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RESEARCH ARTICLE

Thymoquinone inhibits proliferation and invasion of human nonsmall-cell lung cancer cells via *ERK* pathway

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Abstract Thymoquinone (TQ) is the primary bioactive component of *Nigella sativa* Linn seed oil and used as anti-inflammatory, anti-oxidant, and anti-neoplastic agent. Previous studies have shown that TQ exhibits inhibitory effects on multiple cancers. However, the detailed antineoplastic effects and its molecular mechanisms of TQ on lung cancer are not entirely elucidated yet. In the present study, we aimed to investigate the effects of TQ on cell proliferation, migration, and invasion as well as its underlying anti-metastatic mechanisms in A549 cells. Lung cancer cell line A549 cells were treated with different concentration of TQ for different period of time, and the growth-inhibitory effects of TQ was measured by MTT and cell count assays; cell cycle was determined by flow cytometry; wound healing and transwell assays were used to assess the cell migration and invasion activities; Western blot and real-time quantitative RT-PCR were used to determine the expression of proliferation and invasion associated genes as well as MAPKs pathway molecules; gelatinase activity was estimated using gelatin zymography assay. The results show that TQ played a role in inhibiting the proliferation, migration, and invasion of A549 lung cancer cells, it also inhibited the expression level of *PCNA*, *cyclin D1*, *MMP2*, and *MMP9* mRNA and protein in a dose- and time-dependent manner especially at 10, 20, 40 μmol/L concentrations. The cell cycle inhibitor *P16* expression and the gelatinase activities of *MMP2* and *MMP9* were also inhibited by TQ dramatically. TQ reduced phosphorylation of *ERK1/2*; however, the proliferation and invasion inhibitory effects of TQ on A549 cells were neutralized by *ERK1/2* inhibitor PD98059. In conclusion, our study confirmed that TQ could inhibit A549 cell proliferation, migration, and invasion through *ERK1/2* pathway, as proposed the therapeutic potential of TQ as an anti-metastatic agent in human lung cancer treatment.

Keywords Nonsmall-cell lung cancer · Thymoquinone · Proliferation · Invasion · Signal transduction pathway

Abbreviations

TQ	Thymoquinone
siRNA	Small interfering RNA
NSCLC	Nonsmall-cell lung cancer
SCLC	Small cell lung cancer
TIMP	Tissue inhibitor of metalloproteinase
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
FBS	Fetal bovine serum
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Nonsmall-cell lung cancer (NSCLC) is one of the most common cancers and leading cause of tumor-related death worldwide [1, 2]. The two major histological types of lung cancer

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are non-small cell lung cancer (NSCLC) accounting for about 85 % of cases and small cell lung cancer (SCLC) accounting for 15 % of cases. Despite improvements in surveillance and clinical treatment strategies, progressive stages and metastasis of NSCLC still remains the most common cause for the high NSCLC lethality [3]. Although the researchers gradually understand the mechanisms of cell migration and invasion, efficacy of the present drugs designed to block tumor progression by modulating these mechanisms is very limited.

Tumor progression is a multistep process, in which cancer cells uncontrolled growth, detach from the primary tumor, and invade surrounding tissues and intravasate into blood and/or lymphatic systems. Finally, cancer cells settle and colonize at the target organs [4]. Among them, the ability of infinite proliferation is the basis of tumor formation. Marker of cell proliferation, for example PCNA, dramatically increases in various types of tumors. Moreover, abnormal expression of cycle related protein has been linked to proliferation. Similarly, invasion and metastasis were regulated by many genes. Matrixmetalloproteinases (MMPs) play important roles in tumor metastasis. They are widely considered to be a secreted, zinc-dependent endopeptidase which can degrade extracellular matrix (ECM) components such as collagen, fibronectin, proteoglycan, laminin, and elastin in both physiological and pathological processes [5–7]. Among them, MMP2 and MMP9 are considered to be overexpressed constitutively and degrade type IV collagen in many stages of human cancers, especially in highly metastatic cancers, such as lung cancer [8–10]. Therefore, the inhibition of MMP2 and MMP9 may be a therapeutic target in lung cancer cells. It has been reported that the expression of MMPs is regulated by mitogen-activated kinases (MAPKs) pathways [11], which are involved in regulating cell proliferation, migration, and invasion [12, 13].

There have been many reports focused on the effectiveness of chemopreventive or therapeutic drugs from natural products. The Black Caraway seed, also named *Nigella sativa*, which belongs to Ranunculaceae family, is an annual herbaceous plant that grows in countries bordering Mediterranean Sea, Pakistan, and India. It is commonly used traditionally as a natural treatment for numerous diseases for more than 2,000 years [14].

Thymoquinone (TQ, 2-isopropyl-5-methyl-1,4-benzoquinone) was the primary bioactive component of *N. sativa* Linn seed oil and used as anti-inflammatory, anti-oxidant, and anti-neoplastic agent [15, 16]. In the last decade, multiple papers have reported that TQ was able to inhibit a variation of carcinomas including breast, prostate, ovarian, liver, colorectal carcinoma, and so on [17].

Previous studies have shown that TQ exhibits inhibitory effects on multiple process of cancer, including proliferation, apoptosis, migration, invasion, and angiogenesis. In addition, TQ synergistically augments conventional medicine inhibition of cancer cells, such as NCI-H460 non-small cell lung cancer cells [18] and U266 multiple myeloma cells [19]. However, the detailed molecular mechanisms of the antineoplastic effects of TQ are not entirely elucidated yet and the potential therapeutic effects of TQ in lung cancer also remain enigmatic.

In the present study, we aim to investigate the effect of TQ on cell proliferation, migration, and invasion as well as its underlying anti-metastatic mechanisms in A549 cells.

Materials and methods

Chemicals and antibodies

Trypsin-EDTA, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and thymoquinone (TQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 and fetal bovine serum (FBS) were purchased from Hyclone (Loughborough, UK). PD98059 (ERK1/2 inhibitor) was purchased from Promega (WI, USA). BD matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies to PCNA, MMP2, MMP9, TIMP1, TIMP2, JNK, p-JNK, ERK, p-ERK, p38, p-p38, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and treatment

A549 cells were purchased from Cell Resource Center of Life Sciences (Shanghai, China) and grown in RPMI 1640 medium containing 10 % FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C and were passaged three times a week by treating with 0.25 % trypsin containing 0.02 % EDTA.

Cell proliferation assay

Cell proliferation activity was determined by MTT and cell count assays. For MTT assay, briefly, A549 cells were incubated at a density of 1×10^4 cells/well in 96-well plates in six duplications for 24 h. The cells were treated with various concentrations (0, 5, 10, 20, 40, 80, 160 μmol/L) of TQ for 24, 48, or 72 h. The cells were washed with PBS and incubated with RPMI 1640 medium and MTT solution (5 mg/mL) for another 4 h at 37 °C. Then, the medium was totally removed and the MTT-containing medium was replaced with DMSO to dissolve the water-insoluble formazan salt. Then, the absorbance was measured by Multiskan Ascent at 570 nm (Thermo Scientific, Wilmington, DE, USA). Cell survival was expressed using OD value. Cell number counting was conducted on a cell count meter.

Cell cycle analysis

Monolayer cells were trypsinized using 0.25 % trypsin-EDTA (Gibco) and then neutralized and resuspended with 10 % FBS containing MEM (Gibco). After washing with cold PBS, cells were stained with propidium iodide 50 μg/mL PI (Sigma), and incubated for 30 min at 4 °C. DNA content of cells was determined by flow cytometry (FC500MCL, Beckman Coulter) and data were analyzed by using CXP software (Beckman Coulter).

Migration assay

A549 cells were seeded (5×10^4cells/well) into 12-well plates and grown to 80~90 % confluence for the experiment. Monolayer cells were wounded by scratching with 200-μL sterile pipette tips and washed twice with 1×PBS. And then, cells were treated with TQ (0, 5, 10, 20, 40, 80, 160 μmol/L) for 24, 48, or 72 h. The cell migration activity was expressed as the number of cells migrating into the wound.

Transwell invasion assay

Cell invasion analysis was made in a 24-well plate transwell chamber. The transwell (Corning Incorporated, USA) were coated with 30 μL matrigel and incubated at 37 °C for 1 h. A549 cells were treated with of TQ (0, 10, 20, or 40 μmol/L) for 24, 48, or 72 h, and then cells were trypsinized and resuspended in serum-free medium and seeded on the upper chamber of transwell. Meanwhile, 30 μL of RPMI 1640 medium containing 10 % FBS was added to the lower chamber. After incubation of 16 h, a cotton swab was used to wipe matrigel glue and cells in the upper chamber. Membrane was fixed with methanol for 10 min, followed with crystal violet staining for 1 h and rinsed with fresh water. The cell invasion activity was expressed as the number of cells on the lower side of the membrane, as was counted randomly in the six visual fields at high magnification.

Gelatin zymography

MMP2 and MMP9 gelatinase activities were determined by gelatin zymography. Briefly, A549 cells were treated with various concentrations (0, 10, 20, or 40 μmol/L) of TQ for 48 h, then the medium was collected and mixed with non-reducing sample buffer, then were resolved by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 1 mg/ml gelatin. The resulting gel was washed in 10 mM Tris (pH 8.0) containing 2.5 % Triton X-100, and was then incubated for 16 h in a reaction buffer at 37 °C. After staining with Coomassie brilliant blue R-250, and destained until gelatinases were identifiable as clear bands.

Real-time RT-PCR

Total RNA of A549 cells with different treatment were isolated using Trizol Reagent (Invitrogen, California Carlsbad, USA). The quantity of RNA was measured using a NanDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA synthesis was performed using 1 μg total RNA and MMLV reverse transcriptase with oligo dT primer (Fermentas, USA). Real-time PCR was performed on an ABI 7500 PCR system (Applied Biosystems, USA) using the SYBR Green RT-PCR Kit (Applied Biosystems, Foster City, CA) to detect the expression of target genes. β-actin was used as an internal control to evaluate the relative expressions of target genes. PCR initialed by 95 °C for 5 min, followed by 40 reaction cycles with 94 °C, 30s; 58 °C, 30s; 72 °C, 30s. Fluorescence was detected at the end of each cycle. The relative amount of target genes was carried out using the $2^{-\Delta\Delta Ct}$ method. The PCR primers were used as Table 1.

Western blotting analysis

A549 cells with different treatment were harvested and lysed on ice in RIPA buffer (1 % Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 % NP-40) containing protease inhibitor. Protein concentration was determined using a Bradford method (Bio-Rad, Hercules, USA), and 60 μg of protein was separated by 10 % SDS-PAGE and transferred to PVDF membranes (Roche). Membranes were probed overnight at 4 °C with a primary antibody against PCNA, MMP2, MMP9, TIMP1, TIMP2, JNK, p-JNK, ERK, p-ERK, p38, p-p38, or β-actin. Bands were detected with goat-anti-rabbit IRDye800 secondary antibody (Santa Cruz, CA, USA) and enhance chemiluminescence (Pierce). Membranes were incubated for 2 h in a horseradish peroxidase-conjugated secondary antibody (1:10,000 diluted); target bands were detected with the enhanced chemiluminescence (ECL) detection system (Santa Cruz, USA) according to the manufacturer's instructions. β-actin was used as the loading control. The experiments were replicated three times.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical Package for the Social Sciences (SPSS) for Windows (version 13.0; SPSS, Chicago, IL, USA) was used for our statistical analysis, $P<0.01$ was considered as statistically significant.

Table 1 PCR primer sequences

Gene	bp	Forward (5′ to 3′)	Reverse (5′ to 3′)	GenBank no.
PCNA	156	GTGGAGAACTTGGAAATGGAAAC	TTGAAGAGAGTGGAGTGGCT	NM_002592
Cyclin D1	105	CTCGGTGTCCTACTTCAAATGT	TCCTCGCACTTCTGTTCCT	NM_053056.2
P16	76	TTCGCTAAGTGCTCGGAGT	CGGTATCTTTCCAGGCAAG	NM_000077.4
MMP9	87	AGAACCAATCTCACCGACAGG	CGACTCTCCACGCATCTCT	NM_004994
MMP2	62	CAGGAGGAGAAGGCTGTGTT	AGGGTGCTGGCTGAGTAGAT	NM_004530
TIMP1	167	ACTTCCACAGGTCCCACAAC	GCATTCCTCACAGCCAACAG	NM_003254
TIMP2	152	CGACATTTATGGCAACCCT	ATTCCTTCTTTCCTCCAACG	NM_003255.4
β-actin	105	GGTCATCACCATTGGCAA	GAGTTGAAGGTAGTTTCGTGGA	NM_001101.3

Results

TQ inhibited growth of A549 cells

TQ is the main active ingredient of the volatile oil of *N. sativa* Linn, its structure is presented as Fig. 1a.

A549 cells were treated with 0, 5, 10, 20, 40, 80, 160 μmol/L of TQ for 48 h, alternatively, 40 μmol/L for 24, 48, or 72 h, and the growth-inhibitory effects of TQ was measured by MTT and cell count assays. As shown in Fig. 1, proliferation inhibition of A549 cells treated with 10, 20, 40, 80, 160 μmol/L TQ for 24, 48, or 72 h had a dose- and time-dependent manner.

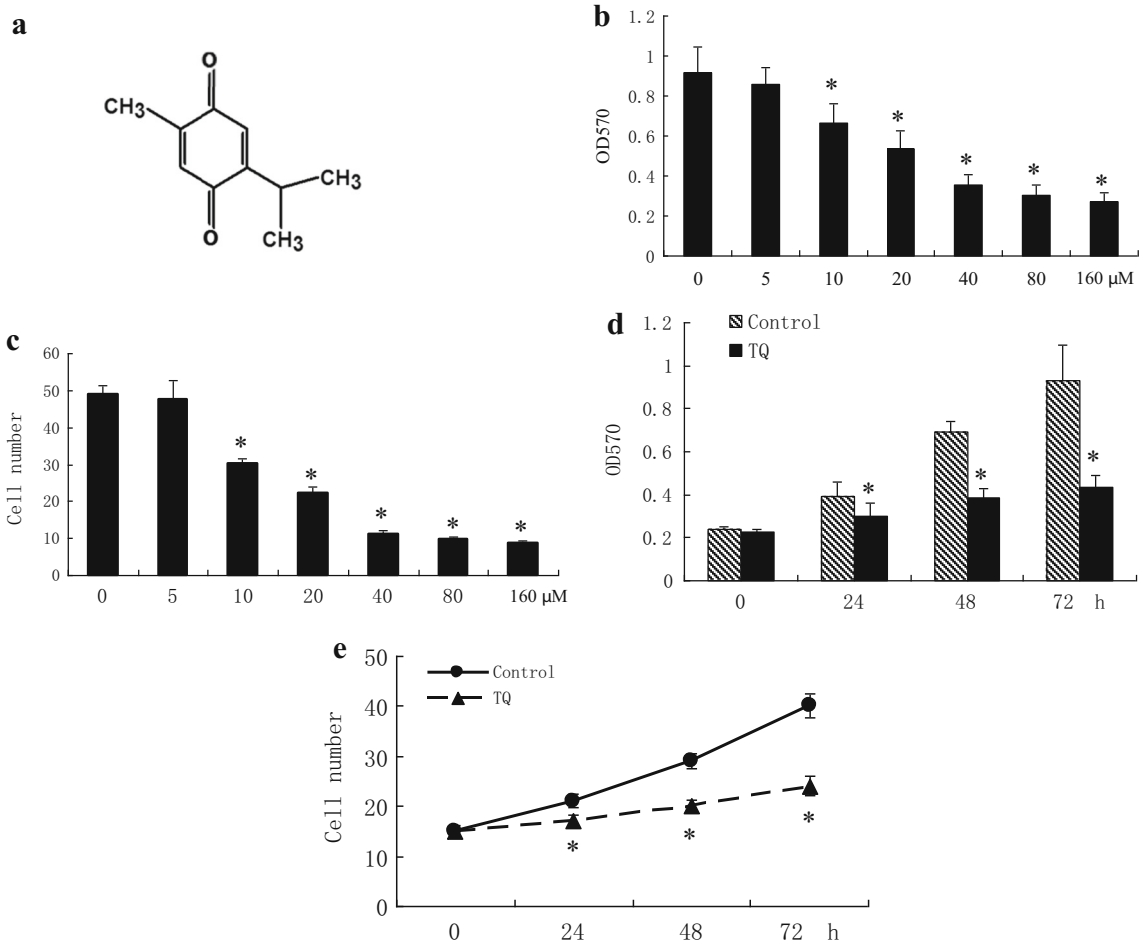

Fig. 1 Effect of TQ on the growth of A549 cells. A549 cells were plated on 96-well for 24 h and treated with different concentrations (0, 5, 10, 20, 40, 80, and 160 μmol/L) of TQ for for 48 h, alternatively, 40 μmol/L for 24, 48, or 72 h. Then, cells proliferation were assessed by MTT assay and cell number counting. **a** Structure of TQ (2-isopropyl-5-methyl-1,4 benzoquinone). Values are mean±S.D. (n=6). *p<0.01 versus control group

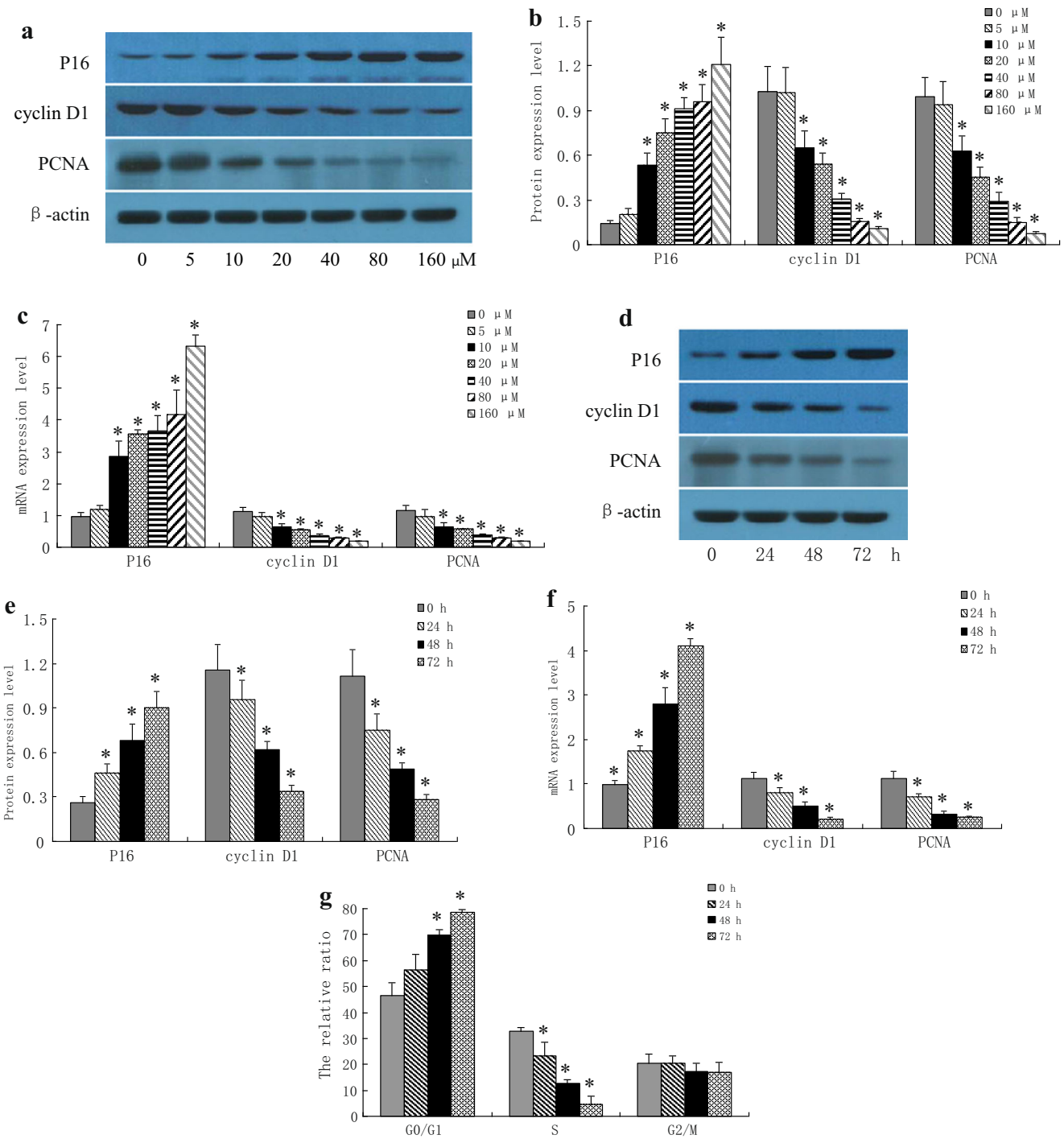

Fig. 2 Effect of TQ on the expression of growth marker genes for A549 cells. A549 cells were plated on 96-well for 24 h and treated with different concentrations (0, 5, 10, 20, 40, 80, and 160 μmol/L) of TQ for for 48 h, alternatively, 40 μmol/L for 24, 48, or 72 h. Then, PCNA, P16 and cyclin D1 expression were assayed by Western blot (**a**, **d**) or real-time quantitative RT-PCR (**c**, **f**). Cell cycle of A549 cells were assayed by flow cytometry (**g**). β-actin was utilized for an endogenous reference to standardize protein expression levels. Densitometry analysis was carried out and normalized to β-actin (**b**, **e**). Values are mean±S.D. (n=3). *p<0.01 versus control group

However, proliferation inhibition rate was not significantly altered in 5 μmol/L TQ treated A549 cells. At the concentration of 40 μmol/L, TQ has a dramatic effect on A549 cell proliferation. The results suggested that TQ played a role in inhibiting the proliferation of lung cancer cells (P<0.01).

We also detected the effect of TQ on the expression of growth marker genes PCNA, P16, and cyclin D1 for A549 cells. As the results of Western blot or real-time quantitative RT-PCR assays shown in Fig. 2, TQ inhibited the mRNA and protein expression levels of PCNA and cyclin D1 in a dose-

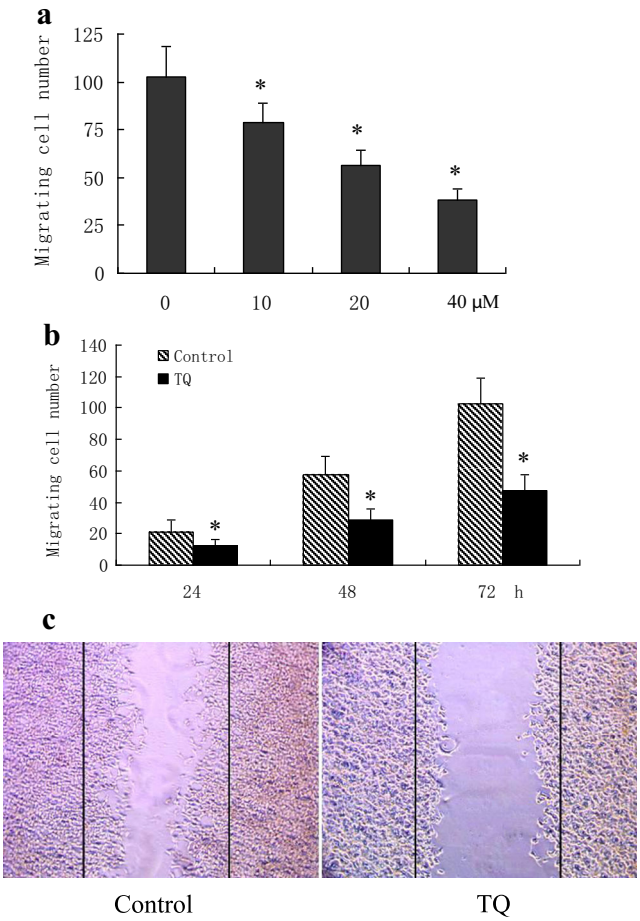

Fig. 3 Effects of TQ on migration of A549 cells. A549 cells were plated on 12-well and grown to 80~90 % confluence for the experiment. Cells were scratched with 200-μL sterile pipette tips and then treated with TQ (0, 10, 20, or 40 μmol/L) for 24, 48, or 72 h. The cell migration activity was expressed as the number of cells migrating into the wound. Values are mean±S.D. (n=3). *p<0.01 versus control group

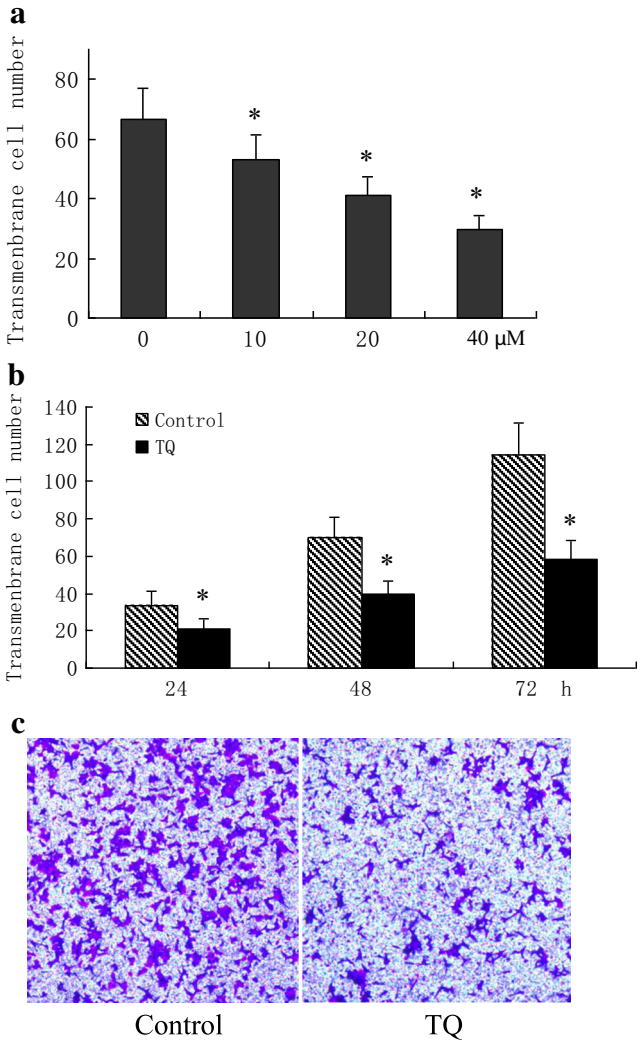

Fig. 4 Effects of TQ on invasion of A549 cells. A549 cells were treated with of TQ (0, 10, 20, or 40 μmol/L) for 24, 48, or 72 h, then cells were trypsinized and resuspended in serum-free medium and seeded on the upper chamber of transwell for 16-h incubation. The invading cells on the lower surface of the membrane were counted. Values are mean±S.D. (n=3). *p<0.01 versus control group

and time-dependent manner especially at 10-, 20-, 40-μmol/L concentrations (P<0.01), while the cell cycle inhibitor P16 expression increased. At the same time, cell cycle was inhibited markedly.

TQ inhibited the migration of A549 cells

A wound healing assay was used to investigate the effects of TQ on cell migration. After treatment with 10, 20, and 40 μmol/L TQ for 24, 48, or 72 h, compared with the negative control group, migration inhibition rate had a dose-dependent increase in A549 cells (P<0.01) (Fig. 3a). In addition, after treatment with 40 μmol/L TQ for 24, 48, or 72 h, compared with the negative control group, migration inhibition rate had a time-dependent increase in A549 cells (P<0.01) (Fig. 3b). The results suggested that TQ played a role in inhibiting the migration of lung cancer cells.

TQ inhibited the invasion of A549 cells

To investigate inhibitory effect of TQ on invasion of A549 cells, changes of cell invasion ability was detected in transwell invasion assay. After treatment with10, 20, and 40 μmol/L TQ for 24, 48, or 72 h, compared with the negative control group, invasion inhibition rate had a dose-dependent increase in A549 cells (P<0.01) (Fig. 4a). Additionally, after treatment with 20 μmol/L TQ for 24, 48, or 72 h, compared with the negative control group, invasion inhibition rate had a time-dependent increase in A549 cells (P<0.01) (Fig. 4b). Collectively, these data suggested that TQ could inhibit the invasion of lung cancer cells.

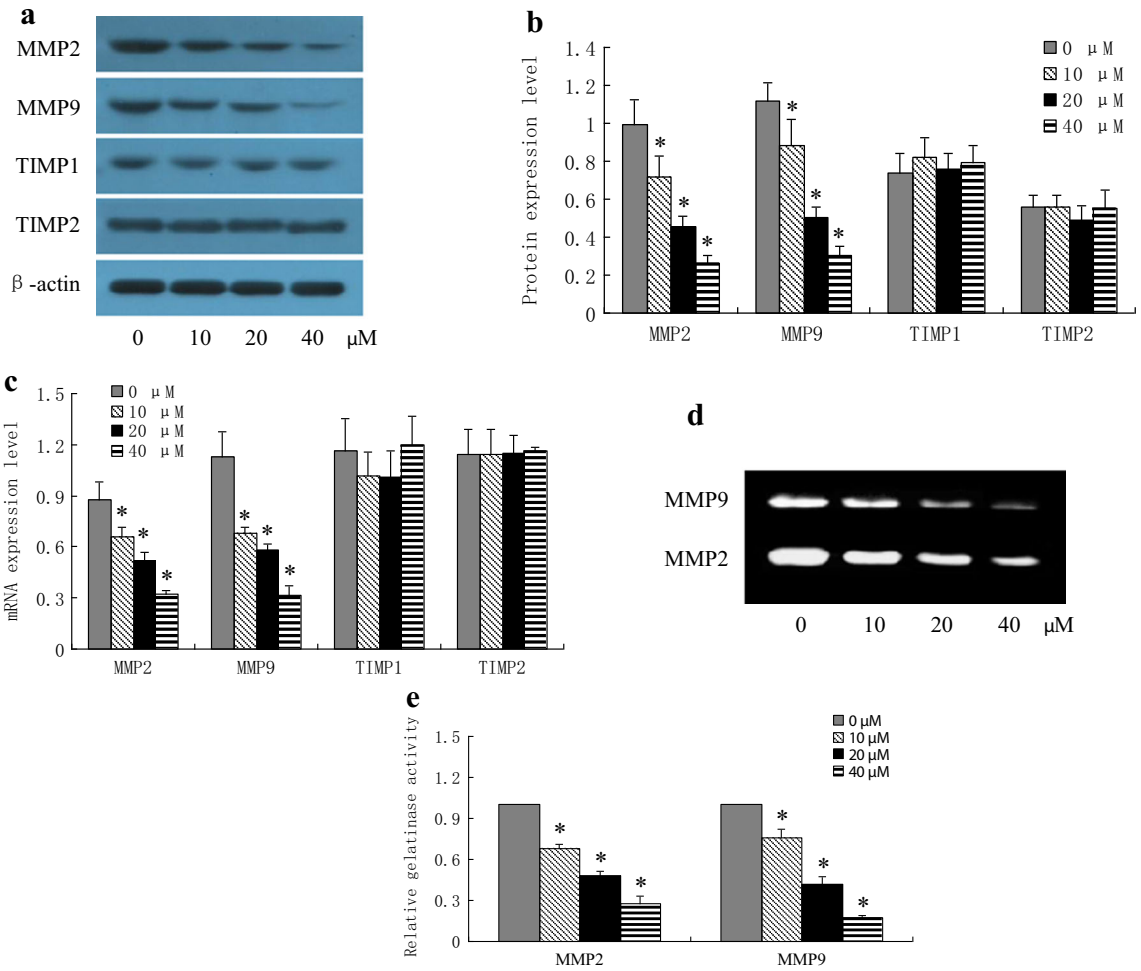

Fig. 5 Effect of TQ on MMP2 and MMP9 expression and activities in A549 cells. A549 cells were treated with various concentrations of TQ (0, 10, 20, or 40 μmol/L) for 24 h, then cells were collected and subjected to Western blot (**a**, **b**) and real-time quantitative RT-PCR (**c**) assays, meanwhile, the conditioned media was collected, and gelatinase activity was estimated using gelatin zymography (**d**). β-Actin was utilized for an endogenous reference to standardize protein or mRNA expression levels. **b**, **d** Densitometry analysis was carried out and normalized to β-actin. Values are mean±S.D. (n=3). *p<0.05 versus control

TQ inhibited the activity and expression of MMP-2, MMP9 in A549 cells

The degradation of extracellular matrix (ECM) is important to cell migration and invasion. Key molecules of ECM degradation, *MMPs*, are also thought to play major roles in cell behaviors such as cell migration and invasion. MMPs and its inhibitors of tissue inhibitors of metalloproteinase (*TIMPs*) constitute a control system for degradation of ECM [20]. Therefore, we detected the expression of *MMP2*, *MMP9*, *TIMP1*, and *TIMP2* using Western blot and real-time quantitative RT-PCR assays. In the results shown in Fig. 5a, b, and c, the mRNA and protein expression of *MMP2* and *MMP9* were inhibited by TQ treatment in a dose-dependent manner at 10, 20, and 40 μmol/L concentrations. However, TQ has no effect on the expression of *TIMP1* and *TIMP2*.

We further assessed the effect of TQ on MMP2 and MMP9 gelatinase activities gelatin zymography. As shown in Fig. 5d, after cells were treated with TQ (10, 20, and 40 μmol/L) for 48 h, the *MMP2* and *MMP*9 activity was suppressed in a concentration-dependent manner in A549 cells (P<0.01).

TQ inhibited ERK1/2 pathway in A549 cells

Above findings show that TQ significantly inhibited proliferation, migration, and invasion of A549 cells. Meanwhile, the expression and activity of *MMP2* and *MMP9* were also inhibited by TQ in A549 cells. However, the signal mechanisms responsible for the inhibitory effect of TQ are still unclear. Hence, the effect of TQ on the signal transductions of MAPKs was further assessed by western blotting analysis. A549 cells were treated with 40 μmol/L of TQ for 0, 4, 8, 12, 24, or 48 h, and then total protein lysates of each sample was collected and then subjected to western blotting with *phospho-ERK1/2*, *ERK1/2*, *phospho-JNK*, *JNK*, *phospho-p38 MAPKs*, and *p38 MAPKs* antibodies. The results of Fig. 6 show that

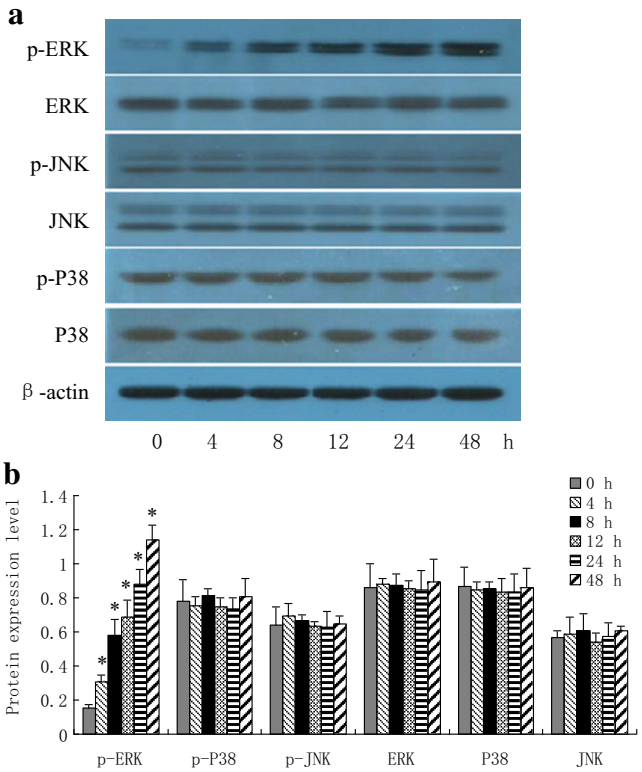

Fig. 6 Effect of TQ on phosphorylation of MAPKs pathways in A549 cells. **a** A549 cells were treated with 40 μmol/L of TQ for 0, 4, 8, 12, 24, or 48 h, then total protein lysates of each sample was collected and then subjected to western blotting with phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38 MAPKs, and p38 MAPKs antibodies. β-Actin was utilized for an endogenous reference to standardize protein expression levels. **b** Densitometry analysis was carried out and normalized to β-actin. Values are mean±S.D. (n=3). *p<0.01 versus control group

TQ reduced phosphorylation of ERK1/2 in a time-dependent manner, whereas TQ had no obvious effects on p38 and JNK1/2 protein and its phosphorylation levels.

TQ inhibits the A549 cell proliferation and invasion through ERK1/2 pathway

To confirm whether the inhibitory action of TQ rely on its suppression of ERK1/2 pathway, we detected the effects of *ERK1/2* inhibitor (PD98059) in combination with TQ on proliferation and invasion for A549 cells. A549 cells were pretreated with 20 μmol/L PD98059 for 2 h and then incubated in the absence or presence of TQ (40 μmol/L) for 48 h, then cells were collected and subjected to Western blot assay to detect the expression of *MMP2* and *MMP9*, and media was collected for gelatin zymography assay. In the results shown in Fig. 7a, TQ decreased MMP2 and MMP9 expression; however, the inhibitory effect was blocked by ERK1/2 inhibitor PD98059. This effect was also confirmed by gelatin zymography assay as shown in Fig. 7c.

We further assessed cell proliferation activity by MTT and cell count assays. In the results shown in Fig. 7e and f, TQ decreased A549 cell proliferation activity; however, the inhibitory effect was blocked by PD98059. Also, cell migration and invasion activities were detected by wound healing and transwell assays. In the results shown in Fig. 7g and h, TQ decreased A549 cell invasion activity; however, the inhibitory effect was blocked by PD98059. In summary, the effects of TQ on proliferation and invasion of A549 cells is possibly exhibited by the modulations of *ERK1/2* pathway.

Discussion

NSCLC has a strong ability of tumor growth, angiogenesis, tumor cell detachment, which is one of the important reasons that lead to poor prognosis. In the last few decades, great advances have been made in the medical sciences to control diseases. But many diseases like cancers are not yet fully cured. To find out new therapies, scientists and clinicians are working with traditional medicines in parallel of modern medicine.

TQ, the major bioactive constituent present in black seed oil, is a safe and effective anti-inflammatory and antioxidant drug widely applied clinically [21]. In addition, accumulating evidence confirmed that TQ had a strong anti-cancer effect. Among them, TQ's mechanism of action and its ability to induce apoptosis and inhibit cancer cell growth were verified by Ivankovic [22]. The chemotherapeutic potential of TQ in the clinic has also been identified [18]. Additionally, it was also reported that TQ inhibits tumor angiogenesis in human prostate cancer (PC3) [23]. It can be inferred that TQ inhibited tumor growth in an extensive and multiple ways.

Moreover, the anti-cancer activity of TQ was also proven in lung cancer cells. For example, exposure of lung cancer LNM35 cells to increasing TQ concentrations resulted in a significant inhibition of viability and invasion [24]. Recent studies have shown that TQ has an anti-neoplastic effect both in a NSCLC and a SCLC cell line [18]. Banerjee et al. [25] have shown that synthetic analogues of TQ were found to be more potent than TQ in terms of inhibition of cell growth, induction of apoptosis. Thus, as a potential anti-cancer drug, TQ's mechanism of action deserves deep study.

In accordance with other studies, we show that TQ is a potent anti-cancer drug inA549 cells. Firstly, we show that the cytotoxic activities of TQ towards A549 cells are selective. In our experimental conditions, TQ could inhibit the proliferation rate of A549 cells in dose- and time-dependent manners. Down-regulated expression of the proliferation marker PCNA and cyclin D1 was observed in A549 cells after TQ treatment. It is confirmed that TQ has a clear antiproliferative effect on A549 cells.

As we know, cancer cells can operate different migration programs under different environmental conditions [26]. While the cancer cell metastases, based on cancer cells migration and their invasion to surrounding tissues and vessels, this require the changes of locomotion related genes and the

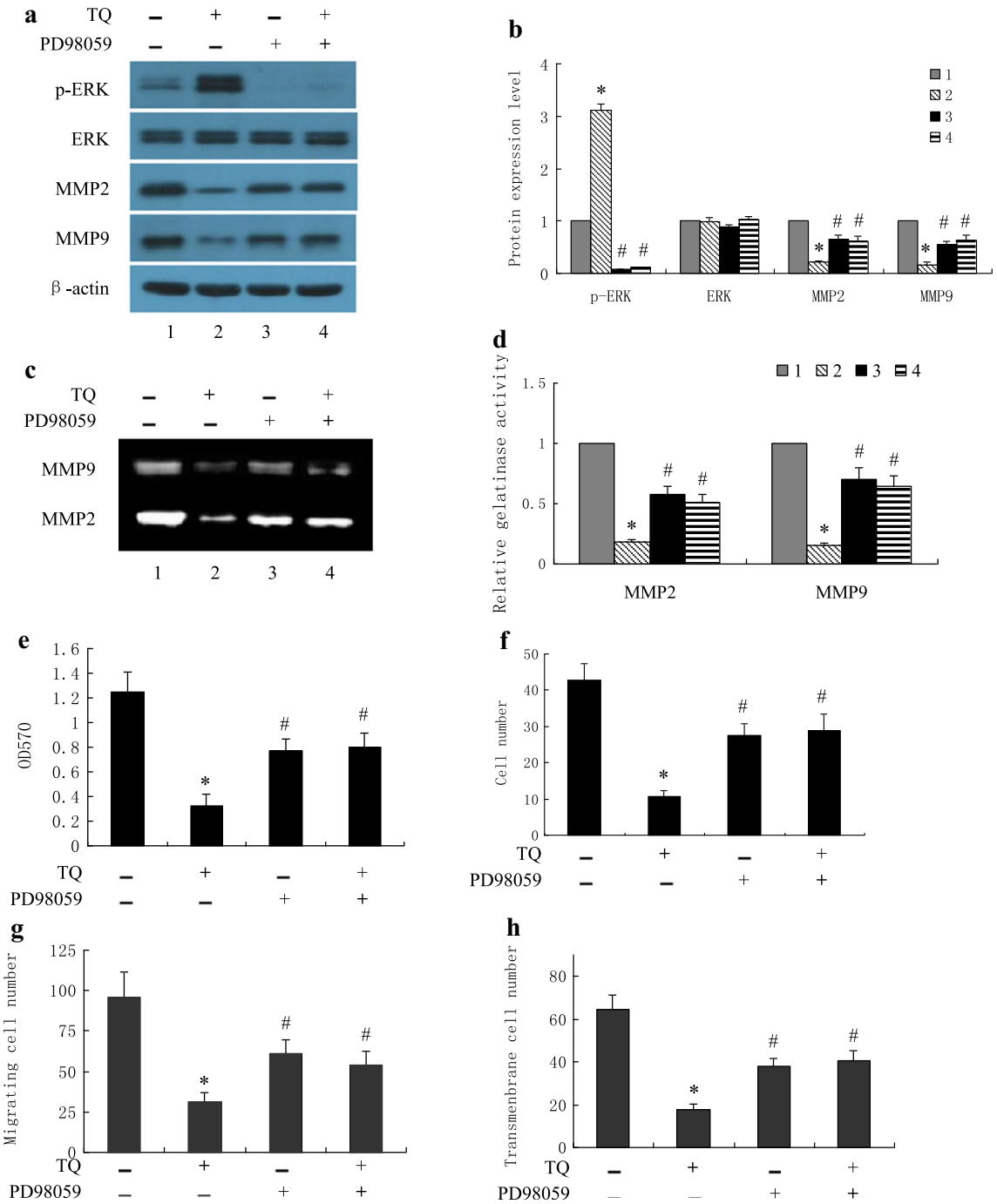

Fig. 7 Effects of ERK1/2 inhibitor (PD98059) in combination with TQ on proliferation and invasion for A549 cells. A549 cells were pretreated with 20 μmol/L PD98059 for 2 h and then incubated in the absence or presence of TQ (40 μmol/L) for 48 h, then cells were collected and subjected to Western blot assay (**a**), and media was collected for gelatin zymography assay (**c**). Cell proliferation activity were determined by MTT (**e**) and cell count (**f**) assays. Cell migration and invasion activities were detected by wound healing (**g**) and transwell (**h**) assays. Values are mean±S.D. (n=3). *p<0.01 versus one group, #p<0.01 versus two groups

upstream signaling pathways [27]. Therefore, comprehensive understanding the effect of new drug, such as TQ on the cancer cell migration/invasion is needed. In this study, the results of wound healing assay and transwell invasion assay significantly show that TQ dose- and time-dependent inhibited A549 cell migration and invasion.

MMPs are one kind of Zn+-dependent endogenous proteinases. MMPs family consists of 25 members, which can almost degrade all components of extracellular matrix except for polysaccharide, and are involved in tumor growth, migration, invasion, and other pathophysiological processes [28]. Numerous studies have shown that the expression level and

activity of MMP2 has a close relationship with lung cancer metastases [29–31]. The higher MMP2 activity in human lung cancer cells, the easier it metastasizes [32]. In this study, we also investigated the expression of matrix metallo proteinases inhibitor 1 and 2. However, both of TIMP1 and TIMP2 has not been changed by TQ. These results suggest that TQ may affect invasion and migration of A549 cells by up-regulating the expression of MMPs rather than TIMPs. Furthermore, blocking of MMPs activity may lead to the effective therapy in metastatic lung cancer patients.

In order to find efficacious drugs to suppress metastasis, many natural products and their pharmacological ingredients were used to identify their anti-metastatic activity than synthetic chemicals. Among them, it has been reported that TQ reduced MMP2 and MMP9 secretion in glioblastoma [33]. In addition, TQ down-regulated the mRNA level of MMP1, MMP3, and MMP13 and up-regulated the mRNA level of tissue inhibitors of metalloproteinase-1 in rabbit chondrocytes [34]. On the cell surface of macrophages, dendritic cells and fibroblast cells, TQ activated Neu4 sialidase via MMP9 [35]. In this study, we found that the activity and expression of MMP2 and MMP9 in A549 cells was gradually decreased with increasing concentrations of TQ.

Numerous studies have shown that MAPKs (JNK 1/2, ERK 1/2, and p38) are involved in cancer cell migration, invasion, and the changes of MMP-2 activity [36]. ERK1/2 pathway plays an important role in the invasive or migratory behavior of a number of tumors, such as prostate cancer, oral cancer, hepatocellular carcinoma, and lung cancer [37–40]. As shown in our study, the anti-proliferation, anti-migration, and anti-invasion effects of TQ are based on its inactivation of ERK1/2 pathway in A549 cells.

In conclusion, our study confirmed that TQ could inhibit A549 cell proliferation, migration, and invasion by the suppression of ERK1/2 signaling pathway to inhibit MMP2 and MMP9 expression and activities, resulting in the down-regulation of cancer cell migration and invasion. These results proposed the therapeutic potential of TQ as an anti-metastatic agent in human lung cancer patients.

Conflicts of interest None

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