Increased multi-drug resistance and reduced apoptosis in osteosarcoma side population cells are crucial factors for tumor recurrence

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Abstract. The present study investigated the characteristic features of cancer stem cells (CSCs) using an aggressive human osteosarcoma cell line OS-65. Hoechst 33342 dye exclusion was used to distinguish the cancer stem-like side population (SP) cells from OS-65 cells. Furthermore, the SP cells were characterized via chemoresistance and cell death assays, reverse transcription-quantitative polymerase chain reaction and immunofluorescence. The present study identified ~3.3% of cancer stem-like SP cells from OS-65 cells whose prevalence is reduced significantly (0.9%) following treatment with verapamil. It was demonstrated that osteosarcoma SP cells are highly efficient at generating additional sarcospheres as transcriptional regulation of stemness genes, including SOX2, OCT-4 and NANOG, is highly upregulated. Notably, these SP cells demonstrated high resistance against chemotherapeutic drugs and apoptosis via elevated transcriptional regulation of several ATPase binding cassette (ABC) transporter and anti-apoptotic proteins, including ABCG2, ABCB1/MDR1 ABCB5, B cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein, respectively. The results of the present study suggested that CSCs may be a novel therapeutic target for the prevention of tumor relapse.

Introduction

Osteosarcoma is the most common aggressive malignancy in children and young adolescents, which accounts for 2.4% of all pediatric malignancies worldwide. The estimated average survival rate of patients with osteosarcoma following diagnosis at metastatic stage is 4-5 years (1,2). Despite recent advances and developments in osteosarcoma treatment strategies, the overall survival rate of patients has not yet improved. According to cancer stem cell theory, the presence of a small population of cancer initiating cells, cancer stem cells (CSCs), are responsible for treatment failure, tumor relapse and metastasis (3-5). These CSCs have been demonstrated to possess increased expression of stem cell surface proteins, including Oct3/4A and Nanog, and ATPase binding cassette (ABC) transporters, including MDR1, also known as ABCB1, ABCC1 and ABCG2, also known as BCRP1, which have an essential role in the maintenance of self-renewal and drug resistance properties of CSCs, respectively (6,7). Therefore, understanding the underlying molecular mechanism and signaling pathways involved in the multi-drug and apoptosis resistance of cancer stem cells is necessary in order to efficiently target CSCs. The Hoechst 33342 dye exclusion assay (8,9) is a well-used method for the purification of CSCs. In this method, the Hoechst 33342 dye is prevented from entering cancer stem cells due to ABC transporter proteins, such as ABCG2. These cells appear as a distinct population on the periphery of the dot plot analysis of the fluorescence activated cell sorting (FACS) profile; hence, they are termed side population (SP) cells (9). Previous studies have demonstrated that SP cells possess the majority of the remarkable features of cancer stem cells, including a high resistance to chemotherapeutic drugs, high tumorigenic potency and the overexpression of stemness genes (6,7,10); therefore, they are referred to as 'enriched cancer stem cells' (5,11,12). Characterizing SP cells and elucidating the pathways associated with drug resistance may help to develop anti-cancer drugs to target CSCs.

In the present study, osteosarcoma stem-like SP cells were purified and characterized from the aggressive human osteosarcoma OS-65 cell line. Furthermore, the level of multi-drug and apoptosis resistance of SP cells was evaluated, along with the transcriptional regulation of multiple ABC transporter and stemness genes, in order to explore the credibility of CSCs as a novel therapeutic target for the prevention of tumor relapse.

Materials and methods

Cell lines and culture conditions. Osteosarcoma cell line OS-65 was donated by Dr Di-Sheng Yang (Department of Orthopaedics of the Second Affiliated Hospital of Zhejiang

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University (Hangzhou, China). OS-65 is an aggressive primary human osteosarcoma (stage-III) with lung metastasis. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (both purchased from Sigma-Aldrich, St. Louis, MO, USA) with antibiotics (GE Healthcare Life Sciences, Logan, UT, USA) and maintained in T-75 flasks (Corning Inc., Corning, NY, USA) at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air. Upon 90% confluency, cells were removed from the culture flask using Trypsin-EDTA (0.25%-53 mM EDTA; Sigma-Aldrich), washed and suspended in 10% DMEM. A cell count was conducted using a hemocytometer (Sigma-Aldrich).

FACS analysis

Experimental groups. Cells were equally divided into two groups: Control group, cells + Hoechst 33342 dye (Sigma-Aldrich) (n=9); and drug-treated group, cells + verapamil drug + Hoechst 33342 dye (n=9). Each sample had ~1x10^6 cells. A total of ~10^6 cells/ml in 10% DMEM were labeled with either 5 μl/ml Hoechst 33342 dye alone or in combination with 0.8 μl/ml verapamil (Sigma-Aldrich), as outlined. Cells were resuspended in 500 μl Hank's balanced salt solution (HBSS; Sigma-Aldrich) containing 10 mM HEPES for FACS analysis. These cells were then incubated at 37˚C for 90 min, centrifuged for 5 min at 300 x g (4˚C) and resuspended in ice-cold HBSS. Then, 2 μg/ml propidium iodide (PI; Sigma-Aldrich) was added to identify dead cells. The cells were then filtered through a 40 μm cell strainer (BD Falcon; BD Pharmingen, San Diego, CA, USA) to obtain single suspension cells. Dual-wavelength analysis and purification were performed using dual-laser cytometry (FACSVantage; BD Biosciences, Franklin Lakes, NJ). A 610 nm dichroic mirror short-pass was used to separate the emission wavelengths. PI-positive dead cells were excluded from the analysis.

Sarcosphere formation assay. Sarcosphere formation assay was performed, according the protocol described by Gibbs *et al* (7). Cells were plated at a density of 60,000 cells/well in ultra-low attachment six-well plates (Corning, Inc.) containing serum-free DMEM/F12 medium supplemented with N_2, 10 ng/ml epidermal growth factor and 10 ng/ml human basic fibroblast growth factor (both purchased from Sigma-Aldrich). The culture was analyzed for sphere formation daily for 7 days, and cell proliferation was measured by checking the absorbance at 450 nm using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following 7 days of culturing, the total number of sarcospheres generated by FACS-sorted SP and non-SP cells was quantified by inverted phase contrast microscopy (Eclipse TS100; Nikon Corporation, Tokyo, Japan).

Immunofluorescent staining. FACS-sorted SP and non-SP cells from the OS-55 cell line were fixed in BD Cytofix solution (BD Biosciences) and incubated for 20 min at 4˚C. Following blocking in donkey serum (Sigma-Aldrich) for 20 min, cells were incubated with goat anti-Oct3/4A polyclonal primary antibody (1:100; sc-8628; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4˚C, and were subsequently incubated with rhodamine red-conjugated donkey anti-goat antibody (1:200; 705-295-003; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). For CD44 and Nanog immunofluorescence analysis, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human Nanog (1:5; 674206; BioLegend, San Diego CA, USA) or CD44 (1:5; 8011-0441; eBioscience, Inc., San Diego, CA, USA) antibodies. Human embryonic stem cells were used as the positive control. Cells were observed under a confocal microscope (LSM 700; Zeiss AG, Oberkochen, Germany). Images were captured and processed by Adobe Photoshop CS4 (Microsoft Corporation, Redmond, WA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from SP and non-SP cells was extracted using TRIzol reagent and treated with RNAase-Free DNase according to the manufacturer's instructions (both purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples were then reverse transcribed using a First-Strand cDNA Synthesis Kit containing Oligo$(dT)_{12-18}$ primers (Fermentas; Thermo Fisher Scientific, Inc.) and 1.5 μg total RNA, according to the manufacturer's instructions. RT-qPCR analysis was subsequently performed using IQ Supermix with SYBR-Green (Bio-Rad Laboratories, Inc.) and a 20 μl reaction volume containing 300 nM forward and reverse primers and 50 ng cDNA template. The thermo cycling conditions were as follows: Initial denaturation and enzyme activation at 95˚C for 2 min, 37 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 50 sec and extension at 72˚C for 30 sec, using instrument default settings for melt curve analyses. Sequences of the human specific primers (Sigma-Aldrich) were as follows: ABCG2 forward, TCAATC AAAGTGCTTCTTTTTTATG and reverse, TTGTGGAAG AATCACGTGGC; ABCB5 forward, CACAAGTTGGAC TGAAAGGA and reverse, ACCACTAGGCATGTCCTT CC; MDR1 forward, ACAGGAAGAGATTGTGAGGG and reverse, TATCCAGAGCTGACGTGGCT; Oct3/4A forward, TGGAGAAGGAGAAGCTGGAGCAAAA and reverse, GGCAGATGGTCGTTTGGCTGAATAGACC; Sox2 forward, CACACTGCCCCTCTCACACAT and reverse, CAT TTCCCTCGTTTTTCTTTGAA; Nanog forward, TCCTCC TCTTCCTCTATACTAAC and reverse, CCCACAAATCAC AGGCATAG; actin, forward GCGGGAAATCGTGCGTGA CATT and reverse, GGCAGATGGTCGTTTGGCTGAATA; Bcl-2 forward, ACACTGTTAAGCATGTGCCG and reverse, CCAGCTCATCTCACCTCACA (13). PCR products were electrophoresed on 1.2% agarose gel and stained with ethidium bromide (both purchased from Sigma-Aldrich). Results were analyzed using CFX Manager Software (version 3.0; Bio-Rad Laboratories, Inc.).

Cell resistance assay. A total of ~1x10^3 cells/plate were cultured in 96-well plates and treated with the following chemotherapeutic agents: 10 μg/ml 5-fluorouracil, 250 mM gemcitabine, 30 ng/ml paclitaxel, 5 mg/ml cisplatin, 10 mg/ml etoposide and 2 μg/ml 100 oxaliplatin (all purchased from Sigma-Aldrich). Mean optical density (OD) was obtained at OD_{450} and represented as a graph. Cell resistance in SP and non-SP cells was calculated using the following formula: Cell resistance rate (%) = (Experimental group OD_{450}/control group OD_{450}) x 100. Values presented in the graph are the mean of three independent experiments.

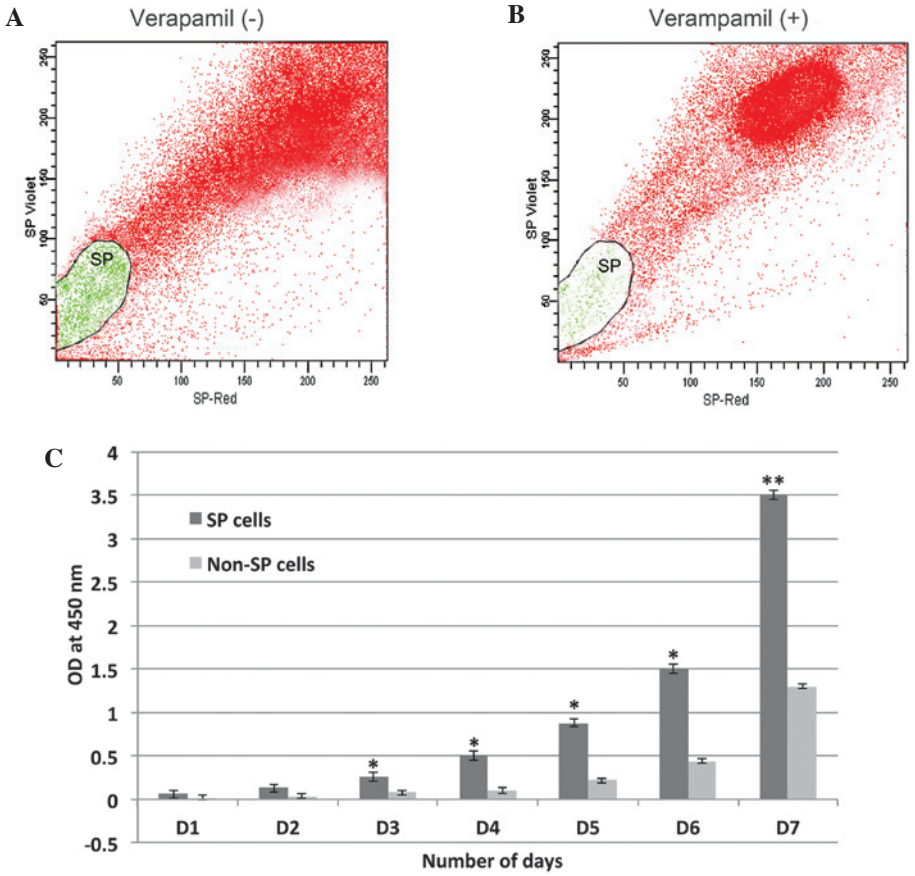

Figure 1. Identification and characterization of SP cells from an OS-65 osteosarcoma cell line. (A) Representative images of dot plot analysis by FACS demonstrating the presence of 3.3% SP cells. (B) SP cells were reduced to 0.3% upon treatment with verapamil. (C) Cell proliferation rate of SP cells was significantly increased after 3 days, as compared with non-SP cells and SP cells were most confluent on day 7. Data are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. non-SP cells. SP, side population; FACS, fluorescence-activated cell sorting; OD, optical density; D, day.

Statistical analysis. One-way analysis of variance and Student's t-test was performed to determine significant differences between the treatment and control groups. Microsoft Excel (version 14.6.1; Microsoft Corporation, Redmond, WA, USA) was used to perform statistical analyses. P≤0.05 was considered to indicate a statistically significant difference.

Results

Purification and characterization of osteosarcoma SP cells. Osteosarcoma samples were examined for the presence of cancer stem-like SP cells using a FACS-based Hoechst 33342 dye exclusion assay. Osteosarcoma samples contained ~3.3% SP cells, and were reduced to 0.3% upon treatment with verapamil, which is an inhibitor of ABC transporters (Fig. 1 and B). Subsequently, the FACS-purified osteosarcoma SP and non-SP cells were subjected to an *in vitro* proliferation assay. Osteosarcoma SP cells exhibited significantly increased cell proliferation from day 3, as compared with the non-SP cells (P<0.05), and were most confluent on day 7, as compared with the non-SP cells (P<0.01; Fig. 1C). In addition, a sarcospheres formation assay was performed in order to determine the self-renewal properties of the osteosarcoma SP cells. SP cells generated significantly more sarcospheres, as compared with the non-SP cells (P<0.01; Fig. 2A). Furthermore, SP cells exhibited significantly increased mRNA expression of stem cell surface genes, including Sox2, Nanog and Oct-3/4A, which are responsible for the maintenance of self-renewal of SP cells (Fig. 2B). As detected by immunofluorescence, SP cells were positive for CD44, Nanog and Oct-4 protein expression, whereas non-SP cells were not (Fig. 3). These results confirmed the presence of a small population of SP cells in the OS-65 osteosarcoma cell line, as demonstrated by their active role in dye exclusion via ABC transporters and their high potency of self-renewal.

Osteosarcoma SP cells are multi-drug and apoptosis resistant. FACS-sorted SP and non-SP cells were subjected to drug resistance and cell death assays. As shown in Fig. 4A, the SP cells were highly resistant to treatment with etoposide, gemcitabine, 5-fluorouracil, cisplatin, paclitaxel and oxaliplatin and their survival rate (>80%) was significantly increased, as compared with non-SP cells (<30%; P<0.01). Consequently, the number of SP cells that underwent apoptosis was significantly decreased, as compared with the non-SP cells (P<0.01; Fig. 4B). Subsequently, in order to address the cause of the high multi-drug and apoptosis resistance exhibited by the SP cells, RT-qPCR was performed to evaluate the transcriptional regulation of various ABC transporters and anti-apoptotic genes. The relative mRNA expression levels of ABCG2, MDR1, ABCB5 and Bcl-2 were significantly upregulated in the SP cells, as compared with

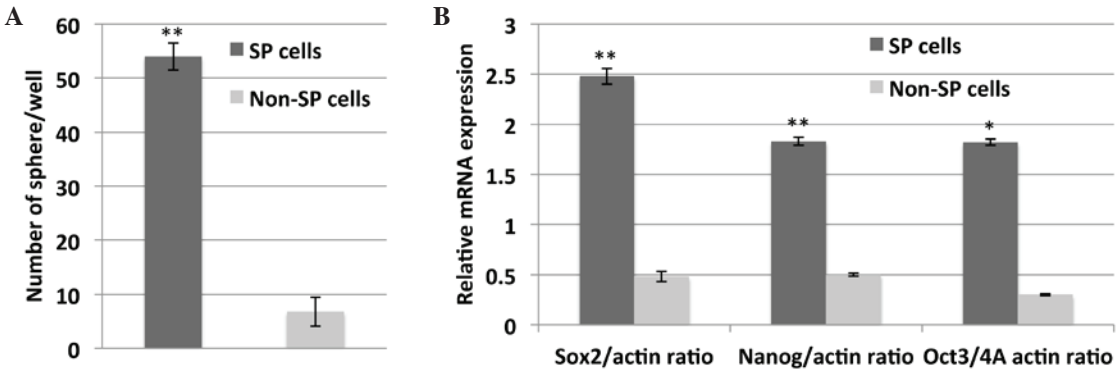

Figure 2. Osteosarcoma SP cells are highly self-renewal. (A) Quantification of clonogenic formation efficiency, demonstrating that SP cells from the OS-65 cell line generated a significantly increased number of sarcospheres, as compared with non-SP cells. (B) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that the relative mRNA expression levels of stemness genes were significantly increased in SP, as compared with non-SP cells. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 vs. non-SP cells. SP, side population.

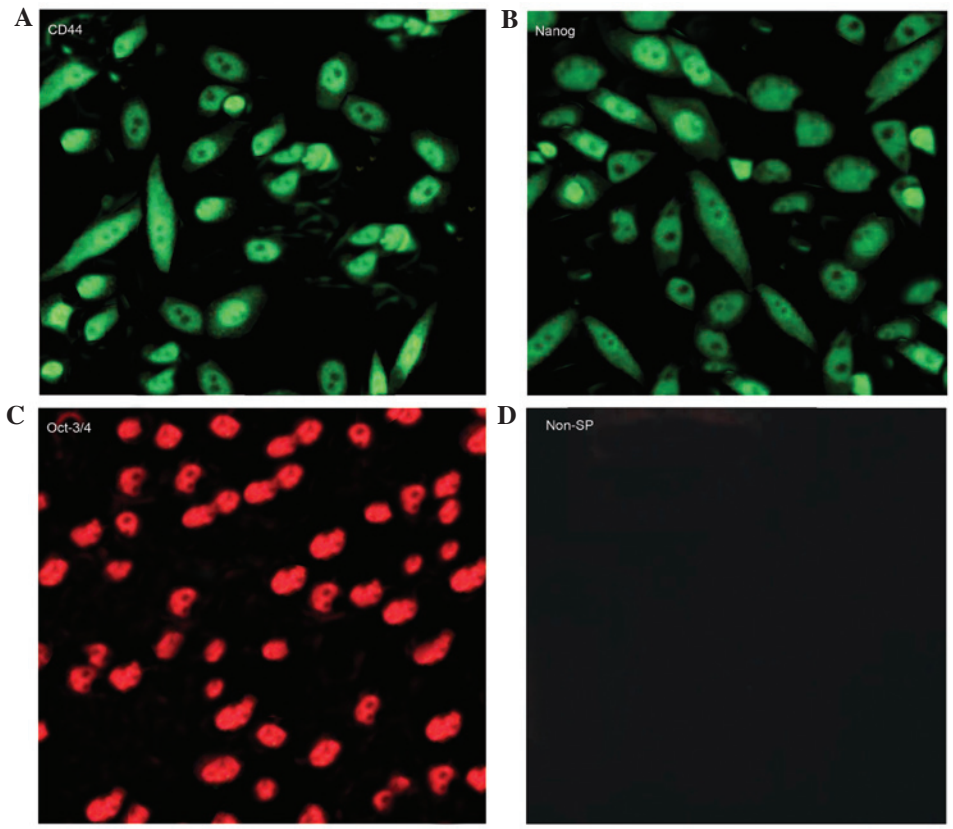

Figure 3. Representative images of immunofluorescence analysis for (A) CD44, (B) Nanog and (C) Oct-3/4A protein expression in human osteosarcoma cell line OS-65 SP cells and (D) non-SP cells. SP, side population.

the non-SP cells (P<0.01; Fig. 5). These results suggested that enhanced drug resistance and downregulated apoptosis in SP cells may be responsible for chemotherapy failure and tumor recurrence.

Discussion

Cancers are heterogeneous and the presence of small populations of CSCs are responsible for initiating the development of tumors. CSCs are characterized by the overexpression of stem cell surface proteins and ABC transporter proteins, which are associated with tumor metastasis and invasion, and multi-drug resistance, respectively (12,14,15). Therefore, elucidating the molecular mechanisms underlying CSC-mediated tumorigenesis is crucial for the development of novel anti-cancer drugs, in order to provide the effective treatment. In order to investigate these mechanisms, it is essential to elucidate the factors and signaling pathways involved in CSC-mediated multi-drug resistance and cancer relapse. The present study demonstrated that the OS-65 human osteosarcoma cell line contains 3.3% cancer stem cell-like SP cells, which were isolated using the FACS-based Hoechst 33342 dye exclusion method. Furthermore, the results of the present study demonstrated that these osteosarcoma SP

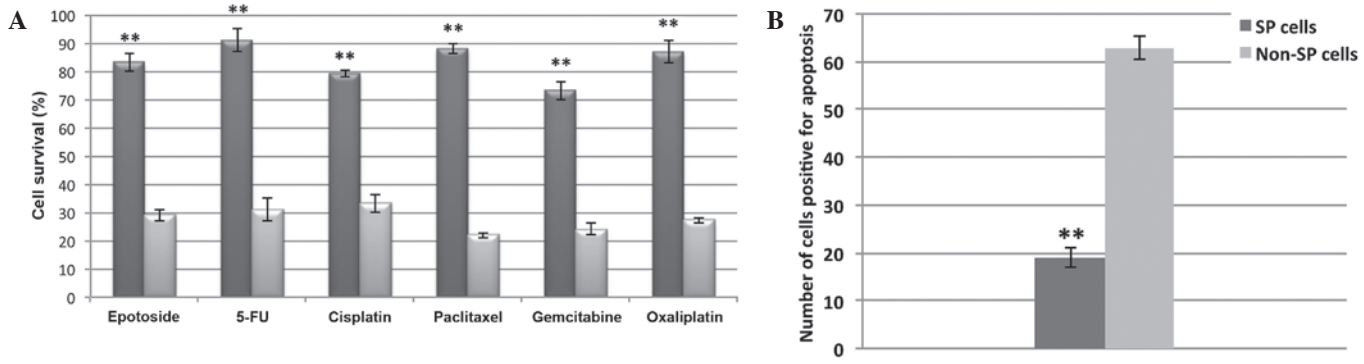

Figure 4. Osteosarcoma OS-65 SP cells are multi-drug and apoptosis resistant. (A) Cell survival rates of SP and non-SP cells following treatment with DNA targeting drugs, including etoposide, 5-FU, cisplatin, paclitaxel, gemcitabine and oxaliplatin. (B) Number of SP and non-SP cells which underwent apoptosis. Data are presented as the mean ± standard deviation. **P<0.01 vs. the non-SP cells. SP, side population; 5-FU, 5-flurouracil.

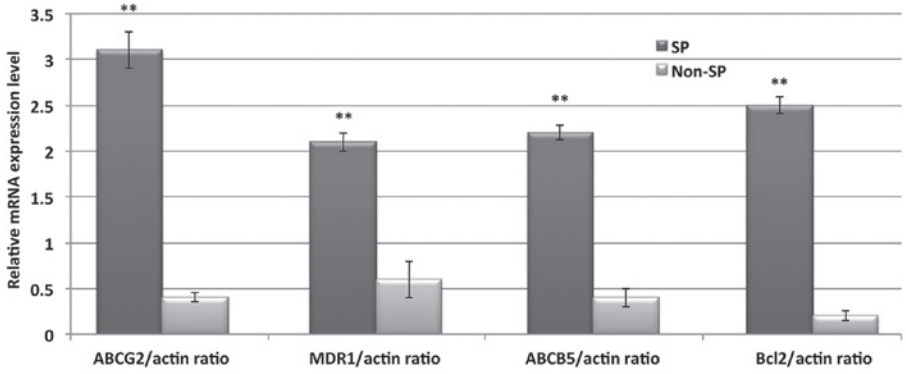

Figure 5. Elevated mRNA expression of ABC transporters and Bcl-2 in osteosarcoma OS-65 SP cells. (A) Quantification of reverse transcription-polymerase chain reaction analysis demonstrated that the relative mRNA expression levels of ABCG2, MDR1, ABCB5 and Bcl-2 were significantly increased in osteosarcoma SP cells. Data are presented as the mean ± standard deviation. **P<0.01 vs. the non-SP cells. SP, side population; ABC, ATPase binding cassette; Bcl, B cell lymphoma.

cells are highly efficient in generating tumor sarcospheres, as they exhibit elevated expression of Oct3/4A, CD44 and Nanog stem cell surface proteins, which have been reported as essential for the maintenance of self-renewal of CSCs during tumorigenesis and tumor invasion (3,15-17). In line with these findings, it has previously been demonstrated that SP cells from the OS99-1 and MG-63 osteosarcoma cell lines exhibit relatively increased mRNA transcriptional levels and are highly capable of generating more sarcospheres (18). Hence, the expression of these stem cell proteins in SP cells may be used to validate the metastatic stage of cancer.

SP cells are also resistant to chemotherapeutic agents and apoptosis. A previous study demonstrated that these effects are induced by the overexpression of multi-drug resistance transporter proteins and anti-apoptotic factors (13). Furthermore, the overexpression of stem cell surface proteoglycans and glycoproteins, including CD44, Oct3/4A and Nanog, may also contribute to tumor metastasis and invasion (19-22). Consistent with these previous findings, the results of the present study demonstrated that OS-65 SP cells exhibited significantly increased expression levels of CD44, Oct3/4A and Nanog, which may be essential for the maintenance of self-renewal and metastasis of osteosarcoma SP cells (3,15-17). In addition, the present study also demonstrated that SP cells possess differential expression of ABCG2, ABCB5 and MDR1 transporter proteins, which are predominantly upregulated, which may explain the resistance of SP cells to chemotherapeutic agents. Similarly, the anti-apoptotic factor, Bcl-2, was significantly downregulated, suggesting that Bcl-2 acts as a survival factor for SP cells. However, the functional association between stem cells surface proteins, anti-apoptotic factors and ABC transporter proteins requires further study.

In conclusion, the results of the present study suggested that presence of cancer stem-like SP cells may be a predominant cause of chemotherapy failure and tumor recurrence. These effects are likely to be due to the overexpression of ABC transporters and anti-apoptotic factors, as these increase the resistance of osteosarcoma SP cells against chemotherapeutic agents and apoptosis. Therefore, designing novel anti-cancer drugs, which suppress the downstream signaling pathways involved in drug and apoptosis resistance may effectively reduce tumor relapse in patients with osteosarcoma.

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