·Cancer Stem Cell Column·

Biologic characteristics of the side population of human small cell lung cancer cell line H446

Bo Wang[1,2], Huan Yang[3], Yu-Zheng Huang[4], Ru-Hong Yan[3], Fen-Ju Liu[5], Jun-Ning Zhang[1]

[1] Department of Radiotherapy, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, P. R. China; [2] Department of Oncology, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215004, P. R. China; [3] Department of Clinical laboratory, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215004, P. R. China; [4] Jiangsu institute of parasitic diseases, Wuxi, Jiangsu 214064, P. R. China; [5] School of Radiation Medicine and Public Health, Soochow University, Suzhou, Jiangsu 215123, P. R. China

[Abstract] Background and Objective: Recently, the theory of cancer stem cells (CSCs) has presented new targets and orientations for tumor therapy. The major difficulties in researching CSCs include their isolation and purification. The aim of this study is to identify and characterize the side population (SP) cells in small cell lung cancer (SCLC) cell line H446, which lays the foundation for the isolation and purification of CSCs. **Methods:** Fluorescence-activated cell sorting (FACS) was used to sort SP and non-SP (NSP) cells from H446. Both subgroups were cultivated to survey the capacity to form into suspended tumor cell spheres. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR were used to evaluate the expression levels of the mRNA of CD133, ABCG2, and nucleostemin in both subgroups. The capacity of proliferation and the differences in drug resistance of both subgroups and unsorted cells were tested by the MTT method. The differentiation ability of both subgroups was determined by FACS. Proliferation was determined by subcutaneous tumor formation in nude mice. **Results:** The percent of Hoechst 33342 negative cells was about (5.1 ± 0.2)% in H446 by fluorescence microscopy. The percent of SP cells was (6.3 ± 0.1)% by flow cytometry. SP cells had a stronger capability of forming into tumor spheres than NSP cells. The mRNA expression levels of ABCG2, CD133, and nucleostemin in SP cells were 21.60 ± 0.26, 7.10 ± 0.14, and 1.02 ± 0.08 folds higher than that in NSP cells ($P < 0.01$, $P < 0.01$, and $P > 0.05$, respectively). In vivo, SP cells showed better proliferative ability and tougher viability when treated with drugs. SP cells can differentiate into NSP cells, but NSP cells cannot differentiate into SP cells. SP cells had a greater ability to form tumors. **Conclusion:** The H446 cell line contained some SP cells with stem cell properties. CD133 and ABCG2 may be cancer stem cell markers of SCLC.

Key words: Small cell lung cancer, side population, tumor stem cells, CD133, ABCG2

In recent years, Goodell *et al.*[1] discovered that there was a small population of cells with relatively weak staining when marrow cells were stained with Hoechst 33342 DNA dye and further assayed by flow cytometry. This population of cells had the abilities of self-renewal and differentiation to a certain degree and had stem-cell-like characteristics. This small population of cells with relatively weak staining was referred to as side population cells (SP cells). SP cells have been found in many adult tissues, in embryos, and in tumor cells[2]. High expressions of the molecular markers of cancer stem cells (CSCs) and ABCG2/BCRP1 are often found in SP cells. In addition, SP cells have strong oncogenic abilities similar to CSCs. Also, SP cells have strong resistance to various chemotherapeutic drugs.

Patients with small cell lung cancer (SCLC) account for 20%–25% of the total cases of lung cancer. Although SCLC is sensitive to chemotherapy and radiotherapy, recurrence and distant metastasis easily occur. The 5-year survival rates of patients with limited-stage SCLC and extensive SCLC have been reported as only 7%[3] and 1%[4], respectively. In the CSC theory put forth in recent years, very few CSCs lurking in most tumors are considered to be the roots of tumor recurrence and metastasis[5]. Currently, most researchers believe that SP cells contain plenty CSCs, so CSCs could be investigated through SP cells where the molecular markers of CSC is otherwise unknown[6]. This study sorted SP cells from the human SCLC cell line H446 and identified their stem-cell-like characteristics. The molecular markers of SCLC stem cells were also explored.

Correspondence to: Jun-Ning Zhang; Tel: +86-512-67780388;
Email: zhangjunning@gmail.com

This paper was translated from Chinese into English by *CJC* Medical Translation and edited by Hope J. Lafferty on 2009-11-03.

The Chinese version of this paper is avaiable at http://www.cjcsysu.cn/cn/article.asp?id=16313.

Received: 2009-06-30; Accepted: 2009-12-28

Grant: National Natural Science Foundation of China (No. 30870585)

Materials and Methods

Materials

Reagents RPMI-1640 medium, fetal bovine serum, and TRIzol were purchased from GIBCO. Recombinant epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were provided by PeproTech EC. RPMI-1640 medium containing 15% fetal bovine serum was used as a serum-supplemented medium (SSM). Fetal bovine serum-free RPMI-1640 medium containing 20 ng/mL EGF and 10 ng/mL bFGF was used as a serum-free medium (SFM). Hoechst 33342, PI, and MTT assays were purchased from Sigma-Aldrich. Verapamil was the product of Tocris Bioscience. The 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was provided by Dojindo Molecular Technologies, Inc. Reverse transcription kits were the product of the Promega Corporation. PCR primers were purchased from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Real-time PCR 50-μL lyophilized powder was provided by Bioneer China.

Instruments Instruments mainly included a FACS Aria II flow cytometer (BD Biosciences), superclean bench (Air Tech Co. Ltd), CO_2 incubators (Thermo Scientific), a fluorescence microscope (Leica Microsystems), PCR instruments (Eppendorf), an Exicycler real-time PCR instruments (Bioneer China), and an inverted microscope (Olympus Corporation).

Cell lines Human SCLC cell line H446 was purchased from the cell bank at the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (CAS).

Experimental animals A total of 36 nude mice (male, 4–6 weeks old, 18–20 g) were purchased from the Shanghai Laboratory Animal Center of CAS [certificate number: SCXK (HU) 2007-0005].

Methods

Observation of Hoechst 33342 staining by fluorescence microscope H446 cells in the logarithmic phase were selected. After the original culture medium was discarded from the culture dish, 2 mL RPMI-1640 medium containing 2% fetal bovine serum was added. Then, Hoechst 33342 was added at a final concentration of 5 μg/mL and incubated at 37℃ for 90 min. After cells were rinsed with phosphate buffered saline (PBS) twice, 1 mL RPMI-1640 medium containing 2% fetal bovine serum was added. A volume of 5 mmol/L CFSE, which was dissolved in dimethyl sulfoxide (DMSO), was diluted with PBS to a concentration of 5 mmol/L. Then, 1 mL of the diluted CFSE was added to the culture dish and incubated at 37℃ for 15 min. After the cells were rinsed with PBS containing 2% fetal bovine serum cooled at 4℃ twice, the cells were observed under an inverted fluorescence microscope . Hoechst 33342 stained negative cells had no or relatively weak blue fluorescence. Five visual fields (×100) were randomly selected, and 100 cells were counted in each field. Finally, the percent of Hoechst 33342 negative cells was calculated according to the following formula:

Percent of Hoechst 33342 negative cells = number of Hoechst 33342 negative cells / number of CFSE positive cells × 100%.

Sorting and purity detection by flow cytometry Cells in the logarithmic phase were selected to prepare the single cell suspension at a density of 1×10^6 cells/mL. The experimental and control tubes were set up. Verapamil was added into the control tube at a final concentration of 100 μg/mL and incubated at 37℃ for 20 min in a water bath. Then, to both the experimental and control tubes, Hoechst 33342 was added at a final concentration of 5 μg/mL. Cells were incubated at 37℃ for 90 min in a water bath, shaken and mixed once every 15 min. Subsequently, cells were centrifuged at 4℃ for 5 min to discard the supernatant fluid. After rinsing with cooled PBS containing 2% fetal bovine serum twice, cells were resuspended in cooled PBS containing 2% fetal bovine serum. PI was added at a final concentration of 1 μg/mL 30 min before detection to remove the interference of dead cells. Finally, SP and NSP subpopulations were sorted by flow cytometer. The entire experiment was carried out in a dark room. Some SP and NSP cells were immediately sorted again to perform purity detection.

Serum-free culture The sorted SP and NSP cells were first cultured with SSM. SP and NSP cells in the logarithmic phase were collected through rinsing with PBS and trypsinization, and then suspended in SFM. Subsequently, SP and NSP cells were seeded in SFM in culture flasks (1:1 inoculation), and then cultured at 37℃ in an incubator with 5% CO_2. Finally, the formation of tumor cell spheres was observed.

RT-PCR and quantitative PCR Total RNA was extracted from the 1×10^6 sorted SP and NSP cells using TRIzol. An amount of 5 μg of total RNA was used to perform the reverse transcription reaction using the reverse transcription kit according to manufacturer instructions. PCR was carried out in a total volume of 20 μL comprising 10 μL 2 × PCR mix, 1.0 μL cDNA template, 1.0 μL forward primer (10 μmol/L), 1.0 μL reverse primer (10 μmol/L), and 7 μL double distilled water (ddH_2O). PCR was performed as follows: denaturing at 94℃ for 5 min, followed by 35 cycles at 94℃ for 30 s, annealing at 55℃ for 30 s (suitable for ABCG2, nucleostemin, CD133, and GAPDH genes), extension at 72℃ for 30 s, and then a final extension at 72℃ for 10 min. Electrophoresis was performed on 8 μL of the amplified products in 1%–2% agarose gel at a constant voltage of 80 V for 30 min. After the gel was stained for 10 min using ethidium bromide (EB), the detection and photographing were performed through a gel imaging system. Primers were designed using the Primer Premier 5.0 software, and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Primer sequences are listed in Table 1. The quantitative PCR was carried out in a total volume of 50 μL comprising the matching real-time PCR lyophilized powder, 1.0 μL cDNA template (1:100 dilution), 2.0 μL forward primer (10 μmol/L), 2.0 μL reverse primer (10 μmol/L), and 45 μL water treated with diethyl pyrocarbonate (DEPC-H_2O). Quantitative PCR was performed as follows: denaturing at 94℃ for 5 min, followed by 40 cycles at 94℃ for 30 s, annealing at 55℃ for 30 s (suitable for CD133, ABCG2, nucleostemin, and GAPDH genes), extension at 72℃ for 30 s and fluorescence collection, and then plotting of the melting curve s at 60℃. The $2^{-\Delta\Delta CT}$ method[7] was

Table 1 Primer sequences of CD133, ABCG2, nucleostemin, and GAPDH genes

Gene	Primer sequence (5′→3′)	Amplification length (bp)
CD133	For: CTGGGGCTGCTGTTTATTATTCTG	337
	Rev: ACGCCTTGTCCTTGGTAGTGTTG	
ABCG2	For: ATGAAACCTGGTCTCAACG	317
	Rev: GAGTCTGCCACTTTATCCA	
Nucleostemin	For: GTTCCAAACAGTGCTCCCT	305
	Rev: CTGGCATCCAACACCTCTA	
GAPDH	For: GGATTTGGTCGTATTGGG	205
	Rev: GGAAGATGGTGATGGGATT	

used to analyze the cycle threshold (*C*t) values of the target and internal control gene s in SP and NSP cells. The data in the NSP cell group were normalized as 1, and the relative differences of expression of each gene in SP and NSP cells were calculated.

Cell proliferation SP, NSP, and unsorted (TOTAL) cells were seeded into a 96-well plate at a density of 1000 cells/well, and 5 duplicate wells were set up for each group. In the blank control group, only complete medium was added. Nine duplicate 96-well plates were set up. Cells were cultured for 1–9 days, and one plate per day was used to perform the MTT assay. In the MTT assay, 20 μL MTT (5 mg/mL) was added and incubated at 37℃ for 4 h. Then, 150 μL DMSO was added, and the plate was gently shaken at room temperature for 30 min. Finally, the absorbance (*A*) value at 570 nm was measured on a fully automated enzyme-linked fluorescent immunoassay meter. The mean value of *A* was calculated, and the value of the blank control was subtracted from the mean *A*. The cell growth rate was calculated according to *A* value in each group of cells per day. Cell growth rate in no. *n* day = *A* value on no. *n* day / × *A* value on the first day × 100%. The cell growth curve was plotted according to the rate of cell growth (*y* axis) at the different times (*x* axis).

Detection of differences in drug resistance SP, NSP, and TOTAL cells were seeded into a 96-well plate at a density of 600 cells/well, and 5 duplicate wells were set up for each group. In the control group, only complete medium was added. In the drug-treated groups, cisplatin was added at a final concentration of 0.2 PPC (plasma peak concentration). At the same time, groups without the drug were set up with the same number of cells. After a 2-week cell culture, MTT assay was performed to determine *A* value at 570 nm (A_{570}), and A_{570} in groups without drug treatment was used as the control. The cell proliferation inhibition rate (CPIR) was calculated according to the following formula:

CPIR = (1 – mean *A* value in experimental group / mean *A* value in control group) × 100%.

Determination of differentiation ability of SP and NSP cells SP and NSP cells were collected after a 10-day cell culture in vitro. Then the single cell suspension of SP and NSP cells was prepared. After redyeing with Hoechst 33342, the percent of SP cells in SP and NSP cells was assayed by flow cytometry.

Analysis of tumorigenic ability in vivo The 36 nude mice were randomly divided into 6 groups. After the single cell suspension of the SP and NSP cells was prepared, 1×10^3, 1×10^4, and 1×10^5 SP and NSP cells were injected into the subcutaneous tissue of the right axilla. Tumorigenic status was observed every day. Also, tumorigenic time was recorded. The mice were killed after 10 weeks and the rate of tumor formation was observed. The longest diameter (a) and the shortest diameter (b) of the tumor body were measured using a Vernier caliper, and the tumor volume was calculated according to the following formula:

Tumor volume (V, mm^3) = $ab^2\pi/6$. Finally, the tumor was weighed.

Statistical analysis

All experiments were repeated more than three times. Data were presented as mean ± standard deviation (SD). The means between groups were compared by *t* test using Graphpad Prism software. Statistical significance was considered if $P < 0.05$.

Results

Hoechst 33342 negative cells observed under fluorescence microscope

Under fluorescence microscope , CFSE-stained cytoplasm and nuclei of all H446 cells showed green fluorescence. In addition, most of the Hoechst 33342-stained nuclei showed blue fluorescence, and a small portion of nuclei had no or relatively weak blue fluorescence. Through cell counting, the percent of Hoechst 33342 negative cells was about (5.1 ± 0.2)% (Figure 1).

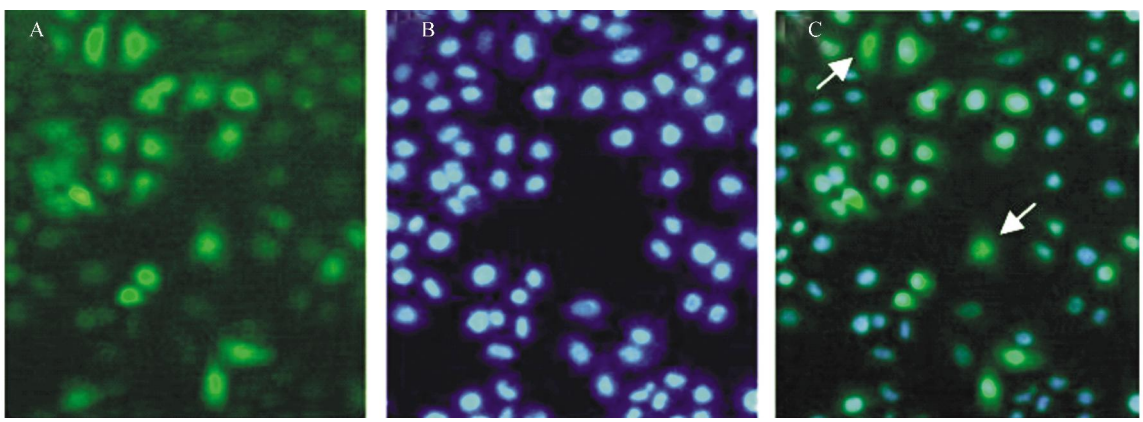

Figure 1 Observation of negative-stained cells in H446 with Hoechst 33342 by fluorescence microscope (×100)
A, all nuclei and cytoplasm counterstained green with CFSF. B, H446 nuclei stained blue with Hoechst 33342. C: A and B merged.

SP cells in H446 cell line detected by FACS

FACS detection results showed that SP cells existed in the H446 cell line, and the percent of SP cells was (6.3 ± 0.1)% (Figure 2A). After ABCG2 was blocked by verapamil, the percent of SP cells decreased to (0.1 ± 0.0)% (Figure 2B). The sorting purity of SP and NSP cells was (98.4 ± 0.1)% (Figure 2C) and (97.6 ± 0.1)% (Figure 2D), respectively. There was no significant morphologic difference between the sorted SP and NSP cells. Finally, the sorted SP and NSP cells were collected for further study under sterile conditions.

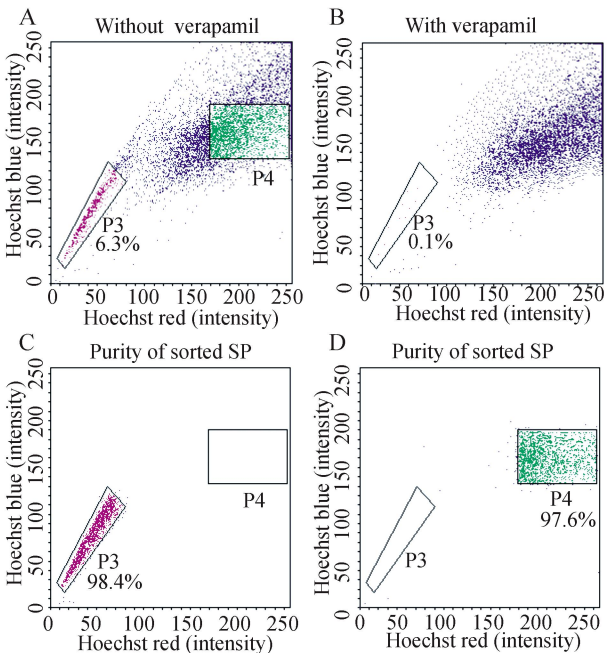

Figure 2 Analysis of side population (SP) cells among H446 cells by fluorescence-activated cell sorting (FACS)

A, hoechst 33342; B, verapamil + Hoechst 33342; C, purity of sorted SP cells; D, purity of sorted NSP cells.

Forming into suspended tumor cell spheres

SP cells had stronger capability of forming into suspended tumor cell spheres in SFM than NSP cells. After the 7-day cell culture, SP and NSP cells could both form into suspended tumor cell spheres in SFM. However, the spheres formed by SP cells were more compact (Figure 3A), and the spheres formed by NSP cells were loose and showed an irregular shape (Figure 3B).

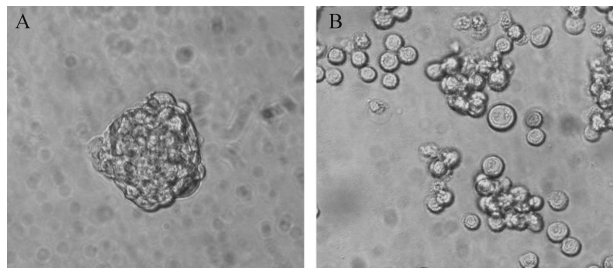

Figure 3 The growth of SP and NSP cells in serum-free medium (SFM) (×200)

A, suspended tumor cell spheres in SP cells; B, suspended tumor cell spheres in NSP cells.

Expressions of CD133 and ABCG2

The results of RT-PCR showed that the expressions of both CD133 and ABCG2 were high in SP cells and low in NSP cells, and the expression of nucleostemin was high in both SP and NSP cells (Figure 4A). The quantitative fluorescent PCR results suggested that the expression levels of ABCG2, CD133, and nucleostemin mRNA in SP cells were 21.60 ± 0.26, 7.10 ± 0.14, and 1.02 ± 0.08 times, respectively, of those in NSP cells ($P < 0.01$, $P < 0.01$, and $P > 0.05$) (Figure 4B). The peaks of the melting curve s of ABCG2, CD133, nucleostemin, and GAPDH all occurred at the same position, which suggests that the quantitative PCR amplification reaction was specific, and no nonspecific bands were amplified. A part of the amplification and melting curves of CD133 and GAPDH in the quantitative fluorescent PCR in SP and NSP cells is shown in Figures 4C and 4D.

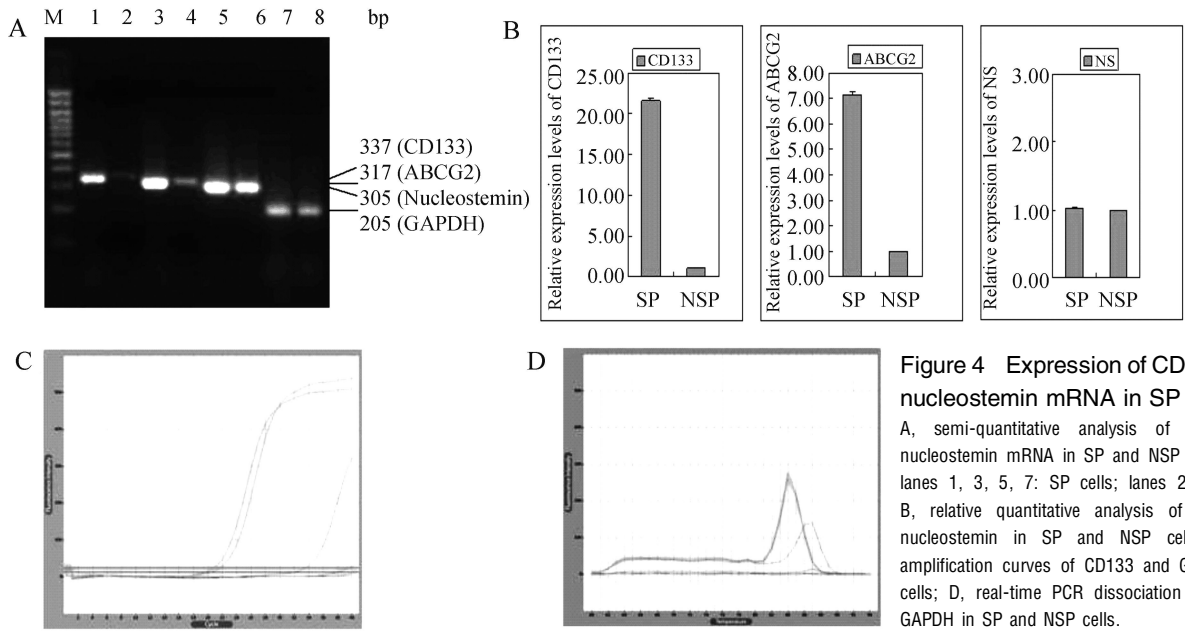

Figure 4 Expression of CD133, ABCG2, and nucleostemin mRNA in SP and NSP cells

A, semi-quantitative analysis of CD133, ABCG2, and nucleostemin mRNA in SP and NSP cells. Lane M, marker; lanes 1, 3, 5, 7: SP cells; lanes 2, 4, 6, 8: NSP cells. B, relative quantitative analysis of CD133, ABCG2, and nucleostemin in SP and NSP cells. C, real-time PCR amplification curves of CD133 and GAPDH in SP and NSP cells; D, real-time PCR dissociation curves of CD133 and GAPDH in SP and NSP cells.

In vitro proliferative ability

In vitro proliferative ability of SP cells was significantly stronger than either NSP or TOTAL cells. The cell growth curve was plotted according to the rate of cell growth (*y* axis) at different time (*x* axis). The proliferative ability in the SP cell group was significantly stronger than the NSP and TOTAL cell groups ($P < 0.01$) (Figure 5).

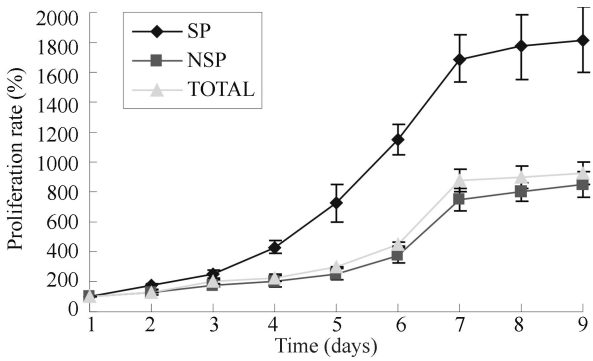

Figure 5 Proliferation curves of SP, NSP, and TOTAL cells

Cell viability with in-vitro drug-resistance

The viability of SP cells with in-vitro drug-resistance was significantly stronger than those of NSP and TOTAL cells. MTT assay results showed that the CPIR of SP, NSP, and TOTAL cells was (32.0 ± 0.8)%, (67.3 ± 1.6)%, and (66.5 ± 1.5)%, respectively. Moreover, the CPIR of SP cells was significantly lower than that of NSP and TOTAL cells ($P < 0.01$).

In-vitro differentiation

SP cells could differentiate into NSP cells in vitro, but NSP cells could not differentiate into SP cells. After the sorted SP and NSP cells were cultured in vitro for 10 days, the cells were stained with Hoechst 33342 again and assayed by flow cytometry. The results showed that the percent of NSP cells in the SP cell group was (20.6 ± 0.1)%, but the percent of SP cells in the NSP cell group was only (0.3 ± 0.0)%. Therefore, it was demonstrated that SP cells could differentiate into NSP cells, but NSP cells could not differentiate into SP cells (Figure 6).

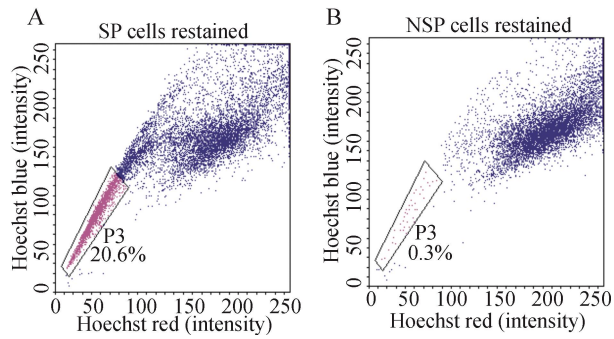

Figure 6 Differentiation of SP (A) and NSP (B) cells

Oncogenicity in SP cells

Accidental death of the mice did not occur during the experiment in any group. The oncogenic time, tumor formation rate, tumor volume, and mouse weight for each group are shown in Table 2. A significant difference in oncogenic ability was found between SP and NSP cells ($P < 0.01$). Tumors could be formed with only 1×10^3 SP cells but needed at least 1×10^5 NSP cells. Moreover, the volume and weight of the tumors formed by 1×10^5 SP cells were both significantly bigger than those formed by 1×10^5 NSP cells. In addition, there was not a significant difference of oncogenic time between SP and NSP cells ($P > 0.05$).

Table 2 Comparison of tumorigenesis in vivo between SP cells and NSP cells

Cell numbers injected	Tumor formation time (days)		Tumorigenesis rate		Tumor volume (mm^3)		Tumor weight (g)	
	SP	NSP	SP	NSP	SP	NSP	SP	NSP
1×10^3	37.5 ± 4.2a	–	2/6[a]	0/6	27.0 ± 3.6a	–	0.14 ± 0.03a	–
1×10^4	30.1 ± 5.5a	–	5/6[a]	0/6	190.0 ± 54.7[a]	–	0.95 ± 0.29a	–
1×10^5	28.7 ± 4.0b	32.9 ± 5.7	6/6[a]	4/6	962.0 ± 267.2[a]	154 ± 41.5	4.87 ± 1.31a	0.75 ± 0.20

[a] $P < 0.01$, [b] $P > 0.05$, vs. NSP cells.

Discussion

Currently, the most authoritative method for sorting CSCs is by first identifying the specific markers of CSCs. Yet, in most cancers, CSCs cannot be sorted by this method because specific markers have not been discovered. However, FACS is a new research approach that can be used to investigate CSCs through the sorting of SP cells without requiring specific markers[6].

Researchers have already sorted SP cells and have further demonstrated their stem-cell-like characteristics in many kinds of cancer cell lines and tissue, including central nervous system cancers[8], ovarian cancer[9], melanoma[10], nasopharyngeal cancer[6], pancreatic cancer[11], and so on. In the present study, SP cells were sorted from human SCLC cell line H446 by flow cytometry, and their biologic characteristics were also detected in vitro, which provide an experimental basis for the further investigation of lung cancer stem cells.

In the present study, SP cells were detected by two methods. First, SP cells were stained with Hoechst 33342, and the characteristics of the SP cells were observed under a fluorescence microscope after redyeing with CFSE. According to Goodell *et al.* [1], nucleus staining with Hoechst 33342 could generate blue fluorescence with various intensities, and the cells

with no or weak staining were SP cells. In Figure 1, nuclei show blue fluorescence with varied intensity, and a minority of nuclei had no or weak staining. Second, FACS was used to detect and sort SP cells. The related principle here is that Hoechst 33342 can generate both blue and red fluorescence under the excitation of ultraviolet light[1]. Moreover, blue and red fluorescence can be simultaneously detected in the analysis of SP cells, and the SP cells can be observed within a two-variable scatter diagram. Comparing the two methods, observing SP cells by fluorescence microscopy was found to give a strong visual impression. However, only one kind of fluorescence was observed with fluorescence microscopy, and the observation could be affected by exposure time. Therefore, the specificity of the observation of SP cells by fluorescence microscopy was inferior to detection of SP cells by FACS.

Currently, it is generally considered that an indivisible relationship exists between the origin of CSCs and normal stem cells[12]. In the present study, basic stem-cell-like characteristics of the sorted SP cells were verified through a series of experiments, including plotting the growth curve, detecting the difference in drug resistance, determining differentiation ability, tumor formation in mice, and so on. The results suggested that SP cells not only had high proliferative activity, strong drug resistance, and multidirectional differentiation abilities in vitro, but also had strong oncogenic abilities in vivo. Moreover, we detected the expressions of stem cells genes CD133, ABCG2, and nucleostemin in SP and NSP cells. CD133 has been reported as a CSCs marker of many types of solid tumors, such as medulloblastoma, colon cancer, and others[13,14]. ATP-binding cassette superfamily G member2 (ABCG2), also called breast cancer resistance protein (BCRP) or mitoxantrone resistance-associated protein (MXR), is a membrane transport protein associated with multidrug resistance, which was separated and identified from human cancer cell line MCF-7 in 1998[15]. The expression of ABCG2 was found on the surface of multiple-source stem cell membranes, but could not be found in most mature cells. At present, ABCG2 is generally considered the determinant of stem cells. The high expression of ABCG2 has been reported to exist in SP cells in breast cancer and glioma cell lines. In addition, the percent of SP cells increased after SP cells were cultured with mitoxantrone. This drug-selection effect may increase the percent of SP cells in tumor cells of patients, which further leads to drug resistance. Therefore, ABCG2 may become a target for cancer therapy[16]. In the present study, significant differences in the expressions of CD133 and ABCG2 between SP and NSP cells were demonstrated, which suggests that CD133 and ABCG2 might be relatively specific molecular markers of human SCLC stem cells.

In addition, CSCs can be induced to form cell aggregates with suspended growth through many kinds of cytokines, including epidermal growth factor (EGF), in serum-free cultures. These cell aggregates are called tumor cell spheres[17]. In the present study, the sorted SP cells were cultured in a serum-free culture medium, and SP cells were found to have stronger capabilities of forming into tumor cell spheres than NSP cells. This phenomenon also suggests that the sorted SP cells contained plenty of CSCs.

Sorting SP cells by FACS is an effective method in studies on CSCs. However, this method has limitations. The main limitation is that SP cells cannot completely reflect the characteristics of CSCs. Although much research has shown that SP cells and stem cells have consistent molecular phenotypes and characteristics, some experiments have not confirmed this. For example, Triel *et al.*[18] demonstrated that SP cells separated from tissue did not always have the characteristics of stem cells. Therefore, the SP cells sorted by FACS are required to be further purified. In the present study, it was suggested that SCLC stem cells may be further purified by FACS sorting combined with CD133 and ABCG2 screening.

In summary, we detected and sorted the SP cells with characteristics of stem cells in SCLC cell line H446. Moreover, we also investigated some of the biologic characteristics of SP cells, which provided an important experimental basis for studies on the origins of SCLC and possible therapies.

References

[1] Goodell MA, Brose K,Paradis G, et al. Isolation and functiona properfies of murine hematopoietic stem cells that are replicating in vivo [J]. J Exp Med, 1996,183(4):1797-1806.

[2] Wang N, Chen L. Advances in the relationship between side population analysis and cancer stem cells [J]. World J Digestol, 2008,25(2):199-202. [in Chinese]

[3] Carbone DP. The biology of lung cancer [J]. Semin Oncol, 1997,24(4): 388-401.

[4] Zhou JC. Practical Medical Oncology [M]. 2nd Edition. Beijing: People's Medical Publishing House, 2003:537-555. [in Chinese]

[5] Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancers tem cells [J]. Nature, 2001,414(6859):105-111.

[6] Wang J , Guo LP , Chen LZ , et al. Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line [J]. Cancer Res, 2007,67(8):3716-3724.

[7] Lival KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 [Delta Delta C (T)] method [J]. Methods, 2001,25(4):402-408.

[8] Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells [J]. Nature, 2004,432(7015):396-401.

[9] Bapai SA, Mali AM, Koppikar CB, et al. Stem and progenitor-like cells contribute to the a ggressive behave of human epithelial ovarian cancer [J]. Cancer Res, 2005,65(8):3025-3029.

[10] Grichnik JM, Burch J A, Schulteis RD, et al. Melanoma, a tumor based on a mutant stem cell [J]. J Invest Dermatol, 2006,126(1):142-153.

[11] Olempska M, Eisenach, Ammerpohl O, et al. Detection of tumor stem cell markers in pancreatic carcinoma cell lines [J]. HePatobiliary pancreat Dis Int, 2007,6(1):92-97.

[12] Chen H, Huang Q, Dong J, et al. Cancer initiating cell theory: popularity and controversies [J]. Chin J Cancer, 2006,25(6):779-784. [in Chinese]

[13] Li MC, Deng YW, Wu J, et al. Isolation and characterizations of brain tumor stem cells in human medulloblastoma [J]. Chin J Cancer, 2006,25 (2):241-246. [in Chinese]

[14] Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells [J]. Nature, 2007,445 (7123):111-115.

[15] Doyle LA, Yang W, Abruzzo LV, et a1. A mutidrug resistance transporter from human MCF-7 breast cancer cells [J]. Proc Natl Acad Sci USA,

1998,95(26):15665-15670.

[16] Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, et al. Ovarian cancer aide population defines cells with stem cell-like character-iatics end Mullerian inhibiting substance responsiveness [J]. Pros Nad Acad Sci USA, 2006,103(30):11154-11159.

[17] Singh SK, Clarke ID, Terassaki M, et al. Identilication of a cancer stem cell in human brain tumors [J].Cancer Res,2003,63(18):5821-5828.

[18] Triel C, Vestergaard ME, Bolund L, et al. Side PoPulation cells in human and mouse epidermis lack stem cell characteristics [J]. Exp Cel Res, 2004,295(5):79-90.