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Original Research

Exploration of pro-apoptotic effect of Thymoquinone on oral squamous cell carcinoma cells through PI3K/Akt signaling pathway

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Abstract: Oral squamous cell carcinoma (OSCC) is the sixth most prevalent cancer type in the world, with a 5-year survival rate of only 40% to 50%. Finding effective anti-tumor drug candidates is quite necessary for the progression of OSCC therapy. In this study, the role of Thymoquinone (TQ) in OSCC cells was studied. It was confirmed that TQ can inhibit the proliferation, migration and invasion of KB cells, and can also achieve apoptosis by inhibiting the activation of PI3K/Akt pathway. Therefore, TQ is an effective candidate for the treatment of OSCC.

Key words: Thymoquinone; Oral squamous cell carcinoma; Pro-apoptotic; PI3K-Akt.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common malignancy in the world, and oral cancer is a subtype of head and neck cancer, defined as the growth of any cancerous tissue located in the mouth (1). Several types of oral cancer have been identified, but approximately 90% are oral squamous cell carcinoma (OSCC), making it the sixth most prevalent cancer type in the world (2). Surgery, radiation therapy, adjuvant chemotherapy or a combination of these methods is the standard of choice for managing OSCC. Despite advances in treatment, the percentage of OSCC morbidity and mortality has not increased significantly over the past 30 years. Because OSCC has local invasion, cervical lymph node metastasis, and malignant biological characteristics such as resistance to various chemotherapeutic drugs, the 5-year survival rate of OSCC patients is only between 40% and 50% (3-5). In addition, There are also many adverse side effects during cancer chemotherapy (6). Therefore, finding effective anti-tumor drug candidates is very necessary for the progress of OSCC treatment.

Finding new anticancer drugs usually focuses on natural compounds, such as plant derived products. Plant sources have long been recognized as a powerful resource for the discovery of new cancer drugs because they contain natural compounds that may cause fewer side effects than synthetic compounds (7, 8). In traditional medicine, the roots of Sanguinaria canadensis L., commonly known as the American blood root grass, are used to treat various diseases, especially bronchitis and asthma (9). S. canadensis root extract added to toothpaste and mouthwash quickly gained worldwide attention in the early 1980s (10). The plant is also widely used in dental care products as a remineralization or anti-caries agent.

Black seed (Nigella sativa, Ranunculaceae) is an annual herb that grows in countries bordering the Mediterranean, Pakistan and India and is one of the most widely studied plants in phytochemistry and pharmacology. The seed has been used in natural remedies for more than 2000 years to promote health and treat diseases. Numerous studies have shown that the seeds and oils of this plant are very toxic (Ali & Blunden, 2003). The chemical composition of black seeds is very rich and diverse. In addition to its active ingredient crystalline nigellone, black seeds contain 15 kinds of amino acids, proteins, carbohydrates, fixed oils (84% fatty acids, including linolenic acid and oleic acid) and volatile oils, alkaloids, saponins, crude fiber, and minerals, etc. (11). Thymoquinone (TQ) is the main bioactive component of black seed volatile oil (54%). Studies have shown that TQ exerts anti-inflammatory, anti-oxidant and anti-tumor effects in vitro and *in vivo* (12, 13). Although the anti-cancer and pro-apoptotic properties of TQ have been reported in many studies, its role in OSCC has not been fully investigated. In this study, we investigated the antitumor activity of TQ in KB cells in vitro. We studied the effects of TQ on cell proliferation, invasion and migration, and studied related pathways.

Materials and Methods

Materials

Thymoquinone (purity >98%, Chengdu Pulis Biotechnology Co., Ltd.), human oral epidermoid carcinoma cell line KB (American Type Culture Collection (Manassas, MD, USA), K562 cells and MCF-7 cells were obtained from KeyGEN BioTECH (Nanjing,

China). DMEM medium, fetal bovine serum (Hyclone, USA), Verapamil (VRP, American Sigma), Tetramethylazole Blue (MTT, American Sigma), formulated with PBS 5 g/L, 4 °C protected from light, dimethyl sulfoxide (DMSO, USA Gibco), rabbit anti-PI3K, p-PI3K, Akt, pAkt, β-actin polyclonal antibody (Abcam, USA).

Cell culture and drug preparation

KB cells, K562 cells and MCF-7 cells were cultured in modified Dulbecco Eagle medium (DMEM, Hyclon, USA) containing 10% fetal bovine serum and 1% penicillin and streptomycin in a 37 °C, 5% CO_2 cell culture incubator. Thymoquinone was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A 10 mM solution was prepared in methanol and then diluted in the medium as needed to achieve the desired concentration.

Cell viability assay

Cell viability was measured using an MTT (Sigma-Aldrich Chemical Co.) assay in which blue formazan crystals were formed by mitochondrial dehydrogenase reduction. Present in living cells. Briefly, KB cells, K562 cells and MCF-7 cells were exposed to TQ for 24 hours prior to the experiment, respectively. The cells were then incubated with 0.5 mg/mL MTT solution and the cells were incubated for an additional 2 hours at 37 °C. The supernatant was removed, and the crystal precipitate was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich Chemical Co.). The absorbance of the formazan product was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

Wound healing assay

To determine whether TQ could alter the migration capacity of KB cells, 1.0×10^4 KB cells were plated in six-well plates for 24 hours, scratched with pipette tips, and then treated with different concentrations of TQ for 48 hours. The cells were photographed using a phase contrast microscope (14).

Cell invasion and motility assays

The cells after 24 hours of TQ treatment were collected and seeded in a Boyden chamber (Neuro Probe, Cabin John, Maryland), cultured in serum-free medium at 1×10^5 cells/well, and cultured for an additional 24 hours at 37 °C. For the invasion assay, Matrigel (10 mL) was applied to a polycarbonate membrane filter with a 8 mm pore size and the bottom chamber of the device contained a standard medium. The invaded cells were fixed with methanol and stained with Giemsa. Cells were photographed and counted using an optical microscope. Triplicate samples were tested and the data was expressed as the average number of cells in the five regions. Motility assay was performed following a similar procedure for the invasion assay except that there was no matrigel coating (15).

Western blotting analysis

Total cell lysates were prepared from cells treated with different concentrations of TQ for 48 h in accordance with previously described methods (16). The total cell lysates were incubated with primary antibodies, washed, and monitored through immunoblotting assays with specific secondary antibodies as previously described (17). The following primary polyclonal antibodies were used: anti-PI3K, p-PI3K, Akt andp-Akt antibodies were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, California), and b-actin antibody was purchased from Cell Signaling Technology Inc. (Danvers, Massachusetts).

Statistical method

It applied GraphpadPrism 6.0 statistical software. The measurement data were expressed as x ± SD, and the comparison between groups was performed by one-way analysis of variance, and the comparison between multiple means was performed by q test. $P < 0.05$ was considered statistically significant.

Results

Cytotoxic effect of TQ on KB cells

TQ produced certain cytotoxic effects on KB cells, K562 cells and MCF-7 cells at all concentrations tested, and this effect was dose-dependent. The greater the concentration, the greater the cytotoxicity. And the IC_{50} value of TQ against KB cells reached 3.41 ± 0.25 μM, which has better anti-OSCC cell proliferation ability.

TQ inhibits the migration and invasion of KB cells

We further examined the inhibitory effect of TQ on KB cell migration and invasion. The scratch results showed that after 24 hours of culture with normal medium without drug, the ability to significantly promote cell migration in the vicinity of scratches and the ability to scratch under the same conditions in cells cultured

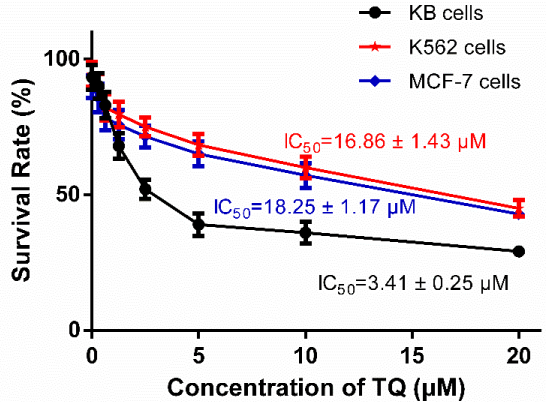

Figure 1. Cytotoxicity of the TQ against KB cells, K562 cells and MCF-7 cells determined by MTT method. Each date point is presented as mean ± SD for three independent tests. The IC_{50} value was determined with GraphPad Prism 6.0.

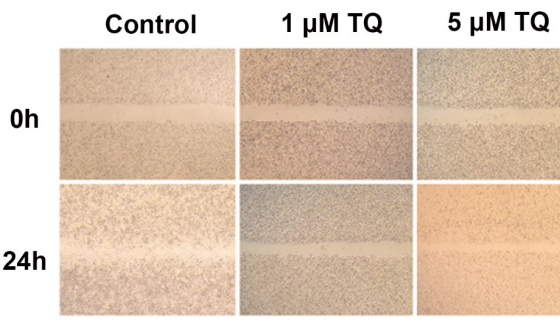

Figure 2. Effects of TQ on the migration abilities of KB cells. Another two independent experiments were conducted.

with TQ-containing medium was observed. Significantly decreased and dependent on the concentration of TQ. These results show that TQ significantly inhibits the migration of KB (Figure 2) cells.

The Boyden assay was used to detect the inhibitory effect of TQ on KB cell invasion. TQ significantly reduced KB cell invasion (cells seeded on Matrigel coated filters) (Figure 3), and inhibition was more pronounced at high concentrations. Quantitative analysis showed that 1 and 5 μM TQ treatment reduced the migration activity of KB cells by 40% ($P < 0.05$) and 78% ($P < 0.01$), respectively, and reduced the invasiveness of OSCC.

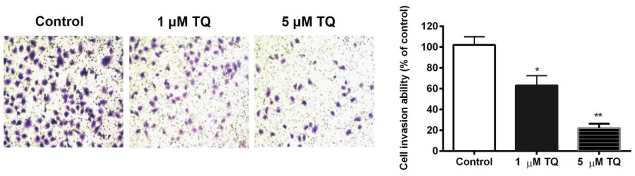

Figure 3. Effects of TQ on the invasion abilities of KB cells. Data represent the mean ± SD of at least three independent experiments. The statistical significance (*, $P < 0.05$, **, $P < 0.01$) of results were determined through Student's t test.

TQ promoted the inactivation of PI3K/Akt signaling pathway in KB cells

To further explore the pathways involved in TQ, we performed expression of related pathway proteins. Western blot results showed that phosphorylation levels of PI3K and Akt were significantly reduced after 48 hours of TQ treatment (Figure 4). However, the total expression levels of PI3K and Akt remained the same throughout the experiment. This indicates that TQ exerts its anti-tumor OSCC effect at least in part by blocking the PI3K/Akt signaling pathway in KB cells.

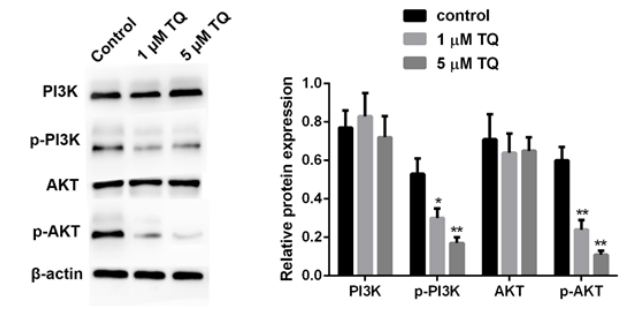

Figure 4. Effect of TQ on PI3K/Akt signaling in KB cells. (*) $P < 0.05$, (**) $P < 0.01$ relative to the control. Representative result is shown here and similar results were obtained in two other independent trials.

Discussion

OSCC is the most common form of head and neck cancer, and its subsequent metastatic tendency is associated with poor prognosis. Oral cancer-related deaths are usually caused by local recurrence or systemic metastasis (18, 19). Therefore, metastasis is a major obstacle to successful treatment of OSCC. Despite advances in treatment, the percentage of OSCC morbidity and mortality has not increased significantly over the past 30 years, and finding effective anticancer drug candidates is essential for the progression of OSCC therapy (20).

Thymoquinone (TQ) is the main bioactive component of black seed volatile oil (54%). Studies have shown that TQ exerts anti-inflammatory, anti-oxidant and anti-tumor effects in vitro and in vivo (21). Although the anti-cancer and pro-apoptotic properties of TQ have been reported in many studies, its role in OSCC has not been fully investigated. This study demonstrates for the first time that TQ can significantly inhibit the proliferation and migration of OSCC cells, inhibit the PI3K/AKT pathway, and induce apoptosis.

In this study, we treated KB cells with different concentrations of TQ and found that they significantly reduced cell viability in a concentration-dependent manner. At the same time, through scratch experiments and invasion experiments, we confirmed that TQ can inhibit the migration and invasion of KB cells. Western Blot experiments show that TQ can also achieve apoptosis through the inactivation of the PI3K/Akt pathway. The PI3K/Akt pathway is a cell survival signaling pathway that blocks apoptosis in a variety of cell types (22, 23). The dysregulation of the PI3K/Akt pathway is associated with chemoresistance (24c). Abnormal activation of this pathway involves the development and progression of several types of human malignancies, including OSCC (25, 26). The pro-apoptotic potential of some anticancer drugs is highly correlated with the inactivation of the PI3K/Akt pathway, suggesting that inhibition of the PI3K/Akt signaling cascade can be an effective strategy for cancer therapy. Therefore, we investigated whether the inactivation of the PI3K/Akt pathway is required for cytostatic inhibition by TQ. We found that phosphorylated PI3K and Akt levels were significantly reduced in TQ-treated KB cells, but total protein levels were almost identical, suggesting that PI3K/Akt may play an important role in TQ-producing anti-KB cell proliferation activity.

In summary, this study demonstrates for the first time that TQ might be a new and effective therapeutic candidate against OSCC cell proliferation and migration, and its anti-tumor effect might be mediated by the PI3K/Akt signaling pathway. This study provides a new theoretical basis and hypothesis for the clinical application of TQ in the treatment of OSCC.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Wei Luo; Xun Ren, Wei Luo collected and analysed the data; Xun Ren wrote the text and all authors have read and approved the text prior to publication.

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