ORIGINAL ARTICLE

Research on the inhibiting effect of tanshinone IIA on colon cancer cell growth via COX-2-Wnt/β-catenin signaling pathway

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Summary

Purpose: To investigate the effects of tanshinone IIA (TSI-IA) on the colon cancer cell growth and to explore the mechanism of TSIIA in regulating the colon cancer cell growth via cyclooxygenase (COX)-2-Wnt/β-catenin signaling pathway.

Methods: Colon cancer cell line HC8693 was exposed to different concentrations of TSIIA. After 24-hr exposure, MTT assay was used to detect the lethal concentration of TSIIA on HC8693 cells. The expression levels of COX-2 and β -catenin were detected by semi-quantitative PCR (sq-PCR). The protein expression levels of COX-2 and β -catenin were detected by Western blot, and the expression of vascular endothelial growth factor (VEGF) was detected by enzymelinked immunosorbent assay (ELISA). Celecoxib, a COX-2 selective inhibitor, was used to inhibit the COX-2 of HC8693 cells, and the inhibiting effect of TSIIA on HC8693 cell growth was assessed.

Results: MTT assay showed that TSIIA concentration of 20µM inhibited significantly the HC8693 cell growth

(p<0.01). With reverse transcription after RNA extraction and (sq-PCR) detection the expression levels of COX-2 and β -catenin were significantly decreased (p<0.01). Western blot showed that the protein expression levels of COX-2 and β -catenin were significantly decreased (p<0.01). ELISA showed that the expression of VEGF was also significantly decreased (p<0.01); after celecoxib (10 μ M) was added, 20 μ M TSIIA had no inhibiting effect on the growth of HC8693 cells (p>0.05). Western blot showed no significant differences in the protein expression levels of COX-2 and β-catenin compared with those in the control group (not exposed to TSIIA).

Conclusions: TSIIA can inhibit the expression of COX-2 and activate the Wnt/ β -catenin signaling pathway, thus downregulating the level of VEGF, and resulting in growth inhibition of colon cancer cells.

Key words: colon cancer, tanshinone IIA, Wnt/β-catenin signaling pathway

Introduction

variety of factors. According to the World Health Organization, the incidence of colon cancer ranks third among malignant tumors [1,2]. With the development of medical technology, great progress has been made in the diagnosis and treatment of this disease. Radiotherapy, chemotherapy and surgical resection have significantly prologned the 5-year survival of colon cancer patients, but the tivities in a variety of tumors. Zhang et al. [7] stud-

Colon carcinogenesis is closely related to a recurrence rate and mortality of colon cancer are still high [3,4], so the development of new treatment methods and chemotherapeutics used for the treatment of this malignancy are particularly important. TSIIA is a monomer component extracted from Salvia miltiorrhiza, with effect in promoting blood circulation [5,6]. A large number of studies have also shown that TSIIA exerts anticancer ac-

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ied and found that TSIIA can significantly reduce the survival rate of colon cancer cells, while Duan et al. [8] found that TSIIA can affect the expression level of COX-2 in breast cancer cells. However, there has been no report on the mechanism of colon cancer cell death caused by TSIIA.

This study aimed to investigate the mechanism of colon cancer cell death caused by TSIIA, and to also clarify its mechanism of affecting the growth of these cells through COX-2-Wnt/ β -catenin signaling pathway, so as to provide new ideas for the development of TSIIA as new clinical treatment option of colon cancer.

Methods

Instruments and reagents

The following instruments and reagents were used in this study: Colon cancer cell line HC8693 was purchased from Shanghai Cell Bank (Chinese Academy of Sciences, China); MTT, DMSO and celecoxib were purchased from Sigma (Sigma, St.Louis, MO, USA); TSIIA was purchased from Shanghai Alladin Biochemical Technology Co., Ltd (Shanghai, China); TRIzol kit and reverse transcription kit were purchased from Invitrogen (Invitrogen, USA); ELISA kit was purchased from R&D System, Minneapolis, USA; rabbit anti-COX2, rabbit anti- β catenin and GAPDH were purchased from Cell Signaling Technology Co Ltd. (Danvers, MA, USA); ECI luminescent solution and inverted fluorescence microscope were purchased from Invitrogen; cell culture bottle was purchased from Corning, NY, USA. Pipette (Eppendorf, NY, USA) and PCR instrument were purchased from ABI (ThermoFisher, Waltham, MA, USA); ultraviolet imagery system was purchased from Biometra (Konrad-Zuse-Strasse 1, Germany), and electronic scales from BP121S, Sartorious, Germany. Other related instruments and reagents are being described in the relevant sections.

Detection of COX-2 and β -catenin expression levels via semiquantitative PCR

After the protein was extracted from the cells treated by 20 µM TSIIA for 24 hrs using TRIzol, the supernatant was centrifuged at 12000 rpm for 10 min. The integrity of RNA was confirmed by agarose gel electrophoresis. The results of electrophoresis showed that the bands of 28S, 18S and 5S were clear, and the brightness of band 28S was about 2-fold of that of 18S, suggesting that the RNA was complete and could be used for follow-up experiments. Reverse transcription kit was used to obtain cDNA. The expression levels of COX-2 and β -catenin were detected by sqPCR with GAPDH as the internal reference. The reaction conditions were as follows: 95°C for 30 s, 64°C for 25 s, 72°C for 30 s, and a total of 35 cycles. The primers were synthesized by Tiangen Biotechnology Co., Ltd. (Beijing, China), and the sequences are shown in Table 1. After the reaction, agarose gel electrophoresis was performed and observed by UV imaging system.

	Sequence
COX-2	Forward primer:
	5'- ATCCAGAGACAAGACATGTAC-3'
	Reverse primer:
	5'- TTCAGATGTTCTAAGCCTACGG-3'
β-catenin	Forward primer:
	5'- TGGCGGTTTGCGGTGGAC-3'
	Reverse primer:
	5'- CCAGTGCAGGGTCCGAGGT-3'
GAPDH	Forward primer:
	5'- GATGATTGGCATGGCTTT -3'
	Reverse primer:
	5'- CACCTTCCGTTCCAGTTT-3'

Detection of the inhibiting effect of TSIIA on HC8693 cell growth via MTT

HC8693 colon cancer cell line was cultured in incubator and the cells at the logarithmic growth phase were inoculated onto the 96-well plate (6000 cells/well) after subculture. After 24 hrs, the HC8693 cells were starved for 2 hrs, and TSIIA with different concentrations (100, 50, 20, 10, 5, 1 and 0.1 μ M) was added; the blank control group and positive drug group (cisplatin) were set up. After incubation with the drug for 24 hrs, MTT 1% was added in the dark and the cells were placed in the incubator and continued to be cultured for another 4 hrs; then the medium was discarded, 150 μ I dimethyl sulfoxide (DMSO) were added to each well, and the microplate reader was used to detect the optical density at 570 nm after shaking for 10 min.

Detection of the protein expression levels of COX-2 and β -catenin via Western blot

Cells at the logarithmic growth phase were inoculated onto 6-well plate, and the blank control group and TSIIA group (20 μ M) were set up. After 24 hrs of drug treatment, the protein was extracted and quantified. Then it was separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membrane. After sealing for 2 hrs, the target band was cut off and incubated overnight at 4°C using target protein antibody, followed by triple washing with TBST (5min/time), and incubated at room temperature for 2 hrs using secondary antibody. A similar triple TBST washing followed, and the appropriate amount of ECL luminescent solution (solution A and solution B were mixed in a ratio of 1: 1) was added in the dark, followed by pressing (the pressing time was determined according to the fluorescence intensity of protein band), development and fixation. The gray value of band was analyzed using Image J software after scanning.

Detection of protein expression level of VEGF via ELISA

Cells at the logarithmic growth phase were inoculated onto the 24-well plate, and the blank control group and TSIIA group ($20 \mu M$) were set up. After 24 hrs of drug treatment, the supernatant was collected and VEGF expression level in cells was detected by ELISA according to the manufacturer's instructions.

Statistics

The data in this study were presented as mean ±standard deviation. SPSS19.0 software was used for data processing. Student's t-test was used for quantitative data and chi-square test was used for data enumeration. One-way ANOVA was used for other numerical data. The homogeneity test was also performed. Bonferroni's method was used for pairwise comparison under the homogeneity of variance, while Welch method was used under the heterogeneity of variance. Dunnett's T3 method was used for multiple comparisons. P<0.05 indicated that the different was statistically significant.



Figure 1. The effect of TSIIA on HC8693 colon cancer cell viability was detected by MTT. When the concentration of TSIIA reached 20 μ M, the survival rate of HC8693 cells was decreased significantly (**p<0.05). This concentration was used as the standard concentration in the follow-up experiments (***p<0.01).

Results

Inhibiting effect of TSIIA on HC8693 cell growth

The inhibiting effect of TSIIA on the growth of HC8693 colon cancer cells was detected by MTT and the results are shown in Figure 1. The concentrations of TSIIA were 100, 50, 20, 10, 5, 1 and 0.1 μ M, respectively, and the blank control group and positive control group (cisplatin) were set up. The results showed that when the concentration of TSIIA reached 20 μ M, the growth of HC8693 cells was inhibited significantly (p<0.01).

Detection of expression levels of COX-2 and β -catenin via sqPCR

After RNA of HC8693 cells was treated by TSIIA, the relative expression levels of COX-2 and β -catenin were detected by sqPCR, with GAPDH as the internal reference. The results showed that, compared with those of the blank control group, the relative expression levels of COX-2 and β -catenin in HC8693 cells treated by TSIIA (20 μ M) were significantly decreased (p<0.01) (Figure 2).

Detection of protein expression levels of COX-2 and β -catenin via Western blot

The protein expression levels of COX-2 and β -catenin in HC8693 cells treated with TSIIA



Figure 2. Detection of influence of TSIIA on the expression quantities of COX-2 and β -catenin via semi-quantitative PCR. Agarose gel electrophoresis image is shown in **A**. The relative expression quantities of COX-2 and β -catenin are shown in **B** and **C**, respectively. mRNA expression levels of COX-2 and β -catenin in colon cancer cells were significantly decreased after treatment with TSIIA (**p<0.01).



Figure 3. Detection of protein expression levels of COX-2 and β -catenin via Western blot. Western blot strip is shown in **A**, and statistical graphs are shown in **B** and **C**. The results showed that the protein expression levels of COX-2 and β -catenin in colon cancer cells were significantly decreased after treatment with TSIIA (**p<0.01).

(20 μ M) were detected by Western blot and the results are shown in Figure 3. Compared with those in the blank control group, the protein expression levels of COX-2 and β -catenin in HC8693 cells treated with TSIIA were significantly decreased (p<0.01).



Figure 4. Detection of expression level of VEGF via ELI-SA. The results showed that the expression level of VEGF in colon cancer cells was significantly decreased after treatment with TSIIA (**p<0.01).



Figure 5. The effect of TSIIA combined with celecoxib on the viability of colon cancer cells was detected by MTT. Celecoxib (CXB) (10 μ M) alone had no effect on the growth of colon cancer cells (p>0.05). TSIIA (20 μ M) could significantly inhibit the growth of colon cancer cells (**p<0.01). When TSIIA (20 μ M) was combined with CXB (10 μ M), the inhibitory effect of TSIIA on the viability of colon cancer cells disappeared (p>0.05).

Detection of VEGF expression level via ELISA

The protein expression level of VEGF in HC8693 cells treated with TSIIA ($20\mu M$) was detected by ELISA and the results are shown in Figure 4. Compared with the blank control group, the protein expression level of VEGF in TSIIA-treated HC8693 cells was significantly decreased (p<0.01).

Effect of TSIIA combined with celecoxib

Cells in the logarithmic growth phase were incubated into 96-well plate, and the blank control group, TSIIA group (20 μ M), celecoxib group (10 μ M) and celecoxib (10 μ M) + TSIIA group (20 μ M) were set up. The cell survival rate was detected by MTT after 24 hrs of treatment. The results are shown in Figure 5. The number of cells in the celecoxib group (10 μ M) + TSIIA group (20 μ M) had no statistically significant difference compared with the blank control group (20 μ M) had a statistically significant difference compared with the blank control group (20 μ M) had a statistically significant difference compared with the blank control group (20 μ M) had a statistically significant difference compared with the blank control group (p<0.01).

Effect of TSIIA combined with celecoxib on the protein expression levels of COX-2 and β -catenin

Cells in the logarithmic growth phase were incubated into the 96-well plate, and the blank control group, TSIIA group (20 μ M), celecoxib group (10 μ M) and celecoxib (10 μ M) + TSIIA group (20 μ M) were set up. The protein was extracted after 24 hrs of treatment, and the protein expression levels of COX-2 and β -catenin were detected by Western blot. The results are shown in Figure 6. The protein expression levels of COX-2 and β -catenin in the celecoxib group (10 μ M) and celecoxib (10 μ M) + TSIIA group showed no statistically significant differences compared with the blank group (p>0.05), but the protein expression levels of COX-2



Figure 6. The effect of TSIIA combined with celecoxib (CXB) on protein expression levels of COX-2 and β -catenin was detected by Western blot. Western blot strips are shown in **A**, and statistical graphs are shown in **B** and **C**. The results showed that the single application of CXB (10 μ M) had no effect on the protein expression levels of COX-2 and β -catenin (p>0.05). TSIIA (20 μ M) could significantly decrease the protein expression levels of COX-2 and β -catenin (**p<0.01). TSIIA (20 μ M) combined with CXB (10 μ M) had no effect on the protein expression levels of COX-2 and β -catenin in colon cancer cells (p>0.05).

and β -catenin in TSIIA group (20 μ M) had statistically significant differences compared with the blank group (p<0.01).

Discussion

Colon cancer cells require a lot of energy for their growth and infiltration process. At present, a large number of studies have shown that reducing the blood and oxygen supply of tumor cells can lead to cell death, which can be exploited for tumor treatment [9,10]. VEGF and angiogenin are necessary factors in the process of angiogenesis in vivo, and they can promote the formation of blood vessels *in vivo* [11]. Angiogenesis inhibitors can lead to the inhibition of vascular growth and there is a certain balance between VEGF and angiogenesis inhibitors in vivo, and when such a balance is destroyed, angiogenesis disorders in vivo will emerge [12,13]. Li et al. [14] studied and found that TSIIA can cause death of lung cancer cells and inhibit their migration and infiltration capacities. TSIIA can downregulate the level of COX-2 *in vivo*, thus leading to decrease of VEGF expression, resulting in insufficient blood and oxygen supply in tumor cells and causing tumor cell apoptosis. Xie et al. [15] also found in a sarcoma model that TSIIA affects the proliferation and migration of tumor cells, and after the addition of COX-2 selective inhibitor, the proliferation and migration capabilities of tumor cells are not affected by TSIIA. In recent years, a large number of studies have shown that the formation of intestinal tumors is closely related to COX- $2/\beta$ -catenin/TCF pathway, and this signaling pathway also affects the formation of vascular growth factors in vivo [16-18].

This study aimed to investigate the effect of TSIIA on the growth of HC8693 colon cancer cells and to explore whether COX-2-Wnt/ β -catenin signaling pathway is involved in the inhibiting effect of TSIIA. When the concentration of TSIIA reached 20 μ M, the growth of colon cancer cells was significantly inhibited, indicating that the inhibiting effect of TSIIA on these cells is definite, therefore carrying the potential of TSIIA development into a new drug for the treatment of colon

cancer. Another finding through Western blot assay was that the expression level of COX-2 could be significantly downregulated by TSIIA. COX-2 is an important rate-limiting enzyme in the synthesis of prostaglandins, which is not expressed or expressed little in most cells. COX-2 can produce prostaglandin PEG₂ after metabolism, which can increase the proliferation of cells and reduce the cell death. TSIIA can downregulate the expression of COX-2, which results in inhibition of colon cancer cells growth. It was later found that TSIIA can also cause a decrease in the expression of β -catenin, and Wnt/ β -catenin signaling pathway is a highly-conserved cell signaling system [19]. Studies [20,21] have shown that TSIIA is closely related to a variety of diseases, the proliferation and migration of tumor cells are closely related to the high expression of β -catenin, and β -catenin can also cause decreased expression of VEGF and lead to blocking of angiogenesis in tumor cells, thus affecting tumor cell growth [22,23]. In this study, the sqPCR showed that the mRNA expression levels of COX-2 and β -catenin were downregulated, paralleling the trend of protein expression. The COX-2 selective inhibitor celecoxib could specifically inhibit COX-2. When both TSIIA and celecoxib were used in colon cancer cells, the inhibiting effect of TSIIA on colon cancer cell growth disappeared, and its effects on the expression of COX-2 and β-catenin also disappeared. These results suggest that TSIIA downregulated the expression of COX-2 and activated the Wnt/β-catenin signaling pathway, leading to decreased VEGF expression, so angiogenesis was blocked, which affects the growth of colon cancer cells. However, there is no in-depth study on downstream proteins of Wnt/β-catenin signaling pathway and VEGF-related proteins in this article, which will be the focus in our future study. It is hoped that the mechanism of action of TSIIA on colon cancer can be fully clarified, so as to provide the experimental basis for the treatment of colon cancer with TSIIA.

Conflict of interests

The authors declare no conflict of interests.

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