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Original Article

Tanshinone IIA suppresses the invasion and growth of human hepatocellular carcinoma HepG2 and BEL-7402 cells by inhibiting Src kinase activity

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Abstract: Tanshinone IIA (T2A), a natural product obtained from plant Danshen (*Salvia miltiorrhiza*), which has a variety of pharmacological effects, including anti-cancer, and selectively inhibiting cell migration in a variety of human cancer cells. However, its mechanism of action needs to be further elaborated. In this study, we investigated the effects of Tanshinone IIA on the cell invasion inhibiting in human HepG2 and BEL-7402 cells. Here, we found that tanshinone IIA can significantly inhibit cell proliferation, cell migration and cell invasion, induced G1/S phase arrest. Moreover, tanshinone IIA inhibited the Src-FAK pathway by decreasing the Src kinase activity. Tanshinone IIA also triggered significant reduction the phosphorylation of p38MAPK and Akt in HepG2 and BEL-7402 cells. Simultaneously, tanshinone IIA down-regulated protein and mRNA expression of p53 and CDK1, increased expression level of p21. These results suggest that tanshinone IIA might be a potential therapeutic candidate for the treatment of hepatocellular carcinoma metastasis.

Keywords: Hepatocellular carcinoma-invasion-Tanshinone IIA-Src

Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors and the third leading cause of cancer-related deaths in the worldwide [1]. Despite advances in surgical techniques, systemic chemotherapy and/or radiotherapy, radical surgery have been made in HCC, the over-all 5-year survival rate of HCC patients has not significantly improved [2, 3]. As shown by the World Cancer Report, hepatocellular carcinoma is still one of the most common cancers with the highest mortality. So effective chemotherapeutic agents are needed to improve the cure rate of patients with hepatocellular carcinoma.

Tanshinone IIA (T2A) is an extract from a widely used traditional Chinese medicine which extracted from the plant Danshen (*Salvia miltiorrhiza*) [4]. It was found to have anti-tumor effect activity in various human cancer cell types, including breast cancer, prostate cancer, colorectal cancer and lung cancer [5-8]. Therefore, tanshinone IIA (T2A) is a natural anti-tumor product potentially having high practical values. However, the regulatory mechanism of T2A in hepatocellular carcinoma cells remains unclear.

In the present study, we tried to investigate the inhibitory effect of T2A on human hepatocellular carcinoma cells, Moreover, the underlying molecular mechanisms of T2A was explored by western blot and qRT-PCR assay.

Materials and methods

Cell lines and cell culture

Human HepG2 and BEL-7402 were purchased from Shanghai Institute for Biological Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium (containing 10% fetal calf serum) and cultured in an incubator under 5% CO_2, 37°C and saturated humidity conditions, followed by digestion with 0.25% trypsin-EDTA for passaging. Cells in logarithmic growth phase were used in all experiments.

Cell viability assay

The effect of tanshinone IIA (T2A) on cell viability was determined using the Cell Counting Kit-8

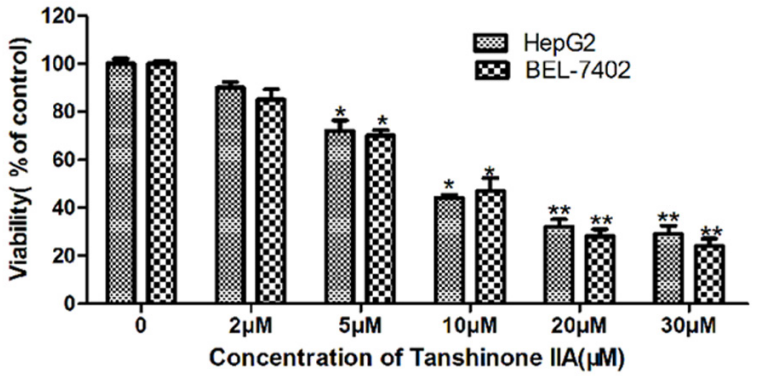

Figure 1. T2A inhibits the proliferation of hepatocellular carcinoma cells. HepG2 and BEL-7402 cells were treated with various concentrations of Tanshinone IIA (T2A) for 24 h. Cell viability was detected using a CCK-8 kit. The 24 h treated IC50 of T2A on HepG2 and BEL-7402 cells was 8.12 μM and 7.01 μM, respectively.*$P < 0.05$ and **$P < 0.01$ where compared with control.

(CCK-8, Dojindo Laboratories, Kumamoto, Japan). After 24 h incubation, HepG2 and BEL-7402 cells were treated with 0, 2 μM, 5 μM, 10 μM, 20 μM, 30 μM of T2A for 48 h, and then the cells were added with 10 μL CCK-8 solution for additional 2 h incubation at 37°C, and plates were read with a microplate reader at 450 nm. The percentage of cell viability was calculated against control.

Invasion and migration assay

Cell invasion assay was performed in a 24-transwell plate (Corning) coated with 50 μl matrigel (Corning), according to the manufacturer's instructions. The 20% fetal calf serum were added in the bottom chambers, 1×10^5 HepG2 and BEL-7402 cells with or without different concentrations of T2A were seeded into the upper chambers. The cells that invaded on the lower side of the insert membranes were fixed and stained with 2% crystal violet in ethanol. The number of invading cells was determined as described previously. The data are the mean ± S.D from three independent experiments.

Flow cytometry

After the cells were treated with various concentrations of T2A for 24 h, the cells were collected and washed twice with cold PBS. For cell cycle assay, cell pellets were resuspended at 1×10^6 cells/mL and fixed in 70% cold ethanol overnight at 4°C. Following the fixation, cells were stained with propidium iodide (PI). For cell apoptosis assay, cell pellets were doubly stained with propidium iodide (PI) and Annexin V, and then analyzed using a FACScan apparatus (Becton Dickinson).

Western blotting assay

Cells were washed with ice-cold PBS for three times and lysed with RIPA lysis buffer for 30 minutes at 4°C. Equal quantities of proteins, determined by using BCA method. Equal amounts of proteins were separated in 12% SDS-PAGE and then, transferred onto PVDF membrane; the membrane was incubated with indicated antibodies at 4°C overnight and then, incubated with specific HRP-streptavidin-conjugated sec Buckinghamshire, UK).

Real-time PCR assay

Total RNA was isolated from cell lines by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out using the Prime-Script RT reagent kit (TaKaRa, Otsu, Japan). Real-time quantitative RT-PCR was performed using the ABI 7900HT system (ABI) according to the manufacturer's instructions. The sequences of the forward and reverse primers for CDK1 (forward) 5'-CATGGCT ACCACTTGACCTGT-3' and (reverse) 5'-AAGCC-GGGATCTACCATACC-3'; p21 (forward) 5'-CATG-GGTTCTGACGGACAT-3'and (reverse) 5'-AGTC-AGTTCCTT GTGGAGCC-3'; p53 (forward) 5'-GC-TCGACGCTAGGATCTGAC-3' and (reverse) 5'-GC-TTTCCACGACGGTGAC'; β-actin (forward) 5'-GG-CATCGTCACCAACTG GGAC-3' and (reverse) 5'-CGATTTCCCGCTCGGCCGTGG-3'. The expression levels of CDK1, p21 and p53 were normalized to the β-actin internal control.

Statistical methods

The experimental data were represented as mean ± SD; SPSS 21.0 software was used for analysis. Student's *t* test was used for comparison. $P < 0.05$ indicated statistically significant difference.

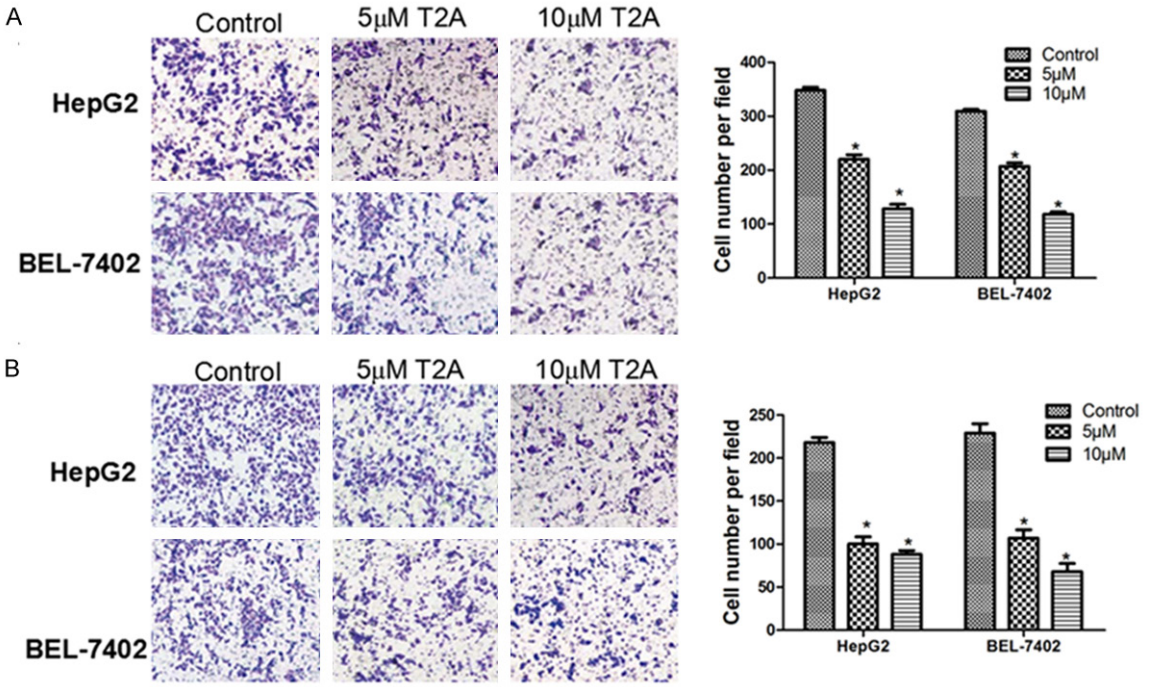

Figure 2. T2A suppressed the invasion of HepG2 and BEL-7402 cells. HepG2 and BEL-7402 cells were seeded in the top chamber and treated with 5 μM and 10 μM T2A for 24 h, cell invasion (A) and migration (B) were detected by transwell, and the invaded cells were stained and photographed. *$P < 0.05$ where compared with control.

Results

T2A inhibits the proliferation of hepatocellular carcinoma cells

To assess the anti-proliferation effect of Tanshinone IIA (T2A) on hepatocellular carcinoma cells. We treated hepatocellular carcinoma cell lines HepG2 and BEL-7402 with 2 μM, 5 μM, 10 μM, 20 μM and 30 μM T2A for 24 h. As showed in **Figure 1**, 24 h T2A treatment resulted in significant cell viability inhibition in both HepG2 and BEL-7402 cells.

T2A inhibits migration and invasion of hepatocellular carcinoma cells in vitro

To evaluate the effect of T2A on migration and invasion, we selected 5 μM and 10 μM concentrations for below experiments. It was shown that T2A suppressed migration and invasion of HepG2 and BEL-7402 cells significantly (**Figure 2**).

T2A Induces cell-cycle arrest in hepatocellular carcinoma cells.

We next analyzed the DNA content and cell-cycle profile of HepG2 and BEL-7402 cells treated with T2A by flow cytometry. As shown in **Figure 3**, HepG2 and BEL-7402 cells displayed distinct changes in the percentages of cells in each cell-cycle phase on treatment with T2A, and the cell cycle was arrested at G1/S phase.

T2A suppresses cell invasion by inhibiting Src-FAK pathways

Src is a non-receptor cytoplasmic tyrosine kinase that is essential for the regulation of cell migration and invasion [12]. To identify whether T2A inhibits Src kinase activity,we examined the phosphorylation of Src in HepG2 and BEL-7402 cells. As shown in **Figure 4**. T2A reduced the phosphorylation levels of Src significantly. Src could bind FAK directly and activated activity of FAK, which is a cytoplasmic tyrosine kinase required for cell migration and invasion. We also found that T2A reduced the phosphorylation levels of FAK. Taken together, these results suggest that T2A inhibits the migration and invasion in HepG2 and BEL-7402 cells by inhibiting the Src-FAK pathways.

T2A inactivates the MAPK pathway by inhibiting the phosphorylation of p38, and AKT

The previous study demonstrated that the MAP pathway played an important role in cell migration. To identify whether T2A inhibits the MAPK

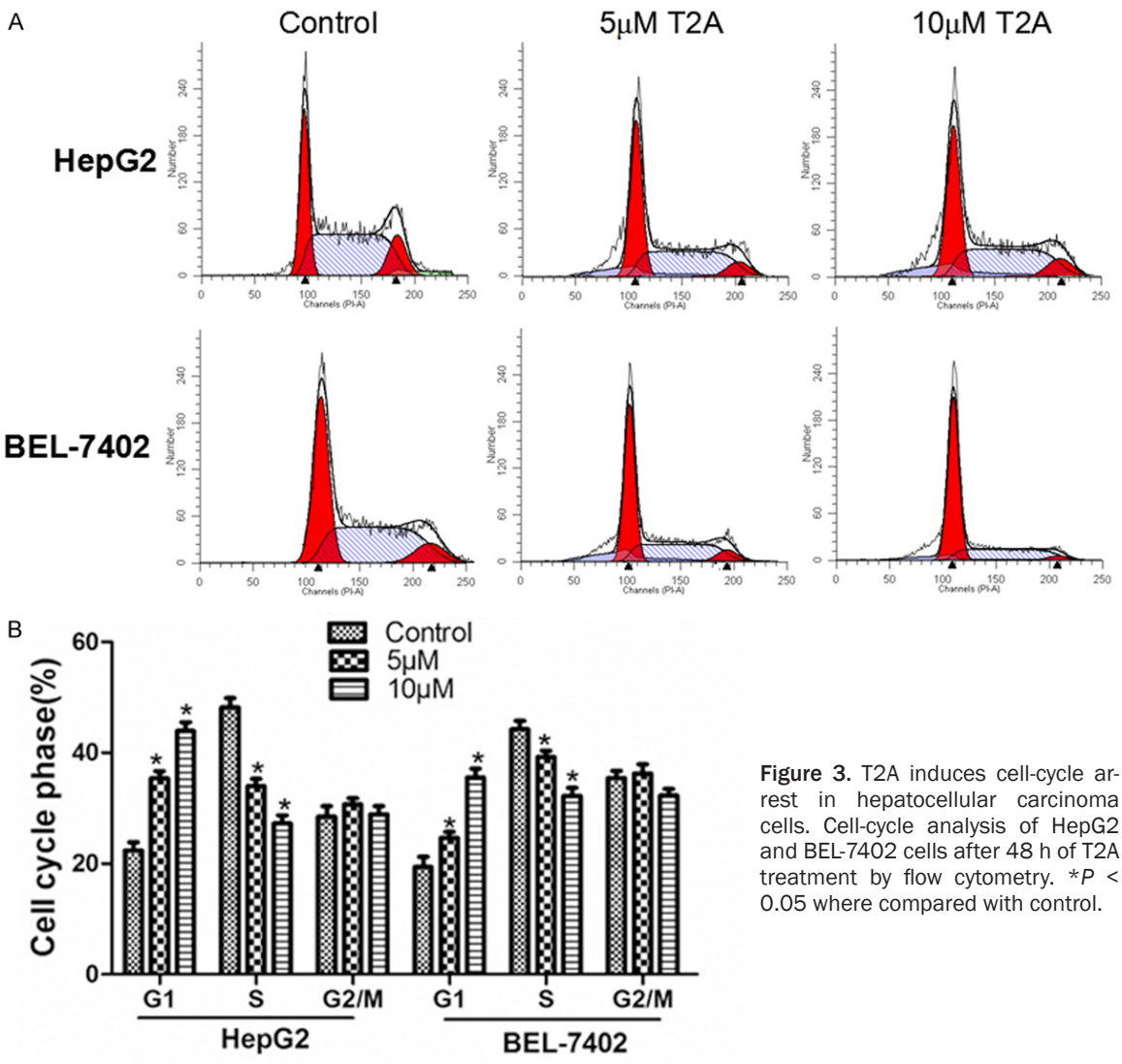

Figure 3. T2A induces cell-cycle arrest in hepatocellular carcinoma cells. Cell-cycle analysis of HepG2 and BEL-7402 cells after 48 h of T2A treatment by flow cytometry. *P < 0.05 where compared with control.

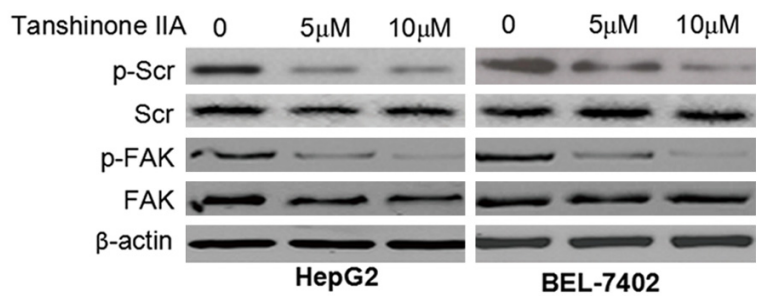

Figure 4. T2A suppresses cell invasion by inhibiting Src-FAK pathways. The HepG2 and BEL-7402 cells were treated without or with various concentrations of T2A for 48 hours and then the Src and FAK kinase activity was detected by western blot assay.

pathway, we examined the phosphorylation of p38MAPK and p-JUN in HepG2 and BEL-7402 cells by western blot assay. As shown in **Figure 5**, T2A reduced the phosphorylation levels of p38MAPK and JUN significantly after 48 h treated.

T2A suppresses the growth of hepatocellular carcinoma cells by regulating the proteolytic activity and mRNA expression of p53, p21 and CDK1

It was reported that p53, p21 and CDK1 play an important role in cell proliferation and cell cycle. To determine whether T2A inhibits the expression of p53, p21 and CDK1, we examined the protein and mRNA expression level by western blot assay

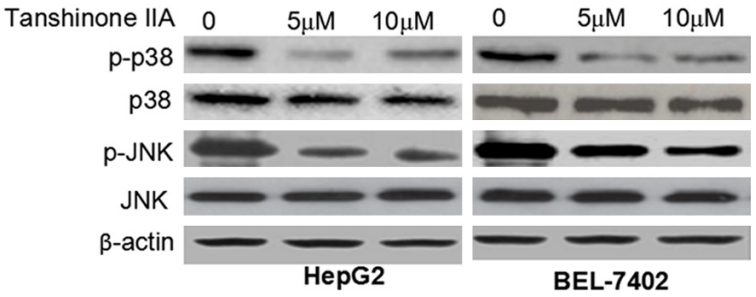

Figure 5. T2A suppresses cell invasion by inhibiting MAPK pathway pathways. The HepG2 and BEL-7402 cells were treated without or with various concentrations of T2A for 48 hours, and then the cell lysates were detected by western blot analysis with specific antibodies against p-JUN, c-JUN, p-p38MAPK, and p38MAPK. b-actin was used as an internal control.

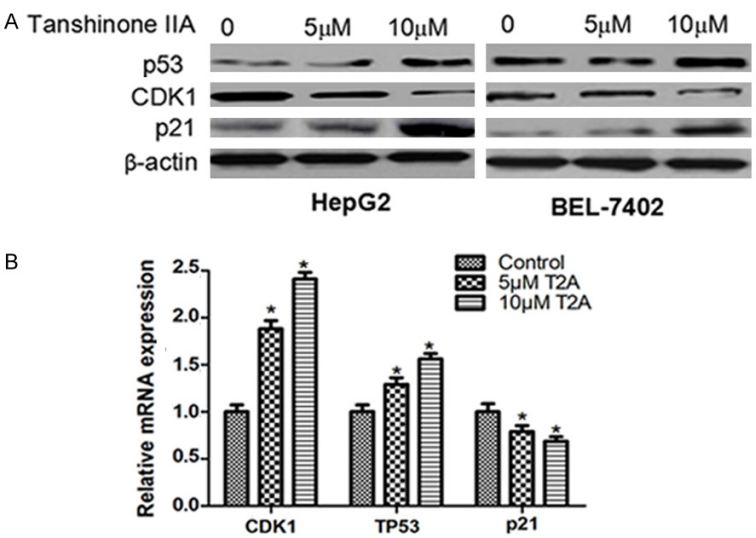

Figure 6. Effects of T2A on the proteolytic activities and mRNA expression levels of p53, p21 and CDK1 in HepG2 and BEL-7402 cells. The HepG2 and BEL-7402 cells were treated without or with various concentrations of T2A for 48 hours, and then the protein and mRNA expression level by western blot assay (A) and qRT-PCR (B). b-actin was used as an internal control.*$P < 0.05$ where compared with control.

and qRT-PCR.As shown in **Figure 6**, the level of p53 and CDK1 increased while p21 decreased after T2A treated.

Discussion

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related mortality worldwide. Prognosis for the HCC patient is poor due to the high rate of recurrence and metastasis [11]. Thus, it is essential to explore the molecular mechanisms under lying recurrence and metastasis for improving patient prognosis, and then developed new strategy for HCC patients by inhibiting specific targets. PTK is a group of compounds capable of catalyzing the phosphorylation of many important proteins by transferring the phosphate group on ATP to the tyrosine residues on these proteins in order to activate various enzyme substrates, consequently affecting cell growth, proliferation and differentiation through a series of reactions [12]. Because Src kinase activity is abnormally high in a variety of tumor cells, PTK is an anti-tumor target with very important significance.

The anti-tumor activity effect of Tanshinone IIA (T2A) has been confirmed in various tumors [13]. T2A also decreases human cancer cells invasion and metastasis. Thus, Tan-IIA can serve as a potential anti-cancer agent in cancer therapy. In this study, we treated hepatocellular carcinoma cells with T2A, and found that T2A can inhibit cell migration and invasion in human HepG2 and BEL-7402 cells. Src family kinases regulate cell migration in normal and oncogenically transformed cells by modifying cell-cell and cell-matrix adhesions, Src overexpression has been observed in a variety of invasive tumors as well as in tumor cell lines [14]. FAK play a crucial role in the transformation and progression of tumor cells. c-Src could bind FAK directly and promotes the phosphorylation of FAK and the activation of multiple intracellular signaling pathways [15]. In this study, it was found that, after human HepG2 and BEL-7402 cells were treated with T2A, the phosphorylation of c-Src and FAK was gradually down-regulated, suggesting that inhibits hepatocellular carcinoma cell invasion and metastasis possi-

bly by inhibiting c-Src kinase activity and FAK activity.

The MAPK signaling pathways have multiple roles in cell proliferation, invasion, and metastasis [16]. It is also one of important downstream pathway of c-Src [17]. p38MAPK and JNK have been reported to contribute to the acquisition of cell invasion and migration [18]. In this study, we found that T2A reduced the phosphorylation of p38, and JNK, suggest that T2A inhibits the invasion and metastasis of HepG2 and BEL-7402 cells by inhibiting the MAPK pathway.

We also found that T2A induced cell cycle arrested at G1/S. Cyclin-dependent kinases (CDKs) are a family of mammalian heterodimeric serine/threonine protein kinases. CDK1 is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle [19, 20]. The gene encoding the CDK inhibitor p21 is a major target for transactivation by the tumor suppressor p53, and introduction of p21 expression constructs into normal and tumor cell lines typically results in cell cycle arrest in G1 [21]. p21 is required for p53-dependent G1 arrest [22]. In this study, we further found that T2A suppressed the expression of CDK1, but increased the expression of p21 and apoptotic related gene p53, which also are Src-regulated genes. These results suggested that suppression of Src kinase activity by T2A also lead to the change of p21, CDK1 and p53 expression, all of which play critical roles in cell cycle progression and cell proliferation.

In conclusion, our data that tanshinone IIA (T2A) may be a therapeutic anticancer agent for cancer therapy, which potently inhibits cell invasion and growth through inhibiting Src kinase activity.

Disclosure of conflict of interest

None.

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