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Original Article

Inhibitory effect of cryptotanshinone on lung cancer cells via Wnt β-catenin signaling

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Abstract: Objective: To explore effects of cryptotanshinone on lung cancer cells (LCCs) through the regulation of WNT/β-catenin signaling pathway. Methods: Human LCC line NCI-H1975 was cultured with various concentrations of cryptotanshinone (20, 40, and 80 μM). The proliferation, migration and invasion of cancer cells were detected using MTT, scratch assay, and Transwell assay. The expressions of β-catenin, Dvl2, Cyclin D1, and GSK-3β were evaluated by RT-PCR and immunoblotting. Results: After treatment with varying concentrations of cryptotanshinone for 24, 48, and 72 hours, compared with the blank control group (BCG), each group treated with cryptotanshinone could inhibit cell proliferation in a concentration-dependent manner ($P<0.05$). Transwell experiments showed that the migration and invasion of LCCs were significantly decreased after cryptotanshinone treatment ($P<0.05$), and the effects were dose-dependent: larger dosage of cryptotanshinone resulted in more obvious inhibition of migration ($P<0.05$). After 48 hours of cell scratching, compared with the BCG, the group treated with greater dose of cryptotanshinone showed slower wound healing rate ($P<0.05$) while the levels of Dvl2, β-catenin, and Cyclin D1 mRNA as well as its corresponding proteins were also significantly decreased ($P<0.05$). On the contrary, GSK-3β mRNA and its corresponding protein level were significantly increased ($P<0.05$). Conclusion: Cryptotanshinone can inhibit NCI-H1975 after disrupting the WNT/β-catenin pathway, which finding provides theoretical guidance for clinical experiments.

Keywords: Lung cancer cell line NCI-H1975, cryptotanshinone, inhibition, WNT/β-catenin signaling pathway

Introduction

Lung cancer is a fast-growing type of cancer worldwide. Prevention and treatment play a key role in reducing the burden of lung cancer [1, 2]. At present, due to the lack of effective treatment strategies, the mortality rate of lung cancer patients has remained high. Biomarkers for early diagnosis of lung cancer have been identified. These biomarkers have been shown to reduce lung cancer mortality, but drug therapy remains to be explored [3]. Chinese medicine has a good prospect in the treatment of lung cancer with low price. Cryptotanshinone has shown anti-inflammatory and antibacterial effects.

Compared with tanshinone IIA, cryptotanshinone has a slow distribution rate and a longer half-life, which is an excellent compound of traditional Chinese medicine and has gradually become the most important research object. Recent studies have further proved that cryptotanshinone is a novel anti-angiogenic agent, and its double bond in C-15 position of dihydrofuran ring may play a key role in inhibiting angiogenesis. It has been reported that cryptotanshinone can inhibit basic fibroblast growth factor (bFGF) in vitro without significant cytotoxicity. Angiogenesis is crucial for embryonic development and pathogenesis, which decides the survival, growth, invasion, and metastasis of primary solid tumors. Inhibition of angiogenesis is one of the important pillars of cancer treatment [4-6]. Cryptotanshinone is isolated from Salvia miltiorrhiza which promotes blood circulation and can significantly inhibit the proliferation of cancer cells, but few studies have explored the role of cryptotanshinone in human lung cancer cells (LCCs) [7, 8]. The immunohistochemistry detection found that expression of Wnt and β-catein and TCF-4 in lung cancer tissues was significantly higher than that in adjacent tissues, indicating that Wnt signaling pathway was activated in lung cancer tissues.

This study aimed to explore the therapeutic effect of cryptotanshinone at different concentrations in LCCs (LCC line NCI-H1975).

Materials and methods

Main reagents and materials

Cryptanthinone was purchased from Guangzhou Aobele Co., Ltd. (HPLC≥98%). Fetal bovine serum and trypsin were purchased from Xi'an Shengjin Co., Ltd. Human lung cancer NCI-H1975 cells were purchased from Wuhan Institute of Cell Biology. Rabbit anti-human Dvl2, GSK-3β, β-catenin, and Cyclin D1 were purchased from Abcam company.

Cell culture and grouping

The NCI-H1975 cells were incubated with DMEM solution (containing fetal bovine serum, penicillin, streptomycin, etc.), cultivated in an atmosphere of >95% humidity and 5% CO_2 at 37°C. The solution of cryptotanshinone with the designed concentration was dissolved in DMSO, sterilized, and stored at -20°C. NCI-H1975 cells were cultured in 6-well plates (10^3/well) and divided into low-dose group (20 μM), middle-dose group (40 μM), high-dose group (80 μM) and blank control group (BCG). 80 μM cryptotanshinone and 1 μM XAV939 were added to the WNT inhibitor group, and 20 μM cryptotanshinone and 20 μM SKL2001 were added to the WNT agonist group. In the above groups, six duplicate wells were set for repetition of experiments.

MTT assay for cell proliferation

NCI-H1975 in the log phase was transferred to a 96-well plate (5×10^3 per well). Each group was added with cryptotanshinone and cultured for 1 d, 2 d, and 3 d, respectively. After discarding the solution, each group was treated with 8 μL, 4 mg/mL MTT. The wells were cultured in an incubator for 5 h, and 180 μL of DMSO was added to each well which was detected by a microplate reader (wavelength 580 nm). Experiments were repeated twice, with each sample in triplicates.

Scratch assay for cell migration

Each group of cancer cells in the log phase was cultured in a six-well plate (2×10^5 per well). A 10 μL pipette tip was used to scratch a wound through the entire center of the well which was washed twice with PBS. The length at 0 h was recorded. Then the cells were cultured in serum-free medium treated with different concentrations of cryptotanshinone. After 48 hours, microscopy was used to take pictures and determine the migration ability.

Transwell assay for cell invasion

The upper surface of chamber on Transwell was coated with a thin layer of Matrigel and left overnight. The NCI-H1975 cells in the log phase were transferred to the Transwell plate, and 200 μL of the medium was added to the upper chamber. Cells treated with varying concentrations of cryptotanshinone were seeded and the lower chamber was added with 600 μL of medium, and left in saturated environment at 37°C, 5% CO_2 for 48 hours. After treatment with paraformaldehyde and methanol separately, the cells were dyed with 1% crystal viole solution for 5 min and washed 2 times with PBS. Numbers of NCI-H1975 cells that crossed through the membrane as well as the migration length were recorded.

Determination of WNT/β-catenin related protein by RT-PCR

The treated NCI-H1975 cells were cultured for 48 hours, and then the extracted cells were fully ground and added with Trizol reagent to extract the total RNA. The purity and concentration of RNA were detected by a micro-nucleic acid analyzer, and the TaKaRa reverse transcription kit was selected to reversely transcribe NRA into cDNA. Finally, the q-PCR assay was carried out according to the instructions of the reverse transcription kit to detect the expression levels of GSK-3β, Dvl2, Cyclin D1 and β-catenin in each group of cells. Primer sequences are shown in **Table 1**.

Western blot analysis of WNT/β-catenin related protein synthesis

NCI-H1975 cells were cultured with varying concentrations of cryptotanshinone. After 48 hours, the supernatant was discarded. Cells in each group were collected, and RIPA lysate was added for lysis. After lysis, the cells were centrifuged at 4°C for 15 min at 12000 r/min. The supernatant protein fluid was collected, and the protein concentration was detected using the BCA kit. The samples were placed on PVDF

Table 1. RT-PCR primers

Gene		Primer sequence (5'-3')	Amplified fragment length
Dvl2	Forward	AGGATACCACCCTTCCGTTG	109 bp
	Reverse	GGCGCCAAGTACTTTTTCAA	
GSK-3β	Forward	GACGCTCCCTGTGATTTATGTC	537 bp
	Reverse	GTTAGTCGGGCAGTTGGTGTAT	
β-catenin	Forward	CGGTACATGCATGACTGAGAC	310 bp
	Reverse	GTCACGTGGTACGACGTCAGAT	
Cyclin D1	Forward	GAGTAGTGCGAAGCATAGGTCT	455 bp
	Reverse	CTAGCACGAGTAGTCGAGCGC	
β-actin	Forward	ACGAGACCIACCTTCAACTCCATC	304 bp
	Reverse	TAGAAGCATTTGCGGTGGACGA	

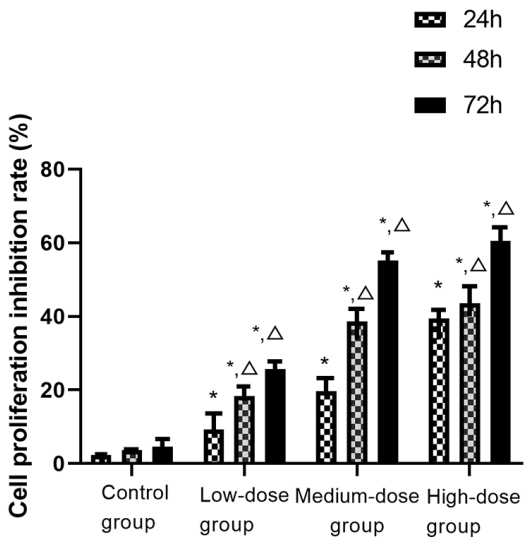

Figure 1. Effects of cryptotanshinone on proliferation of NCI-H1975 cells at different time-points. *: P<0.05, compared with BCG; Δ: P<0.05, compared with values at 24 hours.

membrane and blocked for 1 h. Dvl2, p-GSK-3β, GSK-3β, Cyclin D1, and β-catenin were prepared and the bands were incubated overnight. The secondary antibody solution was added for 2 hours of incubation, washed with TBST, and then developed in a dark room. Quantity One gel analysis software was used to process the detected film, and the absorbance of protein bands of each group was calculated. Each group of samples was processed for 3 times.

Statistical analysis

The obtained data were statistically analyzed by using SPSS 21.2. The data were expressed as mean ± standard deviation (x ± sd), and compared by t-test. The comparison among multiple groups was performed by using One-way variance. P<0.05 was considered statistically significant.

Results

Cryptotanshinone inhibits proliferation of LCCs

After 24, 48, and 72 hours of treatment, the inhibition of NCI-H1975 cell proliferation was significantly increased in the three groups with cryptotanshinone compared with the BCG (P<0.05). Higher doses showed higher inhibitory effects (**Figure 1**, P<0.05).

Cryptotanshinone inhibits migration of LCCs

The results of the Transwell experiments showed that invasion and migration ability of LCCs after treatment with cryptotanshinone were decreased (P<0.05); The higher the dose of cryptotanshinone, the worse the migration ability of LCCs (P<0.05). At 48 hours after the scratch assay, higher dose of cryptotanshinone showed slower healing rate (**Table 2**, P<0.05).

Cryptotanshinone inhibits WNT/β-catenin signaling-related mRNA expression in LCCs

Compared with the BCG, the groups treated with cryptotanshinone showed decreased expression of Dvl2, β-catenin and Cyclin D1 mRNA, and increased expression of GSK-3β mRNA. The higher the dose of cryptotanshinone, the lower the expression level of the Dvl2, β-catenin and Cyclin D1 mRNA and the higher the expression level of GSK-3β mRNA (**Table 3**, P<0.05).

Cryptotanshinone inhibits expression of WNT/β-catenin signaling pathway-related proteins in NCI-H1975 cells

The groups treated with cryptotanshinone showed reduced Dvl2, p-GSK-3β, β-catenin, and Cyclin D1 protein expression levels compared with the blank control (P<0.05), while the expression levels of GSK-3β protein increased significantly (P<0.05); Higher dose of cryptotanshinone resulted in lower expression levels of Dvl2, β-catenin Cyclin D1 mRNA and higher expression level of GSK-3β mRNA (**Figure 2**).

Table 2. Transwell and scratch assay ($\bar{x}$ ± sd)

Grouping	Transwell		Wound healing rate
	Migration (48 h)	No. of cells across the membrane	
BCG	90.43±4.32	76.34±6.64	60.43±4.38
Low-dose	46.19±6.89	42.72±6.06	48.19±5.32
Middle-dose	26.69±5.08	23.88±5.63	35.69±5.13
High-dose	18.32±1.23	16.35±3.19	22.32±4.31
F	106.46	62.339	80.912
P	<0.05	<0.05	<0.05

Table 3. Detection of WNT/β-catenin-related mRNA expression in NCI-H1975 cells by RT-PCR

Grouping	RT-PCR			
	Dvl2	GSK-3β	β-catenin	Cyclin D1
Blank control	1.43±0.01	0.34±0.04	1.21±0.14	1.42±0.11
Low-dose	1.19±0.02	0.72±0.06	1.03±0.02	1.08±0.10
Middle-dose	0.85±0.08	0.98±0.03	0.86±0.10	0.83±0.01
High-dose	0.62±0.13	1.35±0.09	0.52±0.03	0.49±0.05
F	8.46	6.33	9.21	9.52
P	<0.05	<0.05	<0.05	<0.05

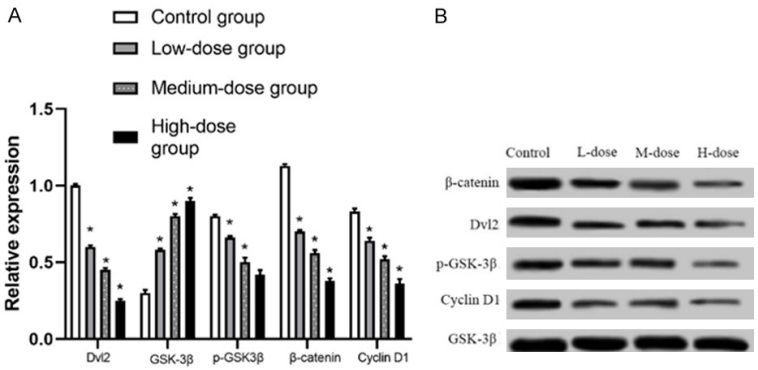

Figure 2. Synthesis of WNT/β-catenin related proteins. Compared with the BCG, the synthesis levels of Dvl2, p-GSK-3β, β-catenin and Cyclin D1 protein in the low dose, medium dose and high dose groups were significantly decreased (P<0.05), and the expression levels of GSK-3β protein were significantly increased (P<0.05) (A). The comparison between different intervention dose groups showed that with the increase of the dosage of cryptotanshinone, the synthesis levels of Dvl2, p-GSK-3β, β-catenin and Cyclin D1 protein showed a significnat decreasing trend, while the expression levels of GSK-3β protein showed an increasing trend (B). * indicates that compared with the BCG, the difference was statistically significant.

Effect of cryptotanshinone on the invasion and migration ability of NCI-H1975 after inhibiting or activating the WNT/β-catenin pathway

Transwell invasion experiments showed that after treatment with SKL2001 as the agonist of the Wnt/β-catenin pathway, the LCCs showed a significantly higher invasive ability than the high-dose group and are close to the BCG.

After XAV939 inhibited the WNT/β-catenin signaling pathway, the number of invasion cells was significantly lower than that of the BCG and closer to that of the high-dose group (**Figure 3**).

Discussion

Biologically active components have been identified in salvia miltiorrhiza, and tanshinone is a representative monomeric compound [9, 10]. Studies found that it has a significant influence on removing oxygen free radicals and improving immunity [11]. In studies of malignant tumors such as prostate cancer, cryptotanshinone could kill tumor cells, but few studies have reported its role in lung cancer treatment [12, 13]. In this study, effects of cryptotanshinone were explored with human LCC line NCI-H1975.

The WNT/β-catenin pathway is a prominent player in multiply biological activities such as cell proliferation and adhesion [14]. The abnormal activation of WNT/β-catenin pathway is often observed in many cancer patients. This phenomenon is related to abnormal WNT protein synthesis as well as the β-catenin nuclear transport [15-17]. WNT protein is a signaling molecule for cell growth. It can interact with Frz protein and activate NCI-H1975 Dvl2 protein under the catalysis of LRP protein, which activates the signal pathway in NCI-H1975 cells, thus the activity of GSK-3β is weakened to inhibit β-catenin degradation [18, 19]. In extracellular matrix, β-catenin protein and E-cadherin could form an adhesion complex, improving cell adhesion [20, 21]. B-catenin in the cytoplasm is transported into

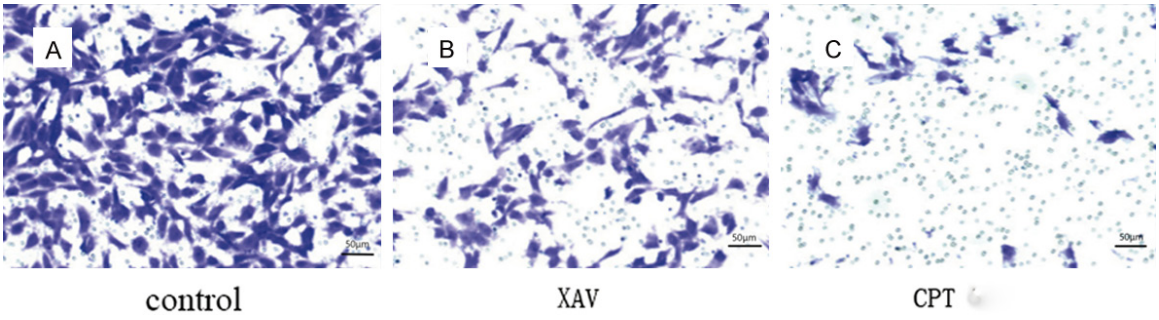

Figure 3. The detection of invasion ability of LCCs in blank control, xav, and high-dose groups of NCI-H1975 cells by Transwell assay (Trypan blue staining × 100) (A-C).

the nucleus, excites target genes such as downstream Cyclin D1, and improves cell proliferation and migration capacity [22-25].

In this experiment, after 24, 48, and 72 hours of treatment, three groups with cryptotanshinone showed significantly increased inhibitory effect of NCI-H1975 cell proliferation than the BCG ($P<0.05$). Higher dosage of cryptotanshinone indicated higher inhibitory effects ($P<0.05$). Transwell experiment showed that after treating with different doses of cryptotanshinone, the invasion and migration ability of LCCs were significantly decreased ($P<0.05$), and the higher dose of cryptotanshinone resulted in the worse migration ability of LCCs, showing significant difference ($P<0.05$).

After treatment with varying doses of cryptotanshinone, the synthesis of NCI-H1975 Dvl2, β-catenin, and Cyclin D1 mRNA in each group was significantly lower than that in the BCG. GSK-3β inhibits the β-catenin protein and thereby inhibits the proliferation of NCI-H1975. It can be speculated that cryptotanshinone may prevent the synthesis of Dvl2 and thus the phosphorylation of GSK-3β. This can further inhibit β-catenin and Cyclin D1, which eventually inhibits cancer cell proliferation.

In order to confirm that cryptotanshinone functions via the WNT/β-catenin pathway, pathway-specific inhibitor and agonist were used to inhibit and activate the WNT/β-catenin signaling pathway, respectively. After the WNT/β-catenin signaling pathway was activated by SKL2001, the invasion ability of LCCs was significantly higher than that of the high-dose group; and after XAV939 inhibited the WNT/β-catenin signaling pathway, the number of invasion cells was significantly lower than that of the BCG and was close to that of high-dose group.

In summary, it was found that cryptotanshinone can effectively block the WNT/β-catenin signaling pathway and inhibit the proliferation and migration of NCI-H1975 cells, which finding provides scientific basis for further administration of cryptotanshinone in the treatment of lung cancer.

Disclosure of conflict of interest

None.

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