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Characterization of a stem cell population in lung cancer A549 cells

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ABSTRACT

We isolated a stem cell subpopulation from human lung cancer A549 cells using FACS/Hoechst 33342. This side population (SP), which comprised 24% of the total cell population, totally disappeared after treatment with the selective ABCG 2 inhibitor fumitremorgin C. In a repopulation study, isolated SP and non-SP cells were each able to generate a heterogeneous population of SP and non-SP cells, but this repopulation occurred more rapidly in SP cells than non-SP. An MTT assay and cell cycle distribution analysis reveal a similar profile between SP and non-SP groups. However, in the presence of doxorubicin (DOX) and methotrexate (MTX), SP cells showed significantly lower Annexin V staining when compared to non-SP cells. Taken together, these results demonstrate that SP cells have an active regeneration capacity and high anti-apoptotic activity compared with non-SP cells. Furthermore, our GeneChip® data revealed a heightened mRNA expression of ABCG2 and ABCC2 in SP cells. Overall these data explain why the SP of A549 has a unique ability to resist DOX and MTX treatments. Therefore, we suggest that the expression of the ABCG2 transporter plays an important role in the multidrug resistance phenotype of A549 SP cells.

According to the cancer stem cell hypothesis, the initiation and progression of tumors are driven by small populations of stem cell-like cancer cells (SCLCCs), which have indefinite proliferation and differentiation potential [1–5]. The ability to purify SCLCC relies on the expression of appropriate cell-surface antigens, which can be isolated by a fluorescence-activated cell sorter (FACS) [6]. Another useful approach for identification and purification of SCLCC makes use of their ability to efflux lipophilic and fluorescent dyes such as Hoechst 33342 [7–9]. To implement this method, cells are stained with dual wavelength Hoechst and, subsequently, sorted for the side population with low red and blue fluorescence [9,10]. The low Hoechst SP phenotype was first identified as a marker for stem cell activity in mouse bone marrow cells [11]. In other studies, the proportion of tumor cells having a side population phenotype can range from 0.04% to 0.2% in human cell lines derived from prostate, breast, colon, glioma, bladder, ovary, cervix, and melanoma [12–15].

The ability of Hoechst to act as a stem cell probe depends on the activity of the ATP-binding cassette (ABC) transporter subfamily members, including MDR1 and ABCG2, which pump dye out of the SCLCC [7,10,16–21]. Consequently, the loss of ABCG2 gene expression leads to a significant reduction in the number of SP cells in bone marrow and skeletal muscle [10,16]. Exogenous expression of ABCG2 in cancer cell lines confers resistance to doxorubicin, mitoxantrone, and related drugs [21–23]. Correlating gene expression and drug sensitivity may improve the therapeutic effectiveness of certain chemotherapeutic agents during cancer treatment. Lung cancers are classified into two major groups, small cell lung cancers (SCLCs) and non-small cell lung cancers (NSCLCs) [24,25]. NSCLCs are frequently chemoresistant and exhibit a multidrug resistance phenotype *in vitro* [24,26]. A549 cells (NSCLCs) are more resistant to DOX [27], contain a higher percentage of SP cells (>10%) [15,19], and have significantly higher expression of lung resistance-related protein (LRP) than other cancer cell lines [27].

In this study, we first confirm that SCLCC can be identified with side population sorting in cultured lung cancer cells. We then used fumitremorgin C (FTC) to disrupt ABCG2 activity and monitor changes to Hoechst staining in A549 cells. Finally, we focus on the importance of efflux activity to the establishment of multidrug resistance in A549 cells.

Materials and methods

Chemicals and cell line. Hoechst 33342, fumitremorgin C (FTC), propidium iodide (PI), doxorubicin, and methotrexate were supplied by Sigma (St. Louis, MO, USA). Methyl-thiazoldiphenyl tetrazolium (MTT) was provided by Roche Diagnostic

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GmbH (Penzberg, Germany). Annexin V-PE was purchased from BD Biosciences Pharmingen (CA, USA). A549 human lung carcinoma cells, a generous gift from Cha Biomedical Centre (Seoul, Korea), were cultured on Ham F12K medium containing 10% FBS and 1% penicillin/streptomycin.

Flow cytometry analysis and sorting. The protocol was based on Goodell et al. [28]. Briefly, A549 cells were suspended in a mixture of Hanks' balanced salt solution, 10 mM HEPES, and 2% FBS to yield a concentration of 1.0×10^6 cells/ml. Hoechst 33342 was added at a final concentration of 5 μg/ml in the presence or absence of fumitremorgin C (FTC) (1 or 10 μM), and the samples were incubated at 37 °C for 90 min. During the incubation time, cells were gently tapped every 15 min. Afterwards, the cells were washed and resuspended in cold HBSS. To exclude nonviable cells from the flow cytometric analysis, propidium iodide (PI) was added at a rate of 2 μg/ml. The Hoechst dye was excited with UV laser at 355 nm, and its fluorescence was measured with a 405/30 BP filter (Hoechst Blue) and a 660 long pass filter (Hoechst Red). A second 488 nm argon laser was used to excite PI.

Repopulation assay. An entire A549 cell population was stained with Hoechst to determine whether the SP cells have the ability to repopulate *in vitro*. SP and non-SP separated by FACS were cultured for 3 or 7 days. After 3 and 7 days, both SP and non-SP cells were restained with Hoechst and analyzed via FACS.

Cell proliferation assay. Growth inhibition by doxorubicin was determined by the MTT assay according to the protocol described by Park et al. [29]. Approximately 2.0×10^3 cells/well were seeded on 96-well plates. After a 24 h incubation, the cells were exposed to various concentrations of DOX (10 nM, 100 nM, and 1 μM) for 1, 2, and 3 days. At the end of each incubation, 10 μl of MTT was added to each well and incubated at 37 °C for 4 h. Afterwards, 100 μl of the solubilization solution was added to each well. The plates were kept overnight in an incubator at 37 °C with humidified air (5% CO_2). The optical density (OD) was measured at 550 nm using an ELISA plate reader (Bio-Rad Model 680, Richmond, CA, USA). To investigate the differences in proliferation, SP and non-SP cells were sorted, and 2.0×10^3 cells were seeded into each well of a 96-well plate. An MTT assay was performed for 3 days without drug treatment as previously described.

Apoptosis assay. Cells were seeded onto a 6-well plate at a density of about 2.0×10^5 cells/well. For 48 h, the cells were exposed to different concentrations of DOX or MTX and washed with PBS. Then, 700 μl cell dissociation buffer was added to the mixture before incubation at 37 °C for 10 min. Afterwards, the cells were washed twice with PBS. Cells were suspended with binding buffer (50 μl), and the Annexin V-PE (2 μl) and PI (2 μl) were added before incubation at room temperature (RT) for 15 min. Cells were washed (1 ml) and resuspended (200 μl) with binding buffer and analyzed *via* FACS.

Cell cycle assay. A549 cells were seeded at 2.0×10^5 cells/well in 6-well plates. The cells were allowed to recover for 24 h and treated with DOX. To analyze the cell cycle distribution, the cells were collected after 24 h incubation and washed with PBS. The cells were fixed in 70% ethanol and stored overnight at 4 °C. For analysis, the cells were transferred into PBS and incubated with Ribonuclease A (50 μg/ml) at room temperature for 5 min. Following incubation, the cells were treated with 10 μg/ml PI and incubated at 37 °C for 10 min. Finally, the cells were analyzed using FACS.

GeneChip analysis. Total RNA from approximately 2×10^6 SP or non-SP cells was isolated with Qiagen One step mini RNA prep kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The RNAs were subjected to GeneChip expression array full service with two-cycle target labeling (Seoulin Bioscience CO., Ltd., Seoul, Republic of Korea). Briefly, cDNA was synthesized from total RNA using T7-Oligo (dT) primers (1st cycle). Biotinylated cRNA was then synthesized by a 2nd *in vitro* transcription from cDNA. Fifteen micrograms of the labeled cRNA was hybridized to a Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA). The array image was scanned and analyzed with GeneChip® Operation Software (Affymetrix, CA, USA).

Statistical analysis. Results are expressed as means ± SD. Student's *t*-test was used to compare the differences between groups. $p < 0.05$ was considered significant.

Results

SP profile in A549 tumor cell line

Mouse bone marrow cells were used as a positive control sample, and FACS analysis revealed clearly defined SP cells constituting 1.49% of the total bone marrow cell population (Fig. 1A). Interestingly, 24.44% of A549 cells were classified as SP cells (Fig. 1B). In the presence of FTC, the SP cells in the bone marrow and A549 cell populations dropped to 1.85% (Fig. 1C) and 0.08% (Fig. 1D), respectively. A549 cells contained SP and non-SP compartments making up 25.97% and 47.55% of the total cell population, respectively (Fig. 1E). After 3 days of culturing, the ratio of SP to non-SP cells was much higher than before sorting. FACS analysis revealed that the cultures derived from SP sorted cells contained 28.3% of non-SP cells. Conversely, sorted non-SP cells yielded cells mostly non-SP (87.1%) (Fig. 1F), but contained a small number of SP cells (3.8%) (Fig. 1G). After 7 days in culture, the approximate proportion of each cell population nearly equaled those present before sorting.

Effect of DOX on A549 cell growth and cycle distribution

An MTT assay was performed to measure proliferation changes in response to DOX treatment in A549. The cells were treated with DOX concentrations ranging from 10 nM to 1 μM for 3 days. As shown in Fig. 2A, DOX significantly inhibited ($p < 0.05$) the proliferation of A549 cells in a time- and dose-dependent pattern. To determine the effect of DOX treatment on cell cycle distribution, DOX was added to A549 media for 24 h, before labeling with PI and analyzing with FACS. Compared with untreated cells, DOX caused an accumulation of cells in the G2/M phase while reducing the number of cells in G0/G1 and S phase. The G2/M cell population in A549 cells increased from 14.38% in control to 32.78% (50 nM DOX) and 67.18% (100 nM DOX) (Fig. 2B).

Cell proliferation and cell cycle distribution

We then looked at changes in proliferation soon after sorting. Hoechst-stained A549 cells were sorted into SP and non-SP fractions. The growth rate for each population was measured with an MTT assay for 3 days. The proliferation rates were not significantly different between SP and non-SP cells (Table 1). In control treatments, the relative cell cycle distributions were similar between SP and non-SP cells (Table 2). In cells treated with 50 nM DOX, the percentage of cells in G2/M increased from 12.28% to 41.98% and 12.88% to 42.38% for SP and non-SP cells, respectively. At 100 nM DOX, the SP cell population was approximately 5% higher than non-SP cells (Table 2).

Apoptosis induction

To evaluate the induction of apoptosis in lung cancer cells (A549), a biparametric cytofluorimetric analysis using propidium iodide (PI) and Annexin V was used following DOX and MTX treatments. Annexin V staining identified the apoptotic cells, and PI staining distinguished dead cells. Non-SP cells showed a higher sensitivity to DOX and MTX when compared to SP cells. The results of the apoptosis assay revealed that non-SP cells treated with 100 nM DOX or 100 and 200 μM MTX were significantly more apoptotic than SP cells receiving the same treatment (Fig. 3).

Gene expression

Comprehensive analysis of gene expression was performed using a DNA chip to investigate the abundance of genes related to multidrug resistance. The data revealed distinct differences between SP and non-SP cells. GeneChip® analysis results have shown that ABCG2 and MRP2 were more highly expressed in SP than in non-SP cells (Fig. 4).

Discussion

Some research has suggested that chemotherapeutic treatments are often unsuccessful because of a small, surviving population of cancer stem cells. The cancer stem cell hypothesis provides a unified explanation for the utility and limitation of cytotoxic anticancer therapies [17]. Several cancer cell lines contain SP cells; however, the SP phenotype is not sufficient to predict the capacity of drug resistance. Drug resistance has a significant impact on the clinical efficacy of anticancer treatment.

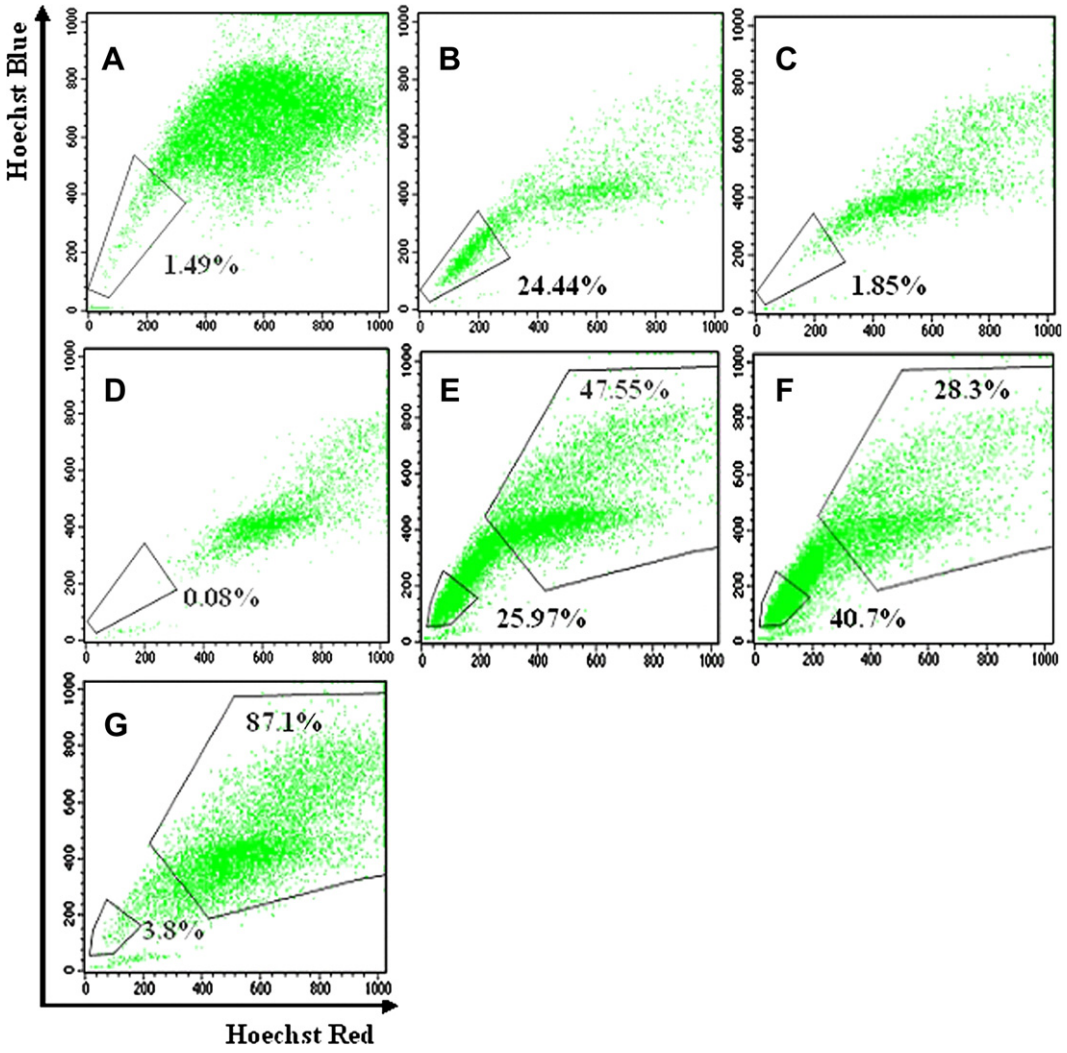

Fig. 1. Hoechst 33342 dye staining profiles of mouse bone marrow cells (A) and A549 cells (B–D). Cells (1×10^6) were stained with Hoechst 33342 and analyzed by FACS. Mouse bone marrow cells (A) and A549 lung cancer cells were stained with Hoechst in the absence (B) or presence of 1 μM (C) and 10 μM (D) FTC. SP cells appear as the Hoechst low fraction and non-SP cells retain high levels of Hoechst staining. Repopulation profile of A549 cell using Hoechst 33342 dye, (E) control, (F) sorted SP cells, and (G) non-SP cells.

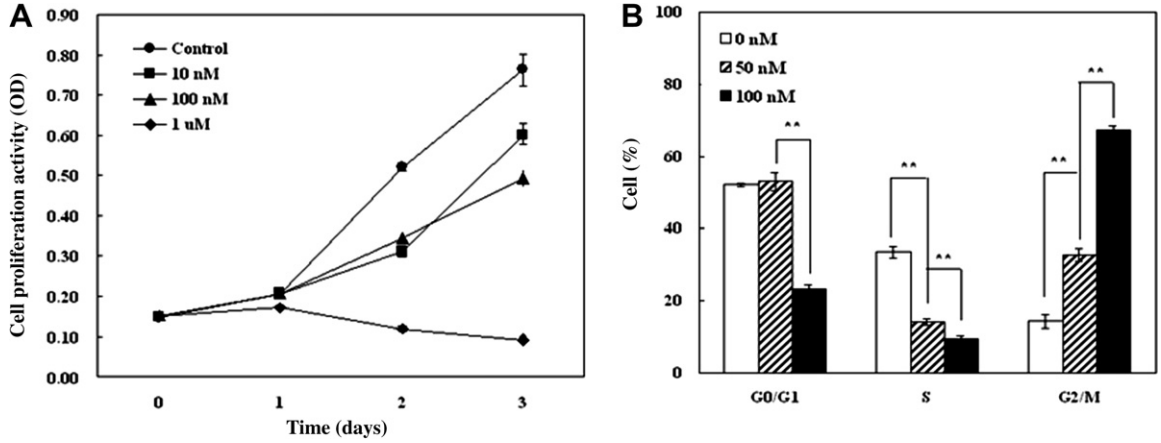

Fig. 2. Effect of DOX on A549 cell growth (A) and cell cycle distribution (B). Cells were seeded in 96-well plates at a density of 2×10^3 cells/well and treated with DOX at specified concentrations for 1–3 days (A). Cells were seeded in 6-well plates at a density of 2×10^5 cells/well and treated with DOX at specified concentrations for 24 h (B) ($n = 3$, $^{**}p < 0.01$).

In the present study, fumitremorgin C effectively decreased the proportion of SP cells in an A549 culture. This finding confirms that the appearance of an SP phenotype depends on ABCG2 transporter efflux [23]. In our repopulation study, we found that both SP and

Table 1
Proliferation differences between SP and non-SP cells

	Cell proliferation activity (OD)		
	1 day	2 day	3 day
SP	0.195 ± 0.004	0.277 ± 0.002	0.459 ± 0.015
non-SP	0.219 ± 0.001	0.312 ± 0.006	0.455 ± 0.012

A549 cells were seeded in 96-well plates at a density of 2×10^3 cells/well. Cell proliferation was assessed by MTT assay.

non-SP cells were capable of generating cells from the other population, consistent with work by Kruger et al. In contrast, non-SP cells can only generate other non-SP cells in some cancer cell lines such as liver and neuroblastoma [12,13]. We suggest that both SP and non-SP cell populations are composed of stem and non-stem cells. In another research report, ABCG2+ cancer cells can generate ABCG2− cells; however, ABCG2− cancer cells can also generate ABCG2+ cells [15]. Furthermore, although SP cells were more clonogenic than non-SP cells, the non-SP cells still maintained some level of clonogenicity [30]. The findings of Mitsutake et al. supported our suggestion that tumorigenic cells are probably enriched in the SP population, but even non-SP cells may contain a small number of tumorigenic cells. In the present study, repopulation occurred more rapidly in SP cells compared to non-SP cells, in agreement with observations made by Hirschmann et al. At seven days post-culturing, the ratio of SP to non-SP cells was almost the same as those present before sorting. The same phenomenon was previously detected in a mammary carcinoma cell line [8].

The relative cell cycle distributions were similar between SP and non-SP cells under control and 50 nM DOX treatments. At

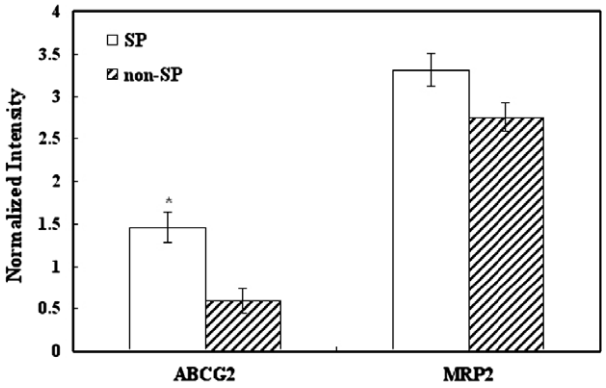

Fig. 4. Comparisons of gene expressions of ABC transporters in SP and non-SP cells. A549 cells were sorted to each population approximately 1×10^6 cells and gene expression data were analyzed using GeneChip® ($n = 3$, $^{**}p < 0.01$).

100 nM of DOX, however, SP cells were far more likely to arrest in G2/M than non-SP cells. Previous work by Lee et al. found that DOX treatment induced overexpression of cell cycle genes such as CDK10, P53, and GADD 45 [31]. We hypothesize that these factors might be more highly expressed in SP cells compared to non-SP cells.

Apoptosis has been identified as a critical determinant of cancer cell responsiveness to both radio- and chemotherapy [27]. Doxorubicin and methotrexate are both examples of anticancer drugs that are effluxed by ABC transporters (ABCC1, ABCC2, and ABCG2) [32,33]. The MTT assay showed that DOX and MTX induced apoptosis more efficiently in non-SP cells compared with SP cells, indi-

Table 2
Comparisons of SP and non-SP cell cycle distributions

	Control		50 nM		100 nM	
	SP	non-SP	SP	non-SP	SP	non-SP
G0/G1 (%)	53.55 ± 1.99	55.17 ± 1.25	47.79 ± 1.46	47.46 ± 1.70	36.58 ± 1.91	43.05 ± 3.32
S (%)	34.18 ± 1.36	31.96 ± 1.76	10.23 ± 0.53	10.15 ± 0.58	8.31 ± 0.794	7.27 ± 3.09
G2/M (%)	12.28 ± 0.62	12.88 ± 3.01	41.98 ± 1.99	42.38 ± 1.22	55.11 ± 1.12*	49.69 ± 0.75

Cells were seeded at 2×10^5 cells/well in 6-well plates and exposed to 50 and 100 nM DOX for 24 h.
$^{*}p < 0.05$.

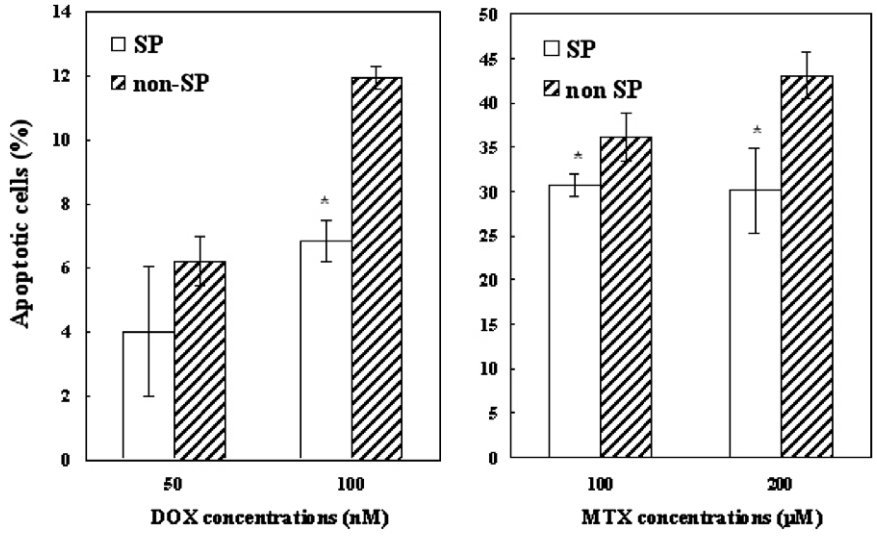

Fig. 3. Differences in apoptosis induction between SP and non-SP cells. Cells were seeded at 2×10^5 cells/well in 6-well plates and exposed to DOX (50 and 100 nM) and MTX (100 and 200 μM) for 48 h ($n = 3$, $^{**}p < 0.01$).

cating that SP cells are less sensitive to drug treatment. It is clear that ABC transporters can confer drug resistance to cancer cells, suggesting that targeting these transporters may improve the efficacy of chemotherapy, consistent with reports in mammary carcinoma and mouse ovarian cancer [8,33].

Classical multidrug resistance is attributed to an elevated expression of ATP-dependent drug efflux pumps that belong to the superfamily ATP-binding cassette (ABC) transporters such as ABCB1 (P-glycoprotein or MDR1) and ABCG2 (BCRP) [12,21]. Therefore, drug efflux mediated by ABC transporters leads to a decrease in cellular accumulation of anticancer drugs. The chemoresistance of SP cells is reportedly dependent on ABC transporters [12,13]. Our GeneChip® analysis revealed that ABCG2 and MRP2 genes were up-regulated in SP cells. Other reports have shown that ABCG2 and MDR1 (ABCB1) are up-regulated in mammary gland and liver cancer SP cells [12], and MRP4 and MRP9 are up-regulated in mammary gland SP cells. Additionally, mouse bone marrow SP cells showed a higher level of ABCG2 RNA compared to non-SP cells [18]. The overexpression of these genes was associated with enhanced drug efflux and the development of drug resistance [13,16,18,20]. The overexpression of ABCG2 gene was reported to confirm resistance to doxorubicin, daunorubicin, and mitoxantrone. Thus ABCG2 expression may serve as stem cell marker, not only in A549 cells but also in other types of cells [9,21]. GeneChip® analysis may be a useful tool for identifying the potential cancer stem cell markers.

We believe that a thorough characterization of SP will advance our understanding of stem cells and will provide an accessible target for limiting drug resistance during cancer therapy.

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