Doxorubicin and 5-fluorouracil resistant hepatic cancer cells demonstrate stem-like properties

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Abstract The efficacy of hepatocellular carcinoma (HCC) treatment is very low because of the high percentage of recurrence and resistance to anticancer agents. Hepatic cancer stem cells (HCSCs) are considered the origin of such recurrence and resistance. Our aim was to evaluate the stemness of doxorubicin and 5-fluorouracil resistant hepatic cancer cells and establish the new method to isolate the HCSCs from primary cultured HCC tumors. HCC biopsies were used to establish primary cultures. Then, primary cells were selected for HCSCs by culture in medium supplemented with doxorubicin (0, 0.1, 0.25, 0.5 or 1 µg/mL), 5-fluorouracil (0, 0.1, 0.25, 0.5 or 1 µg/mL) or their combination. Selection was confirmed by detection of HCSC markers such as CD133, CD13, CD90, and the side population was identified by rhodamine 123 efflux. The cell population with the strongest expression of these markers was used to evaluate the cell cycle, gene expression profile, tumor sphere formation, marker protein expression, and in vivo tumorigenesis. Selective culture of primary cells in medium supplemented with 0.5 µg/mL doxorubicin and 1 µg/mL 5-fluorouracil selected cancer cells with the highest stemness properties. Selected cells strongly expressed CD13, CD133, CD90,

and CD326, efflux rhodamine 123 and formed tumor spheres in suspension. Moreover, selected cells were induced to differentiate into cells with high expression of CD19 and AFP (alpha-fetoprotein), and importantly, could form tumors in NOD/SCID mice upon injection of 1×10^5 cells/mouse. Selective culture with doxorubicin and 5-fluorouracil will enrich HCSCs, is an easy method to obtain HCSCs that can be used to develop better therapeutic strategies for patients with HCC, and particularly HCSC-targeting therapy.

Keywords 5-Fluorouracil · Antitumor drug resistance · Doxorubicin · Hepatic cancer stem cells · Hepatocellular carcinoma

Abbreviations

5-FU	5-Fluorouracil
AFP	Alpha-fetoprotein
CD	Cluster of differentiation
Dox	Doxorubicin
EpCAM	Epithelial cell adhesion molecule
HCC	Hepatocellular carcinoma
HCSCs	Hepatic cancer stem cells
HE	Haematoxylin and Eosin

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of cancer in developing countries, with poor treatment and prognosis with a 5-year survival rate of

less than 5 %. HCC is diagnosed in more than half a million people worldwide each year (Ferlay et al. 2001), and is the fifth most common cancer in men and the seventh most common cancer in women. The efficacy of HCC treatment is low, considering recurrence it can be detected at 12–24 months. The existence of cancer stem cells (CSCs) in malignant tumors has been proposed for some decades, and much evidence supports this hypothesis. Hepatic cancer stem cells (HCSCs) have been identified and isolated from HCCs (Rountree et al. 2011; Mikhail and He 2011; Cao et al. 2011; Cheung et al. 2011; Jia et al. 2012; Yoon 2012; Shen and Cao 2012; Piao et al. 2012; 2012), and characterized by specific properties such as expression of CD133 (Suetsugu et al. 2006; Yin et al. 2007; Ma et al. 2007), CD44, CD133 (Zhu et al. 2010), CD90 (Yang et al. 2008), EpCAM (Epithelial cell adhesion molecule) (Yamashita et al. 2008, 2009), CD13 (Haraguchi et al. 2010), OV6 (Yang et al. 2008) and ALDH (Aldehyde hydrogenase) (Ma et al. 2008).

Antitumor drug resistance is an important property and characteristic of CSCs, which overcomes the effects of chemical therapies. The drug resistance of CSCs is related to several mechanisms, and particularly overexpression of ABCG2 transporters. Collectively, drug resistance and SP phenotypes may be related to the expression of ABC transporters that exclude drugs and dyes. Because many antitumor drugs are substrates of ABC transporters, CSCs can possess multidrug resistance. However, assays based on Hoechst 33342 efflux to detect the SP phenotype may give misleading results. A study has shown that ABCC1-overexpressing HL-60/Adr cells are composed of more than 90 % SP cells (Patrawala et al. 2005) that are not necessarily associated with increased CSC content. The SP phenotype has been used to identify and isolate normal stem cell populations from a variety of tissues including bone marrow (Goodell 2001), mammary glands (Welm et al. 2003), skin (Montanaro et al. 2003), liver (Wulf et al. 2003), lung (Majka et al. 2005), skeletal muscle (Meeson et al. 2004), limbs (Umemoto et al. 2005), heart (Martin and Dargie 2004) and brain (Kim and Morshead 2003). In addition, overexpression of the ABC transporter is usually related to stem cell markers such as CD133, nestin, CD117 (Adhikari et al. 2010), Notch-1 and Nanog (Patrawala et al. 2005; Bourguignon et al. 2008) as well as Oct-4 (Wang et al. 2012). Inhibition of ABC transporters reduces the multidrug resistance of some cancer types (Li et al. 2010; Peng

et al. 2010; Moitra et al. 2011; Tiwari et al. 2011; Meyer zu Schwabedissen and Kroemer 2011). Therefore, a side population (SP) by exclusion of Hoechst 33342 dye was used to isolate HCSC population (Chiba et al. 2006). Another mechanism of CSCs that leads to multidrug resistance is increased doubling time. During long doubling periods, CSCs avoid the anticancer effects of DNA damaging agents, mitotic spindle poisons and antimetabolites (Beier et al. 2008; Ropolo et al. 2009; Thomas and Cannas 2010), whereas differentiated cells with a rapid doubling time are easily affected by these agents. Recent further studies indicate that drug resistance is related to CD44 and PI3 kinase/Akt-related survival pathways as shown in breast tumor cells (Pham et al. 2011; 2012; Cheng et al. 2012; Van Phuc et al. 2011; Tamada et al. 2012). Thus, inhibition of CD44 can sensitize some types of cancer cells to antitumor drugs (Pham et al. 2011; 2012; Tamada et al. 2012) or inhibit tumor growth (Li et al. 2008; Van Phuc et al. 2011). The combination of DNA damaging drugs and inhibitors of the Akt pathway also inhibits CSC proliferation (Sabisz and Skladanowski 2009; Fan et al. 2010; Zhang et al. 2012).

Currently, metastatic HCC is commonly treated by doxorubicin (Dox) alone or in combinatorial therapy (Lin et al. 1997; Lai et al. 1998; Dhanasekaran et al. 2010; Ferrer Puchol et al. 2011; Prajapati et al. 2012). In addition, 5-fluorouracil (5-FU) has been used in combination with other agents for treating metastatic HCC (Ellis et al. 1995; Yamashita 2012; Kirikoshi et al. 2012; Uchino et al. 2012). However, the response rate was too low, from 15 to 20 % and the recurrence of HCC patients was high (Cao et al. 2011). From these results, we proposed that there was a small population of cancer cells resisting to Dox and 5-FU, and they express the cancer stem cell characteristics. Therefore, this research aimed at evaluating the stemness of doxorubicin and 5-fluorouracil resistant hepatic cancer cells and establishing a new method to isolate the HCSCs from primary cultured HCC tumors.

Materials and methods

Primary culture and subculture

Tissues were obtained from consenting hospital patients at Ho Chi Minh City Pharmacy and Medicine University Hospital, Ho Chi Minh City, Vietnam.

Samples were placed in phosphate-buffered saline (PBS) containing a 1× antibiotic-mycotic mix (GeneWorld, Ho Chi Minh City, Vietnam). Tissues were then cut into small fragments (1–2 mm³) with scissors. Then, tissues were placed in a T25 flask (Nunc, Roskilde, Denmark) with 1 mL FBS (fetal bovine serum) for 4 h, followed by culture in DMEM/F12 supplemented with 10 % FBS, 2 mM L-glutamine, 1× antibiotic-mycotic and non-essential amino acids (all purchased from GeneWorld, Ho Chi Minh City, Vietnam) at 37 °C with 5 % CO₂. Medium was exchanged every 3rd day, and cells were subcultured at 70–80 % confluence with 0.25 % trypsin/EDTA (GeneWorld, Ho Chi Minh City, Vietnam). Ten tumors from 10 patients were used in this study.

Selection culture and proliferation

To select the antitumor drug-resistant cell population, we seeded 3,000 cells/well in 96-well plates. Cells were placed into each well by a FACSJazz sorter (BD Bioscience, Franklin Lakes, NJ, USA). The cytotoxic effects of Dox, 5-FU and their combination were measured by an xCELLigence system (Roche-Applied Science, Indianapolis, IN, USA) at 25 doses of 0, 0.1, 0.25, 0.5 and 1 µg/mL Dox, four doses of 0.1, 0.25, 0.5 and 1 µg/mL 5-FU and 16 combinations of Dox and 5-FU. Each sample was run in triplicate. Culture plates were placed into the xCELLigence system and incubated at 37 °C with 5 % CO₂. Cell proliferation was monitored for 8 days with fresh medium changes every 3rd day. Based on the proliferation curve, cancer cells that were still proliferating at the highest doses of Dox, 5-FU or both were harvested and expanded for further analysis. Proliferation medium was similar to primary culture medium.

Immunophenotyping

Cell markers were analyzed following a previously published protocol (Phuc et al. 2011). Briefly, cells were washed twice in PBS containing 1 % bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Cells were then stained with anti-CD13-FITC, anti-CD90-PE and anti-CD133-PerCP; anti-CD326-PerCP monoclonal antibodies (BD Biosciences) for 30 min at 4 °C. Stained cells were analyzed by a FACSCalibur

flow cytometer (BD Biosciences). Isotype controls were used in all analyses.

Rhodamine 123 efflux

Cells were stained with 0.1 µg/mL rhodamine (Sigma-Aldrich) at 37 °C for 30 min, and then washed twice with PBS containing 2 % FBS. Cell populations were analyzed by FITC and PerCP-Cy5.5 filters to detect rhodamine 123. Cells incubated with 50 µM verapamil and 0.1 µg/mL rhodamine 123 for 30 min were used as a positive control.

Tumor sphere culture

Cells were detached with 0.25 % trypsin/EDTA and resuspended in serum-free DMEM/F12 (1:1) supplemented with 15 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 2 mM L-glutamine, 4 U/mL insulin-like growth factor (Sigma-Aldrich, St. Louis, MO, USA) and B27 supplement (1:50) (Invitrogen, Carlsbad, CA, USA). Cells were then cultured at 37 °C with 5 % CO₂.

Gene expression analysis

Total RNA extraction was performed as described elsewhere (Phuc et al. 2011). Briefly, 1 mL TRI Reagent was added into each 1.5 mL tube and mixed by trituration. Then, tubes were centrifuged at 3,000 rpm for 5 min at 22 °C. The supernatant was collected and transferred into a new tube, and then 200 µL chloroform was added. Tubes were incubated at 4 °C for 5 min before centrifugation at 12,000 rpm for 15 min at 4 °C. RNA was precipitated with 500 µL isopropyl alcohol at room temperature for 10 min. HCSCs were analyzed for gene expression of oval cell markers including AFP, NCAM/CD56 and c-kit/CD117, and stem cell markers including Nanog, Oct4 and Sox2. PCR was performed with an Eppendorf gradient S thermal cycler (Eppendorf-AG, Hamburg, Germany) (Table 1).

In vivo tumorigenesis assay

Non-obese diabetic (NOD)/SCID mice (5–6 weeks old) (*NOD.CB17-Prkdcscid/J*) (Charles River Laboratories,

Table 1 Primer sequences

Gene	Accession No.	Sequence
Oct-3/4	NM_002701.3	F:5'-GGAGGAAGCTGACAACAATGAAA-3' R:5'-GGCCTGCACGAGGGTTT-3
Nanog	NM_028016.2	F:5'-ACAACCTGGCCGAAGAATAGCA-3' R:5'-GGTCCCGAGTCGGGTTCAC-3
Sox-2	NM_003106.3	F:5'-TTGCTGCCTCTTTAAGACTAGGA-3' R:5'-CTGGGGCTCAAACCTTCTCTC-3'
AFP	NM_001134.1	F:5'-AAATACATCCAGGAGAGCCA-3' R:5'-CTGAGCTTGGCACAGATCCT-3'
CD117	NM_000222.2	F:5'-TAC TCA TGG TCG GAT CAC AAA-3' R:5'-CCA CTT CAC AGG TAG TCG AGC-3'
CD56	NM_001076682	F:5'-CCCGAATTCATCCTTGTTC AAGC-3' R:5'-TCGGGATCCGGACTGGCTGCGTCTT-3'
GAPDH	NM_002046	F:5'-GGGCTGCTTTTAACTCTGGT-3' R:5'-TGGCAGGTTTTTCTAGACGG-3'

Willimantic, CT, USA) were used in this study. All mice were maintained according to institutional guidelines for animal welfare. Mice were subcutaneously injected with 1×10^6 , 10^5 , 10^4 and 10^3 cells. Three mice were injected for each number of cells. Mice were monitored for tumor formation for up to 1 month.

Statistical analysis

All experiments were performed in triplicate. A value of $p \leq 0.05$ was considered significant. Data were analyzed using Statgraphics software 7.0 (Statgraphics Graphics System, Warrenton, VA, USA).

Results

Primary culture of HCC tissue

Under the condition culture, it was easy to culture primary cells from HCC tissue. After 4 days, cells began to migrate from tissue fragments and adhere to the culture surface (Fig. 1a). There was some heterogeneity in primary cell cultures. Some cells were fibroblast-like cells, while others were oval-like cells. Cells rapidly proliferated and reached 70 % confluence on day 21 (Fig. 1b). Then, cells were easily subcultured and expanded for two passages (Fig. 1c).

Selection and cell proliferation in medium containing high concentrations of Dox and 5-FU

As shown in Fig. 2, Dox and 5-FU inhibited cell proliferation in the dose-dependent manner. At 0.1 and 0.25 Dox, 0.1 and 0.25 5-FU, 0.1 Dox + 0.1 5-FU, 0.1 Dox + 0.25 5-FU and 0.25 Dox + 0.1 5-FU, cell proliferation was affected by drug treatments, but proliferation was not significantly different compared with that of the control. At 0.25 Dox + 0.25 5-FU, 0.25 Dox + 0.5 5-FU, 0.5 5-FU, 0.1 Dox + 1 5-FU, 0.25 Dox + 1 5-FU and 1 5-FU, cell proliferation was significantly decreased compared with that of the control. At 0.5 Dox + 0.1 5-FU, 0.5 Dox + 0.25 5-FU, 0.5 Dox + 0.5 5-FU and 0.5 Dox, cells almost did not proliferate until 6 days after treatment. At 1 Dox + 0.5 5-FU, 1 Dox + 0.1 5-FU, 1 Dox + 0.25 5-FU and 1 Dox + 1 5-FU, significant cell death occurred, and the remaining cells did not proliferate until 7 days after treatment. At 0.5 Dox + 1 5-FU, cell death occurred from day 2 to 4, similar to that of 1 Dox + 0.1, 0.25, 0.5 or 1.0 5-FU. However, after day 4, cells began to proliferate and the cell index increased from nearly 1 to about 3. At 1 Dox + 0.5 5-FU, primary HCC cells did not survive because of antitumor drugs, but the cells that resisted these agents survived and proliferated after day 4. At higher doses of Dox in combination with 5-FU, cells could not overcome these antitumor drugs.

Fig. 1 Primary culture of HCC tissue. Tumor fragments were seeded in T25 flasks (a). After 4 days, cells grew out from tumor fragments (b), and were subcultured to expand cells (c)

Fig. 2 xCELLigence analysis for screening with Dox, 5-FU and their combination. Dox strongly suppressed cell proliferation compared to 5-FU. Their combination efficiently suppressed growth of primary cells. The doses of 0.5 Dox + 1 5-FU selected the cells resisted to these drugs. Results were redrawn from triplicate

Immunophenotype and rhodamine 123 efflux of HCSCs

The HCSC candidate population contained a subpopulation that was positive for CD13 ($7.51 \pm 0.72\%$), and strongly positive for CD326 ($72.87 \pm 1.28\%$), CD133 ($68.09 \pm 2.18\%$), and CD90 ($85.86 \pm 1.59\%$) (Fig. 3a–d). This population could efflux rhodamine 123 in culture. The HCSC candidate population highly exhibited rhodamine 123 efflux with $43.99 \pm 1.67\%$ of cells negative for rhodamine 123 (Fig. 3h–j), whereas $6.20 \pm 1.69\%$ of the unselected population was negative for rhodamine 123 (Fig. 3e–g).

Gene expression and sphere formation of HCSC candidates

We performed analyses of gene expression related to CSCs. Compared with unselected cells, HCSC candidates expressed numerous genes related to stem cells such as *CD117*, *Nanog*, *Sox-2* and *Oct4*, and strongly expressed genes related to HCC such as *AFP* and *CD56* (Fig. 4a). Using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001), the gene expression levels of AFP, CD56, CD117, Nanog, Sox-2 and Oct4 in HCSC candidates were higher at 39.40 ± 0.50 , 92.63 ± 0.82 , 143.68 ± 1.19 , 24.25 ± 0.81 , 40.97 ± 0.17 and

Fig. 3 Immunophenotype and rhodamine 123 efflux of HCSCs. HCSCs weakly expressed CD13 (a), strongly expressed CD326 (b), CD133 (c), and CD90 (d). The HCSC candidate

population highly exhibited rhodamine 123 efflux (h, i, j) compared to primary cells (e, f, g)

31.63 ± 0.96 times, respectively, compared with those of primary cells. HCSC candidates also formed tumor spheres when cultured in serum-free medium (Fig. 4b). These tumor spheres rapidly expanded, and more than half of tumor spheres reached about 200 μ m after 2 weeks.

In vivo tumorigenesis of HCSCs

We evaluated the in vivo tumorigenesis of HCSC candidates by subcutaneous injection into NOD/SCID mice. HCSC candidates caused tumor formation in

mice when 1×10^5 cells were injected (3/3 mice), whereas 1×10^3 HCSC candidates did not form tumors (3/3 mice). Tumors were collected for HE (Haematoxylin and Eosin) staining that clearly showed numerous lobes and cancer cells (Fig. 5).

Discussion

HCC is one of the most common cancers worldwide. Males are affected more than females, and it is

Fig. 4 HCSCs exhibit characteristic gene expression and in vitro tumor sphere formation. **a** HCSCs expressed genes related to oval cells, such as AFP, NCAM/CD56 and c-kit/CD117, and stem cell markers such as Nanog, Oct4 and Sox2

compared to primary cells. **b** HCSCs formed tumor spheres in serum-free medium. The error bar was $\pm$ SEM of the mean. The significance level was labeled with one asterisk ($p < 0.05$)

Fig. 5 Tumorigenesis in NOD/SCID mice injected with 1×10^5 HCSCs. Tumor formed at the injection site in mice (**a**) with large size after 1 month (**b**), and contained cancer cells (**c**)

common between the age of 30 and 50 (Kumar et al. 2003). HCC accounts for approximately 5.4 % of all cancers (Kumar et al. 2004), and cancer causes about 662,000 deaths worldwide every year (WHO 2006).

However, to date, the efficacy of HCC treatment is low. The probability of recurrence is about 50 % within 3 years after successful treatment (Llovet et al. 1999; Koike et al. 2000). Thus, several strategies have been developed to treat and prevent HCC recurrence, such as liver transplantation after successful resection (Sala et al. 2004), and administration of polyphenolic acid (an acyclic retinoid) (Muto et al. 1996), interferon alpha (Kubo et al. 2001) and interferon beta (Ikeda et al. 2000). Some of these therapies got lower HCC rates, but also at a high recurrence rate.

The main reason for HCC recurrence is the existence of HCSCs or stem cell-like hepatic cancer cells in malignant tumors. HCSCs exhibit multidrug- and radio-resistant properties, and easily survive after application of some common therapies. Thus, in recent years, many researchers have tried to establish strategies that target HCSCs. HCSC-targeting therapies aim to kill HCSCs that are considered the origin of

tumors and the reason for HCC recurrence. HCSC-targeting therapies comprise immunotherapies, dendritic cell-based therapies, CSC vaccines and oncolytic viruses. In all novel therapies, HCSCs are significant materials. Thus, our aim was to evaluate the stemness of Dox and 5-FU resistant cells from primary cells, and establish an easy and efficient method to isolate HCSCs that can be applied in further research, and particularly HCSC-targeting therapies.

Based on the multidrug-resistant property of HCSCs and xCELLigence analysis, we established a protocol to isolate HCSCs. xCELLigence is a novel technique that monitors the proliferation of adherent cells in real-time. Unlike the MTT assay and other methods, xCELLigence determines proliferation based on the electric resistance change depending on cell concentration. The resistance change is recorded by electrical nodes at the bottom of culture wells. Cell proliferation can be determined without cell death. Thus, desired cell populations can be isolated after selection or monitoring.

First, we carried out primary culture of cells obtained from HCC tumors. The results showed that

primary cells were easily cultured under our culture conditions. To inhibit differentiation and phenotype changes after long-term subculture, we only subcultured twice to obtain enough cells for subsequent experiments. There were three experimental groups that used two antitumor drugs, namely Dox and 5-FU, and their combination. Based on the proliferation curve, we chose the highest doses of Dox, 5-FU and their combination, in which cancer cells could proliferate. The surviving cells were considered as HCSC candidates. We verified the immunophenotype of HCSC candidates by evaluating HCSC markers. The result showed that HCSC candidates expressed many HCSC markers such as CD13, CD133, CD90, and CD326 (EpCAM). Indeed, HCSCs have been characterized by the expression of CD133 (Suetsugu et al. 2006; Yin et al. 2007; Ma et al. 2007), CD90 (Yang et al. 2008), CD13 (Haraguchi et al. 2010), and CD326 (Terris et al. 2010; Yamashita et al. 2008; Imrich et al. 2012; Yoon 2012). Next, we evaluated the SP characteristic in the HCSC candidate population by rhodamine 123 efflux of HCSC candidates and the unselected population. The results showed that HCSC candidates strongly exhibited the SP phenotype with about $43.99 \pm 1.67\%$ cells that were negative for rhodamine, whereas only $6.20 \pm 1.69\%$ was negative for rhodamine in the unselected cell population. Because CD326 (EpCAM) is an important and more specific marker of HCSCs (Terris et al. 2010; Yamashita et al. 2008; Imrich et al. 2012; Yoon 2012), CD326 positive cell percentage ($72.87 \pm 1.28\%$) was considered as the HCSC percentages in selected cell population.

To evaluate gene expression related to the tumorigenesis of HCSC candidates, we assessed the expression of *CD117*, *Nanog*, *Sox-2* and *Oct4*, which are related to stem cells, and *AFP* and *CD56* that are related to HCC. Using real-time RT-PCR, we found that the expression of *AFP*, *CD56*, *CD117*, *Nanog*, *Sox-2* and *Oct4* in HCSCs was much higher than that in primary cells. Moreover, HCSC candidates also formed tumor spheres in serum-free medium. Indeed, expression of *Sox-2*, *Oct-3/4* and *Nanog* is involved in the self-renewal of undifferentiated embryonic stem cells (Niwa et al. 2000; Mitsui et al. 2003; Chambers et al. 2003), and their proteins have been implicated in the tumorigenesis of adult germ cells (Hochedlinger et al. 2005), with ectopic expression causing tumor formation such as oral squamous cell carcinoma

(Chiou et al. 2008; Tsai et al. 2011), lung cancer (Chen et al. 2008), breast cancer (Liu et al. 2012) and glioma (Guo et al. 2011). Such gene expression has been confirmed in HCC (Zou et al. 2009; Yuan et al. 2010) and HCSCs (Cheung et al. 2011; Xu et al. 2010). The expression of CD117, CD56 and AFP has been also reported in recurrent HCC (Xu et al. 2010) and tumor-propagating cells (Colombo et al. 2011). CD117 is considered as a potential marker of HCC. The CD117 expression was dramatically related to the histological grades and clinical stages. CD117 might play an important role in liver carcinogenesis and the progression of HCC, and they might potentially serve as markers for HCC prognosis (Yan et al. 2011). In fact, in the early discovery CD117 and stem cell factor were considered as hematopoietic factors that induced stem cell maturation and differentiation (Geissler et al. 1991; Galli et al. 1994) in hematopoietic stem cells. They also have been implicated in inflammatory diseases, tissue remodeling—including fibrosis—and in liver regeneration after liver injury (Simpson et al. 2003; Krieg et al. 2006; Reber et al. 2006). However, further studies indicate that, in addition to its physiological functions, in combination with stem cell factor, c-kit plays an important role in carcinogenesis in various tumors (Sun et al. 2006; Yasuda et al. 2007). Following to Mansuroglu et al. (2009), liver cells will upregulate the stem cell factor or/and CD117 during cholangiocarcinogenesis. Therefore, c-Kit as well as stem cell factor may contribute to tumor development, for instance, by inducing proliferation of hepatocytes and of biliary cells (Mansuroglu et al. 2009). The gold standard to confirm CSCs is tumorigenesis in NOD/SCID mice at a low cell number. In this assay, we found that tumors were formed in NOD/SCID mice when 1×10^5 cells were injected. We concluded that selection with Dox and 5-FU was an easy method to enrich the HCSCs. These cells play an important role in anti-tumor drug screening in chemotherapies, producing antigens in immunotherapies and several HCSC targeting therapies.

These results also showed that at the low doses of Dox and 5-FU, the treatment was not only efficient but also enriched the HCSC population. In fact, Dox was commonly used to treat HCC with two strategies. Firstly, Dox of 60 mg/m^2 was delivered as an intravenous infusion every 21 days but not exceed 500 mg/m^2 (Park et al. 2006; Uhm et al. 2009). Secondly, Dox was directly delivered into tumors by drug eluting

beads or liposome. By eluting beads, the Dox concentration ($C_{\max}$) was 78.97 ± 38.3 ng/mL compare to $2,341.5 \pm 3,951.9$ ng/mL in conventional transarterial chemoembolization (Varela et al. 2007) in the serum. In recent research, Namur et al. (2011) showed Dox concentration in tissue from 5 μ M at 8 h to 0.65 μ M at 1 month using drug eluting beads (Namur et al. 2011). This Dox concentration was much lower than the concentration used in this study (5 vs. 862 μ M (0.5 μ g/mL of Dox)). That means that at the concentration of Dox permitted to treat in clinical Dox could not kill all of cancer cells as well as HCSCs. Maybe at this concentration Dox will kill a part of cancer cells and enrich HCSCs. At the extremely high concentration of Dox (862 μ M) used in this research, a minor population also survived; and we confirmed that they expressed cancer stem cell properties. These results suggest that the concentrations of Dox used in the clinic cannot eliminate all HCSCs.

5-FU was used from 300 to 750 mg/m² with a 1-week or 2-week schedule (Suenaga 1988; Mazzanti et al. 2004; Park et al. 2007; Eun et al. 2009). And the max of 5-FU concentration was found to be 3.45 ± 3.21 μ g/mL at 2 h, and this value decreased linearly. The concentrations of 5-FU in the liver tissues were 0.02 ± 0.01 μ g/g and the concentrations of 5-FU in cancer tissues were 0.03 ± 0.01 μ g/g (Suenaga 1988). In another study, the highest concentration of 5-FU was recorded at 1,010 μ g/mL from 14 patients (Maruyama et al. 2003). In similar with Dox, the concentration of 5-FU was too low in patients when compared to the concentrations used in this research. To sum up, the doses of Dox and 5-FU cannot kill HCSCs but also enrich them.

Conclusion

Doxorubicin and 5-fluorouracil resistant hepatic cancer cells demonstrate stem-like properties. By this way, we have successfully isolated HCSCs from HCC. HCSCs obtained by this method exhibit the characteristics of HCSCs. This population is enriched with an SP phenotype, and expresses many specific markers such as CD13, CD90, CD133, and CD326. Our isolated HCSCs also express several genes related to stem cells and CSCs, such as CD117, CD56, AFP, Oct4, Nanog and Sox-2, and can form tumor spheres in

serum-free medium and tumors in NOD/SCID mice upon injection with 1×10^5 cells. These HCSCs can be used for further study, particularly HCSC-targeting therapies such as dendritic cell therapy and CSC vaccination. Our study provides a new method that may advance HCSC research and the development of HCSC-targeting therapies in the future.

Conflict of interest The authors declare that they have no conflict of interest.

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