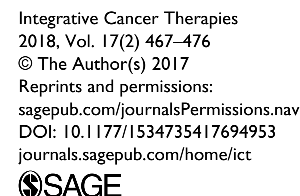
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Molecular Mechanisms Underlying the Inhibitory Effects of Qingzaojiufei Decoction on Tumor Growth in Lewis Lung Carcinoma

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Abstract

Objective: Qingzaojiufei decoction (QD) is an empirical herbal formula from traditional Chinese medicine that is used for the treatment of lung-related diseases. However, the effect of QD on the growth of lung tumor cells has not been investigated. The aim of this study was to examine the antitumor activity of QD in Lewis lung carcinomas (LLC) in vivo and in vitro, and to elucidate the underlying mechanisms. **Methods:** The LLC cells were used to assess the antitumor activity of QD by Cell Counting Kit-8 assay in vitro. In vivo, mice were randomly assigned to 5 groups (n = 10/group): the model control (MC) group was intragastrically administered physiological saline (0.9% NaCl) twice daily from day 2 after tumor implantation for 2 weeks. The QD groups were intragastrically administered QD twice daily from 2 weeks before to 2 weeks after tumor implantation for 4 weeks. The mRNA levels were detected by quantitative polymerase chain reaction, the proteins expression was determined by immunohistochemistry or western blotting. **Results:** Compared with the model group, QD showed inhibition of proliferation of LLC cells and reductions in tumor weight and proliferating cell nuclear antigen protein expression. Furthermore, QD up-regulated p53 mRNA expression, and downregulated c-myc and Bcl-2 mRNA expression, while MMP-9, VEGF, and VEGFR protein expression was suppressed. Phosphorylated ERK1/2 levels were also reduced by QD in a dose-dependent manner. **Conclusion:** Our findings suggest that QD inhibited lung tumor growth and proliferation, by activation of tumor suppressor genes, inactivation of oncogenes, suppressing the potential for invasion and metastasis, and attenuating angiogenesis. The ERK/VEGF/MMPs signaling pathways may play an important role in QD-induced inhibition of malignant tumor cell proliferation.

Keywords

Qingzaojiufei decoction, lung cancer, tumor growth, Lewis lung carcinoma

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Introduction

Lung cancer represents one of the leading causes of death in men and women, both in the Chinese population and worldwide. Furthermore, lung cancer has a higher mortality rate than other types of cancer.[1] Despite advances in therapeutic approaches, the prognosis for patients remains unchanged. The overall 5-year survival rate for lung cancer has risen only 4% over the past 4 decades (from 12% to 16%), resulting in poor quality of life and survival outcomes.[2] Because conventional treatments, including surgery, radiotherapy, and chemotherapy may suppress the immune system, other promising strategies such as traditional Chinese medicine (TCM) have been investigated in patients with lung cancer.[3-6] Furthermore, TCM may be prescribed as a complementary medicine for cancer therapy in Western populations. The use of TCM, which has a long history, has been reported to alleviate clinical symptoms and treatment-related complications, improve quality of life, and reduce the side effects of conventional treatment in several cancer types.[7] Owing to the increased emphasis in modern medicine on concepts such as disease

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prevention and improving physical status, the advantages of TCM have become more obvious in the context of these therapeutic goals. Thus, investigation of the anticancer activity of TCM and elucidation of the underlying mechanisms is urgently required.

The majority of available cancer treatments are targeted toward regulation of tumor-related gene expression, inhibition of tumor invasion and metastasis, and suppression of angiogenesis. Because tumor suppressor genes and oncogenes are involved in physiological processes that regulate both normal cellular homeostasis and cancer cell proliferation,[8] many treatment strategies rely on activation of tumor suppressor genes (eg, p53) and inactivation of oncogenes (eg, c-myc and Bcl-2) to induce apoptosis and inhibit cell proliferation.[9,10]

During the development of lung cancer, invasion and metastasis occurs via detachment, proteolysis, penetration, and intravasation, followed by invasion of cancer cells into new tissue.[11] Matrix metalloproteinases (MMPs), a family of Zn-dependent endopeptidases, degrade an array of extracellular matrix (ECM) proteins, and participate in tumor progression by facilitating growth, angiogenesis, invasion, and migration.[12,13] This suggests that MMP inhibitors may be effective for the treatment of cancer.[14,15]

The induction of angiogenesis, a hallmark of cancer, plays an essential role in the process of primary tumor growth and propagation. Studies have demonstrated that the vascular endothelial growth factor/receptor (VEGF/VEGFR) system is essential and specific for angiogenic processes.[16] Several anticancer drugs target tumor angiogenesis by interfering with the binding of VEGF to VEGFR, which prevents VEGFR activation and suppresses tumor growth.[17,18]

Qingzaojiufei decoction (QD) consists of a complex mixture of natural herbs, minerals and/or animal products, and each component contains various chemical compounds. It is an empirical formula based on the principles of TCM and is used to treat lung-related disease.[19-21] However, the effect of QD on lung tumor growth and the underlying mechanisms of action have not been fully elucidated.

The aim of the present study was to explore the effect of QD on lung tumor growth and proliferation and to investigate the mechanisms of action in a Lewis lung carcinoma (LLC) cells and mouse xenograft model.

Materials and Methods

Reagents and Antibodies

Cyclophosphamide (CTX) was purchased from the Jiangsu Hengrui Medicine Co, Ltd. (Lianyungang, China). ERK pathway inhibitor U0126 were purchased from Sigma-Aldrich (St Louis, MO, USA). Proliferating cell nuclear antigen (PCNA), MMP-9, VEGR, and β-actin antibodies were acquired from Proteintech Group (Chicago, IL, USA). Extracellular regulated protein kinase (ERK) and phospho(p)-ERK antibodies were purchased from Biogot Technology (Nanjing, China). The VEGFR antibody was obtained from Affinity Biosciences (Cincinnati, OH, USA). All other chemicals and reagents were of analytical grade and obtained from local chemical companies.

Composition and Preparation of QD

QD is composed of frost mulberry leaves (9 g), plaster stone (12 g), baked licorice (3 g), *Codonopsis pilosula* root (12 g), donkey hide gelatin (9 g), dwarf lilyturf tuber (10 g), bitter almond (9 g), and loquat leaves (9 g), all of which were purchased from the Pharmacies of Jiangxi Provincial Hospital of Chinese Medicine (Nanchang, China). The QD components were soaked in 10-fold distilled water for 60 minutes, the aqueous mixture was heated to 100°C for 40 minutes, and then the decoction was filtered twice. The filtrates of the raw ingredients of QD were mixed and concentrated to a volume of 73 mL by heating in a water bath at 60°C, and then stored at 4°C until analysis.

Animals and Cells

Pathogen-free 8-week-old C57BL/6 male mice (n = 50; 20 ± 2 g) were obtained from Suzhou Industrial Park, Matt Ireland Technology Co, Ltd., (Suzhou, China; Certificate of Conformity: SCXK (Su) 2014-0007) and were allowed to adapt to the laboratory for 1 week before experiments. The animals were maintained in a pathogen-free facility (22°C ± 2°C, 55% ± 5% humidity) and a 12-hour light/dark cycle with lights on from 07:00 to 19:00 hours. Food and water were provided ad libitum. All animal procedures were performed according to the Animal Care Guidelines issued by the Ministry of Science and Technology of China. The protocols were approved by the Animal Care Committee of Jiangxi Provincial Hospital of Traditional Chinese Medicine.

LLC cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator.

Preparation of Medicated Serum

Medicated mouse serum was prepared according to previous studies with some modification.[22,23] Briefly, the mice were divided into QD-treated (7.6 g/kg QD, n = 10), (QD + CTX)-treated (7.6 g/kg QD, 0.05 g/kg CTX, n = 10), CTX-treated (0.05 g/kg, n = 10), and control groups (7.6 g/kg, 0.9% NaCl, n = 10). Furthermore, mice in the control and QD groups received intragastric doses of the designated

treatment (0.9% NaCl or QD) via gavage twice daily for 3 days. CTX was administered intraperitoneally twice daily for 3 days. On the fourth day, mice were administered required drugs and then 1 hour later, blood was collected from the eyeball. Serum was then isolated from each blood sample, and heat inactivation was conducted at 56°C for 30 minutes. After filtration and repackaging, these "medicated serum" samples were stored at −80°C for future use. The dose of QD (7.6 g/kg) was calculated in accordance with guidelines for correlating dose equivalents between humans and laboratory animals based on body surface area ratios.[24]

Cell Proliferation Assay

Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8) assay kit (Zoman Biotechnology, Beijing, China) according to the manufacturer's protocol. Cells were plated at 1×10^5 cells per well in 96-well plates for 12 hours and then treated with mice medicated serum (5%, 10%, or 20% v/v) for 24 hours. CCK-8 (10 μL) was added to each well and incubated for 3 hours. A 96-well microliter plate reader (Molecular Devices, Sunnyvale, CA, USA) was used to determine the absorbance of CCK-8 at 450 nm.

Mouse Xenograft Models and Treatment Protocols

Solid-type LLC was induced by subcutaneous transplantation of 5×10^6 cells (0.2 mL) into the axilla of C57BL/6 mice (n = 50). The mice were randomly assigned to 5 groups (n = 10/group): model control (MC), QD low concentration (QD-L, 3.8 g/kg), QD mid concentration (QD-M 7.6 g/kg), QD high concentration (QD-H 15.2 g/kg), and CTX (0.05 g/kg). The formula for calculating the difference between humans and mice according to body surface area is: mouse dose (g/kg) = human dose (g/kg) × 37/3.[25] The MC group was intragastrically administered physiological saline (0.9% NaCl) twice daily from day 2 after implantation for 2 weeks. The 3 QD groups were intragastrically administered QD (3.8, 7.6, and 15.2 g/kg) twice daily from 2 weeks before to 2 weeks after implantation for a total of 4 weeks. The CTX group was intraperitoneally administered CTX (0.05 g/kg) twice daily from day 2 after implantation for 2 weeks.

Tumor Weight and Tumor Growth Inhibition Ratio Calculation

After treatment, all mice were sacrificed by cervical dislocation and tumors were extracted to calculate the tumor weight and inhibition ratio (IR). The IR was calculated as: [(Average tumor weight in the MC group − average tumor weight in the treatment group)/average tumor weight in the MC group] × 100%. Tumor tissue was stored at −80°C until further analysis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor tissue sections (4-5 μm) were deparaffinized and rehydrated. The specimens were immersed in 0.01 M citric acid buffer solution at pH 6.0 (LSI Medience Co, Tokyo, Japan) and autoclaved at 121°C for 8 minutes. Endogenous peroxidase was inhibited by incubation with freshly prepared 3% hydrogen peroxide containing 0.1% sodium azide for 10 min at room temperature. Nonspecific staining was blocked with normal goat 10% serum (Sigma, St Louis, MO, USA) for 30 minutes at room temperature. The sections were then incubated with VEGF and VEGFR antibodies at a dilution of 1:500 overnight at 4°C. After incubation with primary antibody, tissue sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA, USA) at a dilution of 1:1000 for 50 minutes at room temperature. The slides were then washed with phosphate buffered saline (PBS) and developed with DAB (3,3′-diaminobenzidine) substrate (Sigma, St Louis, MO, USA) for 5 minutes at room temperature. Tissue sections were counterstained with hematoxylin, and mounted using Permount (Fisher Scientific, Pittsburgh, PA, USA). As negative controls, the primary antibody was substituted for rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or PBS. The sections were examined using a Leica DM 4000B microscope (Leica Microsystems, IL, Germany) and quantitated by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA).

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction

The frozen whole tumors were macerated in liquid nitrogen and 30 to 50 mg of the resultant powder was used to purify total RNA with TRIzol (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were assessed using a Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA integrity (RIN) was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All RNAs had an absorption 260 nm/280 nm ratio >2.0 and a RIN >8.0. Approximately 500 ng of total RNA was reverse transcribed to cDNA using PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). cDNA was subjected to qPCR analysis using SYBR Green I dye reagents (TaKaRa) with an ABI StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Cycling conditions for SYBR Green I PCR were set at 95°C for 2 minutes for the first cycle, 10 seconds at 95°C, followed by 30 seconds at 60°C for the remaining 40 cycles. This was followed by 40 consecutive cycles of 10 s each, starting at 55°C with an incremental temperature increase of 0.5°C per cycle to determine the melt curve, as a method to validate the PCR products. The specific primers were designed using Primer

Table 1. Primers of detected genes.

Genes	NCBI accession No.	Temperature (°C)	Primers
c-myc	NM_010849	60	TCCATCCTATGTTGCGGTCG TGAAGGTCTCGTCGTCAGGA
Bcl-2	NM_009741.5	60	ATAACGGAGGCTGGGTAGGT GTCAGGGGAGCAAAGCTACA
p53	NM_001127233	60	GACCAAGAAGGGCCAGTCTAC GAGTGGATCCTGGGGATTGT
β-actin	NM_007393	60	TGAGCTGCGTTTTACACCCT GCCTTCACCGTTCCAGTTTT

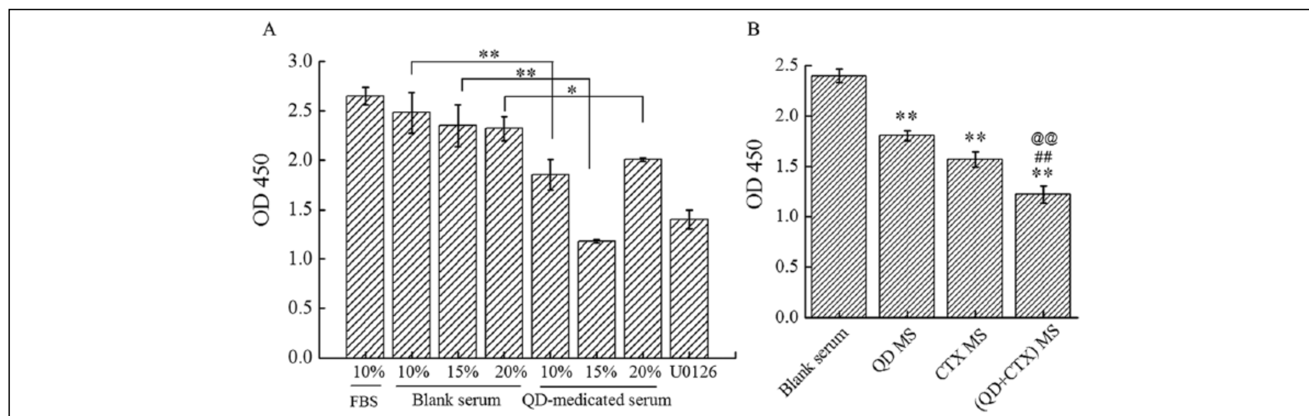

Figure 1. Effect of QD on LLC cell proliferation. Cells were plated at 1 × 10^5 cells per well in 96-well plates for 12 hours and then treated with QD-medicated serum (5%, 10%, or 20%, v/v) for 24 hours. The treatment with FBS (10%), blank serum (5%, 10%, or 20%, v/v) and U0126 (50 μM) served as controls. Cell proliferation was determined by CCK-8 assay. **P* < .05 and ***P* < .01 versus corresponding blank serum groups. ##*P* < .01 versus QD-medicated serum group (QD MS, 15%). @@*P* < .01 versus CTX-medicated serum group (CTX MS, 15%). QD, Qingzaojiufei decoction' LLC, Lewis lung carcinoma; FBS, fetal bovine serum; CTX, cyclophosphamide; CCK-8, Cell Counting Kit–8.

Premier 5.0 software (Palo Alto, CA, USA). Gene expression was analyzed by relative quantification using the $2^{-\Delta\Delta Ct}$ method with normalization against β-actin. The primer sequences for qPCR are shown in Table 1. All primers were obtained from Sangon Biotech (Shanghai, China).

Western Blotting

Proteins extracted from the lung carcinoma cells (LCC) xenograft tumors were analyzed by western blotting. Equal amounts of protein (about 50 μg) were subjected to SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophiresis) and transferred to a PVDF ()polyvinylidine difluoride membrane. Membranes were blocked in 5% skimmed milk or bovine serum albumin containing TBST buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 1% Tween 20) for 2 hours at room temperature, then incubated overnight at 4°C with the following primary antibodies: anti-PCNA (1:1000), anti-ERK (1:1000), anti-pERK (1:1000), and anti-β-actin (1:2000). The membranes were then incubated with HRP-conjugated secondary anti-mouse (1:5000, Kangwei Biotechnology, Beijing, China) or anti-rabbit antibodies (1:5000, Kangwei Biotechnology, Beijing, China). Protein bands on the membrane were visualized by chemiluminescence (Thermo Scientific, Rockford, IL, USA) and quantitated using Quantity One Software (Bio-Rad, Richmond, CA, USA).

Statistical Analysis

All data are expressed as the means ± SD. Statistical analysis was performed using SPSS 19.0 software (IB Corp, Armonk, NY, USA). Data from at least 3 experiments performed in duplicate were subjected to the Student's *t* test or unpaired 1-way analysis of variance with Tukey-Kramer post hoc analysis (0.05). $P < .05$ was considered to denote a significant difference between groups.

Results

QD Inhibited LLC Proliferation and Growth

The effect of QD on cell proliferation was assessed by CCK-8 assay in vitro. After exposure of LLC cells to QD-medicated serum (10%, 15%, or 20%) for 24 hours, cell proliferation was inhibited significantly ($P < .05$) when compared with corresponding blank serum groups (Figure 1A). As a positive control, 15% CTX -medicated serum also

Table 2. Effect of QD on Tumor Weight and Inhibition Rate in Lewis Lung Cancer–Bearing Mice.[a].

Group	Doses (g/kg)	Body Weight (g)	Tumor Weight (g)	Inhibition Rate (%)
MC	–	26.18 ± 1.86	2.50 ± 0.75	—
CTX	0.05	3.17 ± 1.61**	0.4 5± 0.17**	82.1
QD-L	3.8	26.78 ± 2.02##	2.05 ± 0.80*##	18.1
QD-M	7.6	25.37 ± 1.45##	1.37 ± 0.94**##	45.2
QD-H	15.2	24.57 ± 1.20*	1.30 ± 0.53**#	48.2

Abbreviations: MC, model control; QD-L, Qingzaojiufei decoction low, QD-M, Qingzaojiufei decoction mid, D-H: Qingzaojiufei decoction high, CTX, cyclophosphamide.

[a]The MC group was intragastrically administered physiological saline (0.9% NaCl) twice daily from day 2 after tumor implantation for 2 weeks. The QD groups were intragastrically administered QD (3.8, 7.6, or 15.2 g/kg) twice daily from 2 weeks before to 2 weeks after tumor implantation for 4 weeks. The CTX group was intraperitoneally administered CTX (0.05 g/kg) twice daily from day 2 after tumor implantation for 2 weeks. *$P < .05$ and **$P < .01$ versus MC. #$P < .05$ and ##$P < .01$ versus CTX.

suppressed the LLC cell proliferation. Furthermore, the serum from mice treated with the combination of QD and CTX dramatically inhibited cell proliferation compared with QD or CTX serum alone (Figure 1B, $P < .01$).

The effect of QD on lung cancer growth was determined by measuring tumor weight in LLC-bearing mice after treatment with QD-L (3.8 g/kg), QD-M (7.6 g/kg), or QD-H (15.2 g/kg) for 4 weeks. As displayed in Table 2, all 3 dose levels of QD inhibited tumor growth in a dose-dependent manner (18.1%, 45.2%, and 48.2% for QD-L, QD-M, and QD-H, respectively) compared with the MC group ($P < .05$). LLC-bearing mice treated with CTX served as a positive control for inhibition of tumor growth. CTX (0.05 g/kg) inhibited tumor growth by 82.1% compared with the MC group ($P < 0.01$).

To confirm the ability of QD to attenuate lung cancer cell proliferation, protein expression of PCNA, a marker of cell proliferation and tumorigenesis, was assessed by western blot. In the QD-M and QD-H groups, PCNA levels were reduced by 38.0% and 49.3%, respectively, compared with the MC group ($P < .05$; Figure 2).

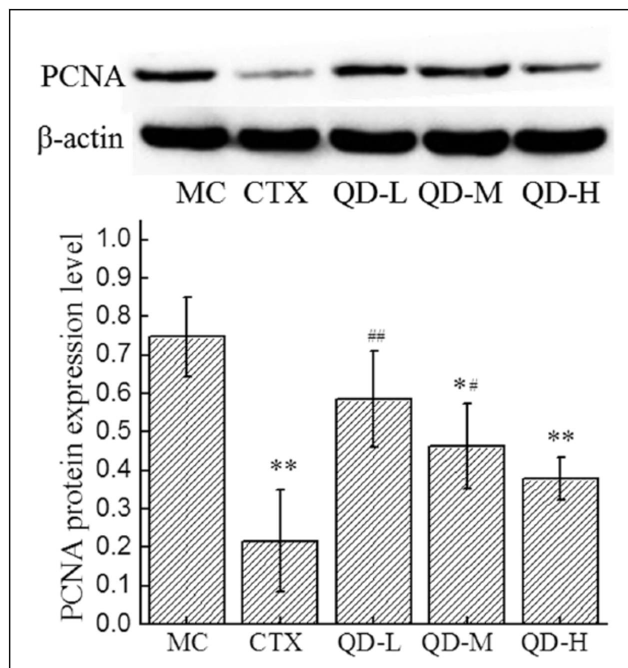

Figure 2. Effect of QD on PCNA protein expression. The MC group was intragastrically administered physiological saline (0.9% NaCl) twice daily from day 2 after tumor implantation for 2 weeks. The QD-L, -M, and -H groups were intragastrically administered QD (3.8, 7.6, or 15.2 g/kg, respectively) twice daily from 2 weeks after tumor implantation for 4 weeks. The CTX group was intraperitoneally administered CTX (0.05 g/kg, a positive control chemotherapy drug for suppressing tumor growth) twice daily from day 2 after tumor implantation for 2 weeks. Equal quantities of total protein (about 50 μg) from LLC mouse tumors were prepared for western blot analysis of PCNA, and β-actin was used as a loading control. Representative blots are shown from one of three independent experiments, and the statistical results are presented in histograms. *$P < 0.05$ and **$P < 0.01$ versus. MC. #$P < .05$ and ##$P < .01$ vs. CTX. QD, Qingzaojiufei decoction; PCNA, proliferating cell nuclear antigen; LLC, Lewis lung carcinoma; CTX, cyclophosphamide.

QD Upregulated p53 Expression and Downregulated c-myc and Blc-2

To explore the underlying mechanisms by which QD inhibited lung cancer growth, mRNA expression of the tumor suppressor gene p53 and the oncogenes c-myc and Bcl-2 was determined by qPCR analysis. As shown in Figure 3, p53 expression was 1.43-fold higher in the QD-H group than the MC group ($P < .01$). However, mRNA expression of c-myc and Bcl-2 were reduced by QD in a dose-dependent manner. In the QD-M and QD-H groups, c-myc mRNA expression was reduced by 52.0% and 71.3% ($P < .01$), respectively, while Bcl-2 mRNA expression was reduced by 53.1% and 69.9% ($P < .01$), respectively.

QD Impaired MMP-9 Expression

To evaluate whether QD was able to reduce the invasive and migratory potential of tumor cells, the effects of QD on MMP expression were investigated. As shown in Figure 4, the QD-M and QD-H groups displayed inhibition of MMP-9

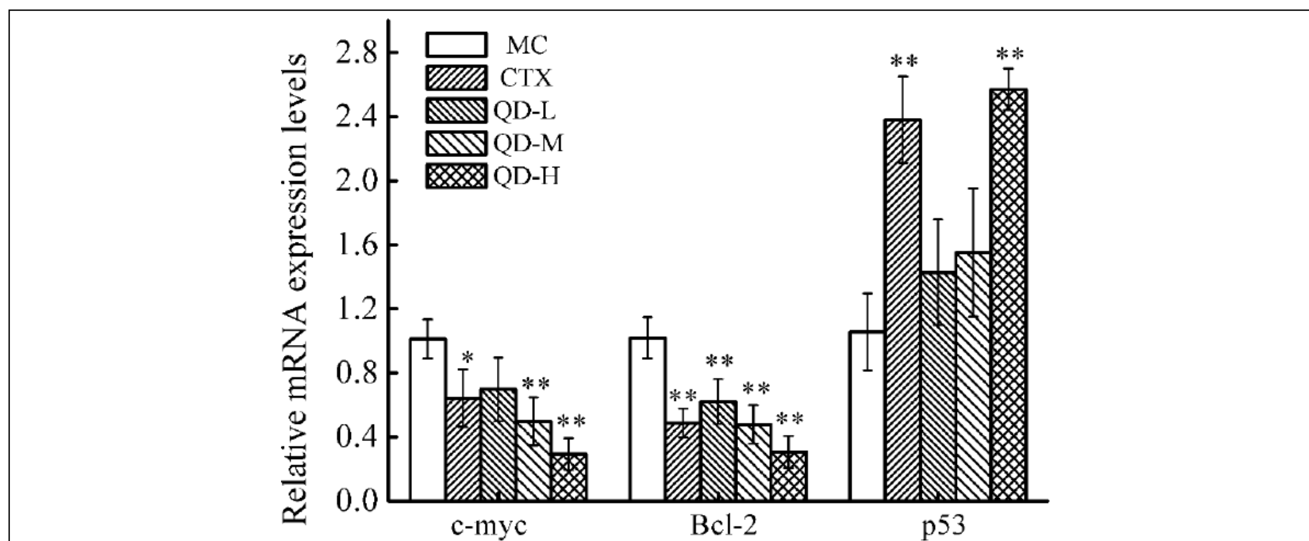

Figure 3. Effect of QD on mRNA expression of tumor suppressor genes and oncogenes. mRNA expression of p53, c-myc, and Bcl-2 were measured by qPCR, and normalized against β-actin after administration of tumor-inoculated mice with physiological saline (MC group), QD-L, -M or -H (3.8, 7.6, or 15.2 g/kg, respectively) or CTX (0.05 g/kg). All experiments were repeated at least 3 times. Data are presented as the means ± SD (n = 3). **P* < .05 versus MC group. ***P* < .01 versus MC group. QD, Qingzaojiufei decoction; qPCR, quantitative polymerase chain reaction; CTX, cyclophosphamide; MC, model control.

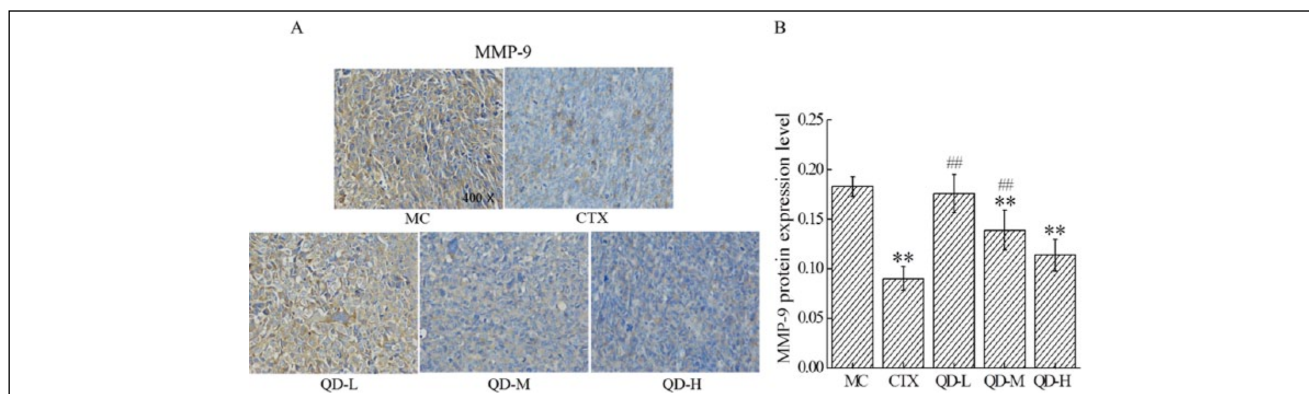

Figure 4. Effect of QD on MMP-9 protein expression. The Lewis lung cancer–bearing mice were administered physiological saline (MC group), QD-L, -M, or -H (3.8, 7.6, or 15.2 g/kg, respectively) or CTX (0.05 g/kg). Protein expression of MMP-9 was analyzed by immunohistochemical staining. Figures represent data from 1 of 3 independent experiments, and the statistical results are presented in histograms. All experiments were repeated at least 3 times. Data are presented as the means ± SD (n = 3). **P* < .05 versus MC group. ***P* < .01 versus MC group. ##*P* < 0.01 vs. CTX group. QD, Qingzaojiufei decoction; MMP-9, matrix metalloproteinase–9; CTX, cyclophosphamide; MC, model control.

protein expression compared with the MC group ($P < .01$). The positive control CTX (0.05 g/kg) also reduced MMP-9 protein expression compared with the MC group ($P < .05$, Figure 4A and B).

QD Reduced VEGF and VEGFR Expression

Expression levels of VEGF and VEGFR, which are crucial for tumor growth and metastasis, were investigated by immunohistochemistry to explore the mechanisms related to QD-mediated inhibition of tumor growth. Protein expression of VEGF and VEGFR was downregulated by QD in a dose-dependent manner (Figure 5). In the QD-M and QD-H groups, VEGF protein expression was reduced by 19.7% and 41.9% ($P < .01$), respectively (Figure 4C), while VEGFR protein expression was reduced by 20.0% and 28.9% ($P < .01$), respectively, compared with the MC group (Figure 5D).

QD Reduced Phosphorylated ERK1/2 Levels

To further elucidate the mechanisms by which QD inhibited tumor growth, the effect of QD on the ERK pathway was

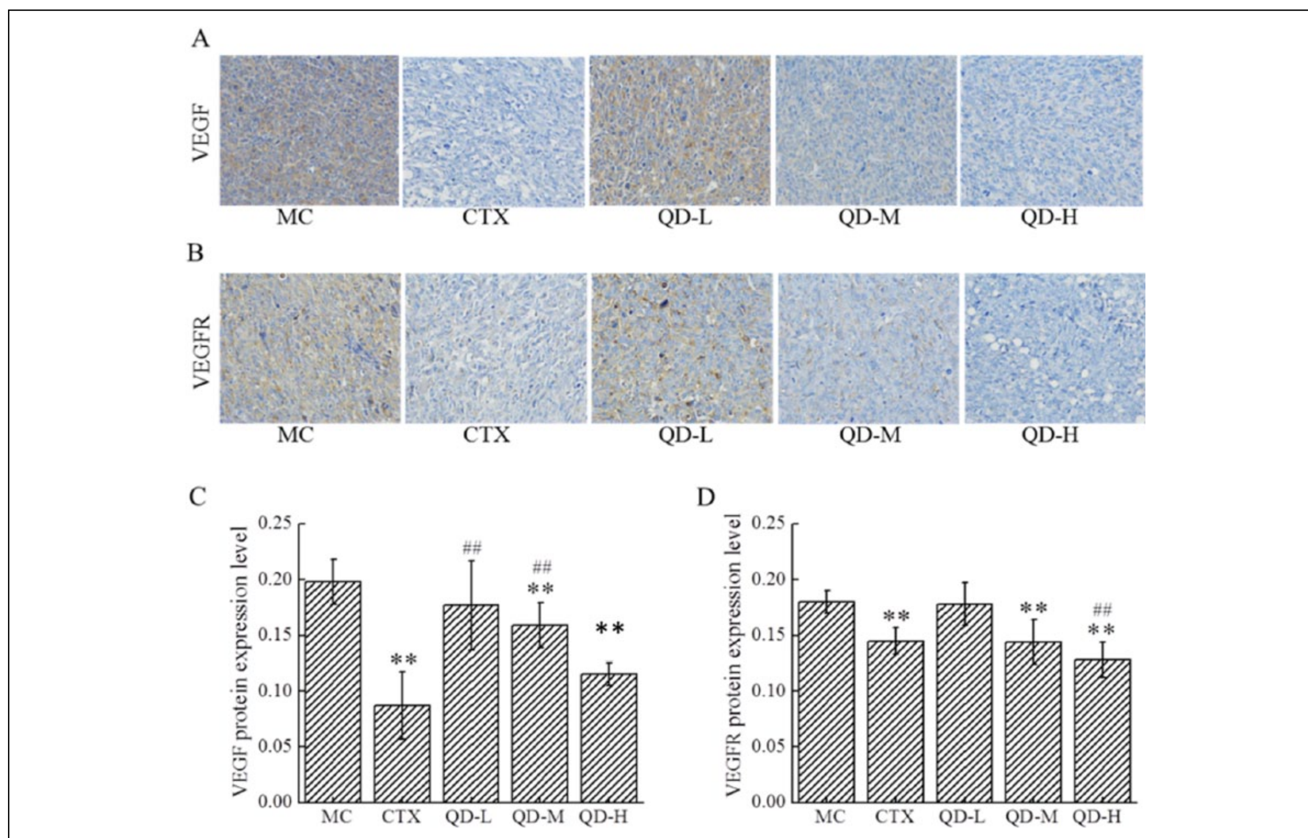

Figure 5. Effect of QD on VEGF and VEGFR protein expression. Lewis lung cancer-bearing mice were administered physiological saline (MC group), QD-L, -M, or -H (3.8, 7.6, or 15.2 g/kg) or CTX (0.05 g/kg). Protein expression levels of VEGF and VEGFR were analyzed by immunohistochemistry. Figures represent data from 1 of 3 independent experiments, and statistical results are presented in histograms. All experiments were repeated at least 3 times. Data are presented as the means ± SD (n = 3). $^{*}P < 0.05$ versus MC group. $^{**}P < .01$ vs. MC group. $^{\#\#}P < .01$ versus CTX group. QD, Qingzaojiufei decoction; VEGF/VEGFR, vascular endothelial growth factor/receptor; MC, model control; CTX, cyclophosphamide.

explored by western blotting. As shown in Figure 6A, total protein expression of ERK was unchanged by QD. However, levels of p-ERK1/2 were significantly suppressed by QD-M and QD-H compared with the MC group. As expected, the positive control CTX reduced p-ERK levels compared with MC in LCC-bearing mice ($P < .01$). The p-ERK1/2 levels of (QD + CTX)-medicated serum were lower than those of cells treated with either QD- or CTX-medicated sera in vitro (Figure 6B). Consistently, treatment with the ERK pathway inhibitor U0126 also decreased the p-ERK1/2 levels and simultaneously inhibited the LLC cell proliferation (Figure 6B and 1A).

Discussion

TCM plays an important role as a complementary therapy for patients with advanced cancer. QD has a long history of being used in clinical settings for the treatment of lung-related diseases, including cough, radiation-induced liver injury, and tuberculosis.[26] However, its therapeutic potential for lung cancer has not been well investigated. In this study, based on preliminary tests and clinical dose levels, QD was administered at doses of 3.8, 7.6, and 15.2 g/kg of body weight. The results demonstrated that QD inhibited lung tumor growth and proliferation in LCC-bearing mice. An in vitro study further confirmed that QD suppressed tumor cell proliferation. Additionally, QD intensified the anti-tumor activity of CTX. The present study showed that the antitumor effect of QD in mice was related to activation of tumor suppressor genes, inactivation of oncogenes, suppression of tumor invasion and metastasis, and the impairment of angiogenesis.

Aberrant expression of tumor suppressor genes and oncogenes contributes to tumorigenesis and cancer progression. p53, one of the most widely expressed tumor suppressor genes, is an established target for the treatment of cancer.[27] In our study, p53 expression was elevated by QD treatment, suggesting that p53 is a possible target of QD-induced inhibition of tumor proliferation. Furthermore, mRNA expression of the oncogenes Bcl-2 and c-myc were

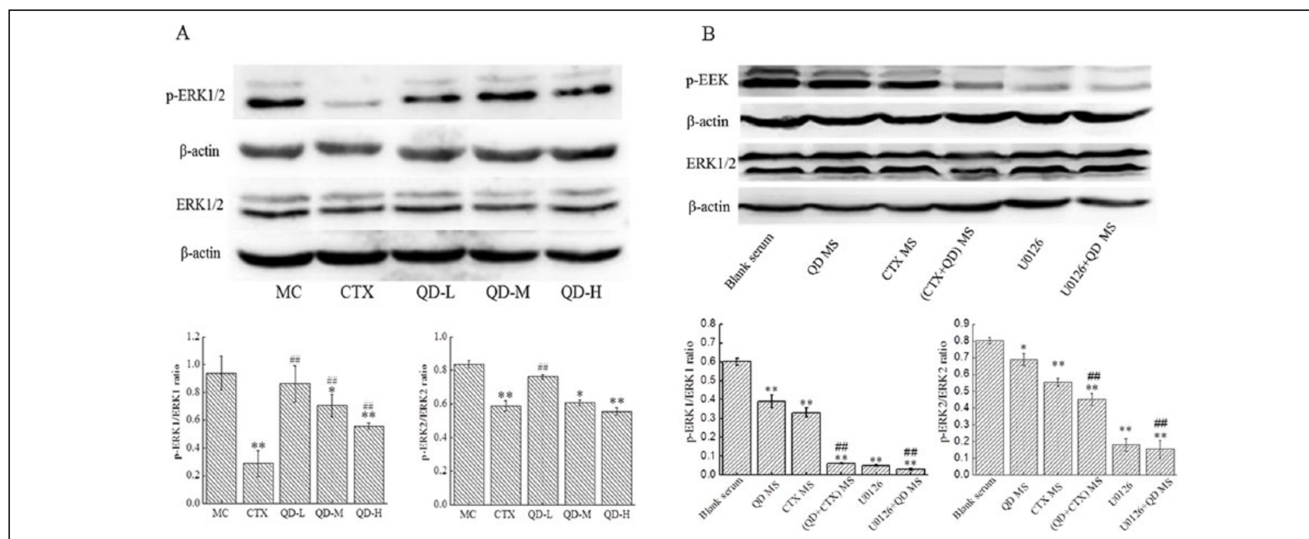

Figure 6. Effect of QD on ERK and p-ERK protein expression. Equal quantities of total protein (about 50 μg) from mice tumors and LLC cells were prepared for western blotting analysis of ERK and p-ERK, and β-actin was used as a loading control. The blots are representative from 1 of 3 independent experiments, and the statistical results are presented in histograms. $^{*}P < .05$ and $^{**}P < .01$ versus MC or Blank serum group. $^{\#}P < .05$ and $^{\#\#}P < .01$ versus CTX or QD-medicated serum group (QD MS). QD, QD, Qingzaojiufei decoction; ERK, extracellular regulated protein kinase; p-ERK, phodpho REK; LLC, Lewis lung carcinoma; MC, model control; CTX, cyclophosphamide.

reduced by QD, suppressing tumor growth. These effects may be attributed to related signaling disorders of p53, Bcl-2, and c-myc, leading to DNA damage and apoptosis of cancer cells.[28] This finding is similar to studies in which an increase in p53 and a decrease in Bcl-2 expression inhibited lung tumor growth, following administration of TCM prescriptions such as Kuan-Sin-Yin decoction[29] and a lung-tonifying and expectorant decoction.[30]

Angiogenesis has been considered an attractive target for cancer therapy owing to its pivotal role in tumor growth and metastasis.[31] Numerous TCM herbs, including *Lithospermum erythrorhizon*,[32] *Viscum album coloratum*,[33] *Chrysobalanus icaco*,[34] and *Cassia garrettiana* heartwood,[35] are known to possess anti-angiogenic activity either in vitro or in vivo. One of the best-studied factors that stimulate tumor angiogenesis is VEGF, which binds to VEGFR on the surface of endothelial cells to promote endothelial cell growth and migration. Our study showed that QD down-regulated protein expression of VEGF and VEGFR, similar to the mode of anticancer action of CTX (a positive control chemotherapy drug that suppresses tumor growth) and Jiedu Xiaozheng Yin, a decoction from TCM.[36] The results demonstrated that anti-angiogenic activity, by down-regulation of VEGF and VEGFR expression, is one of the key mechanisms of QD. A previous study suggested that overexpression of p53 could down-regulate VEGF.[37] Therefore, we proposed that QD-induced upregulation of p53 might contribute to the downregulation of VEGF. Furthermore, VEGF binds to its receptor to promote secretion of MMPs, which are involved in ECM degradation to facilitate cancer cell metastasis and angiogenesis.[38]

Degradation of ECM proteins is required for tumor invasion, and the MMP family is necessary for tumor cell proliferation and metastasis.[15] Therefore, the relationship between MMP levels and the antitumor activity of QD was explored. QD treatment decreased MMP-9 expression, suggesting that QD diminishes the invasive and metastatic capacity of lung tumor cells by modulating MMP-9 expression, which degrades ECM components. Similar to various malignant tumors, human lung cancer cells express high levels of MMP-9.[39,40] Indeed, many anticancer drugs, including TCM, target MMPs to inhibit proliferation of malignant tumor cells.[41-44]

ERK pathways are critical for cancer cell proliferation. The tumor suppressor p53 has been linked with ERK activation[45] and downregulation of oncogenes c-myc follows ERK inhibition[46]. Contrastingly, both c-myc and Bcl-2 are positively regulated by ERK1/2.[47] Additionally, activated VEGF can trigger ERK1/2 signaling to regulate cell proliferation.[48-50] Consistently, QD decreased p-ERK1/2 levels but had no effect on total ERK1/2 protein levels, demonstrating the important role of ERK dephosphorylation in the antitumor action of QD. These findings suggest that crosstalk between molecular events is involved in the antitumor effects of QD. This crosstalk likely results from the multiple compounds present in QD and their involvement in complicated metabolic processes.

Conclusions

These findings suggest that QD has an inhibitory effect on the growth and proliferation of lung cancer in a mouse xenograft model. Evaluation of the underlying mechanisms revealed that QD may act by activation of tumor suppressor genes, inactivation of oncogenes, suppression of tumor invasion and metastasis, and impairment of angiogenesis. The ERK/VEGF/MMPs signaling pathways may be implicated in the inhibitory effect of QD on tumor growth, which may provide a molecular explanation for the anticancer activity of QD. These data reveal that QD may be a potential TCM prescription for use as a complementary treatment for human lung cancer therapy. Future clinical trials should be conducted to confirm the anti–lung cancer activity of QD in humans.

Authors' Note

We state that the views expressed in the submitted article are our own and not an official position of the institution or funder.

Declaration of Conflicting Interests

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Supplementary Material

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