Docetaxel promotes cell apoptosis and decreases SOX2 expression in CD133-expressing hepatocellular carcinoma stem cells by suppressing the PI3K/AKT signaling pathway

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Abstract. Docetaxel is a front-line standard-of-care chemotherapeutic drug for the treatment of cancers. However, the underlying function and mechanism of docetaxel in human hepatocellular carcinoma (HCC) are uncertain. Therefore, the present study aimed to determine the effects of docetaxel on cell apoptosis and SOX2 expression in cultured human HCC stem cells. After human HCC stem cells were treated with docetaxel, cell proliferation was assessed by methyl thiazolyl tetrazolium (MTT) method, the cell apoptotic rate was evaluated by flow cytometry, the expression of CD133 and sex determining region Y-box 2 (SOX2) was determined by RT-PCR and immunohistochemistry, and the protein levels of CD133, SOX2, phosphoinositide 3-kinases (PI3K), AKT and phosphorylated AKT (p-AKT) were analyzed by western blotting. The results indicated that SOX2 and CD133 were highly expressed in patients with HCC while their expression was significantly decreased after patients with HCC were treated with docetaxel. *In vitro*, docetaxel inhibited the proliferation while it enhanced the apoptosis of human CD133-expressing HCC stem cells. Furthermore, lower expression of p-AKT and SOX2 were revealed in the presence of docetaxel. Notably, docetaxel-inhibited SOX2 expression and growth of human CD133-expressing HCC stem cells were partially restricted following the block of the PI3K/AKT signaling pathway using the inhibitor LY294002. The present study collectively indicated that docetaxel promoted apoptosis and upregulated SOX2 expression of human HCC stem cells through the suppression of the PI3K/AKT signaling pathway.

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Abbreviations: HCC, hepatocellular carcinoma; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation

Key words: docetaxel, human liver cancer stem cell, PI3K/AKT, apoptosis, SOX

Introduction

Despite the advances in therapeutic strategies in hepatocellular carcinoma (HCC) treatment, HCC is still one of the most common cancers worldwide (1). Recent studies have demonstrated that HCC stem cells that exist within the tumor mass have the ability to propagate and are considered to play an important role in liver tumor initiation, progression and metastasis (2). In addition, growing evidence has revealed that HCC stem cells are responsible for the resistance of chemotherapy and radiotherapy, and the recurrence of HCC (3). Thus, HCC stem cells are becoming crucial for evaluating new therapeutic strategies and monitoring the progress of HCC therapy.

CD133, also known as prominin-1, was first identified as a potential subpopulation of cancer stem cells (4,5). It is now widely described as a marker of cancer stem cells in the brain (6), esophagus (7), lung (8), colon (9), prostate (10) and ovaries (11). Notably, CD133 is also a marker highly recognized in HCC stem cells (12), which is reported to be an important target for improving chemotherapeutic efficacy of recurrent HCC cells (13). CD133-expressing HCC stem cells have also been demonstrated to be involved in liver tumorigenicity in HCC, conferring radiotherapy resistance due to their high activation of the AKT/Protein kinase B (PKB), B-cell lymphoma 2 (Bcl-2) (14) and mitogen-activated protein kinase (MAPK)/PI3K (15) signaling pathways. In the present study, CD133-expressing HCC stem cells were chosen as an *in vitro* model.

Docetaxel belonging to the taxane family is a promising anticancer agent which is a semi-synthetic derivative from the needles of European yew (*Taxus baccata*) (16). Docetaxel has been widely used to treat breast (17), prostate (18), bladder (19), gastric (20), ovarian (21), head and neck (22) and non-small cell lung (23) cancers. Furthermore, the role of docetaxel in HCC treatment has been recognized due to its low toxicity and high therapeutic efficacy. Presently, docetaxel has demonstrated its ability to reduce the hepatocellular tumor size in nude mice and to inhibit the proliferation of the HepG2 cell

line (24). After intravenously treating mice with 20 mg/kg silica nanorattle-encapsulated docetaxel, the hepatocellular tumor size of the mice was significantly decreased (25). However, the mechanism by which docetaxel maintains its antitumor capabilities in human CD133-expressing HCC stem cells remains to be explored.

The aim of the present study was to elucidate the mechanism by which docetaxel regulated SOX2 expression and cell apoptosis in CD133-expressing HCC stem cells. We revealed that docetaxel inhibited SOX2 accumulation and induced cancer cell death through the suppression of the PI3K/AKT signaling pathway. Collectively, these findings revealed a novel mechanism that mediates the regulation of SOX2 and the anticancer effects of docetaxel in human HCC stem cells.

Materials and methods

Patients and tissue samples. Normal liver tissues and HCC tissues used in this study were obtained between June 2016 and July 2017 from 48 HCC patients (aged 47.36±4.57; 36 male and 12 female patients) who had been treated with 20 mg/kg docetaxel (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) intravenously for a week at our hospital (Department of Oncology, Jingjiang People's Hospital, Jingjiang China). Informed consent was obtained from all patients. All experimental protocols were approved by the Institutional Ethics Committee of Jingjiang People's Hospital, Jiangsu, China (no. 2018-122).

Cell isolation and culture. Human normal liver stem cells and HCC stem cells were isolated from liver tissues of HCC patients at our hospital according to a previous study (26). Briefly, cell suspensions were centrifuged at 300 x g for 10 min and cell pellets were resuspended in 300 μl of buffer/10^8 total cells after aspirating the supernatant completely. Then, 100 μl of FcR Blocking Reagent/10^8 total cells and 100 μl of CD133/CD44/CD24 MicroBeads/10^8 total cells were added, mixed well and incubated for 30 min in the refrigerator. Cells were washed by adding 1-2 ml of buffer/10^8 cells and centrifuged at 300 x g for 10 min. An appropriate MACS Column and MACS Separator was chosen according to the number of total cells and the number of $CD133^{+}/CD44^{+}/CD24^{+}$ cells. The column was placed in the magnetic field of a suitable MACS Separator, and prepared by rinsing with 500 μl buffer MS. The cell suspension was applied onto the column and washed with the appropriate amount of buffer. Unlabeled cells that passed through and combined with the effluent from the MS step were collected. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 10 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA) in an incubator with a humidified atmosphere of 5% CO_2 at 37˚C.

Cell viability assay. Cell viability was assessed using an MTT assay (Bestbio Biotechnology, Shanghai, China). Briefly, human HCC stem cells at a concentration of $2x10^3$ cells/well were seeded in 96-well flat-bottomed tissue culture plates (Corning Inc., Corning, NY, USA) for 24 h. Following two washes with phosphate-buffered saline (PBS), cells were incubated in 100 μl culture medium containing 50 nM docetaxel for 12, 24 and 48 h at 37˚C prior to the MTT assay. Then, a total of 10 μl MTT and 100 μl culture medium was added to each well, and following incubation for 1 h at 37˚C, the optical densities of the samples were measured directly using a spectrophotometric microplate reader (Beyotime Institute of Biotechnology, Haimen, China) at a wavelength of 490 nm.

Cell apoptosis assay. The apoptotic cells were identified by the terminal-deoxynucleotidyl transferase mediated nick end-labeling (TUNEL) apoptosis assay kit (KeyGen Biotech Co., Ltd., Nanjing, China) according to manufacturer's instructions. Cells at a density of $2x10^4$/ml were cultured in 10% FBS-containing DMEM with 50 nM docetaxel for 24 h and harvested and washed twice with cold PBS by gentle shaking. Resuspended cells were added to 1X binding buffer and the cell density was adjusted to 200,000-500,000/ml. Cell apoptosis was analyzed using a FACScan flow cytometric apparatus (BD Biosciences, San Jose, CA, USA) and the percentage of apoptotic cells was analyzed using FlowJo 7.6.1 software (TreeStar, Inc., Ashland, OR, USA).

RT-PCR. The expression of CD133 and SOX2 in human HCC stem cells was assessed by RNA preparation and quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA isolation using TRIzol reagent and cDNA synthesis using Takara PrimeScript II First Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was conducted according to the manufacturers' instructions. Specific primer sequences were synthesized in BioSune Biological Technology Corp. (Shanghai, China), and the sequences of the primers were as follows: CD133 forward, 5'-CCATACCTAGGTCCCCGTCC-3' and reverse, 5'-TTC ACTCAAGGCACCATCCC-3'; SOX2 forward, 5'-AACCAG CGCATGGACAGTTA-3' and reverse, 5'-GACTTGACCACC GAACCCAT-3'; GAPDH forward, 5'-AATGGGCAGCCG TTAGGAAA-3' and reverse, 5'-GCGCCCAATACGACCAAA TC-3'. Data was analyzed using Bio-Rad CFX Manager 1.6 Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA.

Western blot analysis. Following the treatment with 50 nM docetaxel for 6 h, the cells were incubated for an additional 24 h prior to the collection of cells for protein extraction. The examination of the expression levels of CD133, SOX2, PI3K, AKT and p-AKT was then performed separately. Total protein was extracted using Total Protein Extraction kit (Sigma-Aldrich; Merck KGaA). Total protein was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and 30 μg protein/lane was separated via SDS-PAGE on a 6% gel and run on a 10% gel. The separated proteins were subsequently transferred to nitrocellulose (NC) filter membrane and blocked with Tris-buffered saline (TBS) containing 5% milk powder without fat and 0.05% Tween-20. Antibodies of CD133 (dilution 1:1,000; cat. no. sc-33182), PI3K (dilution 1:1,000; cat. no. 4922), AKT (dilution 1:1,000; cat. no. sc-9272) and p-AKT (dilution 1:1,000; cat. no. sc-33437) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies of SOX2 (dilution 1:3,000; cat. no. 3579) and GAPDH (dilution 1:6,000; cat. no. 5174) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA)

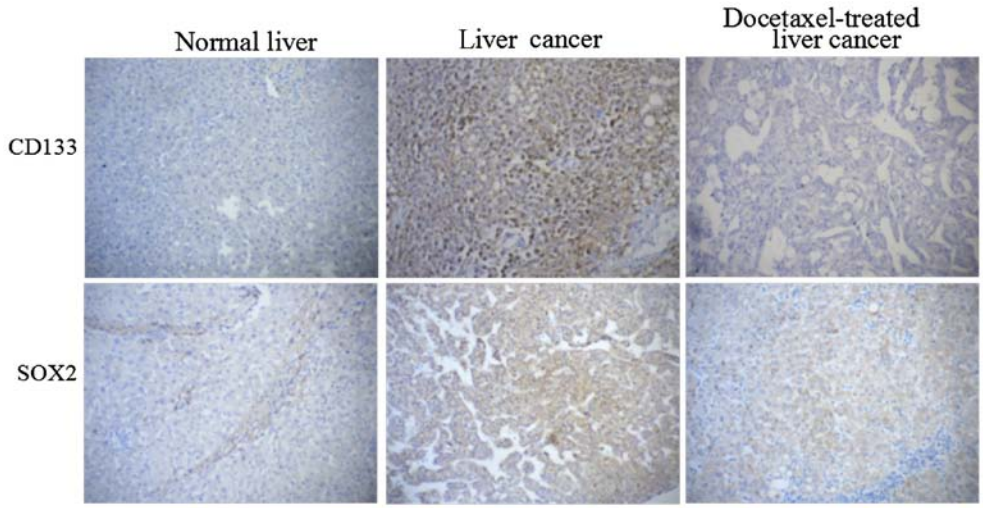

Figure 1. Docetaxel downregulates CD133 and SOX2 expression in human HCC patients. Expression of CD133 and SOX2 was assessed by immunohistochemistry in human HCC tissues and normal human liver tissues following docetaxel treatment. Each experiment was performed in triplicate and repeated six times.

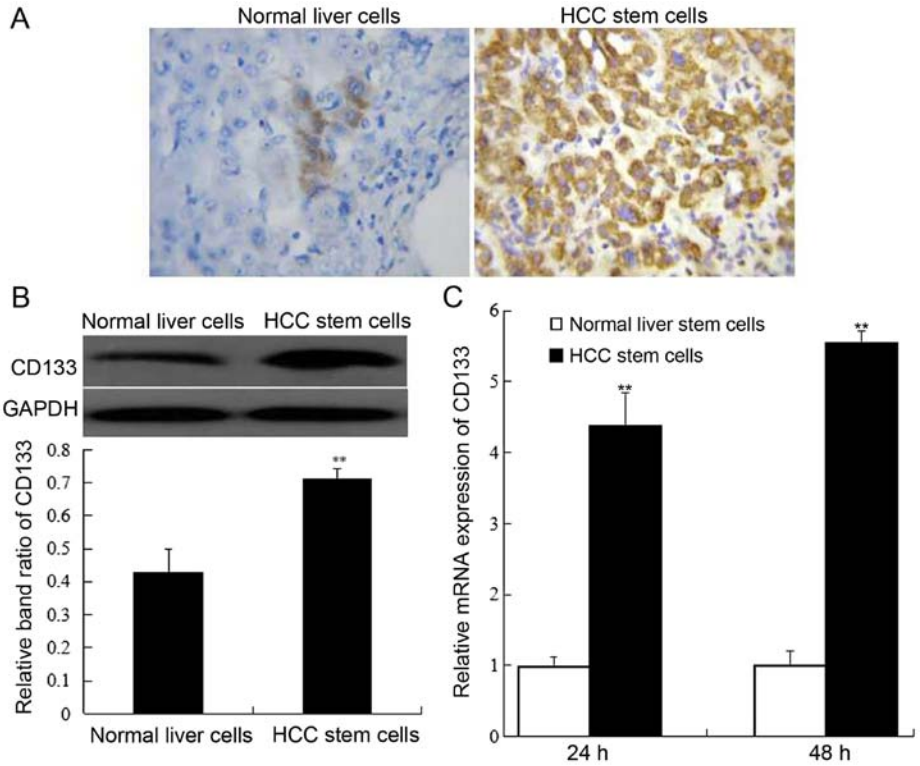

Figure 2. CD133 expression is determined in HCC stem cells. CD133 expression in normal liver stem cells and HCC stem cells was determined by (A) immunohistochemistry, (B) western blotting and (C) RT-PCR. Each experiment was performed in triplicate and repeated three times. Results were presented as the mean ± SD. $^{**}P<0.01$ compared with the control.

and incubated at overnight at 4˚C. Secondary antibodies were conserved in our laboratory and incubated at 37˚C for 1 h. The protein levels were detected using the chemiluminescence reader, ImageQuant™ LAS4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Relative band ratio was analyzed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry. Paraffin-embedded normal liver and cancer liver tissues (3-μm in thickness) were prepared and immunohistochemistry was performed as previously described (27). Primary antibodies against CD133 (dilution 1:500; Santa Cruz Biotechnology, Inc.) and SOX2 (dilution 1:200; Abcam, Cambridge, UK) were used. Data of immunohistochemistry were analyzed using Image-Pro Plus (version 4.1; Media Cybernetics, Rockville, MD, USA).

Block of PI3K/AKT signal using an inhibitor. Human HCC stem cells were treated with PI3K/AKT inhibitor LY294002 at a concentration of 25 μM for 48 h, followed by the addition of 50 nM docetaxel and further incubation for 48 h. Then, cell proliferation, apoptosis, and SOX2 expression were evaluated.

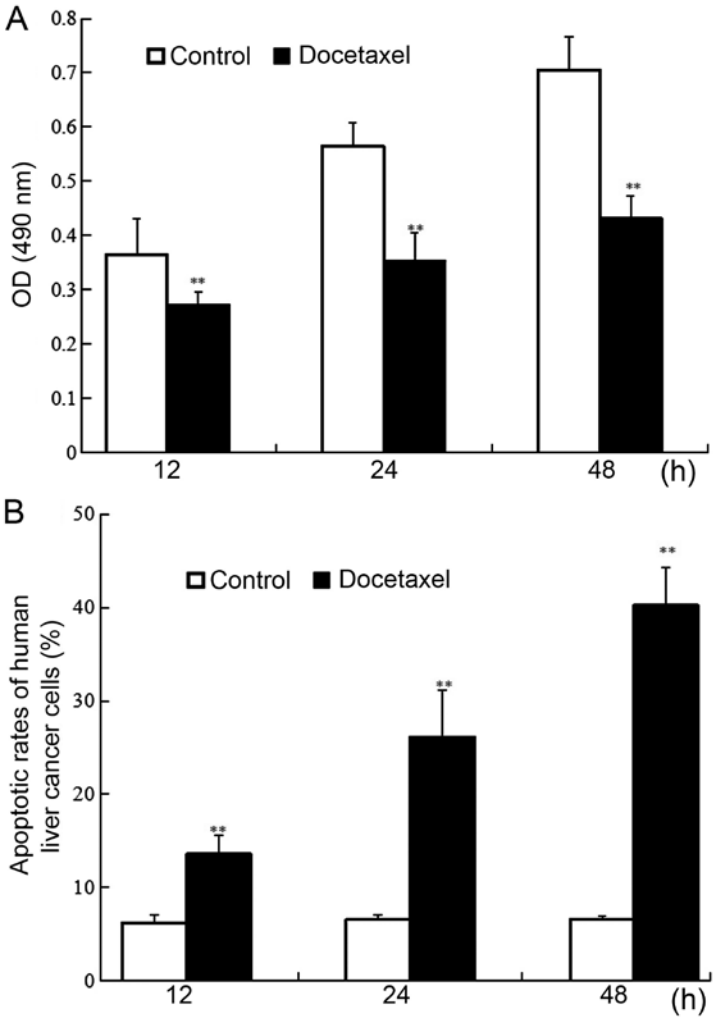

Figure 3. Docetaxel inhibits the growth of human CD133-expressing HCC stem cells. Human HCC stem cells were treated with docetaxel for 12, 24 and 48 h and cell proliferation was assessed by (A) MTT assay, and (B) cell apoptosis was determined by flow cytometry. Each experiment was performed in triplicate and repeated six times. Results were presented as the mean ± SD. **P<0.01 compared with the control.

Statistical analysis. All results were analyzed by one-way analysis of variance (ANOVA) using the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Boferroni's post hoc test was used to determine the statistical differences between the treatment and control groups. P-values were based on the two-sided statistical analysis, and P<0.05 was considered to indicate a statistically significant difference.

Results

Docetaxel downregulates the expression of CD133 and SOX2 in patients with HCC. As shown in Fig. 1, higher expression of CD133 and SOX2 was detected by immunohistochemistry in HCC samples compared with normal liver tissues, indicating that CD133 and SOX2 were two important factors which may be involved in HCC. Moreover, the expression of CD133 and SOX2 in human HCC tissues was significantly downregulated by docetaxel compared to cells without docetaxel stimulation.

Identification of CD133-expressing HCC stem cells. Following 24 and 48 h of cell culture, CD133 expression in normal liver stem cells and HCC stem cells was determined by immunohistochemistry, western blotting and RT-PCR. The results revealed that the percentage of CD133-positive cells was obviously higher in HCC stem cells than that of normal liver stem cells (Fig. 2A). Additionally, compared to normal liver stem cells, CD133 protein expression was highly promoted in HCC stem cells (P<0.01). (Fig. 2B). The assessment of CD133 mRNA expression also supported the conclusion that CD133 expression was increased in HCC stem cells (Fig. 2C).

Docetaxel inhibits the proliferation while promoting the apoptotic rate of human CD133-expressing HCC stem cells. Human CD133-expressing HCC stem cells were respectively exposed to docetaxel for 12, 24 and 48 h, and the role of docetaxel in human CD133-expressing HCC stem cells was assessed through MTT assay and flow cytometry. The results revealed that compared to the control without docetaxel treatment, docetaxel significantly downregulated cell viability in CD133-expressing HCC stem cells in a time-dependent manner (P<0.01) (Fig. 3A). Conversely, as shown in Fig. 3B, docetaxel significantly promoted apoptosis in CD133-expressing HCC stem cells in a time-dependent manner. Approximately 13.58, 26.13 and 40.24% of cells underwent apoptosis after exposure to docetaxel for 12, 24 and 48 h, respectively, and there was a

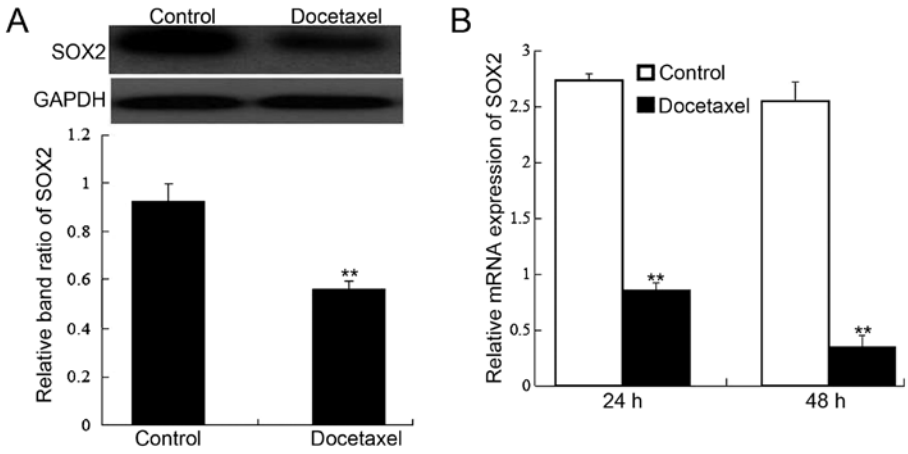

Figure 4. Docetaxel decreases SOX2 expression in human CD133-expressing HCC stem cells. After human HCC stem cells were stimulated with docetaxel, SOX2 protein expression was detected by (A) western blotting and (B) SOX2 mRNA expression was evaluated using RT-PCR. Results were presented as the mean ± SD. **P<0.01 compared with the control.

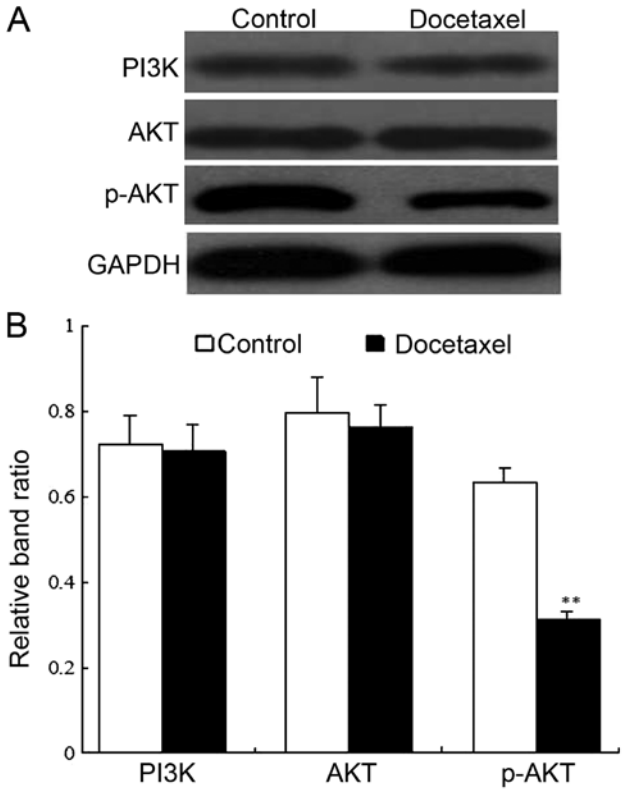

Figure 5. Docetaxel suppresses the PI3K/AKT signaling pathway in human HCC stem cells. (A) After human HCC cells were treated with docetaxel for 48 h, the protein expression of PI3K, AKT and p-AKT was assessed by western blotting. (B) Relative band ratio was analyzed using ImageJ software. Each experiment was performed in triplicate and repeated three times. Results were presented as the means ± SD. **P<0.01 compared with the control.

significant difference between docetaxel-treated groups and the control without docetaxel treatment (P<0.01). The findings above indicated that docetaxel significantly increased the apoptosis in CD133-expressing HCC stem cells.

Docetaxel suppresses the expression of SOX2 in human CD133-expressing HCC stem cells. Since SOX2 expression is associated with HCC, we further detected SOX2 protein and mRNA expression using western blotting and RT-PCR at indicated time-points after docetaxel stimulation. The results demonstrated that SOX2 protein (Fig. 4A) and mRNA (Fig. 4B) expression was significantly inhibited by docetaxel in human HCC stem cells (P<0.05) in comparison with the control.

Docetaxel inhibits the PI3K/AKT signaling pathway in human CD133-expressing HCC stem cells. To further verify the underlying mechanisms of docetaxel in human CD133-expressing HCC stem cells, we explored the expression of PI3K, AKT and p-AKT by western blotting (Fig. 5A). Band analysis indicated that the protein level of PI3K and AKT exhibited no difference

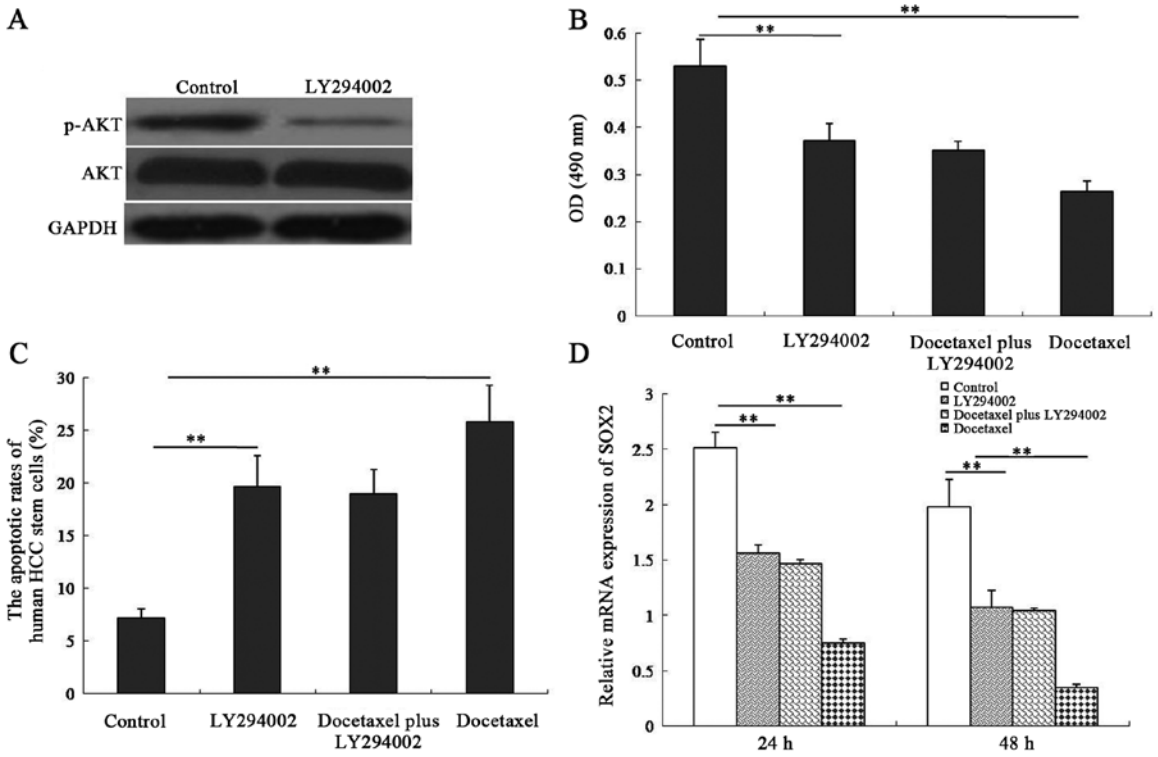

Figure 6. Docetaxel induces cell apoptosis and decreases SXO2 expression in human CD133-expressing HCC stem cells through the PI3K/AKT signaling pathway. After the PI3K/AKT signaling pathway was blocked using LY294002, protein expression of p-AKT was assessed by (A) western blotting. Human HCC cells were stimulated with docetaxel, LY294002 or docetaxel plus LY294002 and (B) cell proliferation, (C) the apoptotic rate and (D) the mRNA expression of SOX2 were determined. Each experiment was performed in triplicate and repeated three times. Results were presented as the means ± SD. **P<0.01 compared with the control.

between the docetaxel-treated group and non-docetaxel-treated group (P>0.05). Moreover, the p-AKT protein level was comparatively low in docetaxel-treated cells in comparison with the control (P<0.01) (Fig. 5B). It was thus suggested that docetaxel could suppress the PI3K/AKT signaling pathway in human CD133-expressing HCC stem cells.

Inhibition of the PI3K/AKT signaling pathway in human CD133-expressing HCC stem cells is required for docetaxel-induced cell apoptosis and decreased SOX2 expression. We next examined the influence of the PI3K/AKT signaling on docetaxel-induced cell apoptosis and -decreased SOX2 expression in human CD133-expressing HCC stem cells. The results revealed that HCC stem cells treated with the PI3K/AKT inhibitor LY294002 exhibited a significant decrease in p-AKT expression compared with untreated cells (Fig. 6A). Cell viability (Fig. 6B) and SOX2 expression (Fig. 6D) were inhibited in both LY294002-treated cells and docetaxel-treated cells. However, there was no difference in cell viability and SOX2 expression between LY294002 plus docetaxel-treated cells and LY294002-treated cells (P>0.05). As demonstrated in Fig. 6C, LY294002 or docetaxel significantly promoted the apoptotic rate in CD133-expressing HCC stem cells (P<0.01). Moreover, the apoptotic rate exhibited no difference between the LY294002-treated group and LY294002 plus docetaxel-treated group (P>0.05). These results indicated that the PI3K/AKT signaling pathway was involved in docetaxel-exerted biological functions in human CD133-expressing HCC stem cells.

Discussion

In the present study, we investigated the mechanisms underlying docetaxel-induced cell apoptosis in CD133-expressing HCC stem cells. We determined that docetaxel induced the suppression of the PI3K/AKT signaling pathway, thereby causing HCC stem cell death and decreased SOX2 expression.

Docetaxel has been used to treat various types of cancers. *In vitro,* docetaxel suppresses proliferation and induces apoptosis by suppressing the mitogen-activated protein kinase (MAPK) signaling in renal cell carcinoma cells (28). Docetaxel induces cell apoptosis and suppresses cell proliferation in non-small cell lung cancer cells by upregulating microRNA-7 expression (29). In a study on HCC treatment with docetaxel, Geng *et al* demonstrated that docetaxel enhanced radiation sensitivity of human HCC cells (30). Additionally, another study from the same authors provided the evidence that docetaxel reduced the proliferation of SMMC-7721 HCC cells *in vitro*, kept their morphology, and induced cell death by apoptosis (31). Docetaxel was revealed to inhibit the growth of hepatoma cells by arresting the G2/M-phase, activating caspases, and fragmenting DNA (32). A recent investigation confirmed that docetaxel inhibited the progression of cultured human hepatoma cells in advanced HCC (33). In the present study, we also revealed that docetaxel inhibited proliferation while promoting apoptosis in CD133-expressing HCC stem cells through the inactivation of the PI3K/AKT signaling pathway.

According to research, SOX2, a major transcription factor is regarded as a stemness-related factor. It has been demonstrated that SOX2 is associated with various types of cancers and has been used as a marker to identify cancer stem cells (34). Furthermore, SOX2 suppression is mandatory for cellular differentiation. For these reasons, SOX2 has been investigated in cancer stem cells in several cancer types. Notably, predictive value of SOX2 in cancers is associated with the prognosis of patients and is regarded as a possible therapeutic target. In a review study, the role of SOX2 as a prognostic marker, indicator of metastasis, or biomarker in cancer pathogenesis was highlighted (35). Recently, numerous studies have provided evidence that SOX2 mRNA expression was significantly higher in patients with small-cell lung (36), gastric (37), breast (38), cervical (39) and ovarian epithelial cancer (40) compared to the healthy controls. A high level of SOX2 expression was revealed to be correlated with metastasis and a low survival rate in HCC (41). In the present study, we attempted to reveal the relationship between docetaxel and SOX2, and demonstrated that protein and mRNA expression of SOX2 exhibited a significant decrease in docetaxel-treated HCC stem cells.

In order to investigate the mechanisms underlying the antitumor effects of docetaxel in CD133-expressing HCC stem cells, the activation of the PI3K/AKT signaling pathway was determined after cells were treated with docetaxel. Our results demonstrated that p-AKT expression was significantly decreased in the drug-treated CD133-expressing HCC stem cells compared with that of the control group, indicating that the suppression of the PI3K/AKT signaling pathway occured in the presence of docetaxel, and this signal suppression was involved in the changes induced by docetaxel in CD133-expressing HCC stem cells. Previous studies have indicated that PI3K is a type of lipid kinase, and its cascades play an essential role in regulating growth, migration, and survival of different types of tumor cells (42). Additionally, accumulating evidence has revealed that PI3K/AKT was associated with tumorigenesis, cancer progression and drug resistance (43). Several drugs targeting PI3K/ATK are currently used in clinical trials to treat cancers (44-46). Previous research has revealed that docetaxel induced the apoptosis in human prostate cancer cells by modulating the PI3K/AKT pathway (47). Thus, docetaxel and its target PI3K/AKT may offer a new direction for cancer therapy.

In summary, we reported for the first time, to the best of our knowledge, that docetaxel inhibited the growth of human CD133-expressing HCC stem cells by causing cell apoptosis. Further investigation revealed that the PI3K/AKT signaling pathway played a key role in docetaxel-induced apoptosis of human CD133-expressing HCC stem cells. These findings may add new insights to HCC stem cells and help to explore their functions in tumor therapy.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XZ and ZT were involved in the manuscript preparation and the conception of the study; XZ, JS and ZT were responsible for the manuscript editing, reviewing and study design, carried out the experimental studies and were involved in the data acquisition; XZ, XL and ZT were responsible for revising the manuscript critically for important intellectual content; XZ, XL, LC and ZT performed the literature research; XL and LC were involved in the clinical studies; XZ, XL and LC were responsible for the data analysis; XZ and LC performed the statistical analysis. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Ethics Committee of Jingjiang People's Hospital, Jiangsu, China (no. 2018-122). Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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