Aberrant Wnt/β-catenin signaling and elevated expression of stem cell proteins are associated with osteosarcoma side population cells of high tumorigenicity

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Abstract. According to the cancer stem cell theory, the presence of a small sub-population of cancer cells, termed cancer stem cells (CSCs), have a significant implication on cancer treatment and are responsible for tumor recurrence. Previous studies have reported that alterations in the Wnt/β-catenin signaling are crucial in the maintenance of CSCs. In the present study, the characteristic features and activation of Wnt/β-catenin signaling in CSCs from osteosarcoma, an aggressive human bone tumor, were investigated. In total, ~2.1% of the cancer stem-like side population (SP) cells were identified in the osteosarcoma samples. The results of subsequent western blot and reverse transcription-quantitative polymerase chain reaction analyses revealed that the protein levels of β-catenin and cyclin D1 were markedly upregulated in the fluorescence-activated cell sorted osteosarcoma SP cells. In addition, the elevated expression levels of stem cell proteins, including CD133, nestin Oct-4, Sox-2 and Nanog were significantly higher in the SP cells, which contributed to self-renewal and enhanced the proliferation rate of the SP cells. Furthermore, the SP cells were found to be highly invasive and able to form tumors *in vivo*. Taken together, these data suggested that the identification of novel anticancer drugs, which suppress the Wnt/β-catenin signaling and its downstream pathway may assist in eradicating osteosarcoma stem cells.

Introduction

Osteosarcoma, a bone sarcoma, is the eighth most common malignancy occurring in all age groups, the prevalence of which is 20% in adolescents and 5% in children (1,2). Bone sarcomas are generally derived from the mesenchymal or non-epithelial tissues and predominantly occur as a high-grade tumor with pulmonary metastases (3). Following diagnosis, the average life span of patients with bone sarcoma at the metastasis stage is <5 years (1,2). Despite improvements in surgery and multi-therapeutic agents, treatment failure and tumor recurrence have major implications on the success of cancer treatment, with failure in treatment strategies ultimately leading to poor survival rates in patients with cancer (4). Therefore, understanding the mechanism of tumorigenesis at the molecular level is crucial to provide effective treatment strategies to completely eradicate the tumor refractory. The molecular mechanisms underlying the drug resistance and tumor recurrence of bone tumors remains to be fully elucidated. Previous investigations in several types of cancer have demonstrated that the presence of a small population of cancer stem cells (CSCs) are the major cause for drug resistance and tumor relapse. These CSCs share the properties of stem cells, including self-renewal, multi-drug resistance, and high proliferation rates and differentiation potential (5-8). In addition, these CSCs are highly tumorigenic and, therefore, have been termed 'tumor initiating cells' (9).

Several studies have reported the existence of CSCs in different types of cancer, based on Hoechst 33342 dye exclusion assays, which is considered to be a valuable technique to isolate CSCs (10-12). The CSCs which exclude this dye are termed 'side population' (SP) cells, and exhibit enhanced overexpression of the adenosine triphosphate binding cassette (ABC) protein transporter, ABCG2 (13). The SP cells have been isolated and characterized in several types of solid tumor and well-established cell lines (12). The SP cells have been demonstrated to possess the features of CSCs and are considered to be enriched CSCs. Notably, previous findings have revealed that stem-like properties of CSCs and their maintenance are regulated by the Wnt/β-catenin pathway in different types of cancer, including breast, liver and colon cancer (14-16). Therefore, the isolation and characterization of SP cells may assist in understanding the molecular mechanism underlying CSC-mediated tumoringeneis. Previous reports concerning the identification and characterization of CSCs in osteosarcoma stem lines are limited, in which CSCs have been

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observed to be highly tumorigenic in mouse models (8,17). These data suggest that osteosarcoma may also contain a small population of CSCs, which are responsible for treatment failure, tumor recurrence and metastasis. Consequently, the present study aimed to investigate the presence of cancer stem-like SP cells from osteosarcoma samples using a fluorescence-activated cell sorting (FACS-based Hoechst 33342 dye exclusion technique. In addition, the sorted SP cells were analyzed for the activation of the Wnt/β-catenin signaling pathways and expression of stemness genes.

Materials and methods

Cell isolation and culture. Human osteosarcoma samples (n=10) were obtained from 10 patients, who had not undergone chemotherapy treatment, at the Department of Traumatology, Linyi People's Hospital (Linyi, China). This study was approved by the Linyi People's Hospital research review and ethics committee (Linyi, China), and informed consent was obtained from all participants. The patients and tumor details were as follows: Patients were aged between 38 and 49 years and included six males and four females. (18). The tumor samples were classified by a pathologist at Linyi People's Hospital (Linyi, China) using the three-tier grading scheme: Low grade (grade 1), intermediate grade (grade 2) and high grade (grade 3). The tumor samples were fibroblastic osteosarcoma in site and were classified as high grade. Following isolation, the samples were washed extensively in phosphate-buffered saline (PBS) and incubated overnight in Dulbecco's modified Eagle's medium (DMEM/F12; Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA), containing penicillin (500 U/ml), streptomycin (500 μg/ml) and amphotericin B (1.25 μg/ml; Gibco-BRL). Enzymatic digestion was performed using collagenase (1.5 mg/ml; (Gibco-BRL) and hyaluronidase (20 μg/ml) in PBS for 1 h. The cells were cultured in DMEM with 10% FBS, supplemented with antibiotics [penicillin (500 U/ml) and streptomycin (500 μg/ml)] and maintained in T-75 flasks at 37˚C in a humidified 5% CO_2/95% air atmosphere. When the cells reached a confluence of 90%, they were removed from the culture flask using trypsin-EDTA (0.25% 53 mM EDTA; Sigma-Aldrich, St. Louis, MO, USA) washed in PBS, and the cells were resuspended in 10% DMEM. The number of cells were counted using a hemocytometer (LW Scientific, Lawrenceville, GA, USA).

FACS analysis. The cells were cultured in DMEM with 10% FBS, supplemented with antibiotics and maintained in T-75 flasks at 37˚C in a humidified 5% CO_2/95% air atmosphere. At a confluence of 90%, the cells were removed from the culture flask using trypsin-EDTA (0.25% 53 mM EDTA), washed in PBS and then resuspended in 10% DMEM. The number of cells was counted using a hemocytometer. The cells were divided into two groups as follows: Control group, cells + Hoechst 33342 dye (n=7). Drug-treated group, cells + verapamil + Hoechst 33342 dye (n=7). The cells (~10^6 cells/ml 10% DMEM) were labeled with Hoechst 33342 stock-bis-benzimide (5 μl/ml; Sigma-Aldrich) either alone or in combination with verapamil (0.8 μl/ml). The cells were then resuspended in 500 μl Hank's balanced salt solution (HBSS) containing 10 mM HEPES for FACS analysis. Finally, the cells were counterstained using 2 μg/ml propidium iodide (PI) and assessed using a flow cytometer (Attune NxT; Life Technologies).

In vitro proliferation activity. The FACS-sorted SP and non-SP cells were seeded in a 96-well plate at 2x10^6 cells/well, and then cultured in a CO_2 incubator. Each group was set up in triplicate. The proliferation activity of the cells was measured every day for 7 days. Each well was supplemented with Cell Counting Kit (CCK)-8 solution (10 μl; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) and incubated in a CO_2 incubator for 2-3 h. The optical density (OD) was then determined at 450 nm. These data were used to calculate the cell growth and produce graphs, based on the mean value of OD_{450} and standard deviation values for each well.

Cell resistance assay. The determine cell resistance, 1x10^3 cells/plate were cultured in 96-well plates and treated with chemotherapeutic drugs at the following concentrations: 10 μg/ml 5-fluorouracil (5-FU), 250 mM gemcitabine, 100 mM oxaliplatin, 30 ng/ml paclitaxel, 5 mg/ml cisplatin, 10 mg/ml etoposide and 2 μg/ml oxaliplatin. The mean OD_{450} values obtained were presented as a graph, and cell resistance in the groups was calculated using the following formula: Cell resistance rate (%) = (experimental group OD_{450} value / control group OD_{450} value) x 100. The values presented are the average of three independent experiments.

Immunofluorescent staining. The FACS-sorted SP cells were fixed onto glass slides using ice-cold 4% paraformaldehyde at 4˚C for 10 min, and blocked with 1% bovine serum albumin for 30 min at room temperature to inhibit nonspecific binding of immunoglobulin (Ig)G. Following washing with PBS, the cells were incubated with mouse anti-human ABCG2 antibody at 4˚C overnight. Following another wash with PBS, the cells were incubated with goat anti-mouse IgG-horesradish-peroxidase (HRP) for 30 min at room temperature. The cells were counterstained using hematoxylin, and were mounted with glycerol vinyl alcohol aqueous mounting solution (19). Under an optical microscope, the $ABCG2^+$cells were stained red. All images were processed using Adobe Photoshop CS4.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted and complementary DNA was prepared using a Reverse Transcriptase kit (Thermo Fisher Scientific, Pittsburgh, PA, USA). RT-qPCR analysis was subsequently performed using IQ Supermix with SYBR-Green (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 25 ng cDNA per sample. The sequences of human specific primers used were as follows: ABCG2, forward 5'-TCAATCAAAGTGCTTCTTTTTTATG-3' and reverse 5'-TTGTGGAAGAATCACGTGGC-3'; Sox2, forward 5'-CAC ACTGCCCCTCTCACACAT-3' and reverse 5'-CATTTCCCT CGTTTTTCTTTGAA-3'; Nanog, forward 5'-CCAACATCC TGAACCTCAGCTAC-3'and reverse 5'-GCCTTCTGCGT CACACCATT); CD133, forward 5'-TCTTGACCGACTGAGA CCCAAC-3'and reverse 5'-ACTTGATGGATGCACCAAG CAC-3'; CCND1, forward 5'-TGATGCTGGGCACTTCAT CTG-3'andreverse5'-TCCAATCATCCCGAATGAGAGTC-3'; OCT-4 forward 5'-GCA ATTTGCCAAGCTCCTGAA-3'and

reverse 5'-GCAGATGGTCGT TTGGCTGA-3'; Nestin, forward 5'-GACGGAGGAGGTAGCCCGCA-3' and reverse 5'-GCCTCCACAGCCAGCTGGAACT-3'; and GAPDH, forward 5'-TCTGCTCCTCCTGTTCGACA-3' and reverse 5'-AAAAGCAGCCCT GGTGACC-3'. GAPDH was used as a housekeeping gene. The parameters used to set the qPCR reactions were as follows: Initial denaturation-95˚C for 15 sec; annealing-58˚C for 45 sec; extension-60˚C for 30-45 sec; cycles-35. The qPCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide (0.5 mg/ml). The data represented are the average values of three independent experiments.

Western blot analysis. The proteins were extracted from the SP and non-SP cells, and protein concentration was determined using a Bradford assay. 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: β-catenin (rabbit polyclonal anti-human, 1:1,000 dilution, sc-7199); ABCG2 (mouse monoclonal anti-human, 1:1,000 dilution, sc-18841); GAPDH (mouse monoclonal anti-human, 1:1,000 dilution, sc-47724). The secondary immunoglobulin (Ig)G antibodies with alkaline phosphatase markers were used with specificity for the appropriate species (goat anti-rabbit, 1:5,000 dilution, sc-2034 and goat anti-mouse, 1:5,000 dilution, sc-2047) and incubated for 2 h at room temperature. All antibodies were purchased from Santa Cruz Biotechnology Inc, (Dallas, TX, USA). Immuno-reacted proteins were detected with using a chemiluminescence reagent kit (ab79907; Abcam, Cambridge, MA, USA). Blots were detected and scanned using a densitometer (Biorad GS-710; Bio-Rad Laboratories, Inc.).

Tumor cell implantation. For tumor cell implantation, 4-6 week-old NOD/SCID mice were obtained from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). All mice were fed a standard chow, and food and water were available *ad libitum*. Animals were housed at the appropriate temperature and with a standard 12-h light-dark cycle. The FACS-purified SP- and non-SP H460 cells containing Matrigel (1:1) at a concentration of $1x10^5$ per 100 μl, were administered to NOD/SCID mice by sub-cutaneous injection. The density of the cells injected and growth of the mice were monitored, according to the previously described protocol (20). The tumor volumes were measured according to the following formula: $V = 1/2\ ab^2$ (where a represents the long diameter and b represents the short diameter of the tumor. After 4-5 weeks, the mice were sacrificed by asphyxiation with CO_2; tumors were harvested and measured, and images were captured.

Sarcosphere formation assay. A sphere formation assay was performed, according to a previously described procedure (5). The cells were plated at a density of 60,000 cells/well in ultra-low attachment six-well plates containing serum-free DMEM/F12 medium, supplemented with N2, epidermal growth factor (10 ng/ml) and human basic fibroblast growth factor (10 ng/ml). The culture was analyzed for sphere formation each day for 7 days and images were captured using inverted phase contrast microscopy (Eclipse Ti-S; Nikon, Tokyo, Japan). Spheres with a diameter of >150 μm were counted. After 7 days of culture, the total number of sarcospheres that were generated by the FACS-sorted SP and non-SP cells were quantified.

Martigel invasion assay. The cellular invasiveness of the SP and non-SP cells was determined using six-well Matrigel invasion chambers (BD Biosciences, Franklin Lakes, CA, USA). The cells were seeded in serum-free medium at a density of $2x10^5$ per well. The outer wells were filled with DMEM containing 5% FBS as a chemoattractant, and incubated at 37˚C for 48 h. Subsequently, the non-invading cells were removed by swabbing the top layer of Matrigel with a Q-tip (21). The membrane containing the invaded cells was stained with hematoxylin for 3 min, washed with PBS and mounted on slides. The entire membrane containing the invading cells was visualized under a light microscope at 40x objective and the number of cells counted, with the values presented as the average value of three independent experiments.

Statistical analysis. 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. Values are expressed as the mean ± standard deviation. $P<0.01$ was considered to indicate a statistically significant difference.

Results

Identification of osteosarcoma SP cells using Hoechst 33342 dye. The present study investigated the presence of cancer stem-like SP cells in osteosarcoma samples. Using FACS, a small population of SP cells of ~2.1% were found to exclude the Hoechst 33342 dye (Fig. 1A; gated region). The action of pumping out Hoechst 33342 dye is actively performed by overexpression of the ABC transporter protein, ABCG2, which was confirmed by treatment of cells with verapamil. Upon verapamil treatment, the population of SP cells were significantly reduced to 0.7% (Fig. 1B; gated region). These data indicated that the SP cells were resistant to drug uptake due to the overexpression of ABC transporter proteins (Fig. 1C).

Phenotypic characterization of osteosarcoma SP cells. In order to characterize the FACS-sorted SP cells, the SP and non-SP cells were subsequently subjected to *in vitro* cell proliferation and multi-drug resistance assays. Notably, the osteosarcoma SP cells (Fig. 2A) underwent rapid cell proliferation, beginning on the third day (D3), and became more confluent on the eight day (data not shown). However, the growth rates of the non-SP cells were significantly lower, compared with the SP cells (Fig. 2A). Similarly, the SP cells were resistant to uptake drugs, including etoposide, gemcitabine, 5-flurouracil (5-FU), cisplatin, paclitaxel and oxaliplatin. Upon treatment with these drugs, the survival rate of the SP cells was significantly higher, compared with the non-SP cells (Fig. 2B). The increased drug resistance of the SP cells was most likely due to overexpression of ABCG2 in the SP cells. As shown in the Fig. 2C, the SP cells were more positive to ABCG2 than the non-SP cells. Therefore, these findings demonstrated that the osteosarcoma SP cells underwent marked proliferation and exhibited enhanced survival rates following chemotherapy.

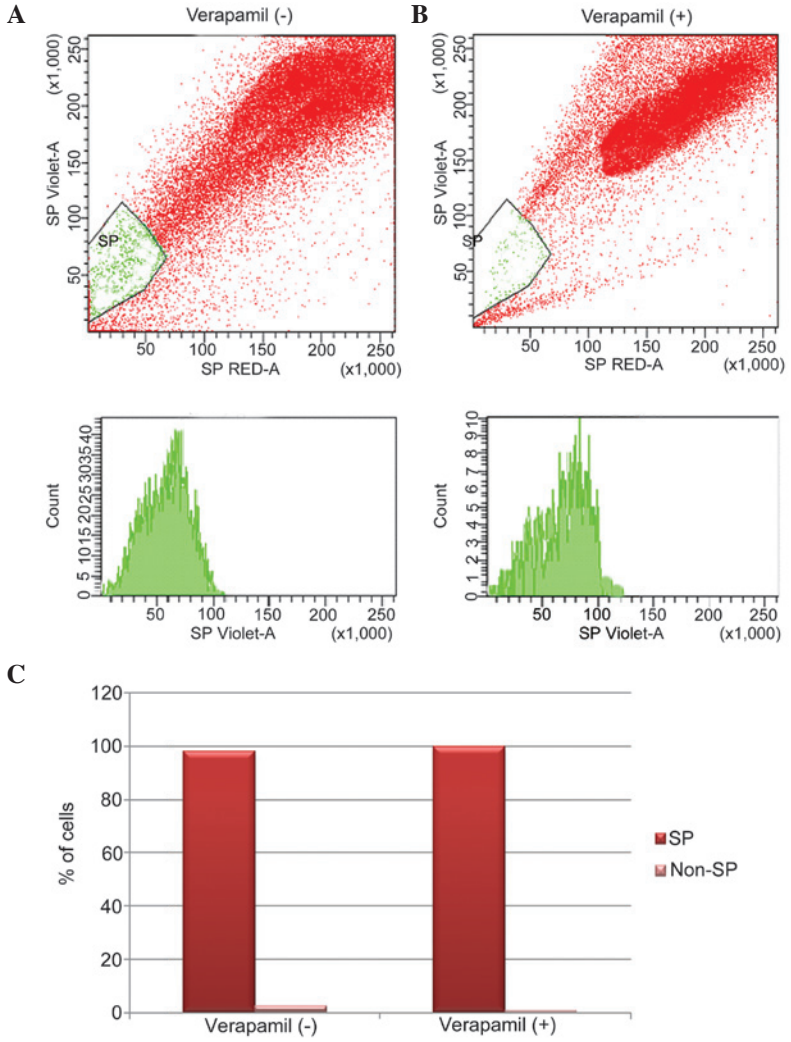

Figure 1. Dot-plot analysis of FACS for sorting of osteosarcoma SP cells. (A) Cells were stained with Hoechst 33342 dye and SP cells of 2.1% were identified and outlined (gated population). (B) Percentage of SP cells was significantly reduced to 0.3% in the presence of verapamil. (C) Graphical representation of the SP cells with and without verapamil treatment. This quantitative graph was constructed with data from the dot blot analysis using FACS. SP, side population; FACS, fluorescence-activated cell sorting.

Elevated Wnt/β-catenin signaling and upregulation of Oct-4 in SP cells. Previous studies investigating different types of cancer have reported that hyperactivation of the Wnt/β-catenin pathway results in elevated expression levels of stem cell surface proteins and its downstream signaling pathways (22,23). Therefore, the presents study evaluated the activation of Wnt/β-catenin signaling and the expression of stemness genes in the in the FACS-sorted SP cells. Western blot analysis revealed that the protein level of β-catenin was higher in the SP cells, compared with the non-SP cells (Fig. 3A). Similarly, the expression of the ABCG2 ABC transporter protein was significantly higher in the SP cells. In addition, the results of the RT-qPCR analysis revealed that the relative mRNA expression levels of the wnt target gene cyclin D1, ABCG2 and stem cell genes, including CD133, nestin Oct-4, Sox-2 and Nanog were significantly elevated in the SP cells, compared with the non-SP cells (Fig. 3B). Therefore, these data suggested that elevated levels of Wnt/β-catenin signaling may be a trigger for the increased expression levels of ABCG2 and stem cell surface proteins, involved in multi-drug resistance and tumorigenic properties of the SP cells.

SP cells exhibit high levels of self-renewal and invasion. In order to compare the clone formation efficiency of the FACS-sorted SP and non-SP cells, sphere formation and invasion assays were performed. The total number of sarcospheres generated by the osteosarcoma SP cells was significantly higher, compared with the non-SP cells (Fig. 4A). Similarly, the sarcospheres generated by the SP cells were increased in size following continuous culture, and attained maximal size on day 6 (Fig. 4B). However, the non-SP cells did not attain a mature size. In addition, the *in vitro* Matrigel invasion assay demonstrated that FACS-sorted SP cells were significantly more invasive, compared with the non-SP cells (Fig. 4C). Taken together, the SP cells were capable of initiating tumor growth and causing tumor invasion.

Tumorigenic potential of osteosarcoma SP cells. The SP cells were able to regenerate tumors even following transplantation at the lowest cell density (5,000 cells), however, the non-SP cells were unable to repopulate the tumor cells at this cell concentration (data not shown). As shown in Fig. 5, injection of the SP cells into the NOD/SCID mice formed tumor

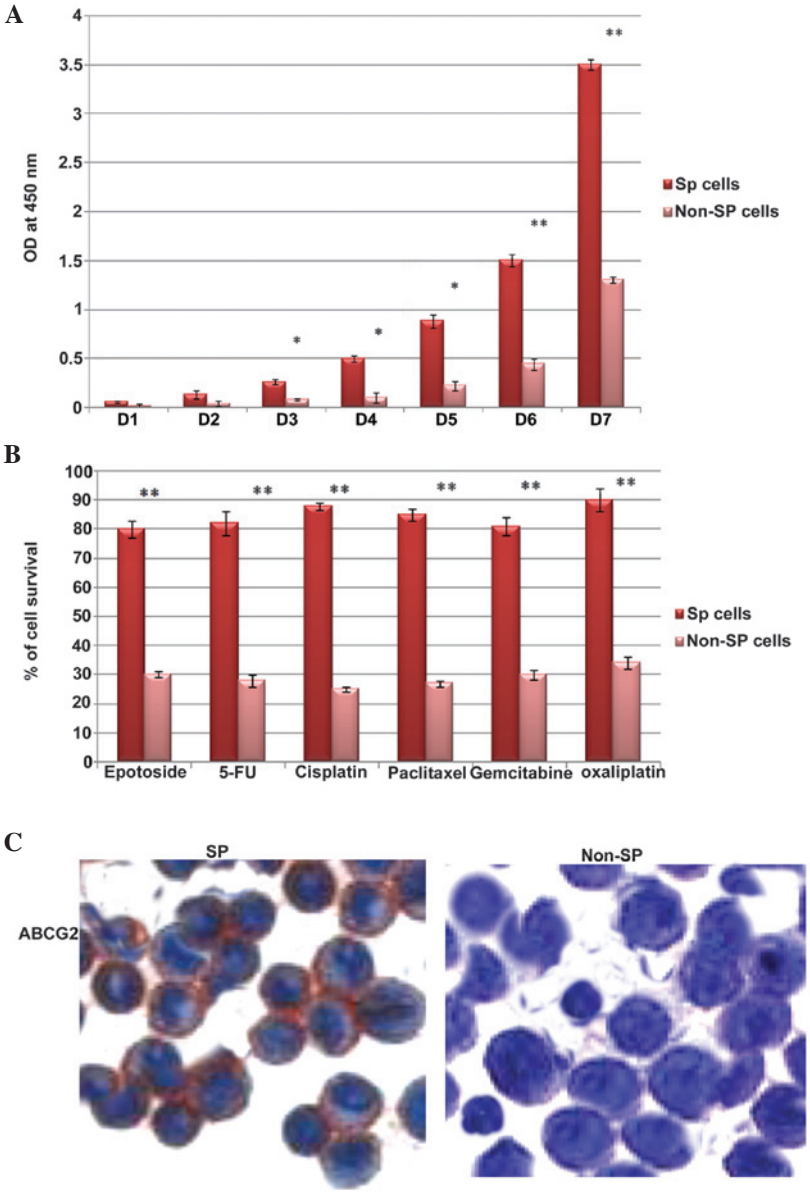

Figure 2. Phenotypic characterization of FACS-sorted osteosarcoma SP cells. (A) *In vitro* cell proliferation assay. The cell proliferation rates of the SP cells were significantly higher than those of the non-SP cells. The x-axis represents days (D) 1-7, the y-axis indicates the corresponding OD value at 450 nm. (B) Comparison of cell survival rates of the SP cells and non-SP cells following treatment with etoposide, gemcitabine, 5-FU, cisplatin, paclitaxel and oxaliplatin. The SP cells exhibited increased resistance and increased survival rates (>80%), compared with the non-SP cells (<35%). (C) Immunocytochemistry analysis of the sorted SP cells (magnification, x100). SP cells exhibited enhanced expression of ABCG2 (stained red), compared with the non-SP cells. Cells were counterstained with hematoxylin. The data are expressed as the mean ± standard deviation. *P<0.05; **P<0.01 vs. non-SP cells. SP, side population; 5-FU, 5-fluorouracil; OD, optical density.

spheres *in vivo* and these tumors grew significantly faster than the tumors formed from non-SP cells. Therefore, the osteosarcoma SP cells exhibited multi-drug resistance, enhanced survival rate and increased tumorigenicity.

Discussion

The CSC theory states that the presence of a small sub-population of cancer cells, termed CSCs, are responsible for treatment failure and minimal residual disease (MDR). These CSCs evade current treatment regimens and are capable of initiating tumor growth following chemoradiotherapy and are considered to be 'tumor initiating cells'. Therefore, it is important to isolate and characterize CSCs to provide effective treatment in subjecting CSCs to apoptosis. Based on Hoechst 33342 dye exclusion, CSCs have been identified in a variety of solid types of tumor, including glioblastomas, and breast, prostate and lung cancers (21,24-27). In the present study, the presence of osteosarcoma-initiating cells was examined using the Hoechst dye exclusion method. The results revealed osteosarcoma SP cells of ~2.1%, which were analyzed for stem cell properties and the Wnt/β-catenin signaling pathways. The SP cells exhibited clonogenic capacities, high levels of tumorigenicity and self-renewal characteristics. The SP cells efficiently excluded the Hoechst 33342 dye from the cell due to overexpression of the ABCG2 ABC transporter protein. Important characteristic features of cancer stem-like SP cells are multi-drug and apoptotic resistance, which are particularly

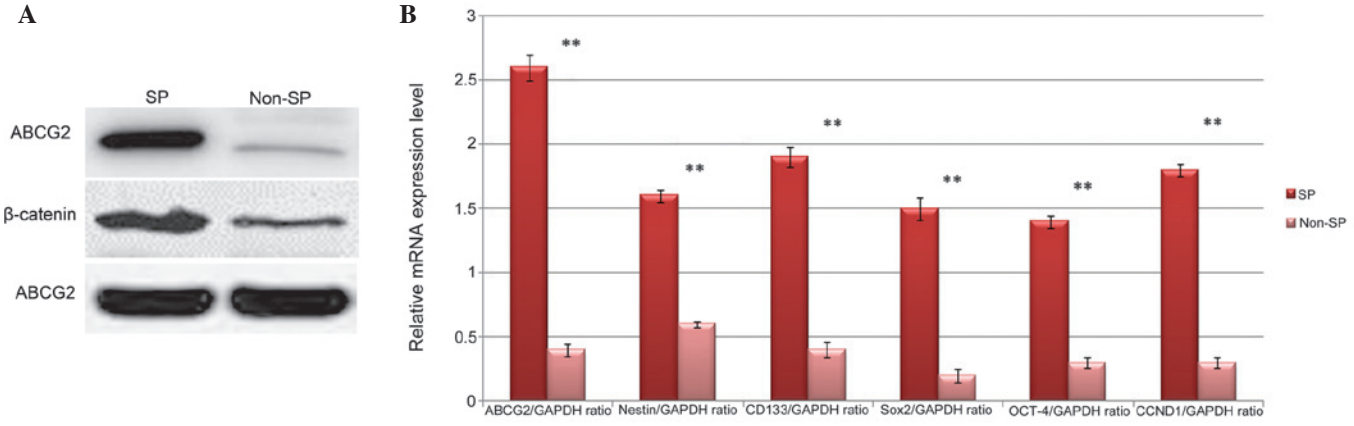

Figure 3. Elevated Wnt/β-catenin signaling and stem cell proteins in SP cells. (A) Western blot analysis of protein expression in SP and non-SP cells. Equal quantities of protein were loaded in each lane. (B) Elevated expression levels of the Wnt target gene, CCND1, and stem cell genes, OCT-4, SOX2, nestin, CD133, Nanog SP and ABCG2 were detected using reverse transcription-quantitative polymerase chain reaction. Quantification was performed using data of three independent experiments. GAPDH was used as a housekeeping gene. Data are expressed as the mean ± standard deviation. **P<0.01 vs. non-SP cells. SP, side population.

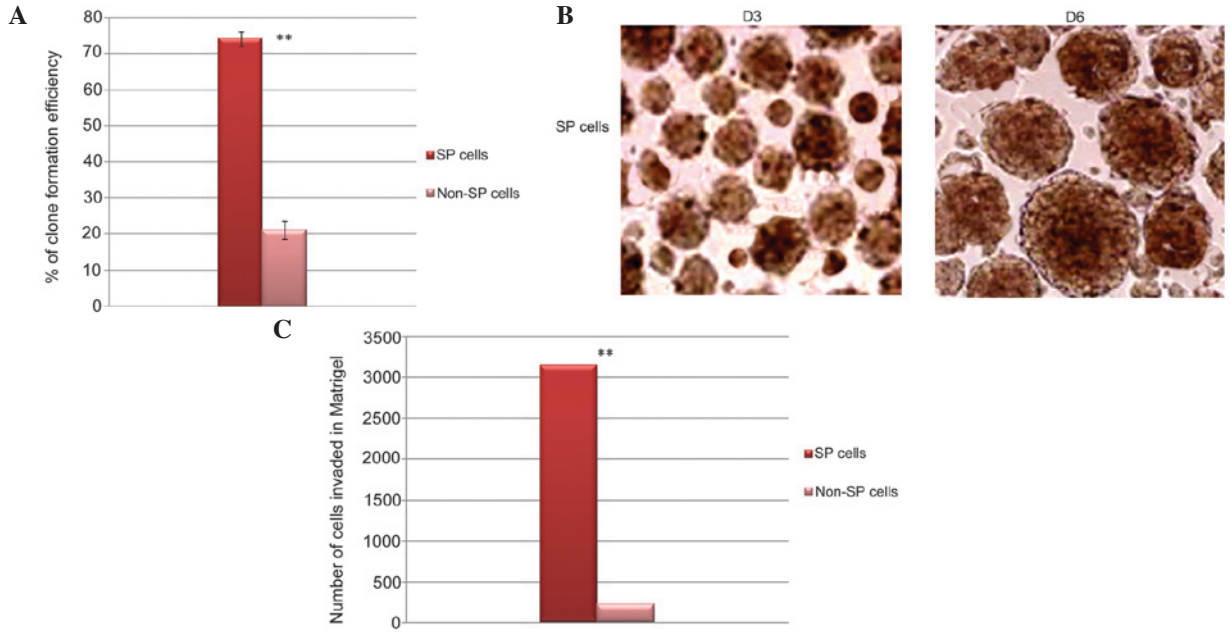

Figure 4. Clone formation efficiency of osteosarcoma SP cells. (A) Total number of sarcospheres generated by osteosarcoma SP cells was significantly higher than those generated by the non-SP cells. (B) Representative images of monoclonal spheres formed by osteosarcoma SP cells (magnification, x100). The sarcospheres were generated rapidly on day 3 (D3) and the size of the spheres were increased on day 6 (D6) . (C) SP cell invasiveness was measured using a Matrigel assay. SP and non-SP cells ($4x10^5$) were seeded and incubated for 72 h. The graph represents the number of cells, which invaded across the membrane. The data are expressed as the mean ± standard deviation. **P<0.01; *P<0.05 vs. non-SP cells. SP, side population.

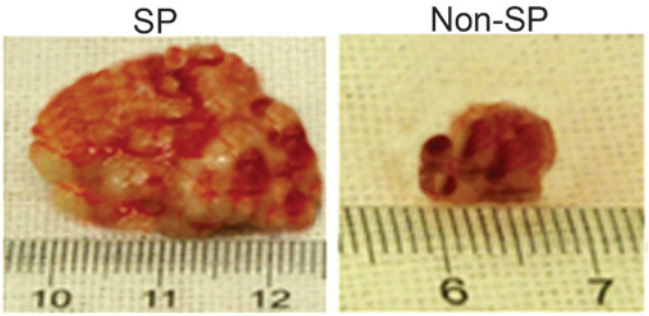

Figure. 5. Tumorigenic potential of osteosarcoma SP cells. Gross tumor images of the tumors derived after 27 days of subcutaneously injected SP and non-SP cells into NOD/SCID mice.

tolerant to cytotoxins. This multi-drug resistance property is predominantly regulated by ABC transporters, which promote efficient efflux chemotherapeutic drug and, therefore, are vital in tumorigenesis. It is also possible that these SP cells also exhibit reduced apoptosis, and these factors together produce CSCs with drug, apoptosis resistance and enhanced survival rates (28). The immunofluorescence and RT-qPCR analysis in the present study demonstrated that the osteosarcoma SP cells had relatively higher expression levels of ABCG2, compared with the non-SP cells, contributing to multi-drug resistance.

The primary difference between the SP and non-SP cells was the ability to generate tumor spheres. The SP cells exhibited marked self-renewal abilities and formed sarcospheres

rapidly, compared with the non-SP cells. Another notable difference was that the SP cells are particularly tumorigenic and were able to initiate tumor growth *in vivo* at the lowest cell concentration, which failed to form tumor growth in the the non-SP cells. The SP cell-derived tumors were identical to primary tumors. Similar to these findings, it was previously demonstrated that SP cells from osteosarcoma cell lines were more clonogenic and exhibited higher self-renewal capacities, compared with non-SP cells (8,29).

Another notable feature of SP cells are the elevated expression levels of stem cell genes. Previously, it was reported that increased expression levels of stemness genes, including CD133, CD44, Oct-4 Nanog, in osteosarcoma SP cells contribute to self-renewal and enhanced the proliferation rate of the SP cells (8,29-31). It has also been demonstrated that the Wnt/β-catenin pathway is one of the most important signal transduction pathways involved in tumorgenesis, tumor progression and maintenance of CSCs (32-34). Consistent with these findings, the present study demonstrated that increased expression levels of β-catenin and cyclin D in the SP cells, compared with the non-SP. In addition, the SP cells exhibited significantly higher expression levels of CD133, CD44, nestin Oct-4, Sox-2 and Nanog, which are important for the self-renewal properties of the SP cells. However, the precise molecular mechanism underlying the Wnt/β-catenin mediated overexpression of stemness genes remains to be fully elucidated.

In conclusion, the present study demonstrated that the osteosarcoma samples contained a small population of SP cells, in which the wnt/β-catenin signaling was markedly elevated, concomitant with increased stem cell surface proteins. Therefore, the wnt/β-catenin signaling is crucial in the maintenance of self-renewal and tumorigenic SP cells and may be a potential target of novel anticancer drugs in order to eliminate chemoresistance and tumorigenicity of SP cells.

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