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Multidrug-resistant hepatocellular carcinoma cells are enriched for CD133$^+$ subpopulation through activation of TGF-β1/Smad3 pathway

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Chemotherapy is a main treatment for cancer, while multidrug-resistance is the main reason for chemotherapy failure, and tumor relapse and metastasis. Cancer stem cells or cancer stem-like cells (CSCs) are a small subset of cancer cells, which may be inherently resistant to the cytotoxic effect of chemotherapy. Some studies suggest that CSCs could be enriched by chemotherapy. However, the mechanism of chemotherapy regulating CSCs remains unknown. Therefore, we investigated whether drug treatment could enrich CSCs in hepatocellular carcinoma (HCC) cells and the molecular mechanism of chemotherapy regulating the expression of CSCs markers. In the present study, a multidrug-resistant (MDR) human HCC subline, Huh7.5.1/PTX, was developed by exposing parental cells to paclitaxel (PTX) repeatedly at a single high concentration. The cell counting kit-8 (CCK-8) assay was used to determine cellular sensitivity of various anticancer drugs. Flow cytometry (FCM) was used to analyze the CSCs markers expression level. Western blotting (WB) was used to analyze the changes of TGF-β1/Smads signaling. Our results show that PTX treatment of HCC cells *in vitro* resulted in a development of subline six months later, and Huh7.5.1/PTX, with stable MDR phenotype. Huh7.5.1/PTX cells enriched CSCs fraction and strongly activated the TGF-β1/Smad3 signaling. Activation of TGF-β1/Smad3 signaling resulted in enrichment of the CSCs population (CD133$^+$ cells), while inhibition of this pathway activity attenuated the percentage of these cells. Taken together, our results suggest that MDR HCC cells are enriched with CSCs, which is partially dependent on TGF-β1/Smad3 pathway. Inhibition of TGF-β1/Smad3 pathway may be useful for targeting CSCs to develop more effective treatments for HCC.

Key words: Hepatocellular carcinoma (HCC) CD133, chemotherapy, cancer stem cells, TGF-β1, Smad3.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, the third leading cause of

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Abbreviations: CSCs, Cancer stem cells or cancer stem-like cells; **HCC,** hepatocellular carcinoma; **MDR**, multidrug-resistant or multidrug-resistance; **PTX**, paclitaxel.

cancer- related deaths and an aggressive tumor with a poor prognosis (Ferlay et al., 2010). Current curative treatments such as liver resection and transplantation are limited to the early disease stage. Chemotherapy has generally not improved overall mortality in HCC except for a recent report using sorafenib, which improved advance stage mortality by less than 3 months (Thomas et al., 2010). Therapeutic strategies against this disease target mostly rapidly growing differentiated tumor cells. However, the result is often dismal because of the chemo-resistant nature (Thomas et al., 2008).

Recent research efforts on stem cells and cancer biology have shed light on new directions for the eradication of CSCs in HCC (Zou, 2010a). The CSCs theory has been proposed to explain the tumor heterogeneity and the carcinogenesis (Reya et al., 2001). According to this model, tumor can be viewed as a result of abnormal organogenesis driven by CSCs, defined as self-renewing tumor cells able to initiate and maintain the tumor and to produce the heterogeneous lineages of cancer cells that consist of the tumor (Clarke et al., 2006). The existence of CSCs was first proven in acute myeloid leukemia (Lapidot et al., 1994), and more recently in many solid tumors including breast (Ponti et al., 2005), brain (Singh et al., 2003), prostate (Collins et al., 2005; Patrawala et al., 2006), pancreatic (Li et al., 2007), colon cancer (Ricci-Vitiani et al., 2007) and melanoma (Schatton et al., 2008). To date, it has been shown that CSCs in HCC can be identified by several cell surface markers, such as CD133 (Ma et al., 2007; Suetsugu et al., 2006; Yin et al., 2007; Zhu et al., 2010) and epithelial cell adhesion molecule (EpCAM) (Terris et al., 2010; Yamashita et al., 2009).

Chemotherapy is a main treatment for cancer, while MDR is the main reason for chemotherapy failure and tumor relapse (Zhou et al., 2009). Cancer often recurs after treatment and this can be attributed to the presence of CSCs. CSCs are a subpopulation of cancer cells, which may be inherently resistant to chemotherapy because of their low proliferation rate and resistance mechanisms, such as the expression of multidrug transporters of the ATP-binding cassette (ABC) superfamily (Dean et al., 2005). Some studies have suggested that chemotherapy has no effect on CSCs and can enrich CSCs (Bertolini et al., 2009; Dylla et al., 2008; Levina et al., 2008; Yu et al., 2007). Two recent reports suggested that pancreatic cancer cells resistant to chemoradiotherapy rich in stem-cell-like tumor cells (Du et al., 2011) and CSCs can be isolated with drug selection in human ovarian cancer cell line SKOV3 (Ma et al., 2010).

TGF-β1 (transforming growth factor- beta1) is a multipotent cytokine that plays an important biological effect on tissue and organ development, cellular proliferation, differentiation, survival, apoptosis and fibrosis (Ikushima and Miyazono, 2010; Kelly and Morris, 2010). In the liver, TGF-β1 is hypothesized to serve as an important link between chronic injury, cirrhosis, and HCC (Matsuzaki, 2009). Previous reports indicate that TGF-β1 expression is decreased in early-stage HCC and increased in late-stage HCC (Abou-Shady et al., 1999; Matsuzaki et al., 2000). A recent report indicated that dysregulation of the TGFβ pathway leads to HCC through disruption of normal liver stem cell development (Tang et al., 2008). Two more recent studies reported that the percentage of SP (side population) cells, a potent marker of stem cell, and CD133+ cells are increased by TGF-β treatment (Nishimura et al., 2009; You et al., 2010). Furthermore, their results suggested that the phenotypic change with increased aggressiveness in HCC cells caused by TGF-β stimulation may be relevant to the kinetics of CSCs (Nishimura et al., 2009; You et al., 2010).

It is believed that CSCs resist the radiotherapy and the cytotoxic effect of chemotherapy (Dean et al., 2005; Zhou et al., 2009). However, the relationship between chemotherapy and CSCs is not clear and needs to be further elucidated. Based on the potential role of TGFβ1 in liver cancer progression and the importance of CSCs in HCC, we hypothesized that chemotherapy can enrich liver CSCs through constituted activation of TGF-β1 pathway. Using Huh7.5.1 HCC cells and PTX, we developed a MDR HCC subline model, Huh7.5.1/PTX. Furthermore, we found that MDR Huh7.5.1/PTX cells showed high percentage of CD133, CD90 and EpCAM positive cells and strongly activated the TGF-β1/Smad3 signaling. Activation of TGF-β1/Smad3 signaling can lead to propagation of $CD133^+$ population, while inhibition of this pathway activity attenuated the percentage of these cells. In summary, our findings propose that CSCs could be enriched in MDR HCC cells, which is partially dependent on TGF-β1/Smad3 pathway.

MATERIALS AND METHODS

Cell line and cell culture

The human hepatocellular carcinoma cell line, Huh7.5.1, was kindly gifted from Dr. Wenyu Lin (Massachusetts General Hospital, Harvard Medical School). Huh7.5.1 cells were cultured in Dulbecco's modified eagle's medium/high glucose (DMEM/H) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and were incubated at 37°C in a humidified incubator with an atmosphere of 5%CO_2.

Reagents

DMEM/H, FBS and Trypsin-EDTA were purchased from Hyclone (Thermo Scientific). CCK-8) was obtained from Beyotime (Hangzhou, China). Paclitaxel (PTX), Cisplatin (DDP), gemcitabine (GEM), 5-fluorouracil (5-Fu), doxorubicin (ADM), and mitomycin (MMC) was obtained Shanghai Xudong Haipu Pharmaceutical Co. Ltd (Shanghai, China). Fluorochrome-conjugated antibodies against human CD29, CD34, CD44, CD54 and CD105 (ICAM-1), and CD133 and associated isotype control antibodies were from eBioscience, Inc (San Diego, CA USA) and CD90, CD326 (EpCAM), and CD338 (ABCG2) and associated isotype control antibodies were from Biolegend (San Diego, CA USA). Antibodies

against CD133, Smad3, Smad4, and phosphorylated Smad3 (pSmad3) were from Abcam Inc. (Abcam,Cambridge, MA). Cytokine TGF-β1 and antibodies against TGF-β1 and β-actin were from R&D Systems INC. (Minneapolis, MN). SIS3, a specific Inhibitor of Smad3[46], was from Merck (NJ, USA).

Establishment of a PTX-resistant Huh7.5.1 cell line (Huh7.5.1/PTX) *in vitro*

Huh7.5.1/PTX was produced by exposing Huh7.5.1 cells to PTX repeatedly at a single high concentration over a period of 12 h. Briefly, Huh7.5.1/PTX was selected by a procedure consisting of six pulse drug treatments with 5 μg/ml PTX. When Huh7.5.1 cells were growing exponentially, they were exposed to PTX for 12 h. The majority of the cells were dead following 12 h exposure to PTX. The treated cells were then washed with phosphate buffered saline (PBS) and cultured in PTX-free growth medium. After two to three days, the dead cells were washed out with PBS and fresh medium was added again. The resistant subclones were isolated by limiting dilution.

After four weeks' incubation at 37°C in a humidified atmosphere containing 5% CO_2, the cells recovered at an exponential rate and were then subcultured. Once cells reached 80-90% confluence, the cells were preserved and treated again as described above. The PTX-resistant subclone was established 6 months after the treatment was initiated, and the resistant phenotype developed. For maintenance of PTX -resistant cells, the Huh7.5.1/PTX cells were grown in the presence of 0.01 μg/ml PTX. Before experimentation, Huh7.5.1/PTX cells were maintained in a PTX-free culture medium and subcultured at least 3 times.

Detection of cellular sensitivity to anticancer drugs using CCK-8 assay

The MDR characteristics of these Huh7.5.1/PTX cells were tested using various concentrations of anticancer drugs including PTX, DDP, GEM, ADM, MMC and 5-FU. The effects of chemotherapeutic agents on the growth of Huh7.5.1 and Huh7.5.1/PTX cells were evaluated with CCK-8. Cells (5×10^3) were seeded into 96-well plates in 100 μL of DMEM/H with 10% FBS incubated at 37°C in a humidified atmosphere containing 5% mL/L CO_2. After 12 h, the medium was removed, and exchanged with media containing a test chemotherapeutic agent at various concentrations. After incubation for 48 h at 37°C, the drug-containing growth medium was replaced with 110 μL medium containing CCK-8 reagent. After 2 h, the absorbance was read at 450 nm with a reference wavelength at 600 nm. The experiment was replicated at least 3 times. The IC_{50}, defined as the drug concentration required to reduce cell survival to 50%, was calculated by probit regression analysis using SPSS 13.0 statistical software.

FCM analysis of cell surface markers expression levels

FCM was used to measure cell surface markers expression levels (CD11b, CD29, CD34, CD40, CD44, CD45, CD54, CD90, CD105, CD133, EpCAM and ABCG2 in Huh7.5.1 and Huh7.5.1/PTX cells). The cultured Huh7.5.1 and Huh7.5.1/PTX cells with or without SIS3, TGF-β1 and anti-TGF-β1 monoclonal antibody stimulation were collected by trypsinization, washed in ice-cold PBS, and then directly immunostained using fluorochrome- conjugated antibodies described above. The isotype control IgG was evaluated in each experiment to determine the level of background fluorescence of negative cells. Mean fluorescence intensity was determined for positively stained cells. Samples and results were analyzed using a Epics XL flow cytometer and WinMDI 2.9 software.

WB

The cultured Huh7.5.1 and Huh7.5.1/PTX cells with or without stimulation were lysed in radio-immuno-precipitation assay buffer. The samples were incubated for 2 h on ice. Samples were then centrifuged at 12 000 *g* for 15 min and protein concentrations were measured in the supernatants using a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Cell extracts were denatured in LDS sample buffer for 5 min at 95°C, and electrophoresed on a 10-20% SDS-PAGE and blotted onto PVDF membranes (0.2 μm, Invitrogen). Membranes were blocked with 5% milk or 5% bovine serum albulin (BSA) in TBS-T (TBS containing 0.05% Tween 20) for 1 h at room temperature and were subsequently incubated overnight at 4°C with primary antibodies described above. After incubation with the respective primary antibodies, membranes were washed three times for 5 min in TBS-T, and then incubated with species-specific horseradish peroxidase (HRP)-labeled secondary antibodies at 37°C for 1 h. The membrane was developed using the ECL Plus WB reagent (Biomiga) with visualization on X-ray films. The expression of β-actin was detected as an internal control.

Statistical analysis

All experiments were run at least three times, and the results are given as mean ± SD. Statistical analyses were performed using either a one-way analysis of variance (ANOVA) or Student T test. The difference was considered statistically significant when the P value was less than 0.05. All statistical analyses were carried out with GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Huh7.5.1/PTX cells show higher chemotherapeutic resistance and MDR

To study the enrichment of CSCs in HCC by chemotherapy, we firstly developed a drug-resistant model. We compared the sensitivity of Huh7.5.1 cells to various drugs and found that Huh7.5.1 cells were most sensitive to PTX (Figure 1A). By exposing Huh7.5.1 cells to PTX repeatedly at a single high concentration over a period of 12 h, the PTX-resistant clones was established six months after the treatment was initiated. To test the resistance to anticancer drugs, we used CCK-8 assay to determine the effects of PTX, DDP, GEM, 5-Fu, ADM and MMC on the growth of Huh7.5.1 and Huh7.5.1/PTX cells. We found that besides PTX, Huh7.5.1/PTX cells were also more resistant to some other anticancer drugs including DDP, GEM, 5-Fu, ADM and MMC. Huh7.5.1/PTX cells showed high resistance to PTX and the IC_{50} (50% inhibitory concentration) of these drugs in Huh7.5.1/PTX cells were significantly higher than those in Huh7.5.1 cells (Figure 1B). Huh7.5.1/PTX cell showed MDR and varying degree of drug-resistance, high degree of PTX and DDP, medium degree of 5-Fu and ADM, and low degree of MMC and GEM concerning that RI (resistance index) of Huh7.5.1/PTX cells to PTX, DDP, GEM,5 -Fu, ADM and MMC was 15.70, 11.41, 5.00, 5.29, 2.26 and 2.31, respectively (Figure 1C).

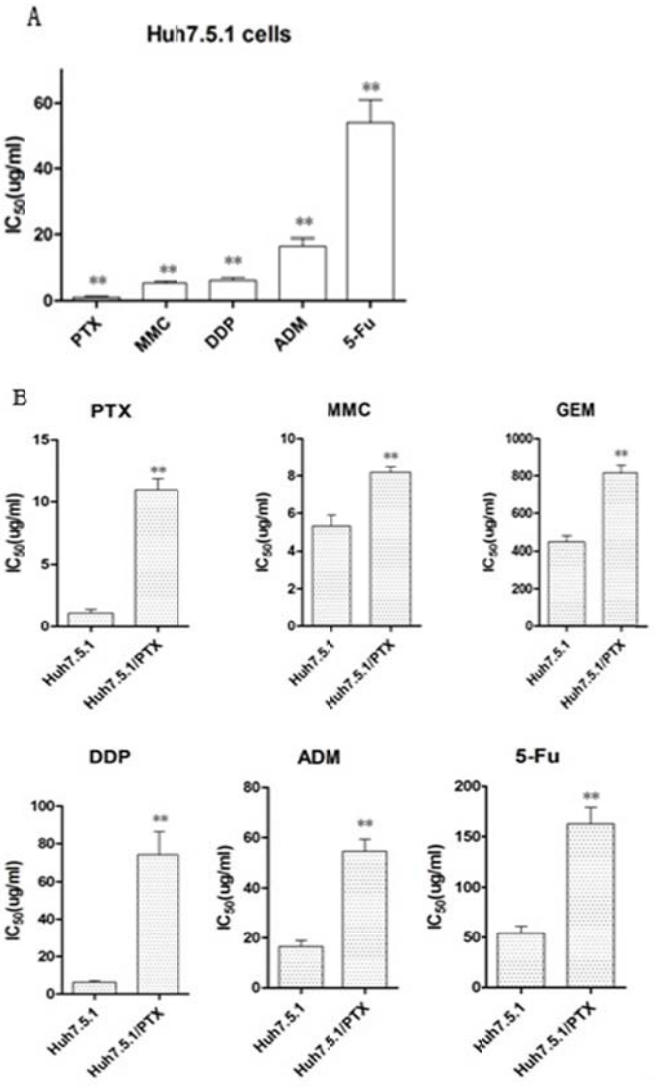
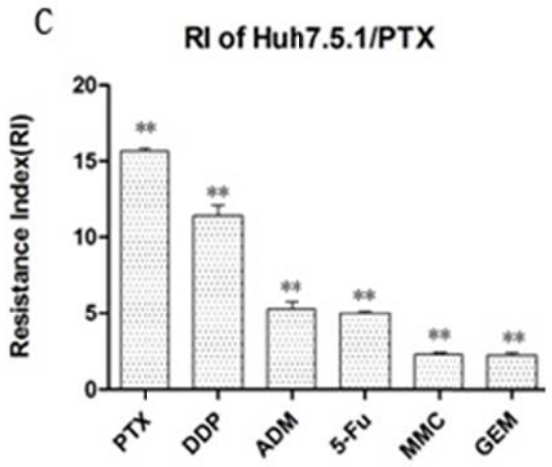


Figure 1. Huh7.5.1/PTX cells show higher chemotherapeutic resistance and have cross-resistance. A. IC_{50} (50% inhibitory concentration) of Huh7.5.1 cells to various drugs (including PTX, MMC, DDP, ADM and 5-Fu). Huh7.5.1 cells are most sensitive to PTX. **p<0.01 (one-way analysis of variance). **B.** Huh7.5.1/PTX cells show more resistance to PTX, DDP, GEM, 5-Fu, ADM and MMC than parental Huh7.5.1 cells. **p<0.01(Student t test). **C.** RI (Resistance Index) of Huh7.5.1/PTX cells to anti-cancer drugs. Huh7.5.1/PTX cells showed highest resistance to PTX and have multi-drug resistance. RI of Huh7.5.1/PTX cells to PTX, DDP, 5-Fu, ADM, GEM and MMC was 15.70, 11.41, 5.00, 5.29, 2.26 and 2.31, respectively. **p<0.01(one-way analysis of variance). Each value represents the mean ± standard deviation for at least three independent experiments.

Huh7.5.1/PTX cells express higher level of CSCs markers

Resistance to chemotherapy distinguishes CSCs from other cancer cells. As mentioned above, Huh7.5.1/PTX cells were resistant to chemotherapy. To examine whether



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chemotherapy might enrich for CSCs, we compared the proportion of CD133, CD90, CD44, CD326, CD338, CD29, CD34, CD54 and CD105 positive cells in Huh7.5.1 and Huh7.5.1/PTX cells by FCM. CD133, CD90, CD44 and CD326 have been reported to isolate stem-like cells from HCC cell lines or HCC patients tissues and they may be the candidate markers of liver CSCs.

Concerning our research results, we found that Huh7.5.1/PTX cells contained high percentage of these markers positive cells: (CD 133 69.9% vs. 19.4%, CD90 6.02% vs. 0.88%, CD44 90.1% vs. 2.57%, CD326 90.7% vs. 24.1%). Besides that, some markers were also expressed by a high level in Huh7.5.1/PTX cells including stemness-and drug-resistance-associated markers: CD338 (81.3% vs. 1.92%), CD34 (23.8% vs. 0.47%) and CD105 (98.7% vs. 3.61%). Huh7.5.1/PTX cells expressed low level of CD54 (64.8% vs. 94.3%) and did not express CD11b and CD45 (data not shown). CD133, CD90, CD44, CD326, CD338, CD34, CD54 and CD105 expression are statistically significant and there is no significant expression of CD29 in Huh7.5.1 and Huh7.5.1/PTX cells (Figure 2).

TGF-β1/Smad3 pathway is activated in MDR Huh7.5.1/PTX cells

To determine the activity of TGF-β1/Smad3 signaling, we compared the protein expression level of TGF-β1, Smad3, Smad4, pSmad3 and CD133 in parental and resistant cells by WB. Compared to the parental cell line, the protein level of CD133, TGF-β1 and pSmad3 were higher in MDR cells and total Smad3 did not changed (Figure 3). Our results show that MDR Huh7.5.1/PTX cells showed higher activity of TGF-β1/Smad3 signal, and these results are concordant with the results of percentage of CD133, CD90, CD326 and CD44 positive cells in MDR Huh7.5.1/PTX cells.

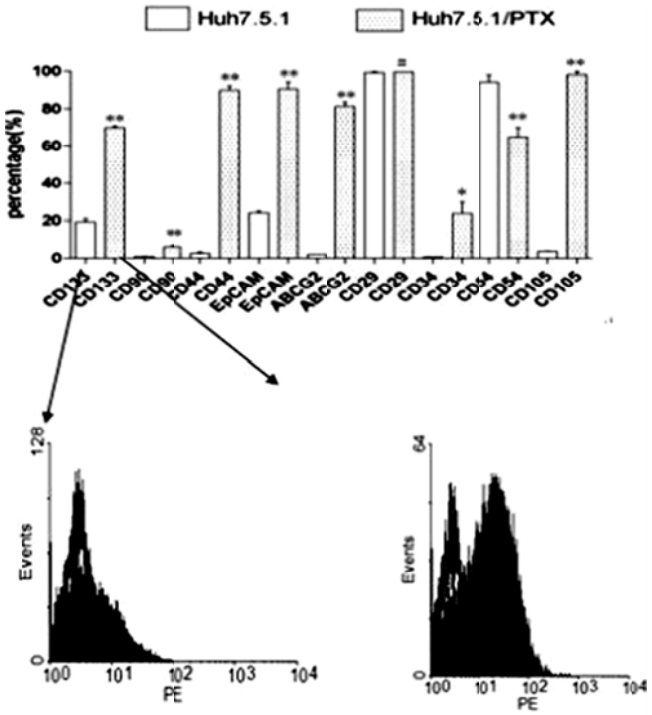


Figure 2. Huh7.5.1/PTX cells are enriched for cancer stem-like cells. The proportion of CD133, CD90, CD44, CD326，CD338, CD29, CD34, CD54 and CD105 positve cells in Huh7.5.1 and Huh7.5.1/PTX cells was examined by FCM.Huh7.5.1/PTX cells contained high percentage of these markers positive cells: (CD133 69.9% vs. 19.4%, CD90 6.02% vs. 0.88%,CD44 90.1% vs. 2.57%,CD326 90.7% vs. 24.1%) and also expressed high level of stemness-and drug-resistance-associated markers including CD338(81.3% vs. 1.92%), CD34 (23.8% vs. 0.47%) and CD105 (98.7% vs. 3.61%). Huh7.5.1/PTX cells expressed low level of CD54 (64.8% vs. 94.3%) and did not express CD11b, CD40 and CD45 (data not sown). There is no significant expression of CD29 (99.5% vs. 99.2%) in Huh7.5.1 and Huh7.5.1/PTX cells. Each value represents the mean ±standard deviation for at least three independent experiments. # p>0.05,*p<0.05, **p<0.01 (Student t test)

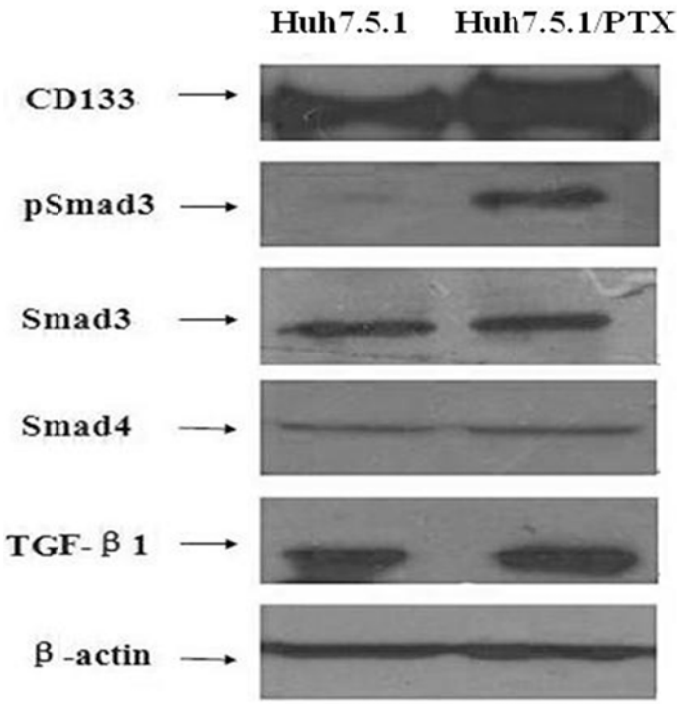


Figure 3. TGF-β1/Smad3 pathway is activated in chemo-resistant Huh7.5.1/PTX cells. The protein level of TGF-β1, Smad4, Smad3, pSmad3 and CD133 was compared between Huh7.5.1/PTX and Huh7.5.1 cells by WB. Huh7.5.1/PTX cells showed elevated expression level of CD133, TGF-β1, and phosphorylated Smad3 compared to Huh7.5.1 cells. Elevated expression of phosphorylated Smad3 in Huh7.5.1/PTX cells was not a result of an increase in total Smad3 protein level. β-Actin was used as a control for equal loading.

CSCs marker-CD133 expression is regulated by TGF-β1/Smad3 pathway

Based on the fact that MDR Huh7.5.1/PTX cells show both high percentage of CSCs and higher activity of TGF-β1/Smad3 signaling, we hypothesized that MDR Huh7.5.1/PTX cells may enrich these cells through activation of TGF-β1/Smad3 pathway. In order to assess whether TGF-β1/Smad3 signaling regulates the expression of CSCs markers, reagents including TGF-β1 and SIS3, were added to the medium in serum-free cultured Huh7.5.1 cells for 48 h.

In cultured Huh7.5.1 cells, CD133 expression was reduced with SIS3 (3 ug/ml) alone stimulation (11.9% vs. 18.6%). CD133 expression was reduced with SIS3 (3 ug/ml) and TGF-β1 (10 ng/ml) co-stimulaton (15.5%) compared with the TGF-β1 alone stimulation (35.3%).

We also found that high concentration of TGF-β1 (10 ng/ml) could up-regulate the percentage of $CD133^+$ cells in Huh7.5.1 cells (35.3% vs. 18.6%) (Figure 4A). Based on this finding, we presumed that CD133 expression was partially dependent on the TGF-β1/Smad3 pathway.

For Huh7.5.1/PTX cells, reagents including monocotlonal anti-TGF-β1 neutralization antibody (TGF-β1 mAb) and SIS3 were added to the medium in normal cultured conditions. FCM analysis showed decreased CD133 expression with SIS3 (3 ug/ml) stimulation (29.9%) compared with CD133 expression of control group cells(69.9%), and reduce expression of CD133 with TGF-β1 mAb (10 ug/ml) stimulation (39.5%) compared to the control group (53.6%), respectively (Figure 5A). Other liver CSCs candidated markers (including CD90, CD44, CD326) have no significant changes with the changes of TGF-β1/Smad3 pathway activity (data not shown). All the FCM results were also demonstrated by WB (Figures 4B and 5B).

Here, we report that CSCs could be propagated by chemotherapy in HCC cell line Huh7.5.1，which may be partially dependent on the activity of TGF-β1/Smad3 pathway.

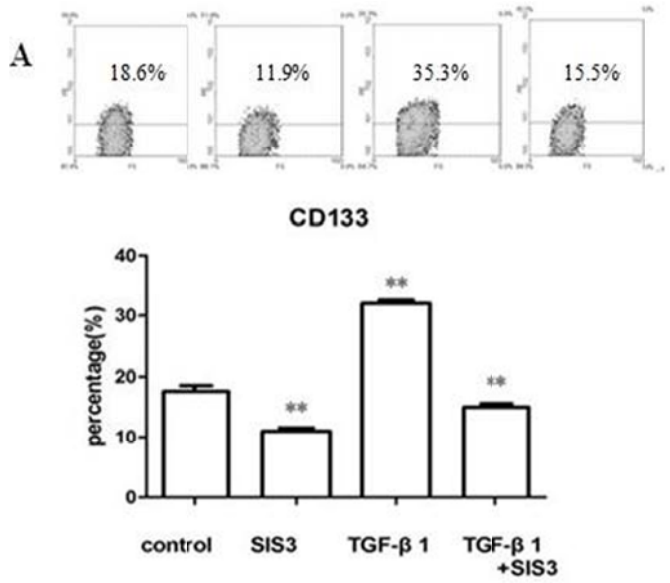
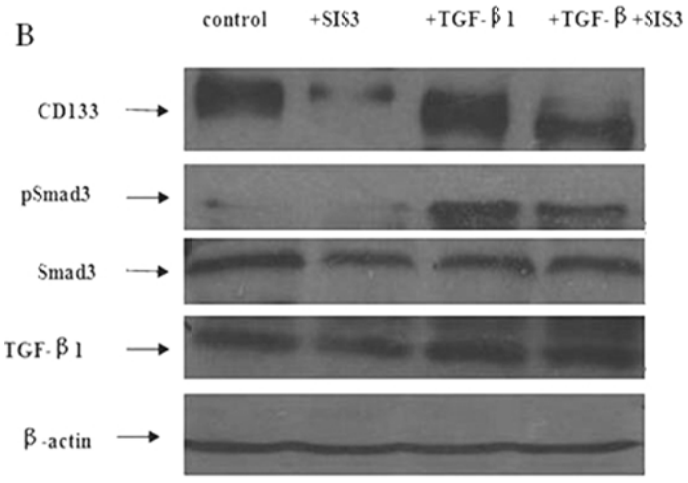


Figure 4. CD133 expression is regulated by TGF-β1/Smad3 pathway in Huh7.5.1 cells. **A.** CD133 expression was reduced with SIS3(3 ug/ml) alone stimulation(11.9%) and increased with TGF-β1 alone stimulation (35.3%)compared with control group(18.6%). CD133 expression was also decreased with SIS3(3 ug/ml) and TGF-β1(10 ng/ml) co-stimulation(15.5%) compared with the TGF-β1 alone stimulation(35.3%). Each value represents the mean ± standard deviation for at least three independent experiments. ** p<0.01(Student t test) Other liver CSCS candidate markers (including CD90,CD44,CD326) show no significant changes (data not shown) . **B.** The results of WB demonstrated our FCM results. TGF-β1 could activate the phosphorylation of Smad3 and SIS3 could inhibit the phosphorylation of Smad3. CD133 expression changes are concordant with the activity changes of Smad3.



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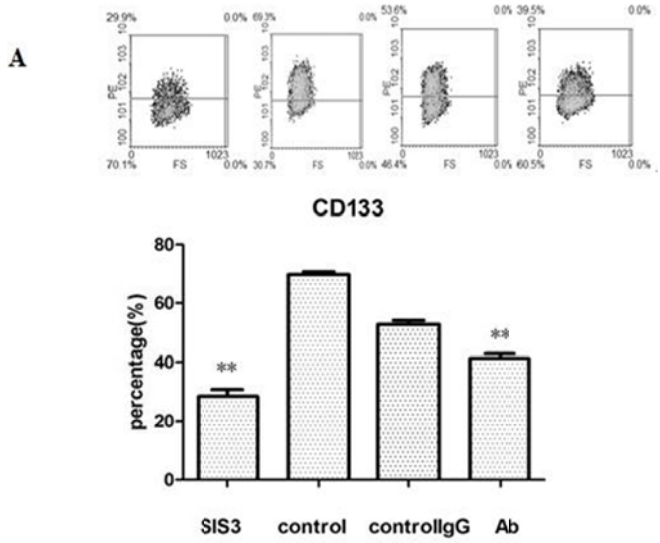
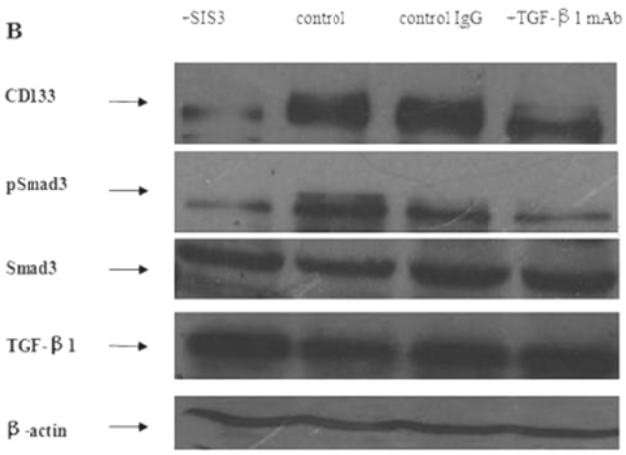


Figure 5. TGF-β1/Smad3 pathway is involved in CD133 expression changes in Huh7.5.1/PTX cells. **A.** CD133 expression was attenuated with SIS3 (3 ug/ml) alone stimulation (29.9%) and TGF-β1 mAb (10 ug/ml) alone stimulation (39.5%) compared with the control group 69.9%, 53.6%, respectively. Each value represents the mean ± standard deviation for at least three independent experiments. **p<0.01 (Student t test). Other markers (including CD90, CD44 and CD326) show no significant changes (data not shown). **B.** The results of WB demonstrated our FCM results. WB results showed that CD133 expression is in conformity with the activity of Smad3.



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The recent discovery of CSCs in solid tumors has played a pivotal role in changing our view of carcinogenesis and chemotherapy (Zou, 2010b). There are two dominant models of carcinogenesis: stochastic organization (clonal evolution model) and hierarchical organization of tumor (CSCs model) (Clarke et al., 2006; Reya et al., 2001). According to the latter, CSCs is at the germinal center of tumor evolution, which is similar to normal adult stem cells and possesses the capacity of self-renewal and differentiation potential (Clarke et al., 2006). Over the past few years, increasing evidence has emerged in

support of the hierarchic cancer model for many solid tumors (Collins et al., 2005; Li et al., 2007; Patrawala et al., 2006; Ponti et al., 2005; Ricci-Vitiani et al., 2007; Schatton et al., 2008; Singh et al., 2003) including HCC (Ma et al., 2007; Suetsugu et al., 2006; Thomas et al., 2010; Yamashita et al., 2009; Yang et al., 2008a; Yang et al., 2008b; Yin et al., 2007; Zhu et al., 2010). The CSCs are posited to be responsible not only for tumor initiation but also for the generation of distant
metastasis and relapse after therapy (Zhou et al., 2009). CSCs are responsible for the formation and growth of neoplastic tissue and are naturally resistant to chemotherapy, explaining why traditional chemotherapy can initially shrink a tumor but fails to eradicate it in full, allowing eventual recurrence (Dean et al., 2005).

Chemotherapy is used to treat unresectable liver cancer with limited efficacy, which might result from HCC cells with stem-like properties and chemo-resistant characteristics (Dean et al., 2005; Zhou et al., 2009; Zou, 2010b). However, the molecular mechanism by which CSCs escape conventional therapies remains unknown. Therefore, investigating the possible molecular mechanism of chemotherapy regulating the expression of CSCs markers is very significant. Some studies have suggested that chemotherapy could enrich CSCs (Bertolini et al., 2009; Du et al., 2011; Dylla et al., 2008; Levina et al., 2008; Ma et al., 2010; Yu et al., 2007). However, in the context of HCC, the relationship between chemotherapy and CSCs remains unclear and the molecular mechanism is unknown. Therefore, we investigated whether drug treatment could enrich CSCs in HCC cells and the possible potential molecular mechanism of chemotherapy regulating the expression of CSCs markers.

Firstly, to test our hypothesis, we established a MDR cell model, Huh7.5.1/PTX. The reasons why we used Huh7.5.1 cells are as follows: (1) There's a moderate percentage of $CD133^+$ cells (19.4% of $CD133^+$) compared to some others HCC cell line in Huh7.5.1 cells (including HepG2, Bel-7402, SMMC-7721, Huh7 and MHCC97-H) (data not shown); (2) If there's a lower or higher percenttage of $CD133^+$ cells in HCC cells, they may not be suitable for enrichment of CSCs. For example, there are almost no $CD133^+$ cells in HepG2 and we found that chemotherapy did not affect the percentage of them (data not shown). Huh7 cells contained high percentage of $CD133^+$ cells (data not shown)and we found that low concentration of chemotherapeutic drugs almost have no effect on this cell, while use of high concentration of drugs in experiments, especially in clinical patients, is no account. Concerning the percentage of $CD133^+$ cells and the sensitivity of cells to drugs, we therefore selected Huh7.5.1cells that contained moderate percentage and PTX to carry out our experiments. The reasons why we used PTX are as follows: (1) Huh7.5.1 cells showed higher sensitivity to PTX at a low concentration (Figure 1A); (2) CSCs are mainly shown in the cell cycle of G0/G1 phase (Kamohara et al., 2008) and PTX mainly kill cells that are in the G2/M phase (Jin et al., 2010). As a result, we selected PTX so that we can kill non-stem cells in cancer to enrich the stem-like cells in HCC cells. Besides that, there are two methods of establishment of drug-resistant model including gradually increasing concentrations of drugs and intermittent administration of high-dose of drugs (Zhang et al., 2010a; Zhang et al., 2010b; Zhou et al., 2010). Concerning the latter, it mimicked the clinical regimen that patients with cancers would receive. As a result, we selected this method to establish our MDR model, which ensured that more than 90% of cells underwent apoptosis or senescence or necrosis with the cells eventually dying, thereby selecting the most resistant clones. Eventually, it took us six months to establish the chemo-resistant model-Huh7.5.1/PTX.

Secondly, to test whether our model is available, we tested the drug sensitivity of Huh7.5.1/PTX. Results demonstrated the availability of the Huh7.5.1/PTX. Huh7.5.1/PTX cells showed high resistance to PTX and had various degree of resistance to other chemotherapeutic drugs. Recent studies have started to link CSCs to chemo-resistance (Dean et al., 2005; Zhou et al., 2009). Therefore, we next compared parental and chemo-resistant Huh7.5.1 cells for cell surface stem cell markers, including CD133, CD90, EpCAM and other stemness-associated markers including (CD29, CD34, CD105, CD308 etc.). We found that MDR Huh7.5.1 cells showed elevated expression of known CSCs markers such as CD90, CD133, and EpCAM in HCC. Recently, the cell surface marker CD133 identifies cancer-initiating cells in a number of malignancies and it has also been used to isolate stem-like cells from HCC cells (Ma et al., 2007; Suetsugu et al., 2006; Yin et al., 2007; Zhu et al., 2010). In summary, these data suggest that chemo-resistant cells derived from cancer cell lines are enriched for CSCs.

Thirdly, we found that chemotherapy can enrich the percentage of CSCs. However, the mechanism of this phenomenon is unknown. Some other reports also suggested that chemotherapy could enrich stem-like cells in breast (Yu et al., 2007), lung (Bertolini et al., 2009; Levina et al., 2008), colorectal (Dylla et al., 2008), pancreatic (Du et al., 2011), and ovarian (Ma et al., 2010) cancer. To the best of our knowledge，the mechanism study of chemotherapy regulating the CSCs is not researched so far. Therefore, we next investigated the potential mechanism of this enrichment. TGF-β1 pathway plays an important role in cell proliferation, apoptosis, and tumorigenesis (Ikushima and Miyazono, 2010; Kelly and Morris, 2010). Recently, a report suggested that $CD133^+$ liver CSCs exhibited relative resistance to TGF-β1-induced apoptosis (Ding et al., 2009). Cells through epithelial-mesenchymal transition by TGF-β could acquire the features of stem cells (Mani et al., 2008; Singh and Settleman, 2010). A recent research reports that dysregulation of the TGFβ pathway leads to HCC through

disruption of normal liver stem cell development (Tang et al., 2008). Two more recent studies reported that the percentage of SP and CD133+ cells were increased by TGF-β treatment in HCC cells (Nishimura et al., 2009; You et al., 2010). Based on the potential role of TGFβ in HCC and CSCs, we hypothesized that chemotherapy resistant cells may have constituted activation of TGF-β1 pathway activity. To validate our hypothesis, we compared the activity of TGF-β/Smad3 pathway in Huh7.5.1 and MDR Huh7.5.1/PTX cells. Our results demonstrate the higher activity of TGF-β/Smad3 pathway in Huh7.5.1/PTX cells.

Eventually, now that MDR Huh7.5.1/PTX cells showed both high percentage of CSCs and higher activity of TGF-β1/Smad3 signaling, we hypothesized that MDRHuh7.5.1/PTX cells may enrich these cells through activation of TGF-β1/Smad3 pathway. In order to assess whether TGF-β1/Smad3 signaling regulates the expression of CSCs markers, we investigated the association of cancer stem markers expression changes and activity of TGF-β1/Smad3 signal. Through activation and inhibition of TGF-β1/Smad3 pathway, we found that CD133 expression was decreased when inhibition and elevated when activation of TGF-β1 pathway. Besides that, we also analyzed other cell surface marker expression such as CD90 and CD326; our results show that there were no significant changes via inhibition or activation of TGF-β1 signal (data not shown). Perhaps, there are other mechanisms involved in regulation of CD90 and CD326 (reported as liver CSCs candidated markers) in MDR Huh7.5.1/PTX cells. We will investigate the possible mechanism in future.

In conclusion, we are the first to report on the mechanism of chemotherapy regulating the expression of $CD133^+$ CSCs in HCC, which is involved in TGF-β1/Smad3 pathway. Taken together, our results suggest that MDR HCC cells are enriched for CSCs, which is partially dependent on TGF-β1/Smad3 pathway. These findings could provide some insight into novel therapy via inhibition of TGF-β1/Smad3 pathway, which may be useful for targeting CSCs to develop more effective treatments for HCC.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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