

# Preliminary analysis of stem cell-like cells in human neuroblastoma

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**Background:** Neuroblastoma is an embryonic neoplasm originating from the neural crest with cellular heterogeneity as one of its oncobiological characteristics. This study was undertaken to determine whether human neuroblastoma contains stem cell-like cells.

**Methods:** Twenty patients with neuroblastoma who have been treated in our hospital since January 2005 were divided into pre-operative chemotherapy (10 patients) and non-chemotherapy (10) groups. Tumor specimens of the patients were taken and paraffin sections were made. The expressions of stem cell markers CD133, ABCG2, CD117 and nestin were immunohistochemically detected in the specimens. Neuroblastoma cells were stained with Hoechst 33342 and PI. The side population (SP) cells were analyzed by the fluorescence-activated cell sorter. The disparity drug resistance to cisplatin (DDP) of SP and non-SP cells was measured with MTT colorimetric assay. The oncogenicity of SP and non-SP cells was identified in nude mice.

**Results:** There was no significant difference in the expression intensity of CD117 and nestin between the two groups of specimens ( $P>0.05$ ). There was a significant difference between the two groups in terms of the expression intensity of CD133 and ABCG2 ( $P<0.05$ ). The SP cells accounted for 0.2%-1.3% of the total human neuroblastoma cells and were decreased to 0.1%-0.5% after verapamil treatment. The SP and non-SP cells showed disparity in cell growth experiment and drug resistance to DDP. Oncogenicity experiment revealed that nude mice could erupt tumor by an injection of  $1 \times 10^6$  SH-SY5Y and WIV SP cells. However, the nude mice could not form tumor by an injection of  $1 \times 10^6$  non-SP cells.

**Conclusion:** Neuroblastoma might contain cancer stem cell-like cells.

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**Key words:** drug resistance; immunohistochemically; neuroblastoma; oncogenicity; side population cells

## Introduction

The cancer stem cell (CSC) theory proposes that the cells within a single tumor are heterogeneous. It is thought that CSCs possess self-renewal, multiple differentiation, and high malignancy.<sup>[1]</sup> Neuroblastoma (NB) is an embryonic neoplasm originating from the neural crest (NC) with cellular heterogeneity as one of its oncobiological characteristics. Recent studies have indicated that cancer stem cells in the tissues and cell lines of NB are closely correlated with the high malignancy of NB. The CSCs have been analyzed using a couple of methods. Surface markers expressed on cancer stem-like cells can be used to isolate these cells. Side population (SP) analysis is another popular method that is used to isolate CSCs. It exploits the efficient efflux of Hoechst 33342 dye from the stem cells to separate the Hoechst-negative cells using flowcytometry (FCM). These Hoechst-negative cells (SP cells) are enriched for stem cells.<sup>[2]</sup> Therefore, SP analysis is suitable for the development of future treatments. The ABCG2 transporter contributes to the exclusion of Hoechst in the SP cells and could be used as a marker to identify the SP cells.<sup>[3]</sup>

To demonstrate the existence of neuroblastoma stem-like cells, we used immunohistochemistry to examine the expression of CD133, ABCG2, CD117, and nestin markers for the stem cell properties in neuroblastoma tissues before and after chemotherapy. The SP cells from the human neuroblastoma SH-SY5Y and WIV cell lines were sorted and the CSC characteristics of the SP cells were systematically identified.

## Methods

### Clinical samples

The patients who had undergone a curative surgery for

neuroblastoma between January 2005 and June 2010 in Xin Hua Hospital of Shanghai Jiao Tong University were included in this study. Specimens were taken from the patients, formalin fixed, and paraffin embedded for analysis during the operation. Ten patients who received pre-operative A/B regimen chemotherapy were compared with another 10 patients who were not treated pre-operatively by chemotherapy.

Data on clinicopathological features, including age, sex, histological subtypes, and pathological stages were obtained from the medical records (Table 1). The study design and procedure involving collection of tissue sample were reviewed and approved by the ethics board of the Xin Hua Hospital.

### Cell lines and animals

The human neuroblastoma SH-SY5Y (from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) and WIV cell lines (presented by Children Cancer Laboratory of University of Chicago) were cultured in DEME/F-12 medium (Gibco, United Kingdom), containing 5% fetal bovine serum (FBS) and were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Four-week-old athymic nude mice (BALB/c-nu/nu) (from Shanghai Slac Laboratory Animal Co., Ltd, Shanghai, China) were housed in laminar-flow cabinets under specific pathogen-free conditions. Animal care and experimental protocols were performed in accordance with the procedures and guidelines established by the Shanghai Medical Experimental Animal Care Commission.

### Immunohistochemistry

Tumor paraffin-embedded blocks were selected and collected. Immunohistochemical studies were conducted using the two-step Super Vision System Kit (Chang Dao, Shanghai, China). The sections of the samples were incubated with ABCG2 (1:200; R&D, USA) CD133 (1:150; Sigma Aldrich, USA), CD117 (1:50; Abcam, USA) and nestin (1:200; Abcam, USA) for 24 hours at 4°C. As for negative controls, phosphate buffer saline (PBS) was used instead of primary antibodies. As for positive controls, known-positive samples were used. Finally, the number of positive cells in 1000 tumor cells within 5-10 microscopic fields at 400×magnification was counted.

**Table 1.** Cell proliferation inhibition rate of different concentrations of cisplatin on SH-SY5Y cells (mean±standard deviation)

Cells	5 μmol/L	10 μmol/L	40 μmol/L
SP	(54.37±1.20)%*	(61.73±3.06)%*	(94.62±2.86)%
Non-SP	(57.13±2.39)%	(65.11±2.81)%	(96.10±3.01)%

\*:  $P < 0.05$ , comparison of side population (SP) and non-SP cells under the same concentration.

### FCM analysis and sorting of SP cells

Exponential growth cells were suspended at  $1 \times 10^6$  to  $1 \times 10^7$  cells per milliliter in DEME/F-12 medium with 2% FBS. The cells were further stained with 5 μmol/L of Hoechst (Sigma, USA) at 37°C in the presence or absence of 50 μmol/L verapamil (Sigma, USA) for a period of 60 minutes. Samples were then washed, centrifuged, and resuspended in the cold DEME/F-12 medium, after which 1 μg/mL propidium iodide (Sigma, USA) was added. FCM analysis and sorting of SP cells were performed using a BD FACS Aria II flowcytometer (BD, USA) as previously described.<sup>[3]</sup>

### FCM differentiation study

After sorting, the SP and non-SP (NSP) cells were cultured in DEME/F-12 medium for two weeks. The cells were reanalyzed by FCM in order to quantify the proportion of SP cells and to determine the differentiation capacity of the two subpopulations. All experiments were performed in triplicate.

### Immunofluorescence

The cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature and were incubated with the primary antibodies, ABCG2 (R&D, USA) and CD133 (Sigma, USA), at 4°C overnight. Antigens were localized by using the goat anti-rabbit second antibody conjugated to TRITC or FITC (Boster Inc., China) for ABCG2 or CD133, respectively.

### Reverse transcription PCR (RT-PCR)

Total RNA was isolated from SP and non-SP cells using TRIzol reagent (Invitrogen, USA) and was reverse-transcribed using a reverse transcription system (Takara, Japan) according to the manufacturer's protocol. PCR amplification of different genes was performed using EXTaq polymerase (Takara, Japan). The cycle was set at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 50-57°C for 30 seconds, 72°C for 30 seconds and extension at 72°C for 10 minutes. The primers used for the RT-PCR included 5'-CGGGTGACTCATCCCAACAT-3' and 5'-CAGGATCTCAGGATGCGTGC-3' for ABCG2, and 5'-CCTGTTATGACAAGCCATCACAA-3' and 5'-CGCAGGTTTCTCTATGAT-3' for CD133.

### Proliferation assay

SP and NSP cells were grown in the 96-well plates and their relative cell number was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) according to the manufacturer's protocol. The absorbance value was then measured at 490 nm using the enzyme-labeled instrument (Bio-Rad, USA).

### Drug resistance assay

Drug resistance was assessed by MTT assay. Briefly, cells were grown in 96-well plates in the presence of cisplatin (DDP) (Qilu Pharmaceutical Company, China). After three days, MTT dye was added to each well and the plates were incubated for an additional four hours. Following the medium in the wells was aspirated. DMSO (Sigma, USA) was added into each well and the plates were shaken for five minutes. The absorbance was then recorded by enzyme-labeled instrument for each well at 490 nm. The cell proliferation inhibition rate (CPIR) was calculated using the following formula:

$$\text{CPIR} = \left(1 - \frac{\text{absorbance of experimental well}}{\text{Absorbance of control well}}\right) \times 100\%$$

### In vivo tumorigenicity assay

Freshly sorted SP and non-SP cells in the aliquots ranging from  $1 \times 10^2$  to  $1 \times 10^6$  cells were suspended in 200  $\mu\text{L}$  of PBS, and were subcutaneously injected into the hind flanks of the mice anesthetized with chloral hydrate. The mice were euthanized after eight weeks and the presence of nodules was confirmed by necropsy. Paraffin-embedded tumor blocks were prepared for further analysis. Each group in the tumor initiation experiments included four mice.

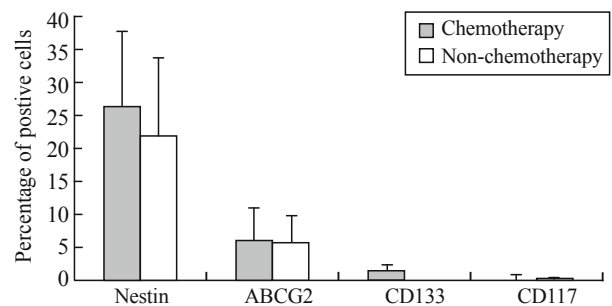
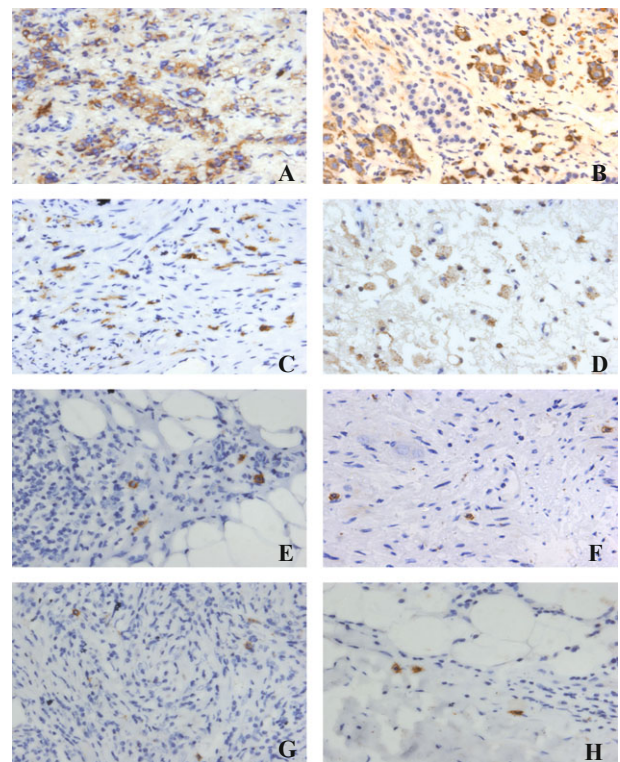
### Statistical analysis

Statistical analyses were performed with SPSS 13.0 statistical software. Data were expressed as the mean  $\pm$  standard error of mean. Student's *t* test was used for comparison between groups.  $P < 0.05$  was considered to be the threshold for statistical significance.

## Results

### Expressions of nestin, ABCG2, CD133 and CD117

Of 20 neuroblastoma tissue specimens, 19 had a positive expression of CD133 in pre-operative chemotherapy (9) and non-chemotherapy specimens (10). Likewise, 19 specimens had a positive expression of ABCG2 in pre-operative chemotherapy (10) and non-chemotherapy specimens (9). As well, 17 specimens had a positive expression of CD117 in pre-operative chemotherapy (9) and non-chemotherapy specimens (8). However, the positive expression of nestin was observed in all of the 20 specimens. Student's *t* test was used to analyze the expression of ABCG2, CD133, CD117, and nestin in the pre-operative chemotherapy and non-chemotherapy neuroblastoma tissues. There was no significant difference in the expression intensity of CD133, ABCG2, CD117, and nestin between the two groups of specimens ( $P > 0.05$ ) (Fig. 1).



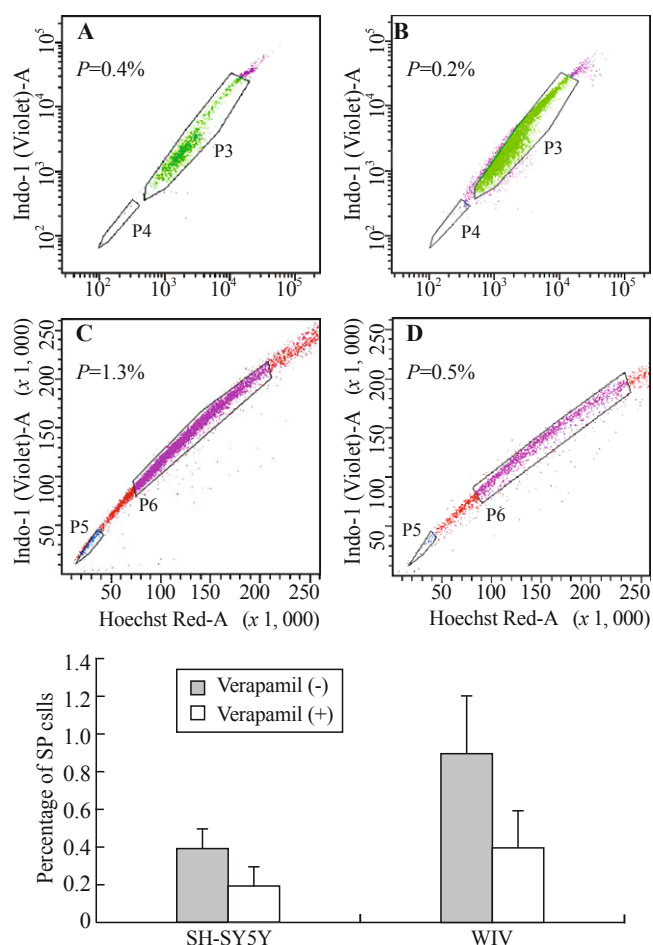
**Fig. 1.** Immunohistochemistry photomicrographs (original magnification  $\times 400$ ): nestin, ABCG2, CD133 and CD117 displayed moderate membranous staining in non-chemotherapy (A, C, E, G) and pre-operative chemotherapy (B, D, F, H) neuroblastoma tissue. Column diagram indicating the percentage of the positive cells in non-chemotherapy and chemotherapy neuroblastoma tissue ( $P > 0.05$ ).

### FCM analysis of SP cells and high levels of ABCG2 and CD133 expression in SP cells

SP and non-SP cells were sorted from the SH-SY5Y and WIV cell lines. The percentage of SP cells of SH-SY5Y and WIV cell lines is shown in Fig. 2A-D after treatment with verapamili. The data indicated that the percentage of SP cells from SH-SY5Y and WIV cell lines decreased from  $0.4 \pm 0.01\%$  and  $1.3 \pm 0.05\%$  to  $0.2 \pm 0.01\%$  and  $0.5 \pm 0.02\%$ , respectively after the treatment.

To examine whether the SP cells could generate both SP and non-SP, the sorted SP and non-SP cells were further cultured *in vitro*. On day 14, the cells were re-stained with Hoechst 33342 and analyzed by flow cytometry. The results indicated that the SP cells from





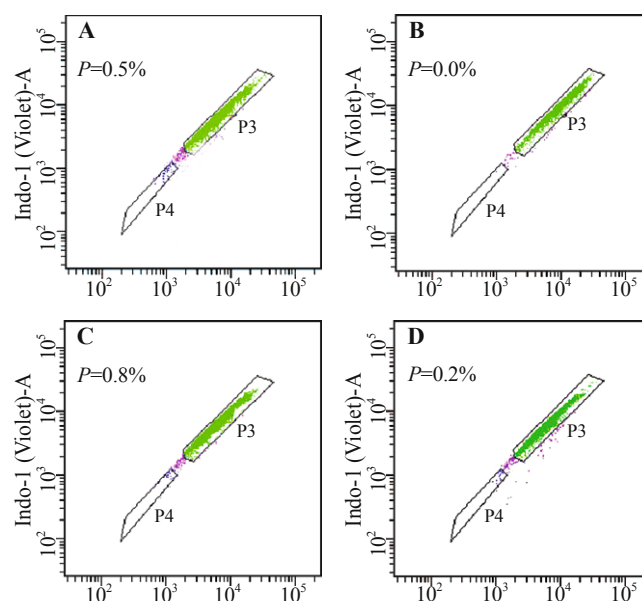
**Fig. 2.** Detection of side population cells in SH-SY5Y and WIV cell lines (A&C). The percentage of side population (SP) cells decreased following co-treatment with 50  $\mu\text{mol/L}$  verapamil plus Hoechst 33342 (B&D). The column diagram indicating that the percentage of SP cells from SH-SY5Y and WIV cell lines decreased from 0.4%±0.01 and 1.3%±0.05 to 0.2%±0.01 and 0.5%±0.02, respectively after treatment with verapamil.

SH-SY5Y and WIV cell lines could regenerate SP cells (Fig. 3 A&C). However, SP cells were not detected in the non-SP cells from SH-SY5Y cell line (Fig. 3B). It was also observed that non-SP cells from WIV cell line could generate a very small subset of SP cells (0.2%, Fig. 3D).

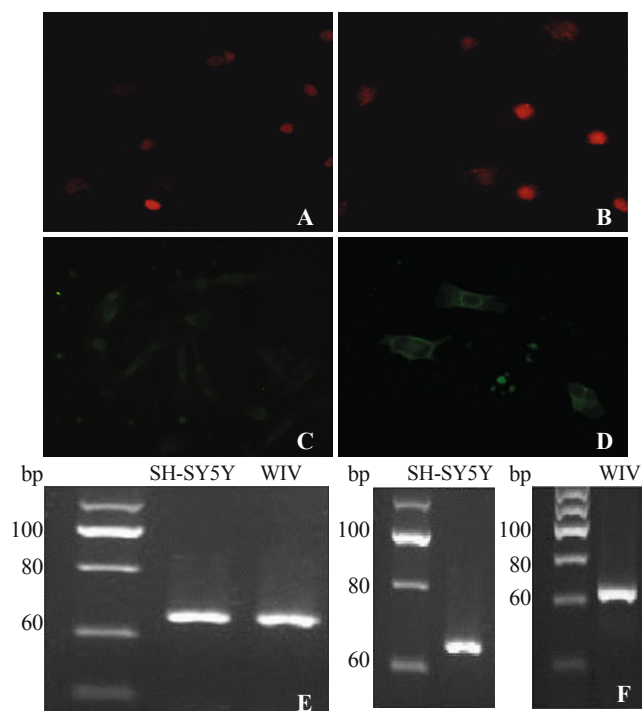
Freshly sorted SP cells of the SH-SY5Y and WIV cell lines had high ABCG2 and CD133 protein and mRNA expressions (Fig. 4).

### High proliferative capacity of SP cells and high malignant potential in SP cells

The average absorbance value of SP and non-SP cells from the SH-SY5Y cell line was 0.405±0.020 and 0.257±0.015, respectively on day two after the sorting ( $P<0.05$ ), and 0.646±0.037 and 0.375±0.029 on day five ( $P<0.05$ ) (Fig. 5A). The average absorbance value of SP and non-SP cells from the WIV cell line was 0.230±0.009 and 0.161±0.003, respectively on day two

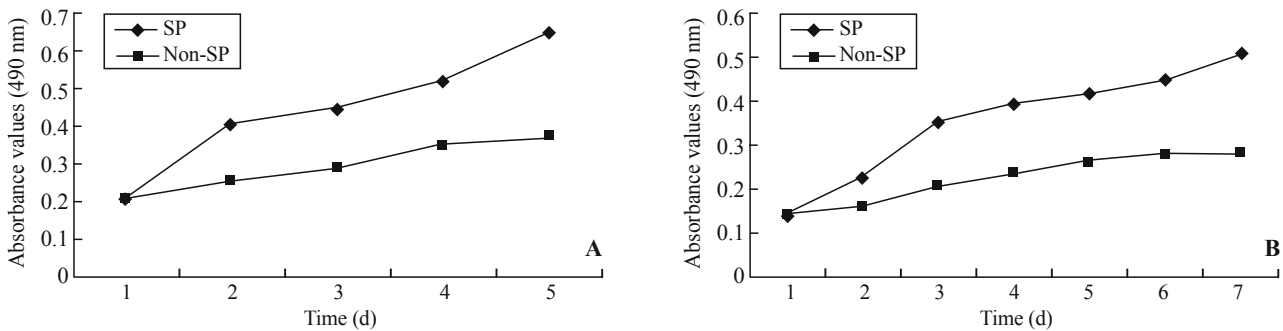


**Fig. 3.** Sorted side population (SP) and non-SP cells from SH-SY5Y and WIV cell lines were reanalyzed by flowcytometry. The SP cells from SH-SY5Y and WIV cells could regenerate SP cells (A&C), whereas non-SP cells from SH-SY5Y couldn't regenerate SP cells (B). Non-SP specimens from WIV cells could also regenerate a very small subset of SP cells (D).



**Fig. 4.** Protein and mRNA expression of CD133, ABCG2 in side population (SP) cells. Immunofluorescence analysis of ABCG2 (A&B, original magnification  $\times 200$ ) and CD133 (C&D, original magnification  $\times 200$ ) in SP cells from SH-SY5Y (A&C) and WIV (B&D) cell lines. Semi-quantitative analysis of CD133 (E) and ABCG2 (F) mRNA in SP cells from SH-SY5Y and WIV cell lines.

after the sorting ( $P>0.05$ ), 0.420±0.027 and 0.263±0.014 on day five ( $P<0.05$ ), and 0.511±0.021 and 0.283±0.016 on day seven ( $P<0.05$ ) (Fig. 5B).



**Fig. 5.** Proliferation of side population (SP) and non-SP cells. The MTT assay was performed at the indicated time points to quantify the number of viable SP and non-SP cells from SH-SY5Y (A) and WIV (B) cell lines.

**Table 2.** Cell proliferation inhibition rate of different concentrations of cisplatin on WIV cells (mean±standard deviation)

Cells	5 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$	40 $\mu\text{mol/L}$
SP	(56.14±1.16)%*	(62.07±1.21)%*	(95.26±3.11)%
Non-SP	(60.63±1.09)%	(69.72±1.27)%	(97.31±2.76)%

\*:  $P < 0.05$ , the comparison of side population (SP) and non-SP under the same concentration.

A total of four, zero and zero of the four SP cells from the SH-SY5Y cell-inoculated mice in each group formed tumors after the implantation of  $1 \times 10^6$ ,  $1 \times 10^5$  and  $1 \times 10^4$  cells, respectively. A total of three and zero of the four SP cells from the WIV cell-inoculated mice in each group formed tumors after the implantation of  $1 \times 10^6$  and  $1 \times 10^5$  transplanted cells, respectively. However, none of the non-SP cell-inoculated mice formed tumors in any of the three groups. Frozen sections of the SP cell-derived tumors stained with hematoxylin and eosin showed the typical features of human neuroblastoma cells. These results supported the hypothesis that the SP cells potentially have a high cancer-initiating ability, similar to that of CSCs.

#### Cell drug-resistance viability

The MTT results showed that the CPIR of SP cells was significantly lower than that of non-SP cells ( $P < 0.05$ ) (Tables 1 and 2). The viability of SP cells from SH-SY5Y and WIV cell lines with *in vitro* drug resistance was significantly stronger than that of non-SP cells.

#### Discussion

Recent studies on several solid tumors have revealed the existence of a rare subpopulation of tumor-initiating cells, known as "cancer stem cells" (CSCs). The CSC model of tumor development and progression indicates that CSCs are responsible for tumor initiation, growth and metastasis.<sup>[4-6]</sup> The detection of numerous cell surface markers has identified the existing of CSCs in several cancers including breast cancer<sup>[7]</sup> and brain

tumor.<sup>[8]</sup> Neuroblastoma is a common childhood tumor. In a recent study, Hirschmann-Jax et al<sup>[9]</sup> used Hoechst 33342 staining method and flow cytometry screening to identify the cancer cells obtained from 23 patients with relapsed, undifferentiated or poorly differentiated NB. They found that 0.8%-51% of the collateral stem cells existed in 15 specimens, which expressed several stem cell surface antigens. We used immunohistochemistry to identify the stem cell surface markers, including ABCG2, CD133, CD117, and nestin in neuroblastoma tissue after chemotherapy and non-chemotherapy. The results revealed that most of the specimens of NB tissue expressed four biomarkers. Moreover, CD133, ABCG2, CD117, and nestin were still expressed in the specimens of neuroblastoma tissue after pre-operative chemotherapy, suggesting that CD133, ABCG2, CD117, and nestin positive cells might be correlated with chemotherapy resistance. This finding indicated that cancer stem cells might exist in NB and be correlated with chemotherapy resistance, and that SP cells from SH-SY5Y and WIV cell lines were stronger in resistance to cisplatin than non-SP cells. Therefore, SP cells might play a crucial role in resistance to anticancer agents.

SP cells were defined by the efflux of Hoechst 33342, a cell permeable DNA-specific bisbenzimidazole dye, through an ABC transporter. Therefore, the SP cells were considered to be resistant to multi-chemotherapeutic agents and were recognized to be responsible for malignant phenotypes of the tumors.<sup>[10]</sup> Hence, SP cells might be a useful tool in the analysis of CSC, especially when specific CSC surface markers are unknown.

According to its features of cellular heterogeneity, three distinct cell types (N-, S- and I-type cells) were previously identified in neuroblastoma cells.<sup>[11-14]</sup> Small N-type cells had scant cytoplasm and neurites, whereas large S-type cells were flat with extensive cytoplasm. The morphologic and growth characteristics of I-type cells were "intermediate" between the N- and S-type cells. The I-type cells expressed the stem cell marker proteins and had a higher tumorigenicity in nude mice

than the N-type cells.

Research into the neuroblastoma stem cells has primarily concentrated on the analysis of the stem cell-like characteristics of the I-type cells.<sup>[3,9,15]</sup> Previous studies of neuroblastoma stem cells using SP analysis have not thoroughly analyzed the CSC characteristics of the sorted N- and S-type SP cells. Toward this goal, we identified and characterized those two different cell types commonly present in many primary human neuroblastoma. We observed the proportions of 0.4%±0.01% and 1.3%±0.05% of SP cells among the NB cell lines of SH-SY5Y (N-type) and WIV (S-type), respectively.

Recent studies have suggested that ABCG2 transporter played an important role in SP cell chemoresistance. The SP cells were identified by their ability to pump out the DNA-binding dyes, and Hoechst 33342 via ABCG2, which was one of the well-studied members of the ATP-binding cassette transporter family. The ABCG2 transporter could also pump out cytotoxic drugs and their overexpression in stem cells inhibited differentiation of these cells.<sup>[9,16,17]</sup> The immunofluorescence results of this study indicated that the SP cells from SH-SY5Y and WIV cell lines expressed ABCG2. The RT-PCR data also indicated that SP cells expressed high levels of ABCG2. These suggested that SP cells might be one of targets for cancer therapy. Further studies are also needed to examine whether ABCG2 expression is systemically correlated with chemoresistance of the SP cells.

To further characterize the SP cells in SH-SY5Y and WIV cell lines, we examined the expression of the previously reported stem cell marker, CD133, which was one of transmembrane proteins known as a hematopoietic stem cell marker.<sup>[18]</sup> It has been used as a cell marker to isolate stem cells of neural crest (NC) and tumors.<sup>[9]</sup> Marzi et al<sup>[19]</sup> found that stem cells were endowed with the immunocytochemical marker, CD133. In our study, immunofluorescence results indicated that the SP cells from SH-SY5Y and WIV cell lines expressed CD133. The RT-PCR data also indicated that SP cells expressed a high level of CD133.

CSCs have the self-renewal and proliferation characteristics. She et al<sup>[20]</sup> reported that SP cells grew clonally and had a self-renewal capability in the conditioned media. The expression of ABCG2, MDRI, Bmi-1 and Oct-4 was different between the SP and NSP cells. Takao et al<sup>[21]</sup> reported that SP cells formed colonies and proliferated on mouse embryonic fibroblast feeder cells. Furthermore, they also reported a long-term repopulating property. In our study, SP cells regenerated both SP and non-SP cells *in vitro*. These results suggested that the SP cells were capable of self-renewal. Furthermore, through MTT assay we

found that the proliferation capacity of SP cells from SH-SY5Y and WIV cell lines was stronger than that of non-SP cells.

Our tumor initiation experiments confirmed that SH-SY5Y and WIV SP cell lines had a higher malignant potential than the non-SP cells. The number of the SP cells from SH-SY5Y and WIV cell lines necessary to form tumors in nude mice was 106. This number was lower than that of the non-SP cells needed to form tumors (107 cells). However, the number of SH-SY5Y and WIV cells necessary to form tumors was higher than that of the SK-N-SH SP cells, an I-type cell line.<sup>[22]</sup> This explains why the SH-SY5Y and WIV cells have a lower malignant potential as compared with the SK-N-SH cells. Therefore, more cells might be required to form tumors in nude mice. *In vitro* study on the SP cells suggested that the malignancy of SH-SY5Y and WIV cells could be largely dependent on the SP cells.

In conclusion, we identified the SP cells in human SH-SY5Y and WIV cell lines. Moreover, we demonstrated that the SP cells had the characteristics of cancer stem-like cells. They were highly proliferative, tumorigenic and chemoresistant. These findings supported the idea that neuroblastoma might contain a certain amount of CSCs. These observations might have potentially important implications for the future therapeutic strategies that target the neuroblastoma cancer stem-like cells.

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**Ethical approval:** This study has been performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

**Competing interest:** No benefits in any form have been received or will be received from any commercial party related directly or indirectly to the subject of this article.

**Contributors:** XLL proposed the study, performed the experiment, and wrote the first draft. SYL analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. LF is the guarantor.

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