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ORIGINAL ARTICLE

Characterization of a stem cell population in lung cancer cell line Glc-82

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Abstract

Objectives: A side population (SP) of cells can be separated from diverse cancer cell lines by a fluorescence-activated cell sorter (FACS) and show stem cell-like characteristics. To gain more information about SP phenotypes, we performed a series of characterizations of SP cells.
Methods: We isolated SP cells from the lung cancer cell line Glc-82 via FACS. Their capability of multilineage differentiation, self-renewal and tumorigenicity were examined both *in vitro* and *in vivo*. Their sensitivity to anticancer drugs was also detected by Methyl-thiazol-diphenyl tetrazolium assay. The expression of cell surface molecules including ABCG2, HERs and CD133 was analyzed with a flow cytometer.
Results: SP cells made up an average of 14.5% of the total cell population and were more tumorigenic than non-SP cells *in vivo*. The growth rate of SP cells was higher than that of the unsorted cells and non-SP cells, and the repopulation of SP cells occurred more rapidly. Moreover, the SP cells expressed elevated levels of ABCG2 protein and showed augmented resistance to multiple chemotherapeutic and targeted drugs, when compared to non-SP cells. In addition, the expression of epidermal growth factor receptor protein and CD133 were higher in SP cells than in non-SP cells.
Conclusions: We suggest that the SP cells in the Glc-82 cell line are enriched with cancer stem cells.

Introduction

Lung cancer is the most common malignancy worldwide with more than 1 million new cases each year.[1] Although new anticancer agents have improved patients' 5-year survival outcome, the overall effect in the last decade has been mainly on palliation rather than reduction in mortality. It was reported that only 15% or fewer of lung cancer patients survived 5 years after treatment and most patients died of local recurrence or distant metastasis.[2] For further improvement of clinical outcomes it is essential to study the biological characteristics of lung cancer to understand its origin and progression, and accordingly modify the therapeutic strategy.

Accumulated evidence has shown that solid tumors as well as hematopoietic malignancies contain a minor population of cancer stem cells (CSC)[3–6] with high self-renewal and multilineage differentiation capacity. According to the cancer stem cell hypothesis inspired by an *in vitro* mouse model, the initiation and progression of tumors are driven by a small population of CSC, which have indefinite proliferation and differentiation potentials and may be the root cause of recurrence and metastasis. Fortunately, recent advances in stem cell biology enable us to identify CSC in solid tumors as well as putative stem cells in normal solid organs.

A subpopulation, called the side population (SP) because of its ability to efflux lipophilic fluorescent dyes such as

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Hoechst 33342 via ABC transporters, can now be identified and purified by a fluorescence-activated cell sorter (FACS).[7–9] The low Hoechst 33342 SP phenotype in mouse bone marrow cells was first identified as a marker for stem cell activity in 1996.[10] In other studies, the proportion of SP cells has ranged from 0.04% to 0.2% in human cell lines derived from prostate, breast, colon, glioma, bladder, ovary, cervix, and melanoma.[11–14] Recent studies have suggested that SP cells may be a source of CSC, although no definite stem markers are available. SP cells have demonstrated high proliferative potential and an anti-apoptotic property compared with non-SP cells. Therefore, if this is the case in human lung cancer, it may be an important target for effective therapy of this disease.[15]

Our study used the Hoechst 33342 dye-based SP technique to verify the existence of CSC in lung cancer and then elucidated their stem-like biological characteristic. A Glc-82 cell line was established from the lung cancer tissue of a female retired worker who had experienced long-term exposure to environmental radon gas pollution. However, there was no evidence to indicate that the SP phenotype existed in Glc-82 cell line. The Glc-82 cell line was selected because of its relatively high proportion of SP cells compared with other non-small-cell lung cancer cell lines, according to our earlier experiments (not shown in this article).

Materials and methods

Chemicals and cell lines

Hoechst 33342, verapamil and propidium iodide (PI) were provided by Sigma (St. Louis, MO, USA). Methyl-thiazol-diphenyl tetrazolium (MTT) was the product of Roche Diagnostics GmbH (Mannheim, Germany). Phycoerythrin (PE)-labeled mouse antihuman ABCG2 and CD133 monoclonal antibody and the corresponding PE-labeled goat antimouse Gigs were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). PE-labeled rabbit antihuman HERs (EGFR, HER2, HER3, HER4) monoclonal antibodies and the corresponding PE-labeled goat antirabbit IgG antibody were purchased from Cell Signaling Company (Beverley, MA, USA). Lapatinib was purchased from GlaxoSmithKline Company (Middlesex, UK). Human lung carcinoma cell line Glc-82, a generous gift from the Chinese Academy of Sciences (Shanghai, China), was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified 37°C incubator with 5% CO_2.

Flow cytometry analysis and sorting

We sorted SP from non-SP cells in the Glc-82 cell line. The cell suspensions were labeled with Hoechst 33342 dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA) using the methods described by Goodell *et al.*[10,16] Briefly, cells were suspended in pre-warmed DMEM containing 2% FBS to yield a concentration of 1×10^6/mL for 20 min, and incubated for an additional 90 min in a shaking bath with 5 μg/mL Hoechst 33342 dye away from light. Control cells were incubated with 50 μM Verapamil (Sigma) for 15 min at 37°C before Hoechst dye was added. Afterwards, the cells were washed with ice-cold phosphate buffered saline (PBS) and then resuspended at 1×10^6/mL. The cells were filtered through a 40-μm cell cribble to obtain single cell suspension, and PI was then added at a concentration of 2 μg/mL 5 min before sorting to exclude non-viable cells. Cell samples were analyzed and sorted using a MoFlo MLS cell sorter (Beckman-Coulter, Hialeah, FL, USA) with UV capabilities and SUMMIT software (North Little Rock, AR, USA) for data acquisition and analysis. The Hoechst 33342 dye was excited with a UV laser at 357 nm and its fluorescence was analyzed in dual-wave length (blue, 402–446 nm; red, 650–670 nm). A second 488 nm argon laser was used to excite the PI.

Cell growth curve and clone-forming experiment

The sorted SP and non-SP cells were inoculated in 96-well plates. From the following day, the growth ratio was routinely documented for 8 days using the MTT assay. Thereafter, the absorbance and time for culture were applied as the ordinate and abscissa respectively to draw the cell growth curve. In a clone-forming experiment, the sorted SP and non-SP cells were counted and plated into 6-well plates (200 cells/well), and cultured for 8 days. When clone formation was complete in the majority of the cells and each clone contained over 50 cells, the clones were counted by crystal violet staining. The clone-forming efficiency was calculated by dividing the number of clones with that of the inoculated cells.

Tumorigenicity test in immune-deficient mice

We did a pretest on the tumorigenicity of the unsorted cells. The planted numbers were 1×10^3, 5×10^3, 1×10^4, 5×10^4 and 1×10^5. The lowest dose for tumorigenicity was chosen as the highest dose for inoculation of SP and non-SP cells, which had been counted precisely and resuspended in PBS. Four preparations of both SP and non-SP cells were made and each volume was uniformly 200 μL. The cells were preserved at 4°C and incubated into the backs of immune-deficient mice subcutaneously (three mice for each dose). The mice were fed *ad libitum* with water and food pellets. The experiment was approved by the regional ethics committee for animal research.

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Assay of multilineage differentiation and self-renewal capacity

To detect the capacity of multilineage differentiation, the sorted SP and non-SP cells were plated into 6-well plates and cultured for 1 week, they were then analyzed by FACS/ Hoechst 33342. To evaluate the ability of SP cells to regenerate the heterogeneity of total population, the tumors in immune-deficient mice were obtained after 4 weeks, minced mechanically and then digested in a shaking incubator with 0.1 mg/mL collagenase for 2 h at 37°C. Afterwards, the cells were sieved through a 100-μm cell cribble to obtain single cell suspension and then layered onto a 70% and 40% Percoll gradient and centrifuged for 20 min at 25 000 rpm at room temperature. Epithelial cells from the 70–40% interface were collected and ready for SP analysis.

Analysis of ABCG2, HERs and CD133 expression

Freshly FACS-sorted cells were collected and washed in PBS. After a 30-min fixation with 1 mL paraformaldehyde (4%), the samples were blocked in 10mmol/L Tris-HCL, 150 mmol/L NaCl, and 0.1% Tween20, pH 8.0 containing 5% bovine serum albumin for 60 min at room temperature. Then PE-labeled corresponding antibodies or isotype control were added to each remaining 100 μL suspension (the SP and non-SP cells were transferred into four tubes separately). After 45 min of incubation at room temperature, the samples were washed with ice-cold PBS and ready for flow cytometer assay (away from light).

Drug sensitivity assay

MTT assay was performed to assess the antiproliferation activity.[17] Approximately 2×10^3 FACS-sorted cells per well were plated into 96-well (12 strips of 8 wells) plates in DMEM with 2% FBS and incubated overnight. Anticancer drugs (carboplatin, cis-platinum, lapatinib, vinorelbine, taxol and docetaxel) at various concentrations were then added to the cells and the cells were incubated at 37°C for 68 h. Afterwards 20 μl of MTT (5 mg/mL) was added to each well and incubated for another 4 h at 37°C. Finally, the medium was discarded and the formazan product from the metabolism of MTT was solubilized in dimethyl sulfoxide (200 μl/well). Optical density was measured at 540 nm, with background subtraction at 655 nm, using the Model 550 Microplate Reader (BIO-RAD, Hercules, CA, USA). The concentration required to inhibit cell growth by 50% (IC50) was calculated from survival curves using the Bliss method.[18]

Statistical analysis

Data were expressed as the mean ± SD from three independent experiments. All of the statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, USA). Student's *t*-test was used to compare the differences among groups. A significant difference was declared if the *P*-value from a two-tailed test was less than 0.05.

Results

SP profile in the Glc-82 tumor cell line

We examined the existence of SP cells in Glc-82 by Hoechst 33342 staining (Fig. 1a,e). As a control, verapamil was added to block the activity of Hoechst 33342 transporter (Fig. 1b). FACS analysis revealed that clearly defined SP cells constituted 14.5% of the total Glc-82 cell population (Fig. 2a) and the proportion of SP cells dropped to 3.6% in the presence of verapamil (Fig. 2b). We also analyzed the purity of sorted SP and non-SP cells by FACS/ Hoechst 33342, and founded that the purity of the SP cells reached 98% (Fig. 1c) and that of the non-SP cells was 96% (Fig. 1d).

SP cells showed more growth advantage and higher clone-forming efficiency

We tested the growth rates using MTT and drew growth curves according to the optical density values of the three groups (Fig. 2). The growth rate of SP cells was higher than that of the unsorted cells and non-SP cells ($P < 0.05$). Whereas the growth rate of non-SP cells showed no obvious difference compared with the unsorted cells in the first 5 days, the growth of non-SP cells turned out to be slower than the unsorted cells from the 6th day (Fig. 2a). To investigate the possible difference between the self-renewal capacity SP and non-SP cells, a clone-forming experiment *in vitro* was performed (Fig. 4). The clone-forming efficiency of SP cells was 55.0 ± 5.6%, significantly higher than 19.8 ± 1.6% of non-SP cells ($P < 0.01$) (Fig. 2b).

SP cells exerted augmented tumorigenicity

To test whether SP cells are enriched with tumorigenic cells, various numbers of SP and non-SP cells from the Glc-82 cell line were injected into the backs of immune-deficient mice subcutaneously (three mice for each dose), and the tumor development was monitored twice per week. As shown in Table 1, 1×10^5 non-SP cells were needed to generate a new tumor in one of three mice tested. However, SP cells could form a tumor when only 5×10^3 cells were used, suggesting that the number of tumor-initiating cells in Glc-82 SP cells is at least 20 times higher than that of the non-SP cells.

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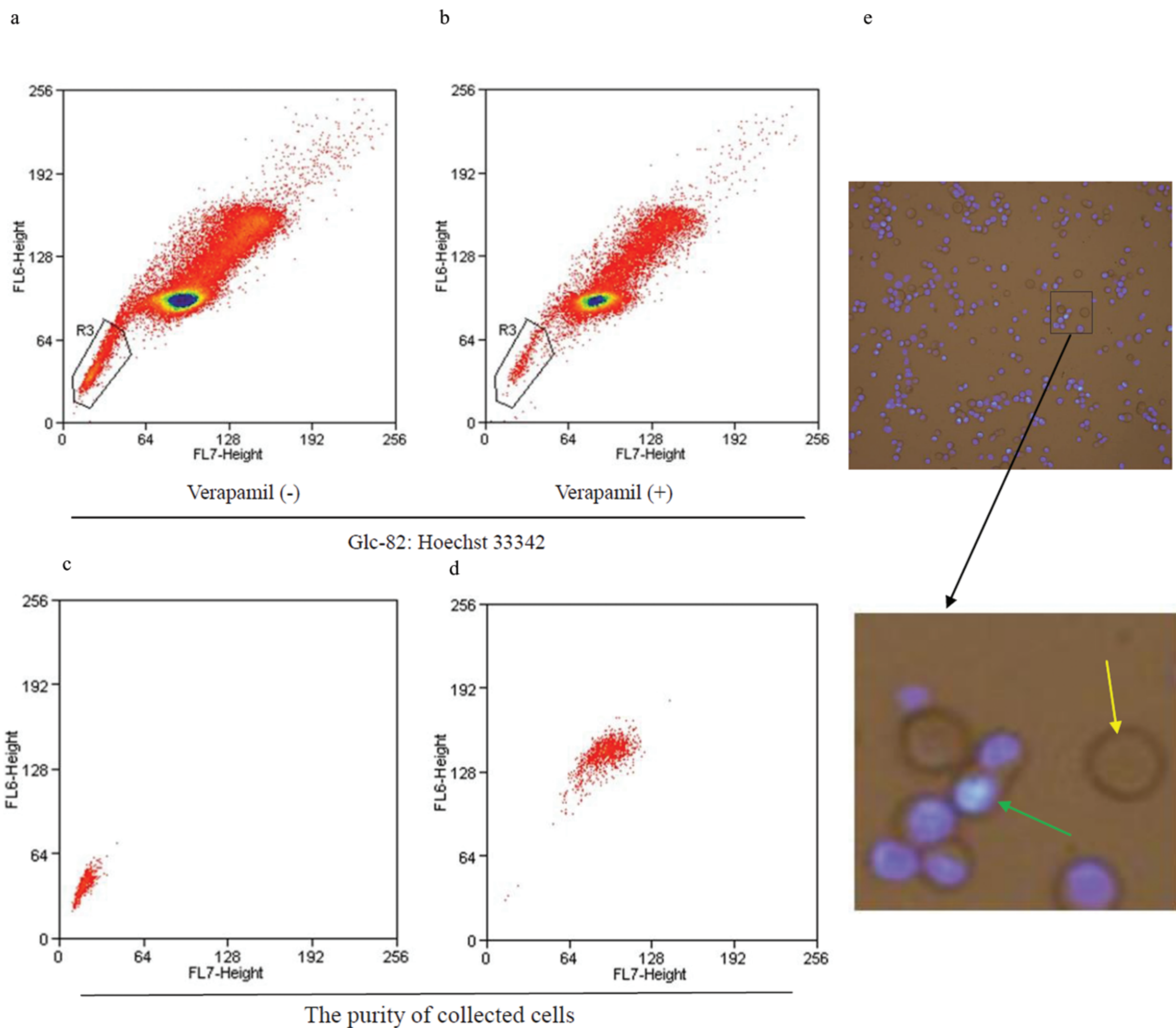

Figure 1 Hoechst 33342 dye staining profiles of human lung cancer cell line Glc-82. Cells were stained with Hoechst 33342 in the absence or presence of 50 μmol/L verapamil. The side population (SP) cells (14.5%) appeared as the Hoechst low fraction and non-SP cells retained high levels of Hoechst staining. This Hoechst-excluding capacity can be blocked by verapamil, where the proportion of SP cells decreased to 3.6%. The purity of collected SP and non-SP cells were also analyzed by flow cytometer. The purity of SP cells reached 98.0% while that of the non-SP cells was 96.0%. (a) Verapamil (-); (b) verapamil (+); (c) purity of SP cells; (d) purity of non-SP cells. After Hoechst 33342 staining, the Glc-82 cells were observed under a fluorescence microscope (e).

SP cells possessed enhanced capacity of multilineage differentiation and self-renewal

The sorted SP and non-SP cells were cultured separately under the same conditions (10% FBS DMEM medium) for 1 week before being restained with Hoechst 33342 dye. We found that the SP cells generated both a SP and a non-SP population with proportions comparable with the original population (Fig. 3a), whereas the non-SP cells produced mainly non-SP cells (Fig. 3b). We also removed the tumors formed by SP cells in the immune-deficient mice after 4 weeks and analyzed the single cell suspension by the Hoechst dye efflux assay. We found that the percentage of the SP cells in the tumors was 9.7% (Fig. 3c), which was comparable with the original proportion of 14.5% (Fig. 1a).

SP cells expressed higher ABCG2, EGFR and CD133

Using flow cytometry assay with ABCG2 and HER antibodies, we found that SP cells displayed higher expression of

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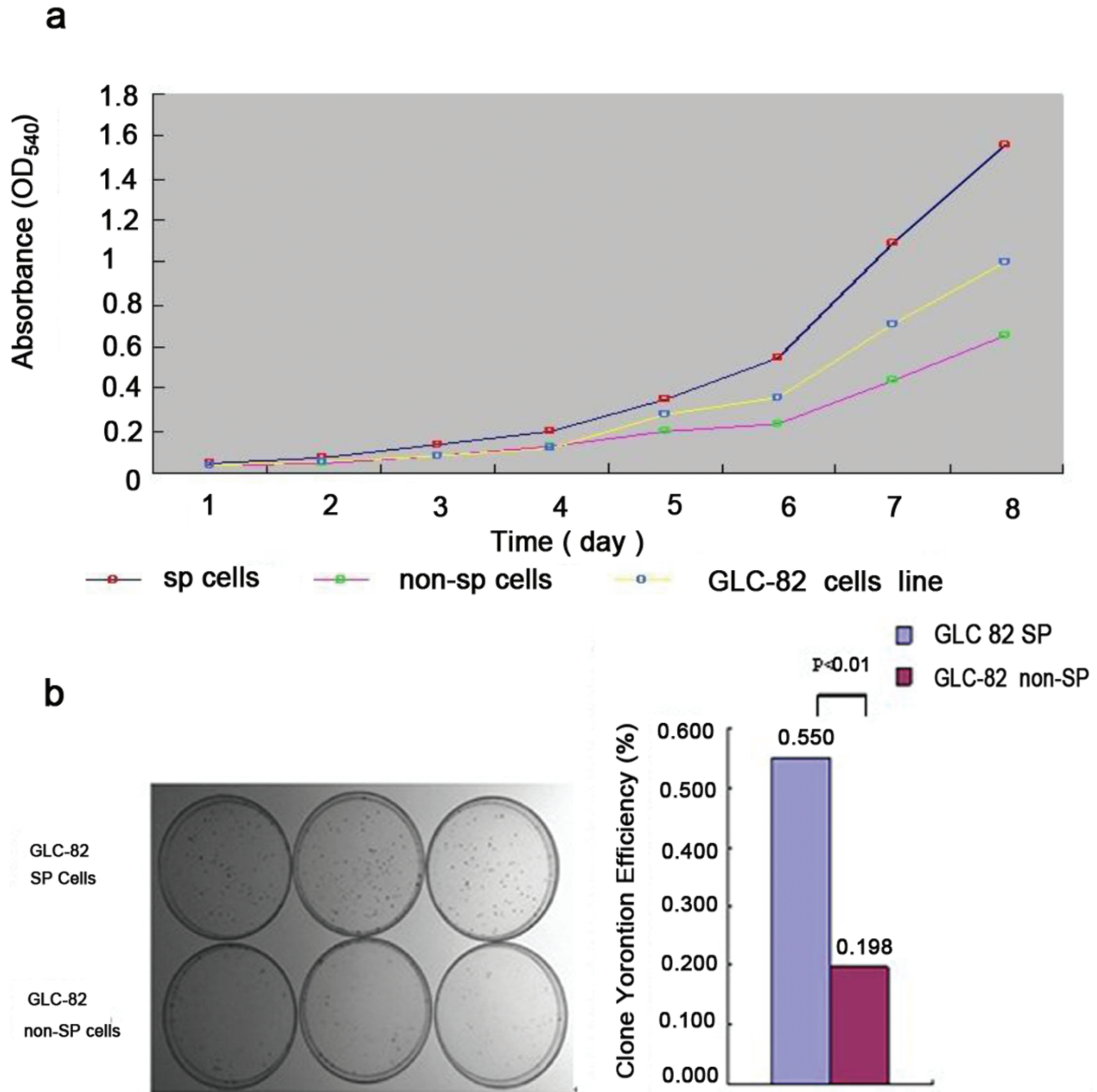

Figure 2 Increasing growth rate shown in side population (SP) cells. (a) The growth curves of SP cells, non-SP and unsorted Glc-82 cells. The growth rate of SP cells was significantly higher than that of the unsorted cells and non-SP cells ($P < 0.05$). The growth rate of non-SP cells showed no significant difference compared with the unsorted cells in the first 5 days. The growth of non-SP cells turned out to be slower than the unsorted cells from the 6th day. (b) The SP cells showed higher clone-formation efficiency (55.0% vs. 19.8%, $P < 0.01$).

Table 1 Evaluation of tumorigenicity of side population (SP) and non-SP cells 4 weeks after injection

Number of injected cells	10^3	5×10^3	10^4	5×10^4	10^5
GLC-82 SP cells	0/3	1/3	2/3	3/3	NA
GLC-82 non-SP cells	NA	0/3	0/3	0/3	1/3

NA, not applicable.

ABCG2 than non-SP cells (2.5% vs. 0.6%, $P < 0.05$, Fig. 4a,c). In addition, both SP and non-SP cells had low expression of CD133. Nevertheless, the expression of CD133 in SP cells was significantly higher than that in non-SP cells (0.6% vs. 0.2%, $P < 0.05$, Fig. 4b,c). We also found different expressions of epidermal growth factor receptor (EGFR) between the SP and non-SP cells (1.33% vs. 0.7%, $P < 0.05$) (Fig. 5a,b,i), whereas no difference in the expression of other HER family members was found (Fig. 5c,d,e,f,g,h,i).

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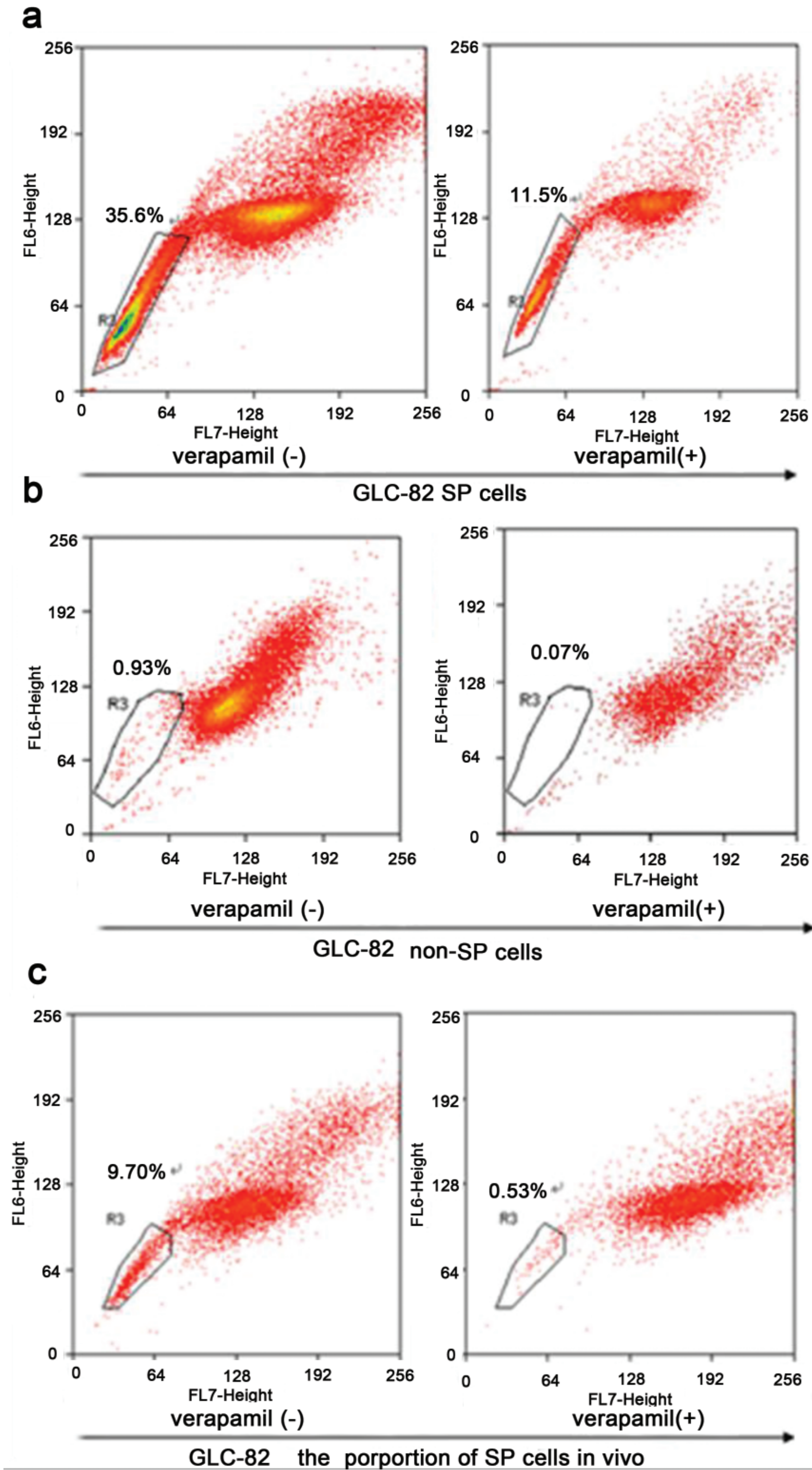

Figure 3 (a) Repopulation of side population (SP) cells *in vitro*; (b) repopulation of non-SP cells *in vitro*; (c) repopulation of SP cells *in vivo*.

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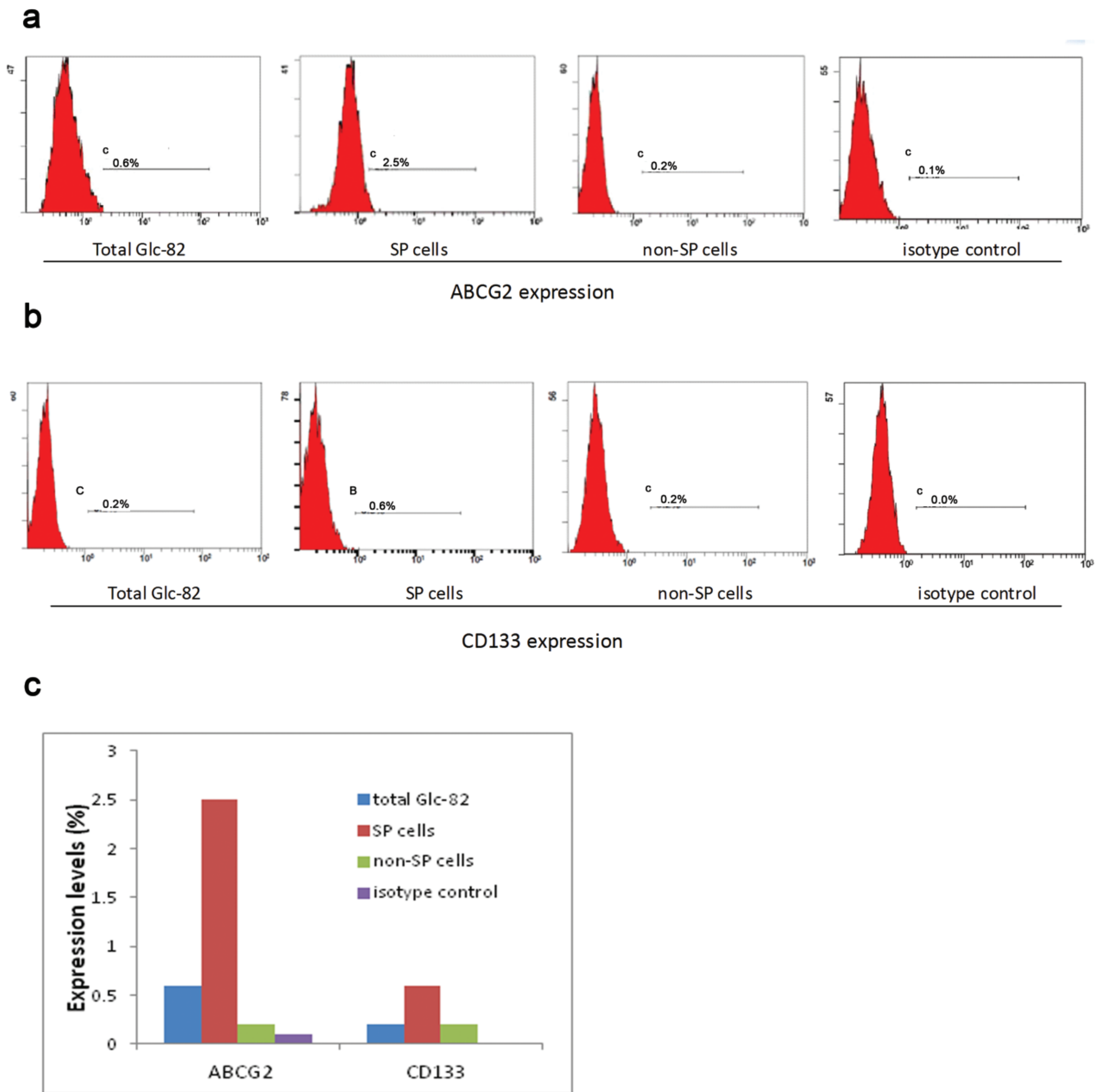

Figure 4 The expression of cell surface molecule ABCG2 and CD133. (a) ABCG2; (b) CD133; (c) bar chart for (a) and (b) See details in text.

SP cells revealed decreased sensitivity to a variety of anticancer drugs

We performed sensitivity assay for six anticancer drugs on SP and non-SP cells of Glc-82 (both populations were recovered in the first 24 h for reliable results). The SP cells exhibited higher resistance to the six drugs than non-SP cells with higher IC50 values ($P < 0.01$) (Fig. 6, Table 2).

Discussion

Our study was in keeping with the literature supporting the existence of SP cells in human lung cancer lines. Some other investigators have observed the existence of SP cells in human lung cancer lines,[15,19] which showed more tumorigenicity than non-SP cells when injected into immune-deficient mice, indicating a significant enrichment of tumor-initiating cells in this small subpopulation.

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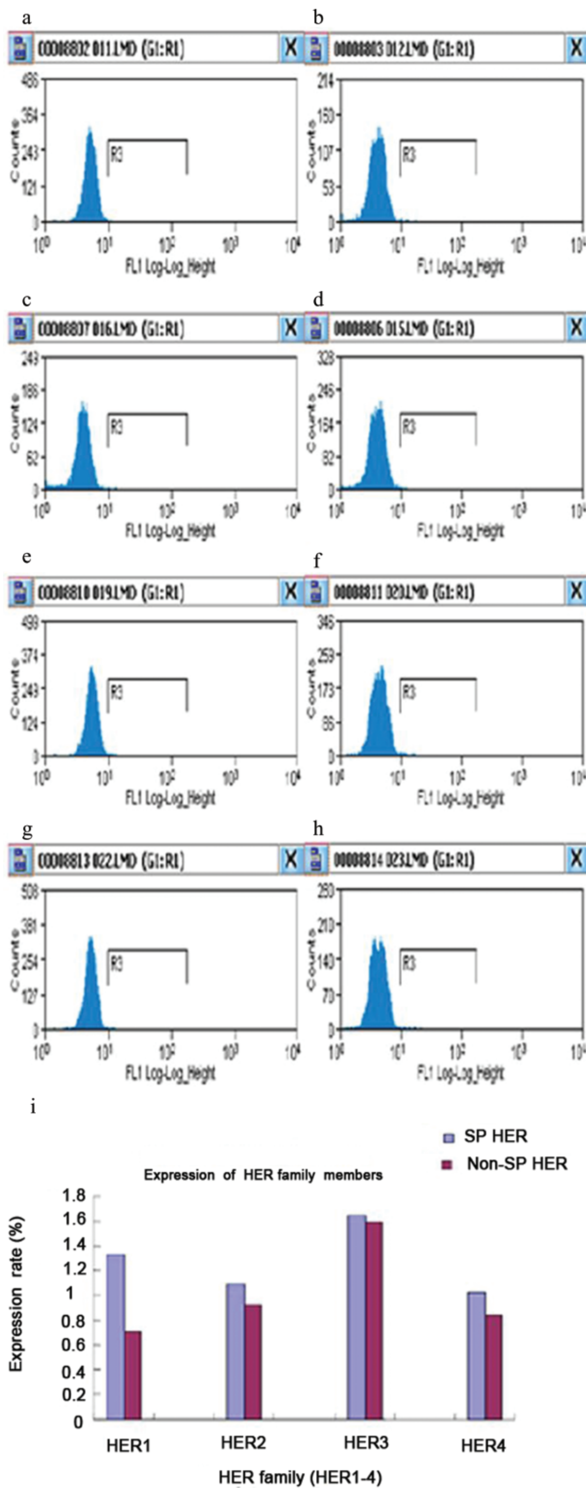

Figure 5 The expression of HER family members (HER1-4) analyzed by fluorescence-activated cell sorter. EGFR (HER1) was expressed higher in side population (SP) cells (1.33%) than non-SP cells (0.7%), although the expression of the other HER family members was not significantly different (i). (a) EGFR of SP; (b) EGFR of non-SP; (c) HER2 of SP; (d) HER2 of non-SP; (e) HER3 of SP; (f) HER3 of non- SP; (g) HER4 of SP; (h) HER4 of non-SP.

We also found evidence for the ability of SP cells to regenerate a population composed of both SP and non-SP cells *in vitro* and *in vivo*, resembling the original unsorted population. Our results also show their augmented multilineage differentiation and self-renewal capacities were very similar to stem cells. Meanwhile, SP cells had the trend of maintaining a certain percentage when cultured in 10% FBS DMEM and injected into immune-deficient mice. A few SP cells also appeared in non-SP cells which may be attributed to pollution.

According to the growth curves in our study, we found that SP cells soon entered a logarithmic phase after being cultured in 10% FBS DMEM and grew faster than non-SP cells and unsorted cells. It has been reported that there is no significant difference in the distribution of cell cycle phases between SP and non-SP.[19] Therefore, it is reasonable to assume that the unlimited proliferation and self-renewal capability, rather than rapid mitosis, actually maintain the fast growth of SP cells. Clone-forming experiments are a common method for testing the characteristics of CSC. Similar to a glioma cell line C6 studied previously,[20] which was considered to contain a large number of CSC because of its strong clone-forming ability, the SP cells in our study also showed a more powerful clone-forming capacity than non-SP cells, indicating they might be enriched with CSC.

The HER family has widely proved its role in tumor proliferation, invasiveness and metastasis in lung cancer, especially in non-small-cell lung cancer. Interestingly, our results also revealed that SP cells had elevated expression of EGFR, which provided the basis for studying the sensitivity to tyrosine kinase inhibitors (TKI), although the expression of other HER family members showed no significant difference in SP and non-SP cells. It is worth noting that the inhibitory effect of lapatinib (targeting both HER1 and HER2) in SP cells did not show significant difference compared with that in non-SP cells. There may be two possible reasons. Firstly, ABCG2 reduces the accumulation of lapatinib in cells. Secondly, as there is no difference in the expression of other HER family members except for EGFR, the downstream signal transduction pathways may not be blocked due to the complementary effect of other HER family members and a corresponding biological effect may remain. This result should be interpreted with caution. After all, relevant reports are still lacking, and further research is required to explore the function of HER family members in SP cells.

Intrinsic and acquired resistance is a major obstacle to cancer chemotherapy. It was reported that ABC transporters such as ABCB1 (P-gp or MDR1) and ABCG2 (BCRP) had the ability to export different chemotherapeutic drugs and were associated with multiple drug resistance (MDR).[21,22] Haraguchi *et al*. reported that ABCB1 and ABCG2 were highly expressed in human breast and liver cancer cell lines.[14] Sung *et al*. found that the mRNA of ABCG2 and ABCC2 were

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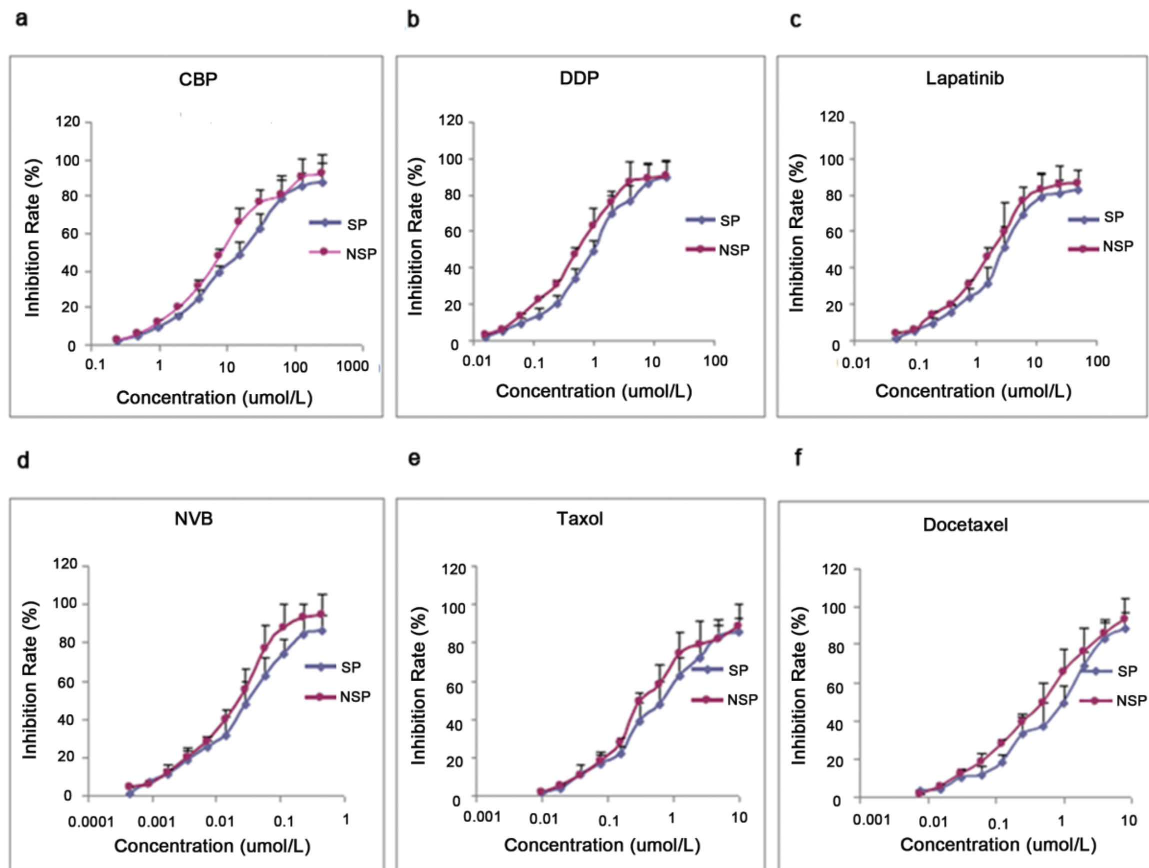

Figure 6 Sensitivity of side population (SP) and non-SP cells to anticancer drugs. (a) Carboplatin; (b) cisplatin; (c) lapatinib; (d) vinorelbine; (e) paclitaxel; (f) docetaxel. Data analysis is shown in Table 2.

highly expressed in SP cells of the A549 human lung cancer cell line under the usage of gene chip technology.[19] Ho *et al.* also reported that high expression of ABCG2 and MCM7 and MDR were found in SP cells isolated from H460, H23, HTB-58, A549, H441 and H2170 lung cancer cell lines, which were predominant in the G_0 phase.[15] Therefore, ABCG2 expression may serve as a stem cell marker based on the cancer stem cell hypothesis, not only in Glc-82 cells but also in other types of cells and could also be a target for therapeutic index. Our study is in keeping with published literature that supports the existence of SP cells in human lung cancer cell lines. Our study is consistent with those showing ABCG2 to be a molecular determinant of the SP phenotype. We found that the expression of ABCG2 protein was markedly higher in SP cells from the Glc-82 cell line, however, the percentage of SP cells is seven times more than that of ABCG2+ cells (14.5% vs. 2.5%). This may be caused by other ABC transporters. In agreement with this, we also found that SP cells showed resistance to multiple anticancer drugs, including: (i) generating antimicrotubules (paclitaxel and docetaxel); (ii) acting on DNA (carboplatin and cisplatin); (iii) inhibiting tubulin polymerization (vinorelbine); and (iv) being directed against EGFR-TKI (lapatinib). Several of these drugs, notably cisplatin, vinorelbine and paclitaxel, are conventionally used as first-line therapy for lung cancer. It is reasonable that the expression of ABCG2 may be an important protective mechanism for resistance of CSC to cytotoxic and targeted drugs.

Table 2 Statistical analysis of IC50 of the six anticancer drugs in side population (SP) and non-SP cells

	IC50 ± SD (μmol/L)		
Drugs	SP cells	Non-SP cells	*P*
Carboplatin	14.6 ± 0.620	9.08 ± 0.525	<0.001
Cisplatin	0.994 ± 0.092	0.561 ± 0.002	<0.002
Lapatinib	2.93 ± 0.474	1.97 ± 0.150	<0.001
Vinorelbine	0.030 ± 0.001	0.018 ± 0.002	<0.0001
Paclitaxel	0.664 ± 0.013	0.442 ± 0.094	<0.001
Docetaxel	0.833 ± 0.011	0.452 ± 0.068	<0.001

IC50, inhibit cell growth by 50%.

It has been reported that tumor recurrence and metastasis might be associated with CSC. For tumors in which CSC

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play a role, three possibilities exist. Firstly, the mutation of normal stem cells or progenitor cells into CSC can lead to the development of a primary tumor. Secondly, during chemotherapy, most of the primary tumor cells may be destroyed but CSC are not eradicated. They may perform refractoriness and lead to recurrence of the tumor. Thirdly, the CSC may emigrate to distal sites from the primary tumor and cause metastasis.[23] Theoretically, identification of the CSC may allow for the development of treatment modalities that target the CSC rather than rapidly dividing cells in cancer. Some molecular markers of CSC have been reported recently; however, none of them have been generally accepted. Membrane antigen CD133 has been found in brain,[24] hematopoietic,[4] prostate[25] and colon[26] CSC, and also exists in normal stem cells of different lineages.[27,28] Thus, some researchers regard CD133 as a marker for lung CSC,[29] although the tumorigenicity of CD133-positive and CD133-negative cells was reported to be comparable.[30] In support of this, our study showed that the expression of CD133 in SP cells was higher than that in non-SP cells. These findings suggest that CD133 might be useful molecular markers for lung CSC, although more relevant research is needed for confirmation.

In summary, our study demonstrates that the SP cells sorted from the Glc-82 cell line are a small subpopulation of potent tumorigenic cells with powerful capability of multilineage differentiation and self-renewal. They might be enriched with CSC marked by CD133 and the elevated expression of ABCG2 may be related to their resistance to multiple anticancer drugs. However, more research is still required to obtain a clearer picture. Nowadays, *in vitro* culture systems or *in vivo* graft of animal models are used to evaluate the therapeutic effect of traditional chemotherapeutic drugs and molecular targeted drugs, and the successful effectiveness of cell death probably occurs in drug sensitive non-SP cells which constitutes the majority of the cancer cell population. It is thus necessary to establish a more pure cell culture system and a clinically feasible evaluation model for SP cells for reliably predicting the effectiveness of therapeutic agents.[31]

Disclosure

The authors declare no conflict of interest.

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