Abstract. Cancer stem cells (CSCs) in hepatocellular carcinoma (HCC) are frequently resistant to current therapeutic regimens and therefore responsible for tumor recurrence. Previous studies have reported that expression levels of dysadherin in CSCs may be used as a prognostic indicator, which is also responsible for treatment failure and poor survival rates. The present study analyzed the association of enhanced dysadherin levels with drug resistance and evasion of apoptosis in human HCC SP cells. An SP of 3.7% was isolated from human HCC cells using fluorescence-activated cell sorting. These SP cells displayed elevated levels of dysadherin and stemness proteins as well as high resistance to chemotherapeutic drugs and apoptosis. In order to reveal the possible link between dysadherin levels and tumorigenesis of SP cells, small interfering RNA technology was used to knockdown the expression of dysadherin in SP cells. Of note, the siRNA-transfected SP cells showed significantly reduced levels of stemness proteins, and were more sensitive to DNA-targeting drugs and apoptotic cell death as compared to non-transfected cells. Furthermore, in vivo experiments in NON/SCID mice indicated that dysadherin-expressing SP cells were highly tumorigenic, as they were able to induce tumor growth. The SP cell-derived tumor tissues in turn showed elevated dysadherin levels. The results of the present study therefore suggested that knockdown of dysadherin suppressed the tumorigenic properties of cancer stem-like SP cells. Hence, dysadherin is a valuable potential target for the development of novel anti-cancer drugs.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer type and has a high mortality rate. During the past 20 years, there has been a net increase of 62% in HCC-associated annual mortality rates (from 463,000 to 752,000) (1). HCC is commonly diagnosed at the stage of metastasis to lungs, adrenal glands, lymph nodes and bones (2). After diagnosis and conventional treatments, the survival rate of individuals is poor due to the fact that HCC is highly resistant to chemotherapeutic drugs and radiotherapy (3,4). The recently proposed cancer stem cell theory suggests the presence of a small distinct population of cancer cells named as ‘cancer stem cells (CSCs)’, which are responsible for minimal residual disease (MDR) after treatment failure (5). These CSCs possess the characteristics of normal stem cells, including high capacity for self-renewal as well as high differentiation, proliferation and cell survival (6). Hence, identification and characterization of CSCs will aid in the discovery of CSC-targeting agents for the complete elimination of MDR. The most commonly used and efficient method for the isolation of CSCs is the Hoechst 33342-dye exclusion method (7). This method is based on the fact that a small population of cancer cells expels the DNA-targeting dye due to the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) transporter proteins; due to their separate appearance in fluorescence-activated cell sorting (FACS) dot plots, they are referred to as side population (SP) cells. SP cells are considered to contain high numbers of CSCs, as they display characteristic features of CSCs and are able to induce tumors in NOD/SCID mice even when administered at low cell concentrations (8).

Furthermore, a large number of studies on various cancer types reported that SP cells displayed an elevated expression of stem-cell surface markers, including CD133, CD44, EpCAM...
and Oct-4, which are responsible for high tumorigenesis and tumor invasion (9-11). Of note, recent clinical experimental studies reported that in CSCs, elevated expression of dysadherin (a membrane glycoprotein) has a vital role in cancer metastasis by upregulating the production of chemokines (12,13). Previous studies on liver cancer cell lines also reported the overexpression of dysadherin in SP cells (13). These studies illustrated that reduced apoptosis and enhanced dysadherin in CSCs are linked to tumorigenesis and cancer metastasis; however, the molecular mechanisms of the regulatory functions of dysadherin in CSCs have remained elusive (14). In order to overcome the therapeutic complications in HCC and to develop novel anti-cancer drugs to target HCC stem cells, the phenotypic characterization of SP cells may be useful. Furthermore, it is known that deactivating mutations of the p53 gene and upregulation of B-cell lymphoma 2 (Bcl-2) lead to the evasion of apoptosis (15). Therefore, the present study analyzed the link between aberrant dysadherin expression and Bcl-2 signaling in SP cells with drug resistance and evasion of apoptosis in order to provide a basis for the development of CSC-specific therapeutic agents.

Materials and methods

Sample collection and cell culture. The hepatocellular carcinoma (HCC) samples were obtained from the patients at the time of surgery at the Department of Hepatic Surgery, The Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). The tumor biopsies were obtained from the patients according to the legal and ethical guidelines approved by the The Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). All patients provided written informed consent. The details of the patient cohort were as follows: Age range, 33-44; number of patients, 19; HCC grade, Edmondson-Steiner grade III; metastasis to lungs was present. The collected tumor samples were minced into fine fragments and cultured in 1 ml fetal calf serum (FCS; Sigma-Aldrich, St. Louis MO, USA). In all assays the cells were obtained from all 19 patients. Cells were subsequently cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) with 10% FCS, supplemented with antibiotics in T-75 flasks at 37˚C in a humidified atmosphere of 5% CO₂ and 95% air. Upon becoming 90% confluent, cells were removed from the flasks using Trypsin-EDTA (0.25%-5 mM EDTA; Sigma-Aldrich), washed, suspended in DMEM with 10% FCS and centrifuged at 4,500 g for 6 min. Cells were re-suspended in DMEM with 10% FCS. Cell counts were determined using a hemocytometer.

FACS analysis with Hoechst 33342 staining. The present study established the following experimental groups: Control, cells + Hoechst 33342 (n=9 samples from 9 patients); and drug-treated, cells + verapamil (Sigma-Aldrich) + Hoechst 33342 (n=9). Cells in staining medium (10° cells/ml DMEM with 10% FCS) were labeled with Hoechst 33342-bis-benzimide stock (final incubation concentration, 5 µl/ml; Sigma-Aldrich) and optionally treated with verapamil (final incubation concentration, 0.8 µl/ml). The cells were mixed and incubated in a water bath at 37°C for 90 min. The cells were then centrifuged (1,800 x g for 10 min at 4°C) and re-suspended in 500 µl Hank's balanced salt solution (Sigma-Aldrich) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma-Aldrich). Finally, the cells were counterstained with propidium iodide (PI; Sigma-Aldrich) 2 µg/ml at 4°C. Cells were filtered through a 50-µm nylon mesh (BD Biosciences, Franklin Lakes, NJ, USA) to remove cell clumps and filled into labeled FACS tubes. Separate tubes with medium (DMEM with 10% FCS) were kept for sterile sorting of SP cells and main population cells. The cells were sorted using a flow cytometer (FACS Aria II; BD Biosciences). The Hoechst 33342 dye was excited at 355 nm and its dual-wavelength fluorescence was analyzed (blue, 450 nm; red, 675 nm).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was isolated by using the RNA isolation kit (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and the RNA samples were treated with DNase I to exclude any DNA contamination. RT-qPCR were performed using the PrimeScript™ RT-PCR kit (Takara Bio, Inc., Otsu, Japan) and SYBR Premix Ex Taq™ kit (Takara Bio, Inc.) according to the manufacturer's instructions, with the following thermocycling conditions using a T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR primers from Shanghai Generay Biotech Co., Ltd (Shanghai, China) were used for RT-qPCR analysis: CD133 forward, 5’-TCTTG ACCGACTGAGAC-3’ and reverse, 5’-ACTCTGATGGATGCA CCAAGCAC-3’; GAPDH forward, 5’-TGAATCCTACAGGAC TACTCAG-3’ and reverse, 5’-ACATGTCTAATATGT TCCA-3’; ABCG2 forward, 5’-AGCTGAAAGGAAAGATC CAA-3’ and reverse, 5’-TCCAGACACACACCAGGATAA-3’; OCT-4 forward, 5’-ATCCCTGCGCTTCTATTGTC-3’ and reverse, 5’-CTCCAGGTGGCCTCCTACTC-3’; EpCAM forward, 5’-CTGAAATGTTGGTGCTATC-G3’ and reverse, 5’-CCAGCTGGTGTGATCTCCTCT-3’; BCL-2 forward, 5’-A CACTGTGTTAGCAGTGCAG-3’ and reverse, 5’-CCAGC TACCTCACAAC-3’; BAX forward, 5’-GGATGCGCT CACACAAAG-3’ and reverse, 5’-ACTCCCGCCACAAG ATG-3’ (16-18). GAPDH was used as an internal control. Using a T100 thermal cycler, the thermocycling conditions were as follows: -95°C for 2 min, 40 cycles of 95°C for 30 sec, 55-60°C for 1 min and 72° for 30-60 sec. PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. The gel was visualized using Bio-Rad ChemiDoc XRS (Bio-Rad Laboratories, Inc.). The band intensity was measured by using Image J 1.0 software (National Institutes of Health, Bethesda, MD, USA), and relative gene expression was quantified using the 2^{-ΔΔCT} method (19). The values presented in the graph are the average values of three independent experiments.

RNA interference. The small interfering RNA (siRNA) specific for dysadherin (GenBank accession no. AB07291) (17,18), was purchased from Dharmaco (Lafayette, CO, USA; cat no. 80026). siRNA transfection was performed according to the manufacturer's instructions (siRNA concentration of 200 nM). The transfected cells were analyzed after 48 h of incubation.
Western blot analysis. Protein was extracted from the SP and non-SP cells, and the protein concentration was determined using the Bradford assay (Pierce™ Coomassie protein assay kit; Invitrogen Life Technologies). Following 10% SDS-PAGE and transfer onto a nitrocellulose membrane (Sigma-Aldrich), the membranes were incubated with primary antibodies overnight at 4˚C. The following primary antibodies were used: Mouse monoclonal anti-human ABCG2 (1:1,000; cat. no. sc-18841), rabbit polyclonal immunoglobulin (Ig)G dysadherin (1:1,000; cat. no. sc-98246) and mouse monoclonal anti-human GAPDH (1:1,000; cat. no. sc-47724). Secondary antibodies with alkaline phosphatase markers were used with specificity for the appropriate species: Goat anti-rabbit IgG (1:5,000; cat. no. sc-2034) and goat anti-mouse (1:5,000; cat. no. sc-2047), incubated for 2 h at room temperature. All antibodies were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Immunoreactive proteins were detected using a Chemiluminescence Reagent kit (cat. no. ab79907; Abcam, Cambridge, MA, USA). Blots were detected and scanned using a densitometer (GS-710; Bio-Rad Laboratories, Inc.).

Multidrug resistance assay. 3,000 cells/well in 96-well plates were seeded and cultured in DMEM/F12 (Sigma-Aldrich) supplemented with the necessary growth factors (Sigma-Aldrich). After 7 h of incubation, SP and non-SP cells were treated with 5 µg/ml carboplatin (Sigma-Aldrich), 5 µg/ml 5-flurouracil (5-FU; Sigma-Aldrich) and 5 µg/ml doxorubicin (Sigma-Aldrich). The mean optical density value at 450 nm (OD450) obtained was expressed as a graph. The drug resistance in the experimental groups was calculated using the following formula: Drug resistance rate (%) = (OD450 experimental group/OD450 control group) x 100.

Immunohisto/cytochemistry. The sorted SP cells and main population cells were seeded onto coverslips in 12-well plates (100,000 cells/well). After 24 h of incubation, the cells were rinsed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in 1X PBS, for 15 min at room temperature. After washing with 1X PBS, cells were blocked with 1% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (TBS; Sigma-Aldrich) with RNase (1:1,000 in 3% BSA-TBS; Sigma-Aldrich). After 1 h of incubation at RT, cells were rinsed with PBS and incubated with the following primary antibodies (Santa Cruz Biotechnology, Inc.) in 1% BSA-TBS: Mouse anti-ABC sub-family G member 2 (ABCG2; 1:1,000), mouse anti-Bcl2 (1:100); mouse anti-Bcl-2-associated X (Bax; 1:200) and mouse anti-dysadherin (1:1000), incubated overnight at 4˚C. After washing with 1X PBS, cells were incubated with secondary antibody conjugated to fluorescein isothiocyanate.
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Following a further wash with PBS, Hoechst 33342 (Bio-Rad Laboratories, Inc.; dilution: 1:100) was used to stain the nuclei. For immunohistochemistry, the cells were processed and stained as described previously (20). The cells were viewed under a confocal laser scanning microscope (Leica TCS; Leica Microsystems, Oberkochen, Germany) and all the images were processed using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

Tumor cell implantation. Mice (20 female and 20 male; age, six weeks-old; weight, 160-200 g) were purchased from the Experimental Animal Center of the Guangdong province (Guangzhou, China). The mice were housed in plastic cages containing corn chip bedding and maintained on a 12-h light-dark cycle (lights on at 7:00 a.m.), at 24-25˚C and 50-60% humidity, with access to food and water *ad libitum*. The mice were sacrificed by injection of 75% alcohol. SP cells isolated by FACS or non-SP cells were mixed with Matrigel (Sigma-Aldrich) and subcutaneously inoculated into NOD/SCID mice (21). The density of cells injected and the tumor size were monitored according to the protocol of a previous study (21). The tumor volumes were calculated according to the formula \( V = \frac{1}{2}ab^2 \) with a being the long diameter and \( b \) the short diameter of the tumor. After 4-5 weeks, mice were sacrificed and tumors were harvested and measured, and their images were captured.

**Statistical analysis.** Values are expressed as the mean ± standard deviation. One-way analysis of variance and Student's t-test were performed to determine significant differences between the treatment and control groups. SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. \( P < 0.01 \) was considered to indicate a statistically significant difference between values.

**Results**

**SP cells are present in human HCC samples and overexpress ABC transporters.** The present study investigated cultured HCC cells from patients for the presence of SP cells using the Hoechst dye exclusion assay. As shown in Fig. 1A, a SP of 3.7% of the total cell population was detected in the HCC cells, whose presence was significantly diminished to 0.3% (Fig. 1B) after treatment with verapamil, an inhibitor of ABC transporters. Immunocytochemical analysis of the SP cells isolated by FACS showed enhanced expression of ABC transporter protein ABCG2 compared with that in non-SP cells (Figs. 1C and 2A). These results clearly indicated that overexpression of ABC transporter proteins in SP cells has a major role in resistance to chemotherapy by pumping the chemotherapeutic drugs out of the cells.

**Dysadherin expression is enhanced in HCC SP cells.** The HCC SP cells isolated by FACS were analyzed for the expression of dysadherin by RT-PCR. In SP cells, an increased level of dysadherin was detected by RT-PCR in SP cells as compared to that of non-SP cells (Fig. 2A). Western blot analysis showed that SP cells exhibited an elevated expression of dysadherin compared to that of non-SP cells (Fig. 2B). Furthermore, the elevated expression of dysadherin in SP cells was confirmed by immunocytochemistry (Fig. 2C).

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**Dysadherin enhances multidrug resistance and lifespan of SP cells.** In line with the findings of previous studies (13,17,18), the present study also reported elevated levels of dysadherin in SP cells (Fig. 2). Next, the present study investigated the role of dysadherin in SP cells by using siRNA technology in order to knockdown dysadherin expression in SP cells. First, the multi-drug resistance capacity of SP cells was tested prior to and after dysadherin knockdown. As shown in (Fig. 3A), SP cells showed a significantly higher survival rate (>80%) following treatment with the chemotherapeutic drugs carboplatin, 5-flourouracil and doxorubicin as compared to that of non-SP
cells. However, after dysadherin knockdown, the survival rate of SP cells upon treatment with the abovementioned drugs was markedly reduced (Fig. 3B). Of note, the relative mRNA expression of ABCG2 was also significantly reduced after silencing of dysadherin compared with that prior to silencing (Fig. 3C and D). These results suggested that

Figure 3. Comparison of cell survival rate of SP cells and non-SP cells treated with carboplatin, 5-FU or doxorubicin (A) before and (B) after dysadherin knockdown. (C and D) Before as well as after dysadherin silencing, the relative mRNA expression of ABCG2 in SP cells was significantly higher than in non-SP cells. Values are expressed as the mean ± standard deviation. **P<0.01 for SP vs. non-SP cells. 5-FU, 5-flourouracil; SP, side population.

Figure 4. Relative mRNA expression of Bcl-2 and Bax (A) before and (B) after dysadherin knockdown (magnification, x100). Values are expressed as the mean ± standard deviation. **P<0.01 for SP vs. non-SP cells. (C) Fluorescence microscopic analysis of Bcl-2 (red) and Bax (green) expression in SP cells and non-SP cells before and after dysadherin-specific siRNA interference (magnification, x120). Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; siRNA, small interfering RNA; SP, side population.
dysadherin has a major role in drug resistance of SP cells, either by direct or indirect regulation of ABCG2.

Dysadherin reduces apoptotic cell death in HCC SPs. Since the silencing of dysadherin efficiently reduced the survival rate of SP cells after drug treatment (Fig. 3), it was speculated whether the dysadherin was involved in regulation of apoptosis in SP cells as well. RT-PCR analysis showed that mRNA expression of anti-apoptotic gene Bcl-2 and pro-apoptotic Bax was upregulated in SP cells compared with that in non-SP cells.
non-SP cells (Fig. 4A). Of note, the dysadherin-silenced SP cells exhibited reduced expression of Bcl-2 and Bax compared with that prior to silencing (Fig. 4B). However, immunocytochemistry indicated that Bcl-2 was decreased and Bax was elevated in SP cells compared with those in non-SP cells, while dysadherin-knockdown led to an increase in Bcl-2 and a decrease in Bax (Fig. 4C). These results indicate that dysadherin affects Bcl-2 and Bax mRNA expression differently from their protein expression; however, the protein expression levels indicated that dysadherin overexpression may, due to upregulating Bcl-2 and downregulating Bax, be associated with drug resistance and evasion of apoptosis in HCC SP cells.

Dysadherin knockdown reduces the expression of stemness genes in SP cells. It is well known that SP cells are have a high capacity for self-renewal and are able to generate an increased number of tumor spheres due to overexpressing stemness genes (22). Therefore, the present study evaluated the expression of stemness genes in SP cells prior to and after dysadherin knockdown. After silencing, the transcription of the stemness genes CD133, Oct-4 and EpCAM, which are essential for the maintenance of CSC self-renewal, was significantly reduced (Fig. 5A and B).

HCC SP cells are highly tumorigenic. Next, the present study performed a xenograft experiment in order to evaluate the tumorigenic potential of SP cells. The results showed that SP cells were able to induce tumor growth in NOD/SCID mice, even when inoculated at low cell numbers (i.e., 5,000); however, the non-SP cells failed to induce massive tumor growth at this cell density (data not shown). Compared with the non-SP cell-derived tumors, the SP cell-derived tumors grew faster and displayed increased sizes (Fig. 6A). Immunohistochemical staining also confirmed that SP cell-derived tumor tissues showed increased dysadherin staining compared with non-SP tumors (Fig. 6B).

Discussion

HCC is one of the major leading causes of cancer-associated mortality worldwide (23). Clinical and experimental studies reported that the presence of CSCs in HCC has a major role in chemotherapy resistance, tumor relapse and metastasis (24). Cancer stem-like SP cells have been identified and characterized in several cancer cell lines, including the MHCC97 HCC cell line, and it has been indicated that SP cells are highly resistant to chemotherapy (16,22,25-27). In addition, SP cells showed elevated expression of ABC transporter proteins, which are the main cause of drug resistance by active drug efflux (28-30). However, to date, the underlying molecular mechanisms and signaling pathways of the regulation of ABC transporter overexpression and multi-drug resistance have remained to be fully elucidated. There may be an indirect link between ABC transporters and apoptotic pathways, which jointly contribute to the capacity of SP cells for drug resistance and evasion of apoptosis resistance.

Elevated dysadherin and its significance in cancer metastasis has been reported in SP cells of HCC cell lines (17,31). Dysadherin, a member of the FXYD family, regulates NaK-ATPase, which is in turn actively involved in cell permeability and polarity and is essential for cell signaling and stem cell dynamics (32-34). The present study reported that SP cells from human HCC displayed increased expression of dysadherin together with elevated expression of ABCG2 and the stemness genes CD133, Oct-4 and EpCAM. Following knockdown of dysadherin by RNA interference, the SP cells displayed increased sensitivity to chemotherapeutic drugs, as the expression of drug resistance-associated ABCG2 was inhibited. It is therefore speculated that dysadherin may also regulate the expression of other multidrug resistance genes, including ABCA1 and ABCB1. Furthermore, following dysadherin knockdown, SP cells displayed enhanced levels of pro-apoptotic protein (Bax) as well as downregulated expression of stemness genes, suggesting that dysadherin has a crucial role in SP-cell maintenance and survival. Similar to the present findings, it was previously shown that dysadherin has a major role in the maintenance of the CSC-like properties of SP cells, while its knockdown efficiently reduced CSC-like characteristics (17). In the present study, the dysadherin-overexpressing SP cells were shown to have a high potential for self-renewal and to be highly tumorigenic. The present study also evidenced that dysadherin-overexpressing SP cells were able to induce tumor growth in NOD/SCID mice and the tumor tissues derived from the mice were highly positive for dysadherin staining.

The key regulator of apoptosis is p53, which is linked to numerous pro-apoptotic genes, including Bcl-2 (anti-apoptotic factor) and Bax. Previous studies on liver cancer cell lines showed that the expression of Bcl-2 and Bax in SP cells was aberrantly altered and therefore, the rate of apoptosis was markedly reduced in SP cells (16). Bcl-2 is an anti-apoptotic protein whose enhanced expression inhibits Bax, which would otherwise provoke the release of cytochrome c from the outer mitochondrial membrane protein, which in turn leads to the formation of apoptosome and caspase activation (35). It is difficult to conclude based on the present study whether the altered ratio of Bcl-2/Bax may lead to lower drug sensitivity. It has previously been reported that Bcl-2 is able to inhibit Bax (35). It is possible that upregulated Bax expression is caused by downregulated Bcl-2 expression, mediated by dysadherin.

The present study showed that the protein expression in Bcl-2 was upregulated and that of Bax was downregulated in HCC SP cells, therefore indicating that SP cells have an enhanced survival rate compared to that of non-SP cells, even after treatment with chemotherapeutic drugs. These results indicated that dysadherin has a pivotal role in drug resistance and evasion of apoptosis in SP cells and thereby, SP cells may be spared by chemotherapy and tumor recurrence may occur.

In conclusion, the results of the present study suggested that dysadherin may enhance the expression of drug efflux pumps and anti-apoptotic mechanisms in SP cells either by direct or indirect interaction. However, the precise underlying molecular mechanism of dysadherin-mediated tumorigenesis remains to be elucidated in future studies. However, the results of the present study indicated that novel anti-cancer drugs targeting the transcriptional regulation of dysadherin may efficiently suppress the activity and drug resistance of SP cells.
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