependently reduce glucose uptake, lactate and ATP levels in A549 cells compared with the control group (all P<0.05; Fig. 2A-C). Similar results were obtained for H446 cells (all P<0.05; Fig. 2D-F). These data indicated that shikonin may inhibit the Warburg effect and regulate glucose metabolism in lung cancer cells.

PFKFB2 expression is regulated by shikonin. To determine the mechanism through which shikonin inhibits glycolysis and the migration, proliferation and invasion of tumor cells, the protein expression levels of key enzymes involved in aerobic glucose metabolism were analyzed in A549 and H446 cells treated with shikonin. As shown in Fig. 3A, western blotting analysis revealed that shikonin downregulated the protein expression levels of PFKFB2 in a dose-dependent manner in A549 and H446 cells, while the expression levels of the other proteins (PDK1, GLUT1, PGK2, LDHA, PKM2, GLUT3, PDH and p-PDH) were not altered by shikonin treatment. These findings indicated that PFKFB2 expression may be regulated by shikonin. In addition, RT-qPCR analysis was performed to analyze the mRNA expression levels of PFKFB2 in A549 and H446 cells treated with 50 μM shikonin. The results revealed that treatment with shikonin significantly downregulated the mRNA

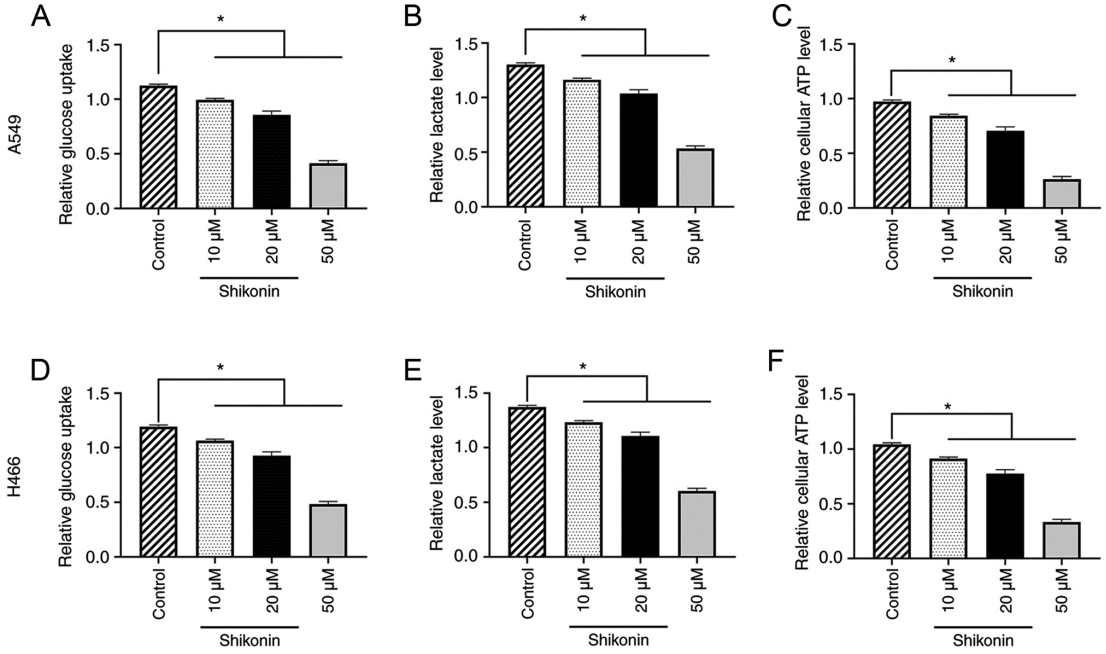

Figure 2. Shikonin inhibits the Warburg effect and regulates the glycometabolism of A549 and H446 cells in a dose-dependent manner. A549 and H446 cells were treated with 0, 10, 20 or 50 μM shikonin for 24 h. Shikonin inhibited (A) glucose uptake, (B) lactate levels and (C) ATP levels in A549 cells. Shikonin inhibited (D) glucose uptake, (E) lactate levels and (F) ATP levels in H446 cells. *P<0.05.

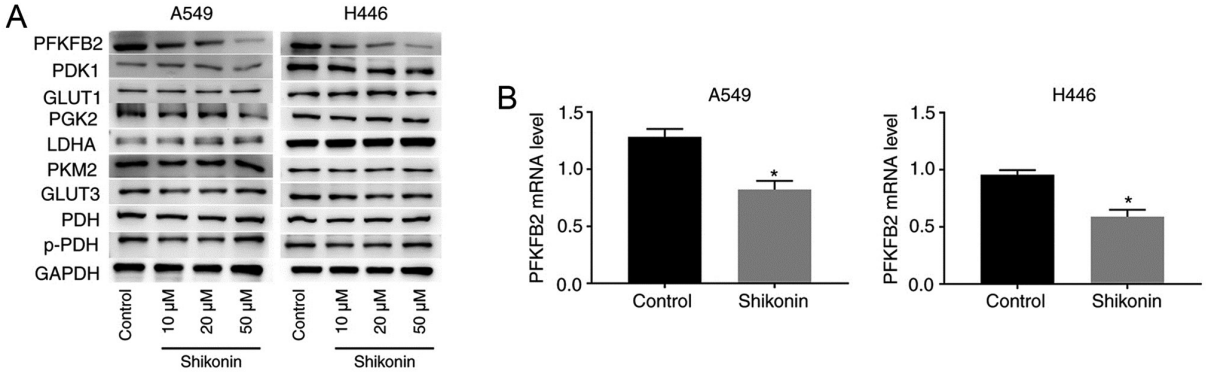

Figure 3. Shikonin downregulates PFKFB2 expression levels in A549 and H446 cells. (A) A549 and H446 cells were incubated with 0, 10, 20 or 50 μM shikonin for 24 h. Expression levels of PFKFB2, PDK1, GLUT1, PGK2, LDHA, PKM2, GLUT3, PDH and p-PDH in A549 and H446 cells were analyzed using western blotting. (B) mRNA expression levels of PFKFB2 were analyzed using reverse transcription-quantitative PCR in A549 and H446 cells treated with 50 μM shikonin. *P<0.05 vs. control. PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; PDK1, pyruvate dehydrogenase kinase 1; GLUT, glucose transporter; PGK2, phosphoglycerate kinase 2; LDHA, lactate dehydrogenase A; PKM2, pyruvate kinase M1/2; PDH, pyruvate dehydrogenase phosphatase catalytic subunit 1; p-, phosphorylated.

expression levels of PFKFB2 compared with the control group (both P<0.05; Fig. 3B).

Expression levels of PFKFB2 are upregulated in lung cancer tissues, and the overexpression of PFKFB2 promotes proliferation, migration and the Warburg effect in lung cancer cells. To investigate the role of PFKFB2, the expression levels of PFKFB2 were first analyzed in 20 NSCLC and adjacent normal tissues. The results demonstrated that PFKFB2 expression levels were significantly upregulated in NSCLC tissues compared with the adjacent normal tissues (P<0.05; Fig. 4A). The expression levels of PFKFB2 were also significantly upregulated in lung cancer cells (A549 and H446) compared with Beas-2B normal lung epithelial cells (both P<0.05; Fig. 4B). In addition, RT-qPCR analysis found that PFKFB2 mRNA expression levels were significantly downregulated in A549 and H446 cells transfected with siRNA-PFKFB2 compared with siRNA-NC (both P<0.05) and significantly upregulated in A549 and H446 cells transfected with pcDNA3.1-PFKFB2 compared with pcDNA3.1-NC (both P<0.05) (Fig. 4C), which indicated that the cell transfections were successful. The results of the CCK-8 and colony formation assays revealed that the overexpression of PFKFB2 significantly increased the proliferation of A549 and H446 cells compared with the pcDNA3.1-NC group, while the knockdown of PFKFB2 significantly inhibited the proliferation of A549 and H446 cells compared with the siRNA-NC group (all P<0.05; Fig. 4D and E). The results of the wound healing assay demonstrated that the overexpression of PFKFB2 significantly promoted the migration of A549 and

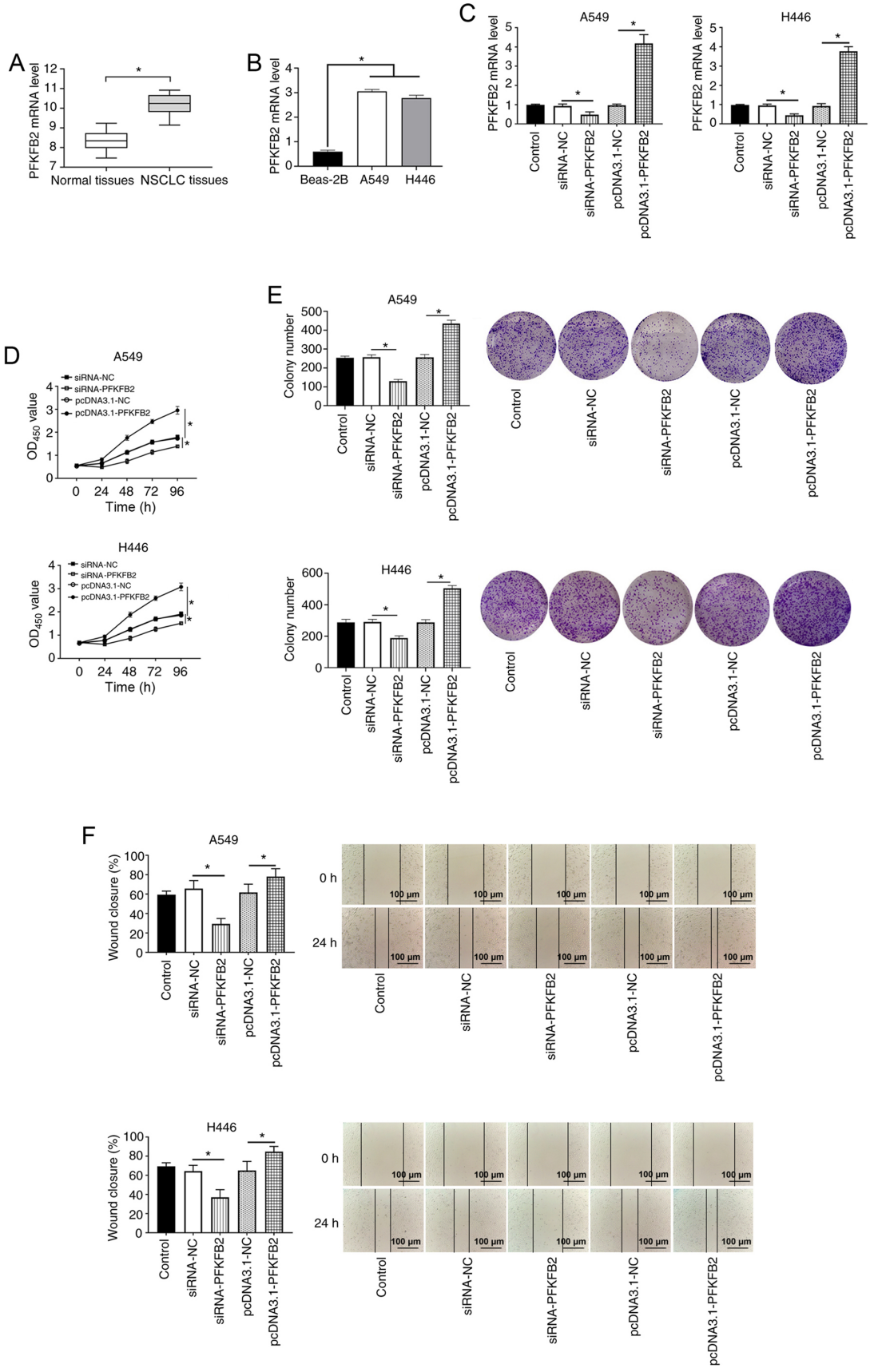

Figure 4. PFKFB2 expression levels are upregulated in lung cancer, and the overexpression of PFKFB2 promotes proliferation, migration and the Warburg effect in A549 and H446 cells. (A) mRNA expression levels of PFKFB2 in NSCLC and adjacent normal tissues were analyzed using RT-qPCR. (B) mRNA expression levels of PFKFB2 were analyzed in Beas-2B, A549 and H446 cells using RT-qPCR. (C) mRNA expression levels of PFKFB2 in A549 and H446 cells following transfections were analyzed using RT-qPCR. (D) Proliferation of A549 and H446 cells was detected using a Cell Counting Kit-8 assay. (E) Colony formation assay was used to measure the colony number in A549 and H446 cells. (F) Migratory ability of A549 and H446 cells was analyzed using a wound healing assay. Scale bar, 100-μm. *P<0.05. PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; RT-qPCR, reverse transcription-quantitative PCR; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; NC, negative control; OD, optical density.

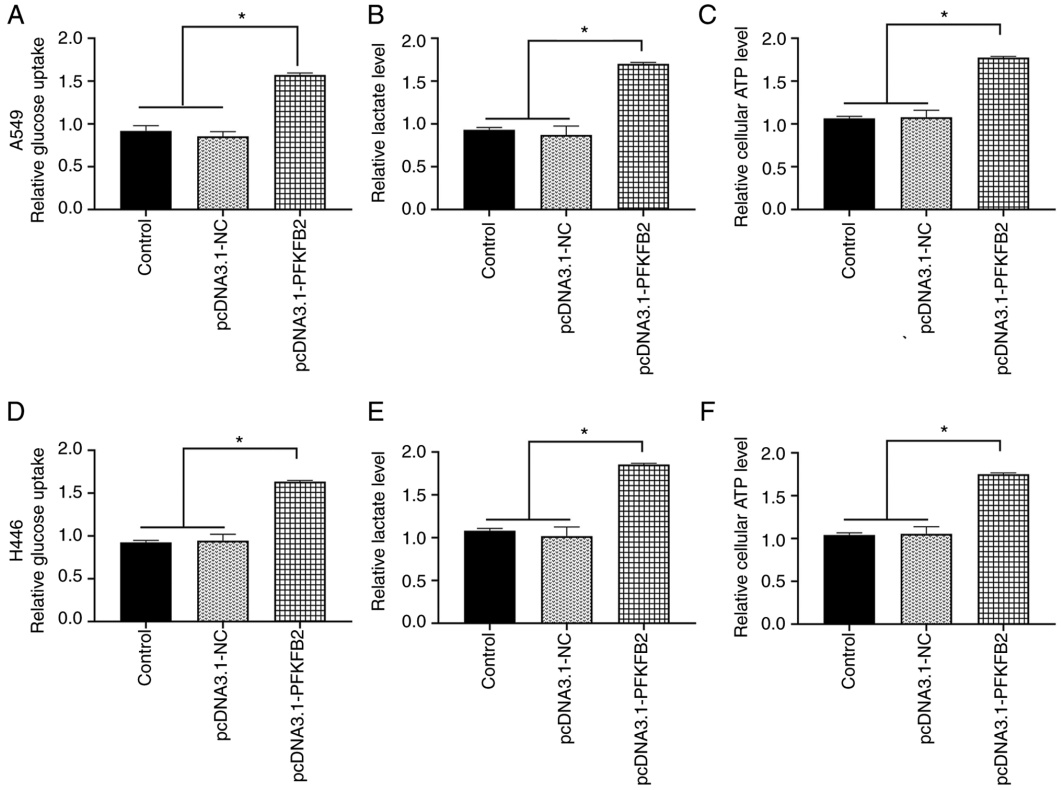

Figure 5. Overexpression of PFKFB2 accelerates the Warburg effect in lung cancer cells. (A) Overexpression of PFKFB2 in A549 cells increased (A) glucose uptake, (B) lactate levels and (C) ATP levels. Overexpression of PFKFB2 in H446 cells increased (D) glucose uptake, (E) lactate levels and (F) ATP levels. *P<0.05. PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; NC, negative control.

H446 cells compared with the pcDNA3.1-NC group, while the knockdown of PFKFB2 significantly inhibited the migration of A549 and H446 cells compared with the siRNA-NC group (all P<0.05; Fig. 4F).

Subsequently, whether PFKFB2 regulated the glycometabolic activity in A549 and H446 cells was investigated. The overexpression of PFKFB2 in A549 cells significantly increased glucose uptake, lactate and ATP levels compared with the control and pcDNA3.1-NC groups (all P<0.05; Fig. 5A-C). Similar results were obtained for H446 cells (all P<0.05; Fig. 5D-F).

PFKFB2 participates in shikonin-induced glycolysis, apoptosis and migration in lung cancer cells. Whether PFKFB2 participated in shikonin-induced aerobic glycolysis, cell apoptosis and migration was subsequently investigated. As shown in Fig. 6A-D, the knockdown of PFKFB2 or treatment of shikonin significantly inhibited glucose uptake, lactic acid production, ATP levels and migration in A549 cells compared with the control group (all P<0.05). Notably, there were no significant differences in glucose uptake, lactate levels, ATP production and migration between A549 cells with knocked down PFKFB2 or treated with shikonin and the knockdown of PFKFB2 in A549 cells treated with shikonin, suggesting that shikonin-mediated glycolysis and migration may be regulated by PFKFB2 in lung cancer cells. In addition, the knockdown of PFKFB2 or treatment of shikonin significantly increased cell apoptosis compared with the control group (P<0.05), while the knockdown of PFKFB2 in shikonin-treated cells did not significantly alter the apoptosis compared with treatment of shikonin or the knockdown of PFKFB2 (Fig. 6E).

Discussion

Lung cancer has become the most commonly diagnosed cancer worldwide, accounting for 11.6% of total cancers diagnosed (1). Currently, due to the lack of effective tumor molecular markers available to guide clinical diagnosis and treatment, the rate of successful treatment in patients with lung cancer remains poor; therefore, it remains an urgent priority to develop novel therapeutic drugs (28). Traditional Chinese medicine has long been used for the treatment of lung cancer and has demonstrated considerable clinical value, such as inhibiting metastasis, enhancing the host immune response and reducing the adverse effects of chemotherapy (29-31). The results of the present study revealed that shikonin inhibited cell proliferation, invasion, and migration, reduced glucose uptake, lactate and ATP levels, increased apoptosis and downregulated the expression levels of PFKFB2 in lung cancer cells.

Shikonin, a natural naphthoquinone compound, is extracted and separated from the dry roots of *Lithospermum erythrorhizon* and has been found to play roles in numerous biological activities, including exerting anti-inflammatory, antiviral and antitumor effects, immune system regulation, promoting wound healing and protecting against multi-drug resistance (32-34). Among these biological activities, the anticancer effect of shikonin has been well reported. For example, a previous study reported that shikonin reduced the tumor diameter by 25% and increased the 1-year survival rate by 47.3% in patients with lung cancer (35). In addition, Zhang *et al* (36) demonstrated that shikonin inhibited the migration and invasion of thyroid cancer cells. Guo *et al* (37) also found that shikonin

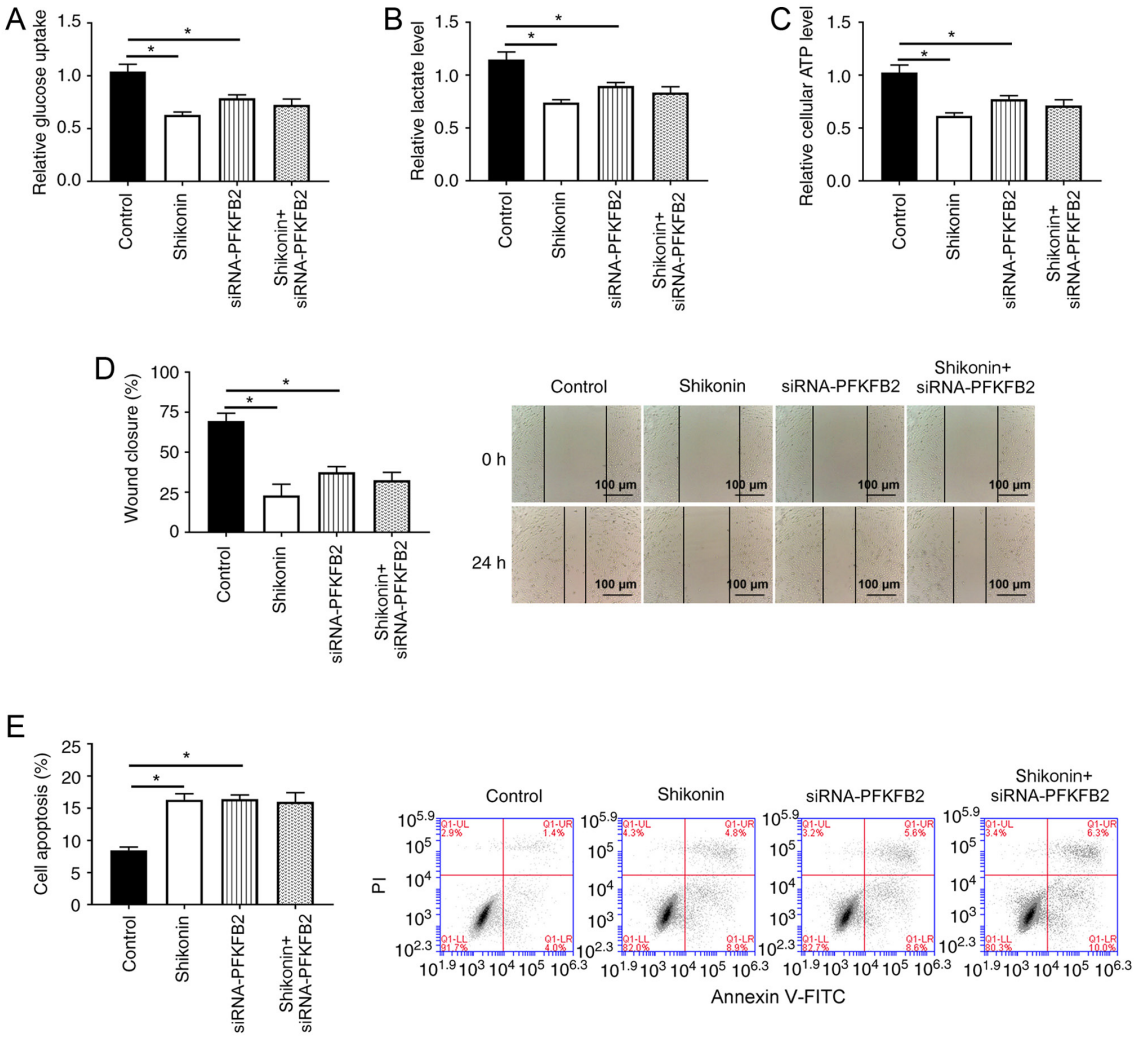

Figure 6. PFKFB2 participates in the shikonin-induced effects on glycometabolism, apoptosis and migration. A549 cells were incubated with 50 μM shikonin for 24 h. (A) Glucose uptake, (B) lactate levels and (C) ATP levels were analyzed in A549 cells. (D) Cell migration ability of A549 cells was analyzed using a wound healing assay. Scale bar, 100-μm. (E) Flow cytometry was used to analyze the apoptosis of A549 cells. *P<0.05. PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; siRNA, small interfering RNA; NC, negative control; NS, non-significant; PI, propidium iodide.

inhibited cell proliferation and induced apoptosis in glioma. Wang *et al* (38) reported that shikonin inhibited the proliferation and promoted apoptosis of breast cancer cells. The results of the present study demonstrated that shikonin could inhibit proliferation, migration and invasion, and increase the apoptosis of A549 cells in a dose-dependent manner, which were consistent with the findings of previous studies (36-38). However, the results of the present study showed a lower apoptosis rate, which is different to the results obtained in the aforementioned studies. Taken together, these results indicated that shikonin might exert antitumor effects in lung cancer.

Cancer cells do not use mitochondrial oxidative phosphorylation, even in the presence of oxygen, but instead use aerobic glycolysis, a phenomenon termed the Warburg effect (39,40). The Warburg effect has been reported to permit tumor cells to overcome metabolic stress and promote energy replenishment, which is vital for the proliferation and survival of cancer cells (41,42). In addition, the Warburg effect was suggested to impact the microenvironment and promote chemoresistance, angiogenesis and metastasis (43,44). Hence, investigating the Warburg effect may be crucial for determining the underlying mechanisms of the development and progression of NSCLC. The results of the present study found that shikonin could dose-dependently reduce glucose uptake, lactate and ATP levels in lung cancer cells. These data indicated that shikonin may inhibit the malignant evolution of lung cancer by inhibiting the Warburg effect.

PFKFB is an important regulator of glycolysis in cancer, and it was previously reported that the PFKFB family served an important role in the development and progression of numerous tumor types, such as lung, pancreatic and gastric cancer (45-47). Previous studies demonstrated that the expression levels of PFKFB2 were upregulated in melanoma, osteosarcoma and thyroid carcinoma (48-50). Furthermore, Liu *et al* (51) reported that microRNA-613 inhibited cell proliferation, invasion and the Warburg effect by regulating PFKFB2 expression in gastric cancer. Ozcan *et al* (22) also found that PFKFB2 inhibited glycolysis and proliferation in pancreatic cancer cells. The present study results showed that the expression levels of PFKFB2 were significantly upregulated in human NSCLC tissues and lung cancer cell lines, and that the overexpression of PFKFB2 increased cell proliferation, migration, glucose uptake,

lactate and ATP levels in lung cancer cells. In addition, shikonin treatment downregulated the expression levels of PFKFB2. Furthermore, there were no significant differences in glucose uptake, lactate levels, ATP production, apoptosis and migration between the knockdown of PFKFB2 or treatment of shikonin and the knockdown of PFKFB2 in cells treated with shikonin. These results indicated that PFKFB2 may play an important role in the effects of shikonin treatment in lung cancer.

To the best of our knowledge, the present study was the first to demonstrate that shikonin inhibited lung cancer cell migration and the Warburg effect by regulating PFKFB2 expression, which may provide a novel insight into the mechanism underlying the anticancer effects of shikonin. However, it is important to note the limitations of the present study. For example, the proportion of apoptotic cells was too low to ascertain that shikonin induced apoptosis. In addition, the current study did not block the Warburg effect to further validate the effect of shikonin on cell proliferation, apoptosis and migration. The above limitations may weaken the conclusions of the present study; therefore, koningic acid, an irreversible and selective inhibitor of GAPDH (a rate-controlling glycolytic enzyme during the Warburg effect) will be used in future studies to block the Warburg effect to further validate the mechanism of shikonin in lung cancer (52).

In conclusion, the results of the present study suggested that shikonin inhibited the metabolism, migration and proliferation of lung cancer cells. In lung cancer tissues, PFKFB2 expression levels were upregulated and promoted the progression of lung cancer. In addition, PFKFB2 was found to participate in the shikonin-induced effects on the glycolysis and migration of lung cancer cells. These data suggested that treatments targeting PFKFB2 may benefit patients with lung cancer. In addition, shikonin may represent a potential novel compound for the treatment of lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HZ conceived and designed the study; LS, ZL and XS performed the experiments; TW, YL and YZ analyzed and interpreted the data; and all authors wrote the manuscript. All authors read and approved the final manuscript. LS and HZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study protocols were approved by the Ethics Committee of The Affiliated Hospital of Qingdao University, and written informed consent was obtained from all patients prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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