

# Side population cells and drug resistance in breast cancer

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**Abstract.** Several studies have demonstrated that the isolated side population (SP) cells from solid tumors exhibit cancer stem cell-like properties, and are responsible for drug resistance during chemotherapy and tumor recurrence. In the current study, cancer stem cell-like SP cells were isolated in the MDU-22 breast cancer cell line using the Hoechst-33342 dye exclusion technique. It was observed that SP cells accounted for 3.8% of cells in the MDU-22 cell line, which was reduced to 0.6% in the presence of verapamil, an inhibitor of the ABC transporter. The sorted SP cells showed an elevated expression of stem cell markers, including *ABCG2*, *OCT-4* and *EpCAM*. Furthermore, it was demonstrated that the isolated SP cells undergo rapid proliferation and have a high survival rate. These results indicate that the coexpression of adenosine triphosphatase binding cassette transporters and stem cell surface markers in SP cells may contribute to chemoresistance, tumor recurrence, metastasis and invasion. Therefore, the isolation and characterization of SP cells may provide novel insights for the development of alternative therapeutic agents to target cancer stem cells.

## Introduction

Breast cancer is one of the most common types of cancer, accounting for 13.7% of cancer-related mortalities in 2008 according to the World Cancer Research cancer report (1). Several factors have a key role in breast carcinogenesis, including ovarian steroids, estrogen and progesterone. Breast tumors are usually screened for hormone receptors, for example estrogen receptor (ER), progesterone receptor (PR) and overexpression of HER2, and based on the expression levels, patients are directed to endocrine or chemotherapy. It

has been determined that the failure of cancer treatment is due to the persistence of cancer stem cells (CSCs) that evade the treatment regimen and are responsible for minimal residual disease (2). These cells have been found to possess characteristics usually associated with stem cells, such as self-renewal, and exhibit a high *in vivo* tumorigenicity, differentiation potential multi-drug and apoptosis resistance (3). Cells that exclude Hoechst 33342 dye are referred to as side population (SP) cells. These cells share characteristics with CSCs; specifically they are enriched for tumor initiating capacity, express stem-like genes and are resistant to chemotherapeutic drugs. Furthermore, SP cells also overexpress the adenosine triphosphatase binding cassette (ABC) transporters ABCB1 [multi-drug resistance transporter 1 (MDR1)], ABCC1 and ABCG2 (BCRP1), which contribute to multi-drug resistance and express stem cell surface markers. SP cells have been identified in several solid tumors and cancer cell lines based on Hoechst 33342 dye efflux by fluorescence activated cell sorting (FACS) (4-6). SP cells have been isolated from malignant and non-malignant tissues and they have been shown to exhibit stem cell characteristics and drug resistance (7-9). It has been reported that the presence of SP cells in mammary gland tissue of breast cancer accounts for 2.0-3.0% of total epithelial cells in mice (8) and 2.0-5.0% in humans (10). These SP cells have overexpression of ABC transporters, including ABCG2 and ABCB1 (MDR1) (11), and a high expression level of stem cell surface markers, which contribute to multi-drug resistance, high proliferation and survival rate, respectively (7). Hence, the sorting and characterization of SP cells would assist in elucidating the mechanisms of oncogenesis and drug resistance (8) and aid in the design of novel therapeutic strategies that may selectively target CSCs. Consequently, the present study is designed to isolate SP cells in the MDU-22 breast cancer cell line and analyze their expression levels of ABC transporters and stem cell surface markers.

## Materials and methods

**Cell line and cell culture.** Samples from breast cancer tissues were obtained from a range of patients at the time of surgery. Written informed consent was obtained from all participants. All the patients were recruited from the Jiangxi Provincial Tumor Hospital (Jiangxi, China). Ethical approval was obtained from the Jiangxi Provincial Tumor Hospital. The patients details were as follows: Age range, 33-43 years; region, ductal mammary gland; grade, 4 (recurrent type). The tumor samples were washed immediately and tissues were

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broken down using collagenase (Sigma-Aldrich, Shanghai, China). The cells were continuously cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) and a cell line, MDU-22, was established. The cell line was cultured in DMEM with 10% fetal bovine serum (Life Technologies, Shanghai, China), supplemented with antibiotics (penicillin and streptomycin; Sigma-Aldrich) and maintained in T-75 flasks at 37°C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere. Once confluent, cells were removed from the culture flask using Trypsin-EDTA (0.25%, 53 mM EDTA) washed, cells suspended in 10% DMEM and centrifuged at 6,200 x g for 6 min. Cells were resuspended in 10% DMEM. Cell count was measured using a hemocytometer (Bio-Rad, Inc., Shanghai, China).

**Labeling with Hoechst 33342.** All reagents mentioned in this paragraph were obtained from Sigma-Aldrich, with the exception of those already specified. The cells were divided into two groups, control cells + Hoechst 33342 (n=4) and verapamil treated cells + verapamil + Hoechst 33342 (n=4). Cells in staining medium (~10<sup>6</sup> cells/ml of 10% DMEM) were labeled with Hoechst 33342 stock (Sigma-Aldrich) and bis-benzimide (5 µl/ml), either with dye alone or in combination with verapamil (0.8 µl/ml). The cells were mixed and placed in water bath at 37°C for exactly 90 min. Subsequently, the cells were spun down (2,000 rpm for 10 min at 4°C) and resuspended in 500 µl of Hank's buffered salt solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Finally, the cells were counter stained with propidium iodide (PI; 2 µg/ml sample) at 4°C. The cells were filtered through a 50 µm nylon mesh (BD Biosciences, Franklin Lakes, NJ, USA) into labeled FACS tubes in order to remove cell clumps. Separate tubes with fresh medium (10% DMEM) were kept for sterile sorting of SP cells and main population cells. The cells were sorted using a FACSAria II flow cytometer (BD Biosciences, Mountain View, CA, USA). A wavelength of 355 nm was used to excite the Hoechst 33342 dye, and its dual-wavelength fluorescence was analyzed (blue, 450 nm; red, 675 nm).

**In vitro proliferation activity assay.** The sorted SP and non-SP cells were seeded into a 96-well plate at a density of 2x10<sup>6</sup> cells/well and cultured in a CO<sub>2</sub> incubator. Each group was set up in triplicate. Cell proliferation activity was measured every day for 7 days. Each well was supplemented with CCK-8 solution (10 µl) and incubated in CO<sub>2</sub> incubator for 2-3 h. The optical density (OD) was determined at a wavelength of 450 nm. These data were used to calculate cell growth graphs based on the mean value of OD<sub>450</sub> and standard deviation values for each well.

**Cell resistance assay.** The obtained SP and non-SP cells were cultured in 96-well plates at a concentration of 1x10<sup>3</sup> cells/plate. After 24 h, 5-fluorouracil (5-FU) was added to all cultures to a final concentration of 10 µg/ml. The plates were placed in a hatch box for 48 h. Each well was supplemented with CCK-8 (10 µl) solution and the plates were incubated for 3 h. The mean value of OD<sub>450</sub> obtained was represented as a graph. Cell resistance in the two groups was calculated using the following formula: Cell resistance (%) = (experimental group OD<sub>450</sub> value/control group OD<sub>450</sub> value) x 100.

**Immunocytochemistry.** The sorted SP cells and non-SP cells were seeded into 35 mm culture plates (~100 µl), maintained in an incubator for 3 h and supplemented with 1 ml DMEM (10%). Following overnight incubation, the cells were rinsed with phosphate-buffered saline (PBS; Life Technologies) and fixed in 4% paraformaldehyde (Life Technologies) in 1X PBS, for 5 min at 4°C. After washing with 1X PBS, cells were blocked with 1% bovine serum albumin in Tris-buffered saline (BSA-TBS; Life Technologies) with RNase (10 µl per 1,000 µl of 3% BSA-TBS; Life Technologies). Following a 1-h incubation at room temperature the cells were rinsed with PBS, and FAK primary mouse polyclonal antibody (Life Technologies) in 1% BSA-TBS was added (dilution, 1:100; 2/200 µl) prior to incubation overnight at 4°C. Once washed with 1X PBS, the cells were incubated with Rabbit Anti-Mouse IgG (H+L) Superclonal™ Secondary antibody (dilution, 1:100 in 1% BSA-TBS), at room temperature for 1 h. The cells underwent a further PBS wash and PI was added (1/200 µl of PBS). The cells were viewed under a confocal laser scanning microscope (Leica TCS; Leica Microsystems, Wetzlar, Germany). Image analysis and figures were prepared using Adobe Photoshop CS4 (Adobe Systems, Inc., San Jose, CA, USA).

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** PCR was carried out as previously described (13). Total RNA was isolated from the SP and non-SP cells using the Ambion RNAqueous® Micro kit (Applied Biosystems, Warrington, UK). cDNA was synthesized using the Bioline cDNA synthesis kit (Bioline, London, UK). qPCR was performed using 2-3 µl cDNA and 2X TaqMan Gene Expression Master mix (Applied Biosystems) in 30 µl reaction volumes. GAPDH was used as a reference. The primer sequences were as follows (14): forward, 5'-AGCTGCAAGGAAAGATCCAA-3' and reverse, 5'-TCCAGACACACCACGGATAA-3' for ABCG2; forward, 5'-ATCCTGGGGGTTCTATTTGG-3' and reverse, 5'-CTCCAGGTTGCCTCTCACTC-3' for OCT-4; forward, 5'-CTGCCAAATGTTTGGTGATG-3' and reverse, 5'-ACGCGTTGTGATCTCCTTCT-3', for epithelial cell adhesion molecule (EpCAM); and forward, 5'-ATGTCGTGGAGTCTACTGGC-3' and reverse, 5'-TGACCTTGCCCA CAGCCTTG-3' for GAPDH. The amplified products were separated by electrophoresis on ethidium bromide-stained 1.2% agarose gels. Band intensity was measured by ImageJ (National Institutes of Health, Bethesda, MD, USA) from two independent experiments.

**Statistical analysis.** A one-way analysis of variance test was performed to determine any differences between the treatment groups. Student's t-tests were performed to compare the effect of different treatments between the SP and non-SP populations. \*P<0.05 and \*\*P<0.01 were considered to indicate a statistically significant difference.

## Results

**Analysis of SP Cells FACS using Hoechst 33342.** The live cell population (P1 gated region) was selected against PI as the P1 gated population. PI is used to exclude the dead

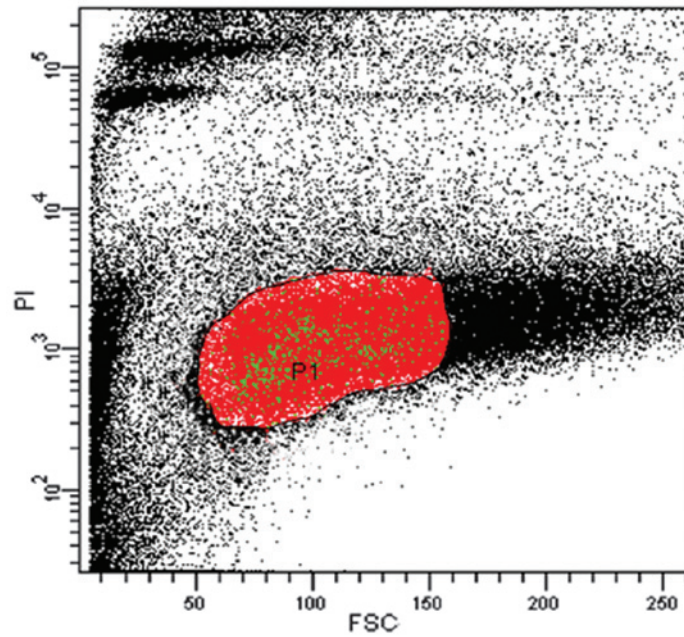


Figure 1. A dot blot analysis by fluorescence activated cell sorter (FACS) clearly showing the live cells in the P1 gated population. Cells were stained with propidium iodide (PI) to exclude the dead cells.

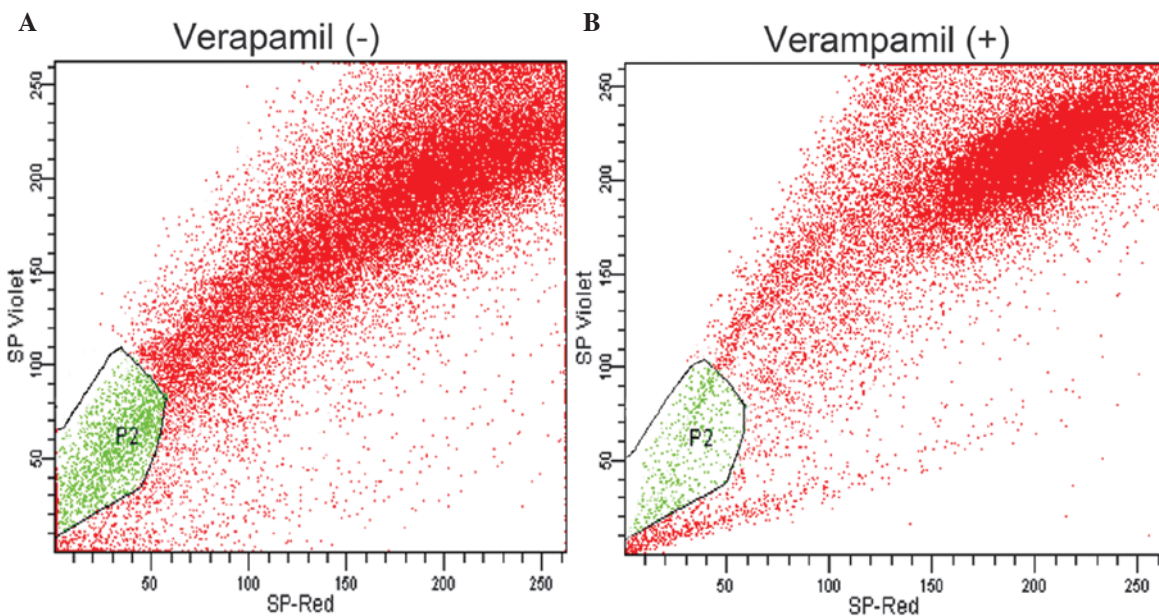


Figure 2. Analysis of side population (SP) cells in the breast cancer cell line. (A) MDU-22 cells were stained using Hoechst 33342 dye and analyzed using flow cytometry. SP cells are outlined (P2-gated population) and also shown as a percentage (3.8) of the total cell population. (B) Percentage of SP cells was significantly reduced (0.6) in the presence of verapamil.

cells from the sample (Fig. 1). SP cells were sorted out from the gated live cell population (P1) using Hoechst 33342, which is a DNA binding dye. Hoechst 33342 emits light at wavelengths of ~450 nm (SP-Violet) and 675 nm (SP-Red) following UV excitation, and a set of cells were observed that displayed low blue and red fluorescence. This distinct cell population (P2) found towards the SP-violet region of the dot plot of the FACS profile are the so-called SP cells (Fig. 2A). The exclusion of Hoechst 33342 by SP cells is an active process involving MDR1, a member of the ABC transporter transmembrane proteins (Fig. 2A). The number

of cells collected was  $\sim 62 \times 10^3$ , which is  $\sim 56\%$  of the initial cell count. Of the  $62 \times 10^3$  cells (P1) analyzed, Hoechst dye was effluxed by 3.8% of cells in the P2 gated region (Fig. 2A). Verapamil-treated cells of the same cell line were sorted out with the same Hoechst 33342 efflux. The resulting P1 cells obtained were  $\sim 48,800$  in number, which was  $\sim 44\%$  of the initial cell count. Following treatment with verapamil, the percentage of SP cells (P2 gated) was reduced from 3.8 to 0.6% (Fig 2B). Verapamil is a MDR1 transporter protein inhibitor, which blocks the drug efflux action by the cells. Hence, the sorted SP cells are highly resistant to drug uptake,

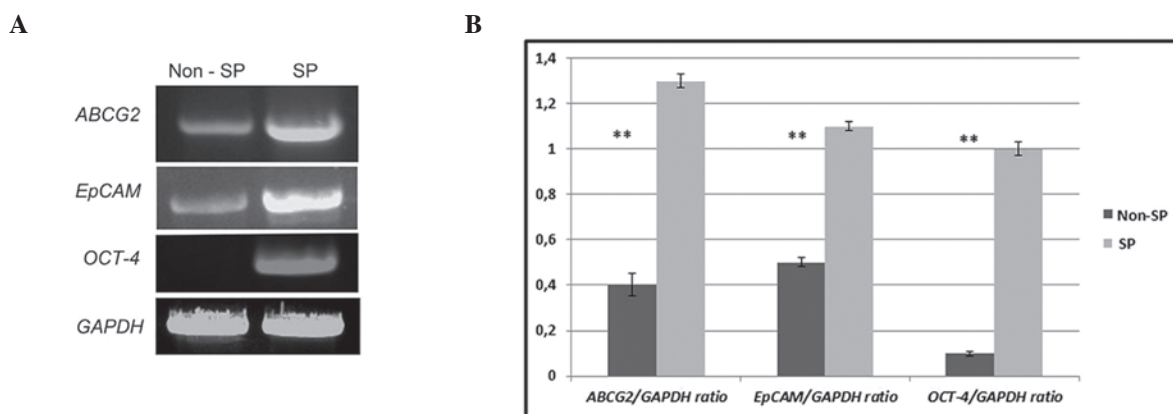


Figure 3. Expression of stem cell markers in side population (SP) and non-SP cells. (A) Elevated expressions of ABCG2, OCT-4 and EpCAM genes in derived MDU-22 were detected by reverse transcription-quantitative polymerase chain reaction. (B) Quantification graph represents the data of two separate independent experiments. The bars represent standard deviation. \*\* $P < 0.01$  compared with parental and non-SP cells, respectively.

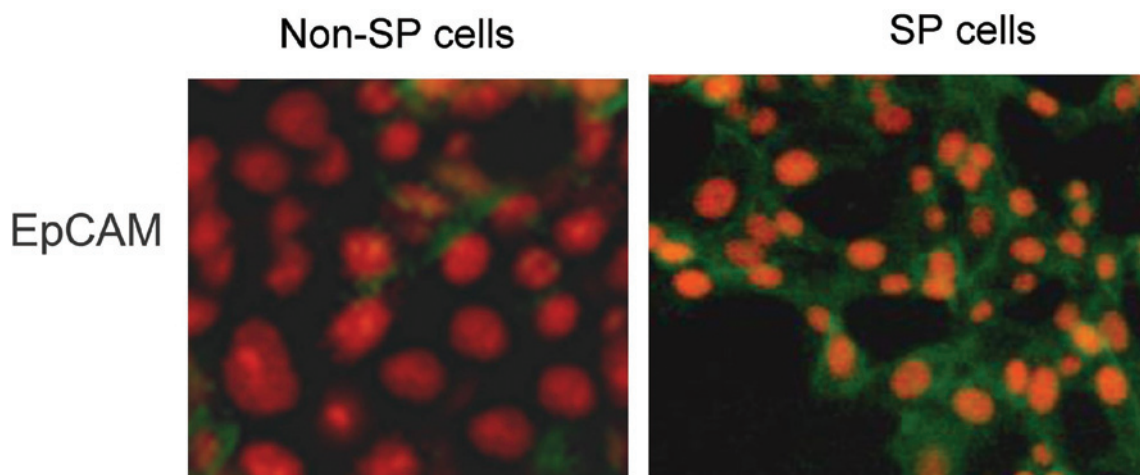


Figure 4. Fluorescence microscopic analysis of cell surface markers. The fluorescence intensity of EpCAM is highly enriched in side population (SP) cells whereas this fluorescence intensity is reduced in non-SP.

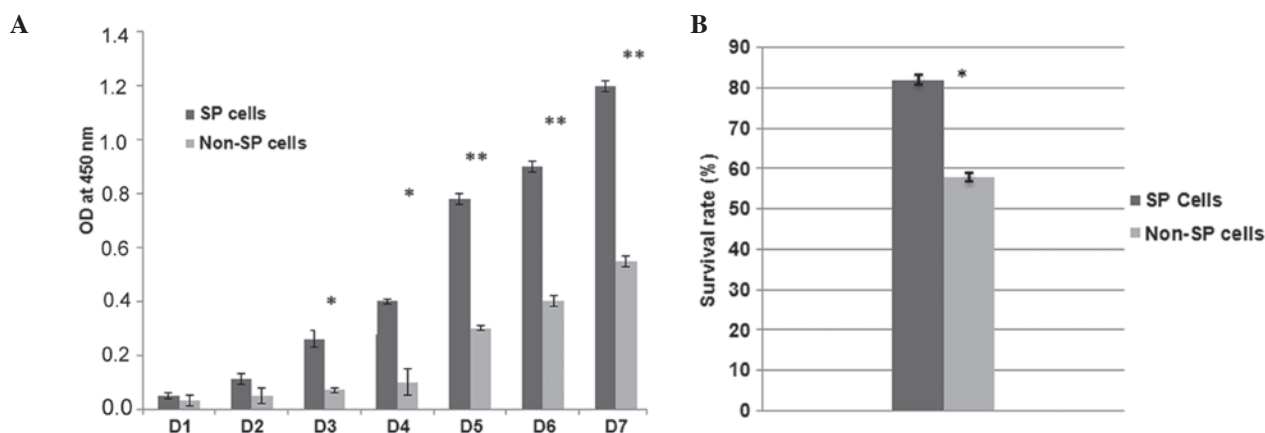


Figure 5. (A) Cell proliferation rate for side population (SP) and non-SP cells. The x-axis represents time, while the y-axis indicates the corresponding optical density (OD) value at 450 nm with \* $P < 0.05$ ; \*\* $P < 0.01$ . (B) Resistance of SP cells and non-SP cells to 5-fluorouracil (5-FU). The y-axis shows the survival rate of the two groups following treatment with 10  $\mu\text{g/ml}$  5-FU for 3 h.

which may be due to the over expression of ABC transporters. Therefore, these cells were further analyzed for the expression of ABC transporters and stem cell surface markers.

*Analysis of ABCG2 expression and stem cell surface markers in SP cells.* It has been previously reported that ABCG2 is expressed by malignant breast tissue, and it was observed that

ABCG2 expression increases with higher tumor grades (15,6). Therefore, to examine and compare the expression level of the *ABCB2* gene and other stem cell genes, including *OCT-4* and *EpCAM*, between SP and non-SP cells, the extracted RNA was analyzed using RT-qPCR. The *ABCB2*, *EpCAM* and *OCT-4* levels were higher in SP cells compared to non-SP cells (Fig. 3A). The quantification graph clearly shows that the levels of these genes in SP cells are significantly higher (Fig. 3B). GAPDH was used as a reference gene. Notably, fluorescence microscopic analysis revealed that, compared with the non-SP cells, the SP cells displayed a greater positive expression of cell surface proteins such as EpCAM (Fig. 4) which further confirms the RT-qPCR results. These results suggest that a high expression level of *ABCG2* and *OCT-4* in SP cells may act as a crucial factor in drug resistance and the massive proliferation of cancer cells.

**Characterization of sorted SP cells.** FACS sorted SP and non-SP cells were further subjected to *in vitro* cell proliferation and cell survival assays. The isolated breast carcinoma SP cells underwent rapid cell proliferation starting from the third day and becoming more confluent on the eighth day (data not shown) (Fig. 5A). However, the growth rates of the non-SP cells were significantly lower when compared with those of the SP cells (Fig. 5A). The measured growth rate of SP cells at 450 nm was significantly higher than non-SP cells (\* $P < 0.05$ ; \*\* $P < 0.01$ ). Following the *in vitro* proliferation assay, the SP cells were further subjected to a drug resistance assay. The survival rate of the SP cells (83%) following exposure to 10  $\mu\text{g/ml}$  5-FU were significantly higher (Fig. 5B) compared with that of the non-SP cells (58%). Hence these data suggest that the sorted SP cells have high proliferation rate and an increased resistance to chemotherapeutic agent.

## Discussion

CSCs have the ability to resist chemotherapeutic agents via increased expression levels of efflux pumps, and they have an enhanced DNA repair activity. The theory of cancer stem cells hypothesizes that treatment failure and minimal residual disease are caused by the presence of small population of CSCs (7,8), which are responsible for tumor growth, metastasis and tumor relapse. Hence the eradication of CSCs is an important goal for providing effective cancer treatment and long term disease-free survival.

SP cells have been observed in several types of solid tumors as well as a number of breast cancer cell lines (7,9). SP cells were first identified in primary human breast cancer cells, and they demonstrated the expression of *HER2* signaling which is involved in drug efflux via ABC transporters (16). In the present study, we demonstrated that SP cells was identified and isolated from breast carcinoma cell line (MDU-22). SP cells were sorted out based on Hoechst 33342 dye exclusion by FACS. The exclusion of Hoechst 33342 by SP cells is due to highly induced *ABCG2* (MDR1), a member of the ABC transporter transmembrane proteins that are involved in multi-drug resistance (Fig. 2A). The results of the current study demonstrated that, following treatment with verapamil (a MDR1 protein inhibitor), the percentage of SP cells was reduced from 3.8 to 0.6%

(Fig. 2B). This is a confirmatory test for the presence of MDR1 protein in MDU-22 cells. Furthermore, the results of the RT-qPCR showed that the expression levels of genes such as *ABCG2*, *OCT-4* and *EpCAM* are significantly higher in SP cells than those in the non-SP population (Fig. 3). A previous study has shown that the *ABCG2* protein is over expressed in the MCF-7 cell line, and that these cells were more drug resistant compared with the MCF-7 non-SP cells (6). Therefore, the results of the current study fit well with the recent study by Britton *et al* (6), 2012 and with other previous studies (9,17) where a differential increase in the expression levels of mRNA encoding *ABCG2* was observed in the SP population when compared with that of the non-SP cells. Similarly, the *OCT-4* gene, a member of the POU family of transcription factors, was shown to be involved in the proliferation potential (18) and survival of CSCs partly through the *OCT-4/Tcl1/Akt1* pathway (19). Hence the results of the current study indicate that the overexpression of *ABCG2* may act as a suitable marker for the identification of SP cells in breast cancers. Subsequently, the results revealed that SP cells displayed a greater positive expression of cell surface markers such as epithelial cell adhesion molecule (EpCAM), a CSC marker (Fig. 4). In line with the present results, CD44<sup>+</sup> cells in the head and neck squamous cell carcinomas of mice were proven to be enriched with tumorigenic CSCs able to propagate tumor formation, whereas CD44<sup>-</sup> cells were not able to form tumors (5).

Furthermore, the *in vitro* proliferation and drug resistance assays revealed that MDU-22 SP cells have an increased proliferation capacity and are highly resistant to 5-FU, hence they have increased survival rate compared with that of the non-SP cells, which are very sensitive (Fig. 4). It has been previously reported that MDA-MB-231 cell lines have a significantly increased survival rate when treated with doxorubicin, methotrexate and 5-FU, compared with untreated cells (20). Taken together, the results of the present study indicate that the sorted SP cells show elevated expression levels of *ABCG2*, *OCT-4* and *EpCAM*, hence, the interactions of these genes may be collectively involved in the drug resistance and enhanced survival rate of SP cells. However, the signaling pathways and cascade of events involved in expression of these genes in CSCs remains speculative. However, the identification and characterization of SP cells provides a strategy to design novel therapeutic targets with the eventual goal of eliminating residual disease and preventing tumor recurrence.

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