Original Article
BCAT1-inducing chemoresistance to cisplatin was partially suppressed by ATO in HCC cells

Huanyu Ju¹, Chengwu Tang¹², Xiaogang Dong¹, Yi Dai¹, Jianping Zhang¹

¹Department of General Surgery, The Second Affiliated Hospital, Nanjing Medical University, Nanjing, China; ²Department of General Surgery, First People’s Hospital Affiliated to Huzhou University Medical College, Huzhou, China

Received October 13, 2016; Accepted November 30, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Hepatocellular carcinoma (HCC) is the fifth main cancer in the world. Most patients will require several forms of chemotherapy. Here we show that the expression of BCAT1 was up-regulated in HCC tissues and induced chemoresistance to cisplatin in HCC cells. Moreover, our results demonstrated that arsenic trioxide (ATO) inhibited BCAT1 protein expression in a dose-dependent manner and time-dependent manner. And ATO inhibited BCAT1 protein expression via suppressing c-myc in HCC cells. However, BCAT1-overexpressing cells with ATO treatment showed less sensitivity to cisplatin and a higher proliferation or a lower apoptosis rate than ATO-treated cells. In conclusion, we identified that the BCAT1-inducing chemoresistance to cisplatin was partially suppressed by ATO in HCC cells. Based on our findings, BCAT1 may act as a therapeutic target for the human HCC, especially with high chemoresistance to cisplatin.

Keywords: HCC, BCAT1, arsenic trioxide, cisplatin, chemoresistance

Introduction
Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide. And only a handful of HCC patients are diagnosed at the early stage. To date, the main treatment for HCC is surgical resection. Eventually, most patients will require several forms of chemotherapy [1]. Cisplatin, one of the most widely used chemotherapy agents, demonstrates lower efficacy [1]. Thus, more research on molecular pathways and new targets for possible new drug development are needed. Clinically, arsenic trioxide (ATO) has been used to treat several types of cancers [2]. Previous study has shown that low concentrations of arsenic trioxide lead to increased sensitivity to cytostatic drugs. When adding arsenic trioxide prior to treatment with cisplatin, doxorubicin, 5-fluorouracil and interferon α-2b (PIAF), the results show a reduced survival of tumor cells in a dose-dependent manner for HCC [3].

Branched-chain aminotransferases (BCATs) have a catabolic function, catalyzing the transamination of all three BCAAs to the corresponding branched-chain a-ketoacids and glutamate [4, 5]. BCAT1 can take part in the regulation of cell cycle progression, cell proliferation, apoptosis and differentiation. Recent studies were indicative for the important role of BCAT1 in the progression of several malignancies, including medulloblastomas, nonseminomas, colorectal and nasopharyngeal carcinomas and glioblastomas [6-10]. Previous study has shown that BCAT1 promotes HCC cell proliferation and induces chemoresistance to cisplatin [5].

This prompts us to investigate whether ATO inhibits the expression of BCAT1 and BCAT1-inducing chemoresistance to cisplatin in HCC cells. Here we present experimental data indicative for strong BCAT1 overexpression in HCC tumors, which correlates with chemoresistance to cisplatin in HCC cells. We also show that the decreased BCAT1 expression by ATO is partially mediated by the down-regulation of the c-myc level.

Materials and methods
Tissues and cell culture
We analyzed tumor specimens from 35 patients with hepatocellular carcinoma (HCC) who
underwent surgery for excision of a primary tumor in the Second Affiliated Hospital of Nanjing Medical University (Nanjing, China). Written informed consent was obtained from the patients, in accordance with the institutional guidelines, and the study was approved by the Committees for the Ethical Review of Research at the Nanjing Medical University. Cells were maintained in 5% CO₂ at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml, Life Technologies, USA). Cisplatin and arsenic trioxide were purchased from Sigma-Aldrich (St. Louis, USA).

**Real-time PCR**

Total RNA was isolated via using TRIzol (Invitrogen, USA) according to the manufacturer’s recommendations. Total RNA was transcribed into cDNA via using AMV Reverse Transcriptase (Promega, USA). Real-time PCR was performed with an Applied Biosystems StepOnePlus and SYBR Green Master Mix (Takara, Japan). GAPDH was used as control for normalization. The primers for mRNAs real-time PCR were shown as below. BCAT1: Forward primer: 5’-AGCCCTGCTCTTTGTACTCTT-3’, Reverse primer: 5’-CCAGGCTCTTTACATACTTGGA-3’.

**Western blotting**

Protein from cell lysates were subjected to SDS-PAGE electrophoresis, and proteins were transferred to PVDF membranes (Millipore, USA). The antibodies which are against BCAT1, c-myc and GAPDH were used according to the manufacturer’s instructions, and were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). After using the secondary antibodies (Santa Cruz, USA) at 1:4,000 (v/v) dilutions in PBS + 0.1% Tween 20, the signals were detected by SuperSignal Chemiluminescent Substrate kit (Pierce, Rockford, USA) according to manufacturer’s instructions. For densitometric analyses, protein bands on the blots were measured by the use of Eagle Eye II software.

**MTT assay**

Cells were seeded (1 × 10⁴ cells/well) in 96-well plate. After 48 h, cells were treated with different doses of cisplatin, or ATO. Then, 50 μl of MTT solution (Sigma-Aldrich, USA) was added to each well, and incubated for 2 h, followed by addition of 100 μl of DMSO to each well. Absorbance at 570 nm was measured immediately.
ATO suppressed BCAT1-inducing chemoresistance

Apoptosis assay was evaluated with an Annexin V-PI assay kit (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. The flow cytometry with a FACS Calibur (BD Bioscience, USA) was performed to assess the result.

Cell transfection

Control siRNA and BCAT1 siRNA were purchased from Santa Cruz Biotechnology. Cells were transiently transfected with the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer’s instructions.

Statistical analysis

All the histogram was evaluated by performing GraphPad Prism, version 4.0 (GraphPad Software, USA). All statistical analyses were performed with SPSS 16.0. Values are showed as the means ± SEM. The student’s t-tests were used to analyze significant differences. Values of $P < 0.05$ were considered statistically significant.

Results

The expression of BCAT1 was increased in HCC tissues and cell lines

We examined the expression levels of BCAT1 in 35 HCC clinical samples via utilizing real-time PCR. Our results suggested that the relative expression levels in tumor tissues were significantly higher compared with those in para-tumor tissues (Figure 1A). And, the relative expression of BCAT1 protein levels in tumor tissues were also significantly higher compared with those in para-tumor tissues (Figure 1B). Then we observed the expression levels of BCAT1 in HCC cell lines (HepG2 and 97H) and normal hepatic cells (LO2) via using real-time PCR and western blotting. The relative expression levels of BCAT1 mRNA and protein in HepG2 and 97H cells were significantly higher compared with LO2 cells (Figure 1C and 1D). These results suggest that the expression of

Figure 2. Effects of BCAT1 on the chemosensitivity of HCC cells to cisplatin. A. Proliferation assay was performed using scramble and BCAT1 siRNA transfected HCC cells HepG2 and 97H with 20 μM of cisplatin treated for 48 h. The results obtained from three independent experiments were presented as the mean ± SEM. *$P < 0.05$ by student’s t-test. B. Apoptosis assay was performed using scramble and BCAT1 siRNA transfected HCC cells HepG2 and 97H with 20 μM of cisplatin treated for 48 h. Flow cytometry assay was performed to analyze cell apoptosis. The results obtained from three independent experiments were presented as the mean ± SEM. *$P < 0.05$ by student’s t-test.
BCAT1 was up-regulated in HCC tissues and cell lines.

**BCAT1 induced chemoresistance to cisplatin in HCC cells**

In order to assess the chemosensitivity, MTT assay and apoptosis assay were performed in HCC cells. HepG2 cells were treated with different concentrations of cisplatin for 48 h, BCAT1 siRNA-treated cells exhibited more sensitivity to cisplatin and a lower proliferation (Figure 2A) or a higher apoptosis (Figure 2B) rate than control cells. Similarly, the suppression of BCAT1 expression in 97H cells enhanced their chemosensitivity to cisplatin, reduced their proliferation rate and increased their apoptosis. The results demonstrated that the overexpression of BCAT1 result in HCC cells more resistant to cisplatin.

**Arsenic trioxide inhibited BCAT1 protein expression via suppressing c-myc in HCC cells**

To determine whether the BCAT1 expression could be inhibited by arsenic trioxide (ATO), we treated HCC cells with 2.5, 5, and 10 μM of ATO, and examined the protein expression of BCAT1 at 48 h by western blotting assay. The results showed that cells with ATO treatment significantly decreased the BCAT1 protein expression in a dose-dependent manner (Figure 3A). Besides, 5 μM of ATO treatment repressed the BCAT1 expression in time-dependent manner (Figure 3B). It has been described previously that ATO treatment decreased c-myc expression, which is the trans-acting factor of BCAT1 gene. So HepG2 cells were treated with 5 μM of ATO or/and 20 μM of cisplatin for 48 h. Western blotting assay was conducted to measure the expression of c-myc and BCAT1. The results showed that 5 μM of ATO inhibited the c-myc and BCAT1 expression (Figure 3C). The results showed that the decreased BCAT1 expression by ATO treatment is partially mediated by the down-regulation of the c-myc level.

**BCAT1-inducing chemoresistance to cisplatin was partially suppressed by ATO in HCC cells**

In order to assess the chemosensitivity, MTT assay and apoptosis assay were performed in HCC cells. When HepG2 and 97H cells were treated with 5 μM of ATO or/and 20 μM of cisplatin for 48 h. ATO-treated cells show a lower proliferation and less sensitivity to cisplatin (Figure 4A) or a higher apoptosis (Figure 4B) rate than normal cells. However, exogenous BCAT1-overexpressing cells with ATO treatment
ATO suppressed BCAT1-inducing chemoresistance

Figure 4. BCAT1-inducing chemoresistance to cisplatin was suppressed by ATO in HCC cells. A. Proliferation assay was performed to analyze cell apoptosis. The HCC cells were performed using BCAT1-expressing vector, then HepG2 and 97H cells were treated with 5 μM of arsenic trioxide (ATO) or/and 20 μM of cisplatin for 48 h. The results obtained from three independent experiments were presented as the mean ± SEM. *P < 0.05 by student’s t-test. B. Flow cytometry assay was performed to analyze cell apoptosis. The HCC cells were performed using BCAT1-expressing vector, then HepG2 and 97H cells were treated with 5 μM of ATO or/and 20 μM of cisplatin for 48 h. The results obtained from three independent experiments were presented as the mean ± SEM. *P < 0.05 by student’s t-test.

Discussion

BCAT1 is overexpressed in various human cancers tissues than in the corresponding normal tissues [11]. In addition, we found that the BCAT1 expression was up-regulated in HCC tissues. The results show that BCAT1 is a hopeful target for new therapeutic drugs for HCC.

BCAT1 can take part in the regulation of cell cycle progression, cell proliferation, apoptosis and differentiation. Although the inhibition of BCAT1 has been reported to decrease HCC cell proliferation, the effect on HCC cell apoptosis has not yet been reported. Here, we found that the suppression of BCAT1 in 97H cells reduced their proliferation rate, increased their chemosensitivity to cisplatin and increased their apoptosis. The results demonstrated that the overexpression of BCAT1 resulted in HCC cells more resistant to cisplatin. Thus, the suppression of BCAT1 may be a potential therapeutic strategy to improve the effects of chemotherapy and increase clinical outcomes in patients with HCC.

However, the mechanisms of BCAT1 expression are not yet clearly. To data, we just know that BCAT1 is a direct target of c-myc [12]. In MCF-7 cells, estrogen stimulated BCAT1 expression increased via inducing c-myc expression [13]. In primary NPC, c-myc up-regulation leads to the overexpression of BCAT1, and BCAT1 overexpression induces cell proliferation, migration and invasion [10]. The c-myc knockdown resulted in evident suppression of the BCAT1 protein expression in epithelial ovarian cancer cells [9]. Previous studies have shown that ATO down-regulated the expression of hTERT, c-Myc, Sp1, NF-κB and USF2 at both mRNA and protein levels [2, 14]. ATO pretreatment with cisplatin show a reduced cell survival rate in a dose-dependent manner for HCC cells [3], so we next aim to study whether ATO inhibited BCAT1 protein expression via suppressing c-myc in HCC.
ATO suppressed BCAT1-inducing chemoresistance

cells. The results showed that 5 μM of ATO inhibited c-myc and BCAT1 expression. The decreased BCAT1 expression by ATO treatment is partially mediated by the down-regulation of the c-myc level.

Furthermore, we developed a series of rescue experiments to further study whether the functional effect of ATO on chemoresistance to cisplatin in HCC cell lines was exerted via targeting BCAT1. ATO-treated cells showed a lower proliferation and less sensitivity to cisplatin or a higher apoptosis rate than normal cells. However, exogenous BCAT1-overexpressing cells showed less sensitivity to cisplatin and a higher proliferation or a lower apoptosis rate than ATO-treated cells. Interestingly, the findings confirmed our hypothesis that proliferation and apoptosis indeed reversed to some extent when we overexpressed BCAT1 in HCC cells with cisplatin treatment.

In conclusion, we identified that the BCAT1-inducing chemoresistance to cisplatin was partially suppressed by ATO in HCC cells. Based on our findings, BCAT1 may act as a therapeutic target for the human HCC, especially with high chemoresistance to cisplatin.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (No. 81572325).

Disclosure of conflict of interest

None.

Address correspondence to: Jianping Zhang, Department of General Surgery, The Second Affiliated Hospital, Nanjing Medical University, Jiangning District, Nanjing 211166, China. Tel: +86 25 5850 9888; Fax: +86 25 5850 9990; E-mail: nydefyjp@163.com

References


ATO suppressed BCAT1-inducing chemoresistance

