The CD133+ subpopulation of the SW982 human synovial sarcoma cell line exhibits cancer stem-like characteristics

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Abstract. Several soft tissues sarcomas have been reported to contain cancer stem-like cells (CSCs) or tumor-initiating cells, based on their ability to initiate and sustain tumor growth. However, these cells have not yet been identified in the human synovial sarcoma cell line SW982. CD133, a surface glycoprotein specific to stem and progenitor cells, has been described as a CSC marker in different tumor types. In the present study, we identified a CSC subpopulation in SW982 cells using the CD133 cell surface marker. CD133-positive ($CD133^+$) cells were identified in SW982 cells (8.59%); these cells showed an increased ability to form spherical colonies and could self-renew in serum-starved culture conditions, compared to CD133-negative ($CD133^-$) cells. Real-time PCR analysis of stemness genes revealed that the $CD133^+$ subpopulation expresses higher levels of *Bmi1, c-Myc, Nanog, Oct3/4* and *Sox2*. $CD133^+$ cells showed increased resistance to cisplatin (CDDP) and doxorubicin (DXR), possibly due to upregulation of the *ABCG2* drug transporter gene. *In vivo* studies revealed that the $CD133^+$ subpopulation is highly tumorigenic. These findings indicate that $CD133^+$ SW982 cells have characteristics similar to CSCs. This discovery may lead to the development of novel therapies that specifically target $CD133^+$ synovial sarcoma CSCs.

Introduction

Synovial sarcoma (SS) is a high-grade malignant tumor that accounts for almost 10% of all adult soft tissue sarcomas (1). It can occur at any age and anatomical site, but is most commonly found in the para-articular regions in adolescents and young adults (2). SS is characterized by the presence of a chromosomal translocation t(X;18) (p11.2;q11.2) between chromosomes X and 18 that leads to the formation of a fusion protein (3).

Currently, SS is classified as a miscellaneous tumor of uncertain histological origin (4), which is thought to arise from undifferentiated mesenchymal cells. A recent report indicates that neural crest cells, which can differentiate into both ectoderm and mesoderm, are potential progenitors of SS (5). However, the cellular origin of SS has not been definitively resolved.

Clinically, SS appears as deep-seated slowly growing mass, with more than half of patients developing metastases, mainly to the lungs and sometimes to the lymph nodes and bone marrow (6). The 5- and 10-year survival rates are as low as 36 and 20%, respectively (7). Thus, SS remains a cancer associated with high morbidity and therefore, improved therapies are necessary.

The cancer stem-like cell (CSC) hypothesis holds that tumor cells are hierarchically organized according to their potential to initiate and sustain tumor growth. Only a small population of tumor cells, defined as CSCs or tumor-initiating cells, can form tumors in serial xenotransplantation assays, are resistant to chemotherapy and have the ability to re-establish the hierarchical cell organization and heterogeneity of the parental tumor at each passage *in vivo* (8,9). These cells were first described in acute myeloid leukemia (10) and subsequently in breast cancer (11), prostate cancer (12), glioblastoma (13), melanoma (14) and other types of cancer (15-18). Thus, the identification of the CSCs has significantly improved our understanding of tumor biology and may lead to more effective tumor therapies.

Recent technical advances have enabled the identification of CSCs by specific surface markers that are selectively expressed on these cells, but not on the majority of tumor cells. Of these, CD133 is a pentaspan membrane glycoprotein that was first described as a surface antigen specific to human hematopoietic stem and progenitor cells (19), but has also recently been recognized as a stem cell marker in brain (20), prostate (21), pancreatic (22), lung (23) and ovarian cancers (24). CD133 expression has also been identified in various sarcomas, including osteosarcoma (25) and Ewing's sarcoma (16). $CD133^+$ Ewing's sarcoma cells express significantly higher levels of the stemness genes *Nanog* and *Oct3/4* than

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their CD133- counterparts. Recently, the presence of CD133+ cell populations was reported in SS (26). However, until now it was unknown whether CD133+ cells with CSC properties could be isolated from the human SS cell line SW982.

Therefore, in the present study we used CD133 as a marker to identify CSCs within the SW982 cell population. We then examined the self-renewal, differentiation capacity and tumorigenicity of the SW982 CD133+ cell population using a spheroid formation assay and xenograft model in nude BALB/c mice. We also compared chemoresistance and examined expression of the specific drug transporter, *ABCG2* and stemness genes in different subpopulations of SW982 cells. Our results allowed us to define the phenotype of SW982 CSCs, which may contribute to the development of more effective therapies for SS.

Materials and methods

Cell culture. The human SS cell line SW982 was obtained from the American Type Culture Collection (USA) and maintained in Leibovitz-15 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT, USA) at 37˚C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 3-4 days and cells were trypsinized and replated when 85% confluency was reached.

Magnetic- and fluorescence-activated cell sorting. CD133+ and CD133- populations were isolated from SW982 cell cultures by magnetic bead sorting using a magnetic-activated cell sorting (MACS) CD133 cell isolation kit. Briefly, SW982 cells were resuspended in MACS buffer [phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, supplemented with 0.5% BSA and 2 mM EDTA] at a concentration of $3.3x10^8$ cells/ml. Single-cell suspensions (300 μl, containing $1x10^8$ cells) were incubated with 100 μl FcR blocking reagent and 100 μl CD133 microbeads for 30 min at 4˚C. After washing, cell populations were separated by passing through LS columns, which retain CD133+ cells, according to the manufacturer's instructions. Before and after separation, cell samples were analyzed by fluorescence-activated cell sorting (FACS) using anti-CD133/2 phycoerythrin (293C3) and isotype control mouse IgG2b phycoerythrin antibodies using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). All reagents were purchased from Miltenyi Biotec.

Spheroid formation assay. Spheroid formation assays were performed as described by Fujii *et al* (27) and Gibbs *et al* (28) with some modifications, using single-cell SW982 suspensions (dissociated using 0.25% trypsin/0.05% EDTA at ~70% confluency) and both CD133+ and CD133- cell populations isolated by MACS. Briefly, cells were resuspended in B27-supplemented Leibovitz-15/1% methylcellulose medium without serum and containing human recombinant epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor (bFGF; 10 ng/ml; PeproTech, Rocky Hill, NY, USA) and plated at $6x10^4$ cells/well into ultra-low attachment 6-well plates (Corning Inc., Corning, NY, USA). B27 was purchased from Invitrogen. Fresh aliquots of EGF and bFGF were added every second day. After 10-14 days, colonies containing >50 cells were counted using inverted phase contrast microscopy (Olympus CKX41, Shinjuku, Tokyo, Japan). Spheroid formation was investigated through at least five cycles of dissociation and re-culturing in anchorage-independent, serum-starved condition on 96-well ultra-low attachment plates.

Chemosensitivity assays. To assess the effects of cisplatin (CDDP) and doxorubicin (DXR) (Sigma), which are frequently used for sarcoma chemotherapy, on the CD133+ and CD133- cell populations, the cells were dissociated, resuspended in Leibovitz-15/10% FBS at a concentration of $2x10^4$ cells/ml, inoculated into 96-well microtiter plates (Corning Inc.) at 100 μl/well and incubated at 37˚C. After 24 h, cells were exposed to 0-10 μM CDDP or DXR for 48 h. The viability of treated and control cells was measured by the MTS assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

For a more detailed characterization of the stemness properties of the CD133+ cell population, we compared drug resistance in CD133+ spheroids and adherent cells. CD133+ adherent cells were grown in medium containing serum, as previously described. CD133+ spheroids ($5x10^4$ cells/ml) were resuspended in B27-supplemented Leibovitz-15/1% methylcellulose medium, plated into 96-well ultra-low attachment microplates at 100 μl/well and incubated at 37˚C for 14 days to induce spheroid formation. CD133+ spheroids and adherent cells were then treated with 0-10 μM CDDP or DXR and cell viability was measured by the MTS assay after 48 h. Triplicate wells were used for each treatment group.

Western blotting. Western blotting was performed as described previously, with some modifications (28). Briefly, cells were washed twice with cold PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1% Na deoxycholate, 1 mM Na vanadate and protease inhibitors (5 mg/ml pepstatin, 1 mM PMSF, 10 mg/ml leupeptin and 1 mM NaF; Sigma) for 1 h on ice. Lysates were clarified by centrifugation at 13,000 g for 10 min at 4˚C and protein concentrations were measured using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Samples (30 μg total protein) were separated using 10% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Sigma). After blocking with 5% skimmed milk for 1 h at room temperature, membranes were incubated overnight at 4˚C with primary antibody diluted in Tris-buffered saline (TBS) containing 0.1% Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA) and 5% bovine albumin (Sigma). After washing three times in TBS with 0.1% Tween-20, blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactive bands were visualized using ECL Plus SuperSignal West Pico (Thermo Scientific, Rockford, IL, USA). Protein levels were normalized to GAPDH. Primary antibodies used are as follows: anti-ABCG2 (1:1000), anti-Bmi1 (1:1000), anti-c-Myc (1:1000), anti-Nanog (1:1000), anti-Oct3/4 (1:1000) and anti-Sox2 (1:1000) were purchased from Cell Signaling Technology. GAPDH (1:5000) was purchased from Santa Cruz Biotechnology.

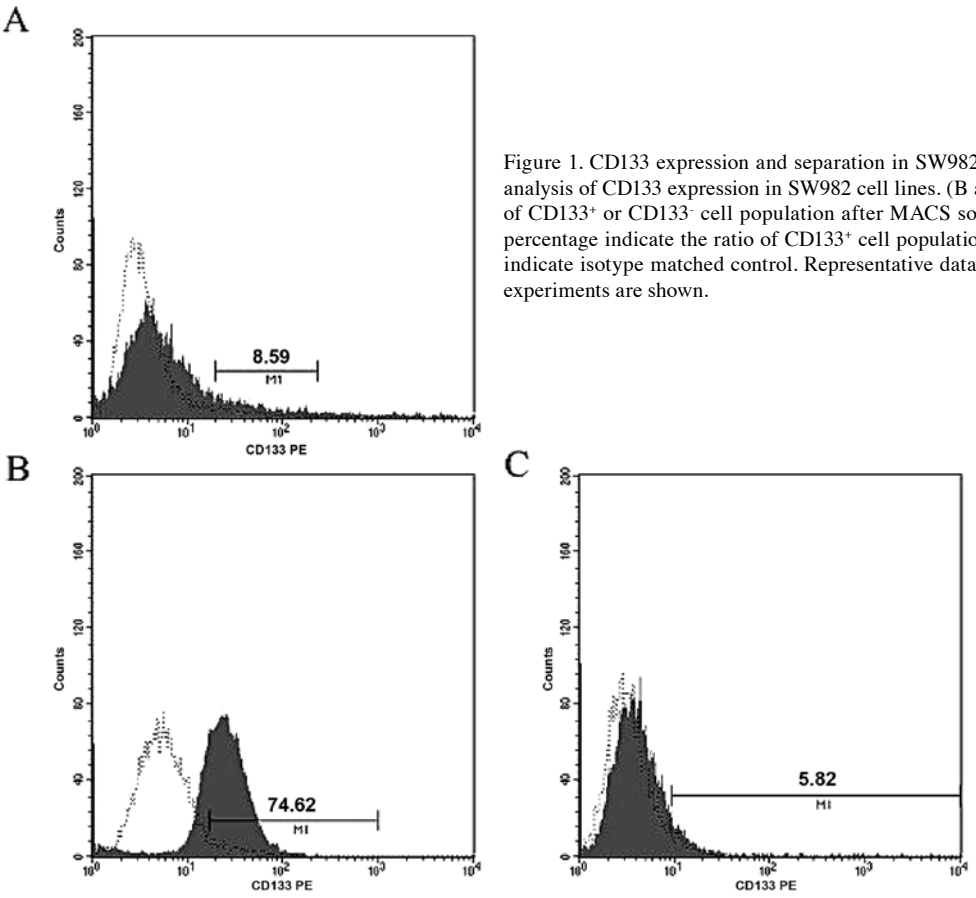

Figure 1. CD133 expression and separation in SW982 cell lines. (A) FACS analysis of CD133 expression in SW982 cell lines. (B and C) FACS analysis of CD133$^+$ or CD133$^-$ cell population after MACS sorting. Black bars and percentage indicate the ratio of CD133$^+$ cell population. Black broken lines indicate isotype matched control. Representative data of three independent experiments are shown.

Real-time PCR. Total RNA was extracted from the CD133$^+$ and CD133$^-$ cell populations using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Sigma). Real-time PCR was performed according to the manufacturer's instructions using an ABI PRISM 7900HT sequence detection system (Applied Biosystems); mRNA expression was normalized to GAPDH. The primer sequences are listed in Table I.

Xenograft experiments. To evaluate the tumorigenic potential of the CD133$^+$ and CD133$^-$ cell populations, 1x10^3-1x10^6 cells were subcutaneously injected into the right side of the back of 6-8-week-old female BALB/c nude mice, obtained from the Animal Research Center, Harbin Medical University, China. All surgical procedures and animal treatments were performed in accordance with institute guidelines. Mice were monitored daily for tumor growth. Twelve weeks after injection, all mice were euthanized, tumors were aseptically excised and samples were processed for histopathological and immunohistochemical analysis. In addition, tumor samples were digested using collagenase II (Sigma) and the CD133$^+$ cells were isolated and immediately re-injected into mice to generate second-round tumors. Data were obtained from at least three independent experiments.

Histopathological and immunohistochemical analysis of xenografts. Excised xenograft tumors derived from CD133$^+$ and CD133$^-$ cells were fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (HE) using a standard protocol to assess tumor type. Immunohistochemistry was performed using an anti-CD133 antibody (Abcam, Cambridge, UK), according to standard immunohistochemical procedures. All microscopy images were captured.

Statistical analysis. Statistical software SPSS19.0 was used in data processing and analyzing. Data are expressed as mean ± SD, comparison between experimental groups of real-time PCR analysis were tested by one-way ANOVA. The results of the chemosensitivity assay was calculated with the survival rates and compared by using the χ^2 test. A $p<0.05$ was regarded as statistically significant.

Results

Isolation of SW982 CD133$^+$ and CD133$^-$ cell populations. We assessed CD133 expression in human SS SW982 cells using FACS analysis and found that CD133$^+$ cells comprised 8.59% of the total population (Fig. 1A). We then isolated enriched CD133$^+$ and CD133$^-$ cell populations using MACS. Using this technique, the CD133$^+$ cell population could be enriched by ~13-fold over CD133$^-$ cells (74.62 vs. 5.82%; Fig. 1B and C).

SW982 CD133$^+$ cells form spheroids in suspension culture. Suspension culture in serum-free medium was initially developed as a method to select neural stem cells through neurosphere formation, but has been widely adapted as a general tumor-initiating cell selection method. Both normal cells and CSCs

Table I. The primer sequences of the tested genes.

Gene	Forward (5'-3)	Reverse (5'-3')
ABCG2	GATAAAGTGGCAGACTCCAAGGT	CCAATAAGGTGAGGCTATCAAACA
Bmi1	CTCCACCTCTTCTTGTTTGC	GATGACCCATTTACTGATGATTT
c-Myc	GCATACATCCTGTCCGTCCA	CAAGAGTTCCGTAGCTGTTCAAG
Nanog	AGGCAAACAACCCACTTCT	TCACACCATTGCTATTCTTCG
Oct 3/4	TATTCAGCCAAACGACCATCT	TCAGCTTCCTCCACCCACTT
Sox2	ATCACCCACAGCAAATGACA	CAAAGCTCCTACCGTACCACTA
GAPDH	GGGAAACTGTGGCGTGAT	GAGTGGGTGTCGCTGTTGA

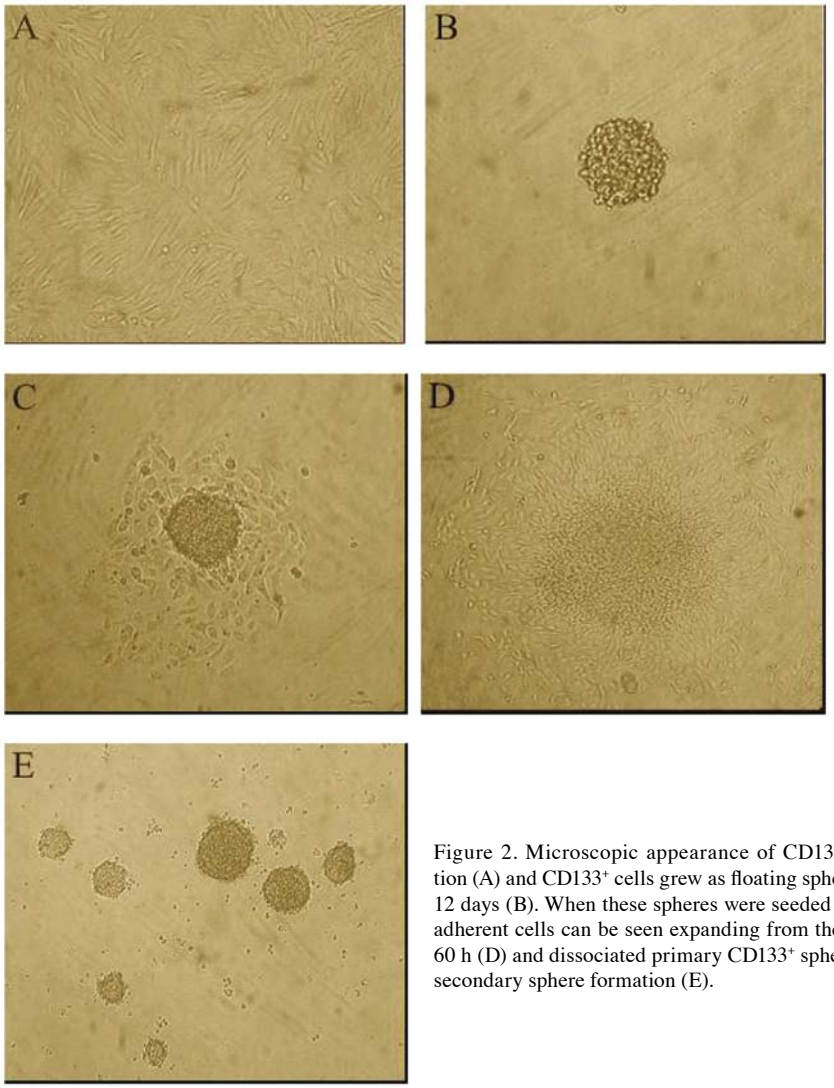

Figure 2. Microscopic appearance of CD133+ cells in monolayer condition (A) and CD133+ cells grew as floating spheres in serum-free medium for 12 days (B). When these spheres were seeded in serum containing medium, adherent cells can be seen expanding from these spheres after 20 h (C) and 60 h (D) and dissociated primary CD133+ sphere cells showed a capacity for secondary sphere formation (E).

from epithelial organs can be grown as spherical clones in serum-free EGF/bFGF-supplemented medium that favors the proliferation of undifferentiated cells (18).

We therefore evaluated SW982 cell self-renewal and ability to generate spherical clones under serum-starved culture conditions. Our results indicated that the CD133+ subpopulation proliferated as floating spheres (Fig. 2B), while the CD133- subpopulation did not form spheres. When CD133+ spheroids were seeded into serum containing Leibovitz-15 medium, they developed the spindle-like features of the original cell culture (Fig. 2A, C and D). Primary CD133+ spheroid cells that were enzymatically disaggregated after 10-14 days of culture and replated as single-cell suspension went on to generate second passage spheres (Fig. 2E). The ability to form spheres was maintained over five serial passages using this procedure.

CD133+ SW982 subpopulations are resistant to chemotherapeutic agents. We found that CDDP and DXR inhibited the

Table II. Cell survival rates (%) after 48 h CDDP and DXR treatments of $CD133^+$ and $CD133^-$ cells.

	CDDP			DXR		
	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
$CD133^+$	93.80±1.23	81.10±2.26	75.54±1.83	90.10±2.21	76.87±1.23	70.87±2.21
$CD133^-$	76.50±1.59	57.63±1.64	45.63±1.30	77.13±0.85	48.53±2.52	40.50±1.44
χ^2	11.84	12.87	18.71	6.16	17.11	18.60
p-value	0.001	0.000	0.000	0.013	0.000	0.000

Values expressed as means ± SD. χ^2 test, $p<0.05$, statistical significance.

Table III. Cell survival rates (%) after 48 h CDDP and DXR treatments of sphere and adherent $CD133^+$ cells.

	CDDP			DXR		
	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
Sphere $CD133^+$	96.42±2.20	90.91±2.17	86.30±1.20	95.00±1.35	89.33±1.41	81.10±1.20
Adherent $CD133^+$	93.80±1.23	81.10±2.26	75.54±1.83	90.10±2.21	76.87±1.23	70.87±2.21
χ^2	0.73	4.06	3.774	1.74	5.55	2.84
p-value	0.394	0.044	0.049	0.187	0.018	0.092

Values expressed as means ± SD. χ^2 test, $p<0.05$, statistical significance.

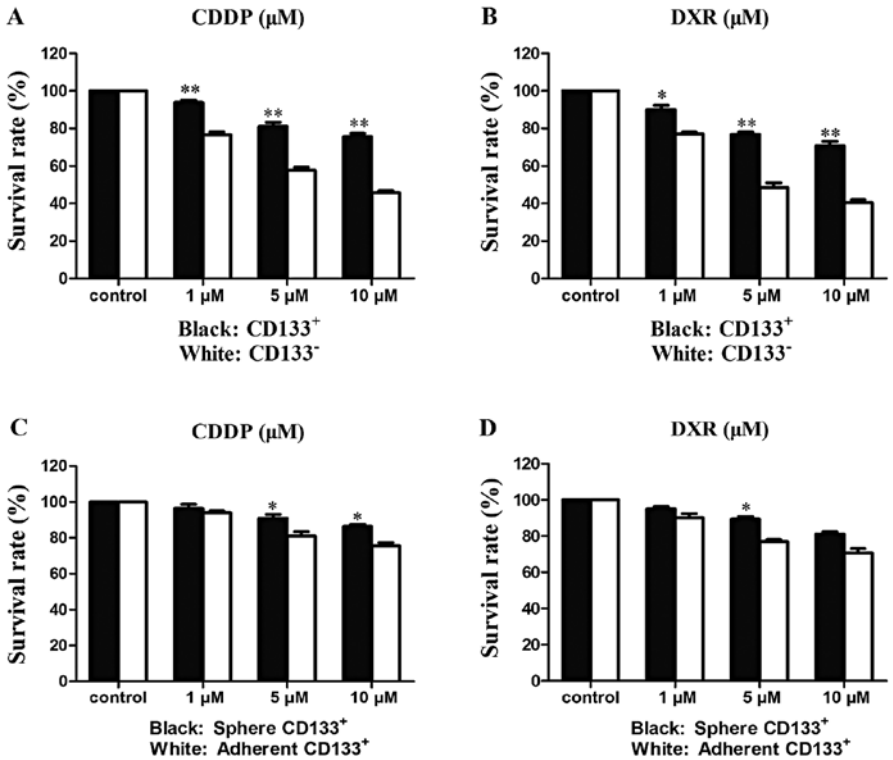

Figure 3. Drug resistance of $CD133^+$ and $CD133^-$ cells. The $CD133^+$ cells were highly resistant to CDDP (A) and DXR (B) in comparison with the $CD133^-$ cells. The spheroids were significantly resistant to CDDP (C) and DXR (D) in comparison with the adherent $CD133^+$ cells (± SD; $^*p<0.05$; $^{**}p<0.01$).

proliferation of both $CD133^+$ and $CD133^-$ SW982 cell populations in a dose-dependent manner. The survival rates of $CD133^+$ and $CD133^-$ cells after 48-h drug treatment were measured (Tables II and III; Fig. 3) and $CD133^+$ cells were found to be significantly more resistant to both CDDP and DXR than $CD133^-$ cells (Fig. 3A and B): 10 μM CDDP inhibited $CD133^-$

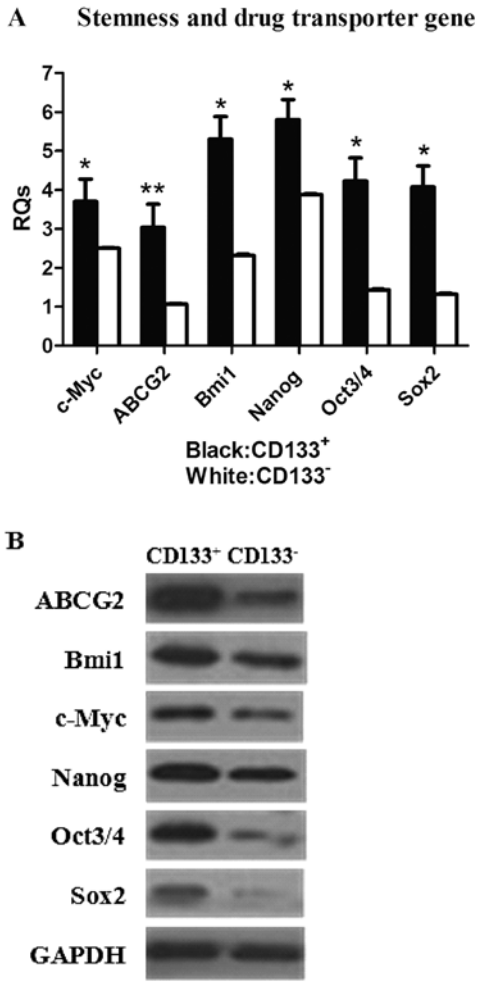

Figure 4. The gene expression profile of CD133+ and CD133- subpopulations. (A) The real-time PCR analysis of stemness and drug transporter gene expression in the CD133+ and CD133- subpopulations (± SD; *p<0.05; **p<0.01; RQ, relative quantity). (B) Western blot analysis of the stemness and drug transporter gene, GAPDH was used as a loading control.

and CD133+ proliferation by ≤54.4 and 24.5%, respectively; 10 μM DXR inhibited CD133- and CD133+ proliferation by ≤59.5 and 30.2%, respectively. These results indicate that CD133+ cells are resistant to both CDDP and DXR, the chemotherapeutic drugs most commonly used for sarcomas. We also compared the drug response in CD133+ spheroids and adherent cells. Maximal growth inhibition of CD133+ spheroids was 13.7% for CDDP and 19% for DXR (Fig. 3C and D), demonstrating spheroid cells were more resistant than adherent cells to both drugs.

Increased expression of stemness and drug transporter genes in SW982 CD133+ subpopulations. Expression levels of stemness and drug transporter genes were examined in both the CD133+ and CD133- subpopulations by real-time PCR and western blotting. We observed that the expression of genes that play a prominent role in stem cell maintenance and nuclear reprogramming, including *Bmi1, c-Myc, Nanog, Oct3/4* and *Sox2* (29-31) were consistently higher in the CD133+ subpopulation compared with their CD133- counterparts (Fig. 4A). However, *c-Myc* and *Bmi1* protein expression was not significantly increased in CD133+ cells in comparison with CD133- cells (Fig. 4B). In addition, both gene and protein expression of the *ABCG2* drug transporter was significantly increased in CD133+ cells compared with CD133- cells.

Table IV. Tumor initiating capacity of CD133+ and CD133- subpopulations.

	Cell number for injection				
	$1x10^3$	$5x10^3$	$1x10^4$	$1x10^5$	$1x10^6$
CD133+	0/5	2/5	4/5	5/5	
CD133-		0/5	0/5	1/5	5/5

Increased cancer initiation by CD133+ cells in vivo. To investigate the tumorigenic potential of CD133+ and CD133- cells, both subpopulations of SW982 cells were injected into BALB/c nude mice to generate xenografts. A difference in tumorigenicity was observed between the CD133+ and CD133- cell populations (Table IV). Tumor formation was observed after the injection of as few as $5x10^3$ CD133+ cells (Fig. 5A and B). In contrast, only one out of five mice injected with $1x10^5$ CD133- cells and none of the mice injected with ≤$1x10^4$ CD133- cells developed tumors. CD133- cells (≥$1x10^5$) were required to initiate a tumor, suggesting that the *in vivo* tumorigenicity of CD133- cells is lower than that of CD133+ cells. In addition, CD133+ cells initiated both secondary and tertiary tumors, whereas CD133- cells did not.

We performed HE staining and immunohistochemistry to demonstrate that the xenografts were derived from the injected CD133+ and CD133- cells. However, both CD133+ and CD133- tumors had similar histological characteristics and levels of CD133 expression (Fig. 5C and D).

Discussion

CSCs are present in numerous types of cancer (15) and are capable of self-renewal and differentiation and spheroid formation and have a high tumorigenicity and resistance to current treatments. In many cases, cancer therapy failure may be caused by a lack of effect of current therapies upon CSCs, which are able to survive such treatments and regenerate the tumor. It is therefore necessary to be able to detect and characterize CSCs, in order to develop new CSC-targeted therapeutic strategies. The major problems involved in isolating CSCs are their rarity and the absence of specific markers to enable their purification.

In early investigations, the side population (SP), defined by Hoechst dye exclusion, was identified as a distinct subset of cells; these cells were subsequently isolated and designated CSCs, possess stemness characteristics and are responsible for tumorigenesis in several cancer types (32-35). Some SS have been shown to contain SP with enhanced tumorigenic potential (36). We originally tried to identify SP cells in the human SS cell line SW982, but failed, possibly because of inappropriate culture conditions or lack of a stem cell population in this cell line. Aldehyde dehydrogenase (ALDH) functions as a detoxifying enzyme, has been proposed as a marker of both normal and cancer stem cells (37) and has been successfully employed

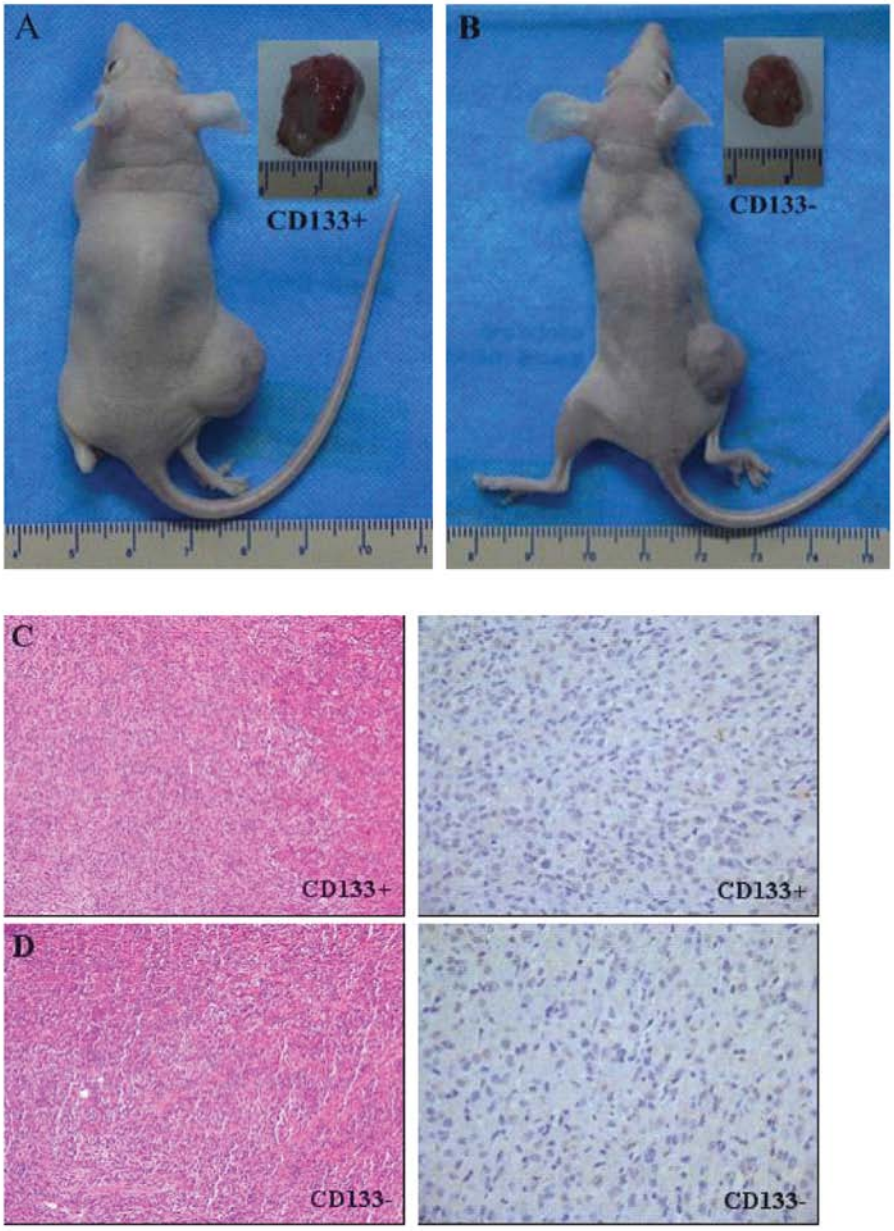

Figure 5. Analysis of tumors grown from CD133^{+} and CD133^{-} subpopulations. (A and B) CD133^{+} and CD133^{-} cells (1x10^{5}) were subcutaneously injected into BALB/c nude mice and gross appearance of resected tumors. (C and D) CD133^{+} tumor revealed histologic characteristics similar to CD133^{-} tumor. Left, H&E staining; right, expression of CD133.

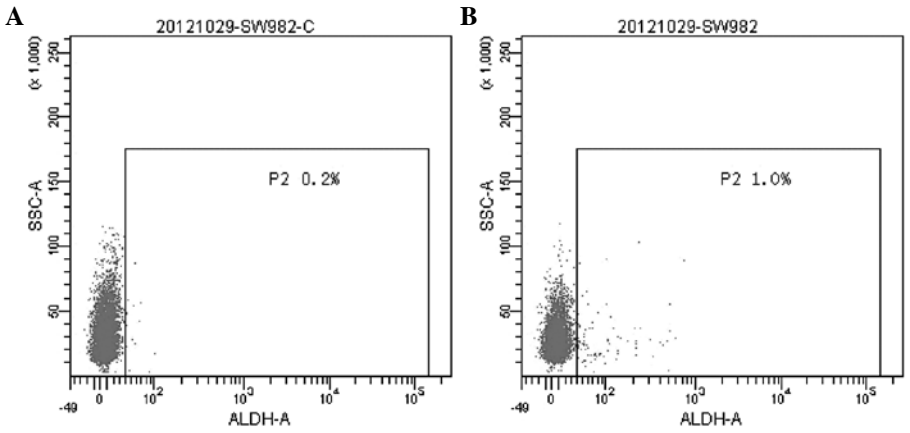

Figure 6. ALDH expression in SW982 cell lines. (A) FACS analysis of SW982 with ALDH inhibitor DEAB as a control. (B) Aldefluor assay showing 1.0% of the cell population in SW982 express high ALDH1 activity.

as a stem cell marker in breast (38), prostate (39), colon (40) and lung cancer (41). We demonstrated that SW982 cells contain a subpopulation with high ALDH activity, comprising ~0.8% of the total cell population (Fig. 6). We also found that the CD133+ subpopulation comprises 8.59% of SW982 cells (Fig. 1A). Therefore, the CD133 antigen is potential CSC marker in SW982 cells; using this marker, we were able to isolate a population containing a high proportion of CD133+ cells. To our knowledge, this is the first report of the analysis of CD133 expression in SW982 cells.

Two SS cell lines have been reported to exhibit sarcospheres (42). In the present study, we identified many differences between the CD133+ and CD133- subpopulations; for example, CD133+ cells were better at forming spherical colonies and self-renewal in anchorage-independent, serum-starved culture conditions. Moreover, the CD133+ subpopulation expressed high levels of stemness genes. We compared the expression of genes that play a prominent role in stem cell maintenance, self-renewal and nuclear reprogramming, including *Bmi1, c-Myc, Nanog, Oct3/4* and *Sox2*, in the CD133+ and CD133- subpopulations. The *Oct3/4* transcription factor is critically involved in self-renewal and the maintenance of pluripotency in undifferentiated embryonic stem (ES) cells (43). The *Sox* family of transcription factors plays an essential role in cell differentiation and development. *Sox2* was originally thought to be the only Sox protein expressed in ES cells (44) and associating with *Oct3/4* to maintain ES cell self-renewal. *C-Myc* is a dominant-acting oncogene that encodes a transcription factor thought to regulate the G0-G1 cell cycle transition. *Bmi1* is a member of the mammalian polycomb group (Pc-G) gene family (45), which contributes to the proliferative capacity and self-renewal of both normal and malignant stem cells (46). *Bmi1* recently emerged as a Myc-cooperating oncogene (47). As both genes play an important role in stem cell maintenance, *Bmi1* and *c-Myc* expression may correlate with self-renewal and anti-apoptotic mechanisms in CSCs. Another highly expressed gene in CD133+ cells is *Nanog*, which encodes a recently identified divergent homeoprotein that maintains ES cell self-renewal (48). All five stemness genes were consistently and significantly overexpressed in the CD133+ subpopulation by real-time PCR. Although we did not demonstrate a relationship between expression of the encoded proteins and the genesis of SS, these data provide compelling evidence that a CD133+ subpopulation with stem-like properties exists in SW982 cells.

CD133+ subpopulations were more resistant to the cytotoxic effects of both CDDP and DXR, showing improved survival compared to CD133- subpopulations. In addition, CD133+ subpopulation-derived spheroids were more drug-resistant than adherent cells. Increased expression of ABC transporter proteins, in particular ABCG2, is reported to significantly contribute to the CSC phenotype, strongly correlates with drug-resistance and is associated with a poor clinical outcome (49,50). In our study, we revealed that *ABCG2* mRNA and protein expression was elevated in CD133+ subpopulations; this may be responsible for high levels of CSC multi-drug resistance and therefore constitutes a good target for clinical cancer therapy.

In addition, using a BALB/c xenograft model we observed that the CD133+ subpopulation had a higher tumorigenic potential *in vivo* than the CD133- subpopulation. Furthermore, slower tumor formation was observed by the CD133- subpopulation, despite injection of more cells. This observation suggests the existence of other cell populations with tumor-initiating potential, although we cannot exclude the possibility of CD133+ cell contamination in the CD133- subpopulation during cell sorting procedures.

In conclusion, this study is the first to successfully isolate the CD133+ subpopulation from the human SS cell line SW982 and reveals that this CD133+ subpopulation exhibits several characteristic CSC properties, including high clonogenicity and self-renewal, increased chemotherapeutic drug resistance, elevated expression of stemness and drug transporter genes and high tumorigenic potential. Therefore, CD133 may represent a new stem cell marker for SS and may lead to novel approaches to develop therapies that selectively attack CSCs without affecting normal tissue stem cells and thus improve the prognosis for patients with SS.

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