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A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem cell characteristics

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We used flow cytometry and a DNA-binding dye efflux assay to isolate a side population (SP) of cells with stem cell characteristics from the human pancreatic carcinoma cell line, PANC-1. Non-obese diabetic/severe combined immunodeficiency mouse xenograft experiments showed that SP cells were enriched in tumor initiating capability compared with non-SP cells. Cultured SP cells were able to differentiate into daughter cells and non-SP cells, through asymmetric division. Our study demonstrated that SP cells had high drug-resistance, both in vivo and in vitro. SP cells also showed significantly higher levels of mRNA expression for CD133, ABCG2 and Notch1, when compared to non-SP cells. Furthermore, xenografted tumors derived from injected SP cells and treated with gemcitabine had more CD133+ cells than untreated ones. We therefore suggest that these SP cells from the PANC-1 cell line were enriched with cancer stem cells.

Keywords. Cancer stem cell, PANC1 cell, side population (SP), CD133, ABCG2

According to the cancer stem cell hypothesis, cancer stem cells (CSCs) are regarded as malignant cells with the capacity for self-renewal, the potential to develop into any cell type in the overall tumor population, and the proliferative ability required to drive continued expansion of the malignant population of cells expressing specific cell-surface antigens, which can be isolated by fluorescence-activated cell sorting (FACS) [1–4]. The efflux of fluorescent dyes, such as Hoechst 33342, can also be used to identify and purify CSCs [5]. Analysis of cell side populations (SP) can be used to identify CSCs [6]. The low Hoechst SP phenotype was first identified as a marker for stem cell activity in mouse bone marrow cells [7]. In addition to the identification of SP cells in bone marrow obtained from patients with acute myeloid leukemia, which might be candidate leukemic stem cells, they have also been identified in various human solid tumors and cancer cell lines. To date, the existence of an SP phenotype has been demonstrated in cancers of the human head and neck, nasopharynx, lung, liver, esophagus, stomach, colon, rectum, and breast, and in gliomas [8–13]. The ability of Hoechst to act as a stem cell probe depends on the activity of the ATP-binding cassette (ABC) transporter subfamily member, ABCG2, which can pump dye out of the CSCs [14].

We used flow cytometry and a DNA-binding dye (Hoechst 33342) efflux assay to isolate a stem cell subpopulation from the PANC-1 human pancreatic carcinoma cell line. We then analyzed and identified the sorted SP cells. The results indicated that SP cells were enriched in tumor initiating capabilities, compared with non-SP cells. Cultured SP cells were able to differentiate into daughter cells and non-SP cells, through asymmetric division. SP cells also demonstrated increased drug-resistance in vivo and in vitro, and expressed higher levels of mRNA for CD133, ABCG2 and Notch1, compared to non-SP cells. Xenografted tumors derived from injected SP cells had more CD133+ cells when treated with gemcitabine than untreated ones. We therefore suggest that the SP of PANC-1 is enriched with CSCs with high drug-resistance.

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Abbreviations: CSCs, cancer stem cells; NOD/SCID, Nonobese diabetic/severe combined immunodeficient; SP, Side population; ABC, ATP-binding cassette; PCR, Polymerase Chain Reaction; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide(methyl thiazolyl tetrazolium); FACS, fluorescence-activated cell sorter.

Materials and methods:

Materials. Epidermal growth factor (EGF), basic fibroblast growth factor-4 (FGF-4), insulin solution, propidium iodide, methyl thiazolyl tetrazolium(MTT), verapamil, and Hoechst 33342 dye were purchased from Sigma-Aldrich (Beijing, China); the monoclonal anti-CD133 antibody was from Miltenyi (Beijing, China); fetal bovine serum (FBS)was from Invitrogen (Beijing, China); Dulbecco's modified eagle medium (DMEM) was from Hyclone (Beijing, China). The human pancreatic carcinoma cell line, PANC-1 (ATCC number: CRL-1469), was from American Type Culture Collection (ATCC) (Manassas, Virginia) and was maintained in culture medium recommended by ATCC. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were from the Experiment Animal Center, Capital Medical University (Beijing, China). The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the Capital Medical University. NOD/SCID mice aged from 4–8 weeks were used to test the tumorigenic potential of sorted cells from PANC-1 cell lines.

Cell culture. PANC-1 cells were cultured in an incubator filled with 5% CO_2 at a temperature of 37°C. The cells were cultured in serum-containing medium (SCM) consisting of a mixture of DMEM with 10% FBS, and 2mM L-glutamine in culture flasks (25 cm^2 or 75cm^2). To induce sphere formation, SP and non-SP cells (separated by FACS after staining with Hoechst 33342) were cultured in serum-free medium (SFM) consisting of DMEM supplemented with 10ng/ml FGF-4, 20ng/ml EGF, and 0.2 U/ml (5 μg/ml) insulin in culture flasks. SP and non-SP cells were plated in SCM in culture flasks and in 6-well culture dishes for 7–10 days to induce differentiation.

Hoechst 33342 dye and FACS. In order to identify and isolate SP and non-SP fractions, PANC-1 cells were removed from the culture flasks with 0.25% trypsin-0.05% EDTA, and washed with DMEM containing10% FBS and 1mM HEPES. Cells were pelleted by centrifugation, washed with 0.01 M phosphate-buffered saline (PBS), and resuspended at 37°Cin DMEM containing 2% FBS and 1mM HEPES. The cells were then labeled with Hoechst 33342 at a concentration of 5μg/ml, with 50μg/ml verapamil. The labeled cells were incubated for 90 minutes at 37°C in 5% CO_2 in a culture container. After staining, the cells were suspended in DMEM with 2% FBS and 1mM HEPES, passed through a 40-μm mesh filter, and maintained at 4°C until flow cytometry analysis and sorting. The cells were then incubated with 2 μg/ml propidium iodide to exclude dead cells. SP analysis and sorting were performed using a FACSVantage SE (BD Biosciences, San Jose, CA). The Hoechst dye was excited using a UV laser and its fluorescence was measured with both a 675/20 nm filter (Hoechst Red) and a 424/44 nm filter (Hoechst Blue).

Real-time reverse transcription-polymerase chain reaction analysis (real-time RT-PCR). The SP, non-SP, and PANC-1 cells were harvested. Total RNA was extracted from all three cell types using the RNeasy Micro kit (Qiagen, Beijing, China). cDNA was synthesized from 1μg of total RNA, using an oligo (dT) primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega M170A, America), according to the manufacturer's instructions. Real-time RT-PCR was performed to detect the expression of human CD133, ABCG2 and Notch1. β-actin was used as a control. Primers were designed to generate PCR products (Table 1). For reverse transcription, 2μg mRNA and 2.5μl oligo (dT) were added to 6.5μl distilled water. After annealing for 5 min at 70°C and immediate cooling on ice, 5.0μl of 5 × M-MLV buffers, 1.25μl of 10mM dNTP, 0.625μl of RNase inhibitor (40 U/μl) and 0.5μl reverse transcriptase were added to give a total reaction volume of 20μl. The reaction was allowed to proceed for 60 min at 42°C, followed by 15 min at 70°C to inactivate the enzyme. The cDNA sample was obtained after cooling on ice. Real-time PCR assays were performed in triplicate. The total PCR volume consisted of 1μl cDNA, 1μl SYBRGreen PCR I, 5μl 10 × buffers, 1.6 μl primers, 7μl $MgCl_2$, 0.5μl Taq DNA Polymerase, 1μl dNTP and 33μl distilled water. After denaturation of the enzyme for 2 min at 94°C, PCR assays were carried out for 45 cycles, with denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. Fluorometric

Table 1 Sequences of human primers used for real-time PCR experiments

Target mRNA	primer sequence	accession No.*	size(base pair)
CD133	5'-TTGTGG CAA ATC ACC AGGTA-3'	NM_ 006017	162bp
	5'-TCAGAT CTG TGA ACG CCTTG-3'		
ABCG2	5'-CACCTT ATT GGC CTC AGGAA-3'	NM_ 004827	206bp
	5'-CCTGCT TGG AAG GCT CTATG-3'		
Notch1	5'-ACTGTGAGG ACC TGG TGGAC-3'	NM_ 017617	196bp
	5'-TTGTAGGTG TTG GGG AGGTC-3'		
β-actin	5'-TGACGT GGA CAT CCG CAA AG-3'	NM_001101	205bp
	5'-CTGGAAGGT GGA CAG CGAGG-3'		

* Cited from NCBI Reference Sequences (Gene Databases)
(http://www.ncbi.nlm.nih.gov/sites/entrez)

PCR was performed using the Line-gene real-time PCR detection system (Bioer Technology Co. Ltd, China). The level of expression of each transporter protein gene was determined, relative to the β-actin gene.

Tumorigenec capabilities. SP and non-SP PANC-1 cells were injected into NOD/SCID mice using a limiting dilution assay. Groups of mice were inoculated with SP cells at 5×10^4 and 1×10^4, or with non-SP cells at 5×10^4 and 1×10^4 (three mice per group). Additionally, each of two NOD/SCID mice was injected with PANC-1cell at 6×10^6 respectively. Tumor growth was monitored every 2 days after the third week of inoculation. The mice were sacrificed at week 15, or when the tumors reached a maximum of 1,000 mm^3. Tumor volume was calculated by the formula: $0.52 \times \text{length} \times \text{width}^2$ [15]. The tumors were surgically removed and digested in 0.1Wünsch U/ml collagenase IV**,** according to the manufacturer's instructions, before re-analysis by the Hoechst 33342 dye efflux assay, as described above.

Drug resistance MTT assay. We isolated SP and non-SP cells from the PANC-1 cell line and seeded them on 96-well plates at approximately 5×10^3 cells per well. We incubated them in culture medium, as described above, at 37°C in an atmosphere containing 5% CO_2 for 48 hours. The cells in both populations were treated with 5-fluorouracil (5-FU; 1, 10, or 100 μg/ml) or gemcitabine (10, 100, or 1000 μg/ml). After 72 hours of exposure to the chemotherapeutic agents (the doubling time of PANC-1 cell is 52 hours; http://www.atcc.org/), cell viability was determined using the MTT assay. 20μl MTT (5 mg/ml) was added to each well and incubated at 37°C for 4 hours. Afterwards, 150μl DMSO solution was added to each well. The optical density (OD) was measured at 490 nm for each well using a microplate reader (Diagnostics Pasteur LP 400 system, France). The trial was performed in triplicate in three independent experiments.

For the in vivo animal experiments, six mice were divided into two groups, when the animal's tumor volume had reached 100 mm^3. The control group received an intraperitoneal injection of PBS (200–300μl) (n=3), and the treated group received an intraperitoneal injection of gemcitabine (100 mg/kg, every second day, six times in total) (n=3). Tumor diameter (measured using a microcaliper) and body weight was measured every 2 days [15]. Tumor volume was calculated using the formula: $\text{length}\times\text{width}^2\times 0.52$. At the end of treatment, the tumors were excised for molecular expression analysis.

Immunohistochemistry. Immunohistochemical detection of CD133 was performed using the transplanted tumors. Paraffin was removed from formalin-fixed, paraffin-embedded tissue, and samples were then blocked and incubated with first antibody-CD133 antibodies overnight at 4°C. The antibody was detected using Elivision Plus reagent kits (Maixin Biotec Ltd., China). All sections were counterstained with hematoxylin. Primary mouse CD133 monoclonal antibody (Miltenyi Biotec) was used at a dilution of 1:200.

Statistical analysis. Data are presented as the mean ± SD. Student's t-test was used to compare the differences between groups. P values <0.05 were considered significant.

Results

Presence of SP cells in pancreatic carcinoma cell line and xenograft tumor cells. Cultured PANC-1 pancreatic carcinoma cells were stained with Hoechst 33342, which is actively extruded by ABC transporters. The results of flow cytometry are shown in Figure 1. PANC-1 cells contained SP cells. The ratio of SP cells to the total cell population was from 0.9% to 1.5% when incubated with verapamil (Fig. 1B) or from 4.0% to8.4% without verapamil (Fig. 1A). The xenografted tumors were surgically removed and digested in 0.1Wünsch U/ml collagenase IV**,** according to the manufacturer's instructions, before reanalysis by the Hoechst 33342 dye efflux assay. SP cells constituted about 55% of the tumors treated with gemcitabine (Fig. 1D) and about 11.4% of untreated control tumors (Fig. 1C).

SP cells have the capacity for asymmetric division. The sorted PANC-1 cells, SP, and non-SP cells were cultured in 6-well plates in SCM. SP cells were able to divide asymmetrically. After restaining with Hoechst 33342, we found that for every SP cell unstained a further three cells were stained (Fig. 3A and Fig.3 B).

Expression of genes, including CD133, ABCG2 and Notch1, in the sorted PANC-1 cells. The expression of mRNA was analyzed in the sorted PANC-1 cells, using the $2^{-\Delta\Delta CT}$method [16, 17]. Genes including CD133, ABCG2 and Notch1 were significantly expressed and were upregulated in the SP cells. Expression of these genes was significantly lower in non-SP cells than in SP cells (Fig. 2). Many $CD133^+$ cells were detected in the tumor xenografts using immunohistochemistry (Fig. 4A and Fig. 4B). We selected four slides each from the treated and control groups, and analyzed them using the Image-Pro plus 6.0 system (http://www.mediacy.com). There were more $CD133^+$cells in the treated xenografted tumor sections than in the in the control ones (p=0.013) (Fig. 4C).

SP cells show high resistance to gemcitabine. After 72 hours of exposure to the chemotherapeutic agents (5-FU or gemcitabine), the viability of the cells was determined using an MTT assay. There was a significant difference in OD values between SP and non-SP cells incubated with gemcitabine, but no significant difference between the two types of cells after incubation with 5-FU (Table2).

SP cells and PANC-1cells can generate xenograft tumors. The implanted SP cells began to form pancreatic tumors after 4 weeks. However, in the first 2 weeks, we also found small tumors, but these had disappeared by the third week. This phenomenon may have been due to an inflammatory reaction. The non-Sp cells were unable to generate pancreatic carcinomas when injected at either 5×10^4 or 1×10^4 cells (Fig. 3C). The injected PANC-1 cells also could generate xenograft tumors. However, these tumors grew very slowly (Fig.5).

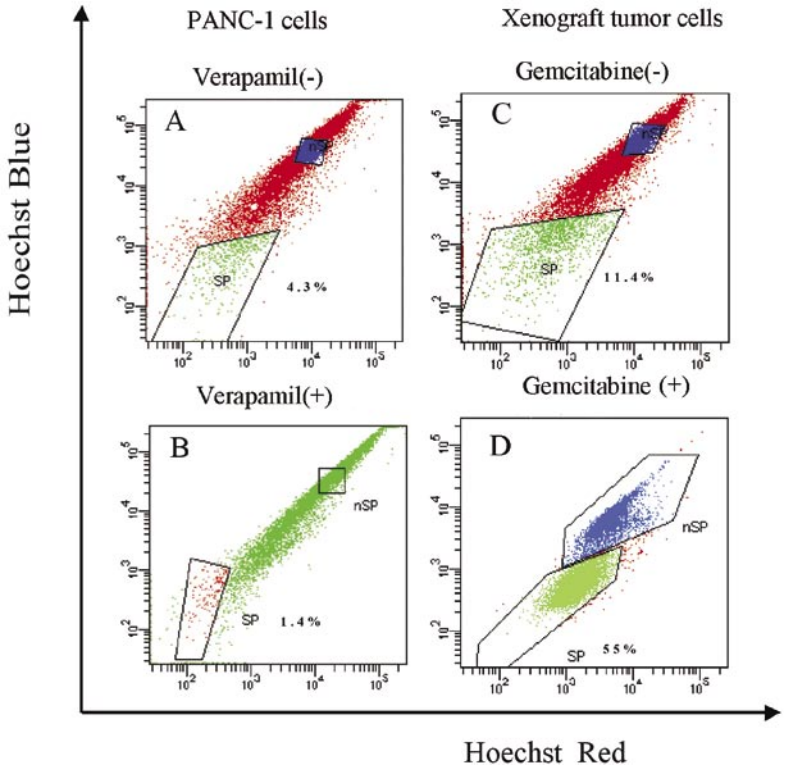

Fig. 1. Hoechst 33342 dye staining profiles of human PANC-1 pancreatic carcinoma cells (A and B) and xenograft tumor cells (C and D). Cells (10^6/ml) were stained with Hoechst 33342 and analyzed by fluorescence-activated cell sorting. Non-SP cells were stained with Hoechst, but side population (SP) cells were unstained or only lightly stained. PANC-1 cells were stained with Hoechst and incubated without (A) (n=6) or with (B) (n=6) 50 μg/ml verapamil. Xenografted tumor cells were stained with Hoechst and treated without (C) (n=3) or with (D) gemcitabine (n=3).

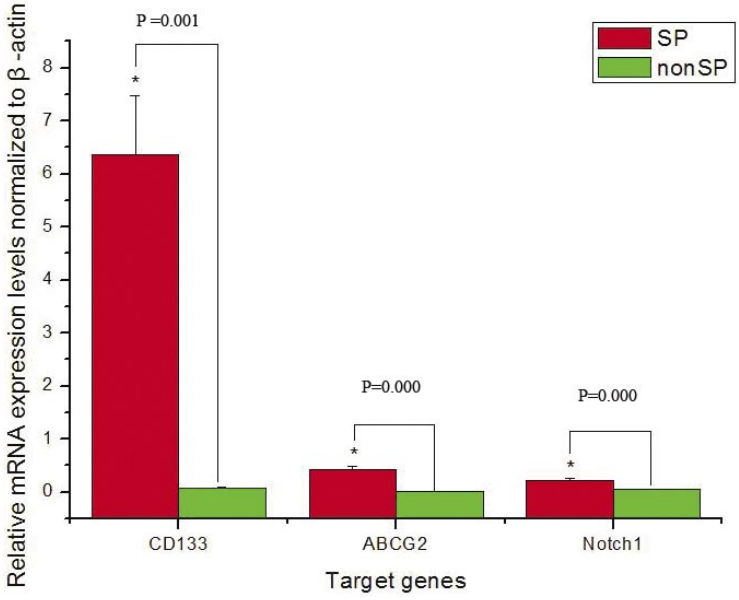

Fig. 2. Relative mRNA expression of genes in side population (SP) and non-SP PANC-1 cells. Relative expression levels of CD133, ABCG2, and Notch1 in each subpopulation were determined using real-time polymerase chain reaction. Expression levels were normalized to that of the housekeeping gene, β-actin. The mRNA expression of genes in PANC-1 cells was used as a standard. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression levels of CD133, ABCG2, and Notch1 in SP and non-SP cells. The data were derived from three independent experiments. Student's t-test was used to analyze the results. The expression levels were significantly different between the two cell types.

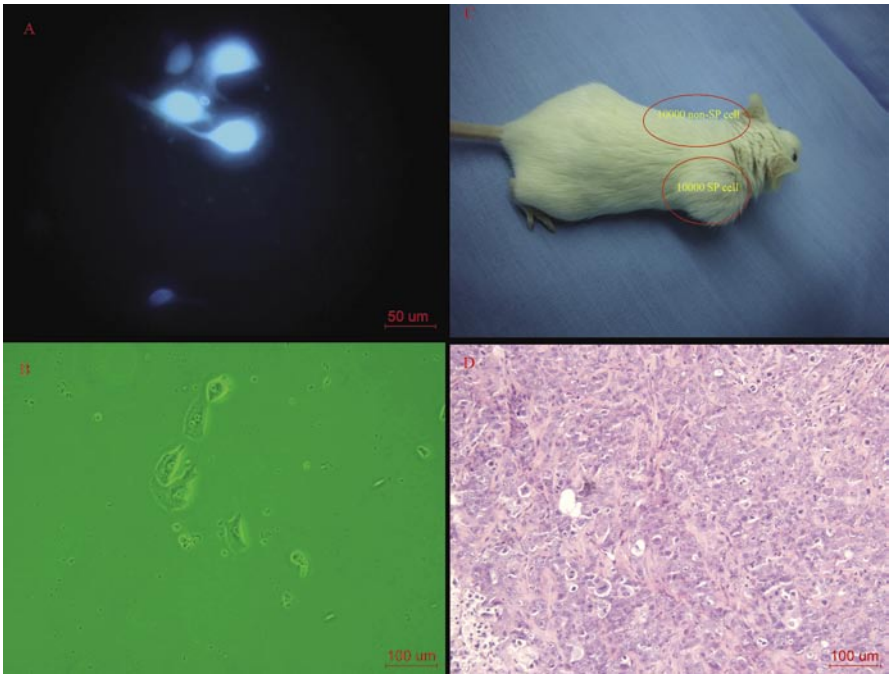

Fig. 3. The divding and tumorigenic capabilities of side population (SP) cells. After culture in serum-containing medium to day 6 (A) and day 7 (B), SP cells were found to be able to divide asymmetrically. They were then restained with Hoechst 33342, and for every SP cell that was unstained, another three cells were stained with Hoechst. SP and non-SP cells were injected separately into NOD/SCID mice at 1× 10^4 cells. The implanted SP cells began forming pancreatic tumors after 4 weeks. Tumor after 8 weeks (C). Xenograft tumor section stained with hematoxylin and eosin (D).

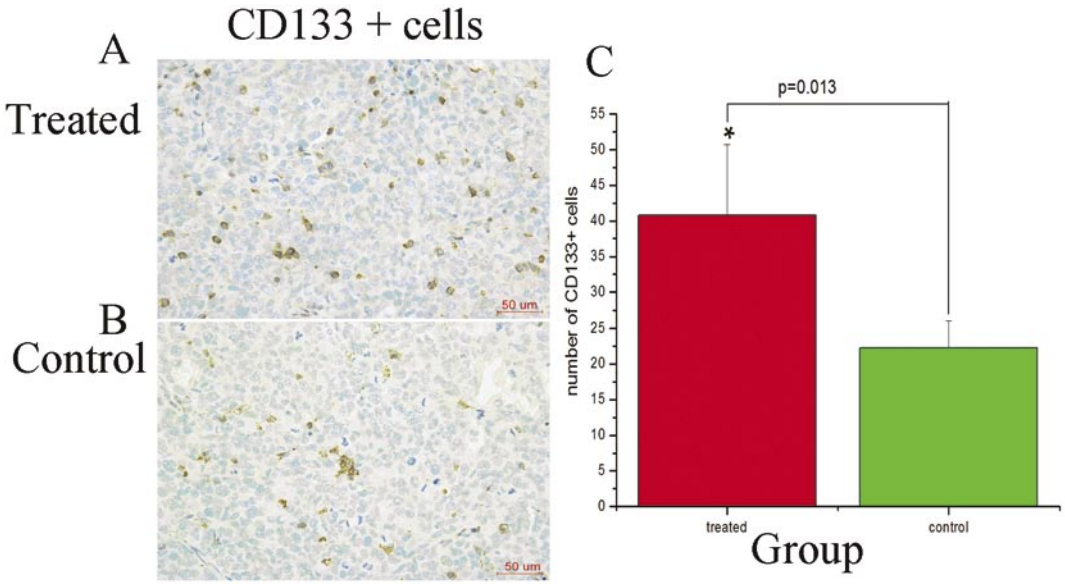

Fig. 4. Comparisons of CD133+ cells between the xenograft tumors treated with (A) or without (B) gemcitabine. The CD133+ cells were counted using IPP 6.0 software (n=4) (C). Student's t-test was used.

Table 2. Resistance to chemotherapeutic drugs in sorted PANC-1 cells (mean±SD, OD values)

	5-FU(μg/ml)			Gemcitabine(μg/ml)		
	1	10	100	10	100	1000
SP(n=3)	0.246±0.019	0.188±0.006	0.154±0.027	0.231±0.017	0.196±0.022	0.221±0.011
Non-SP (n=3)	0.187±0.003	0.181±0.016	0.154±0.007	0.185±0.012	0.156±0.010	0.171±0.022
t values	5.36	0.65	0.000	3.851	2.828	3.468
P values	0.006*	0.551	1.000	0.018*	0.047*	0.026*

Note: Values expressed as mean ± SD, the MTT assay was performed in triplicate in three independent experiments. Resistance quantified as OD values after drug exposure for 72 hours. Student's t-test was used to analyze the results. Statistical significance (P <0.05) indicated by asterisks.

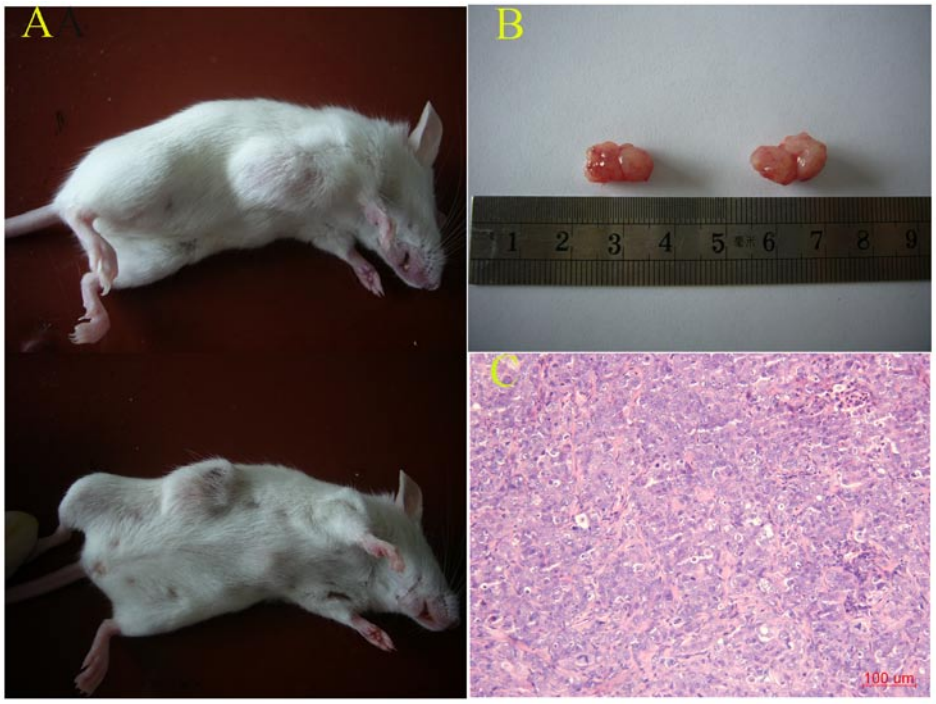

Fig 5. The tumorigenic capabilities of PANC-1 cells. Each SCID mouse was injected with PANC-1 cells at 6×106 respectively. The implanted PANC-1 cells began forming pancreatic tumors after 4 weeks. Tumor after 15 weeks (A and B). Xenograft tumor section stained with hematoxylin and eosin (C).

Discussion

In this study, we found that SP cells maintained the abilities to self-renew and to differentiate into conventional pancreatic carcinoma cells, but they showed different morphologic characteristics from non-SP cells. We isolated CSCs from the human pancreatic carcinoma cell line (PANC-1) using the SP analysis technique and found that SP cells from the PANC-1 cell line were enriched with CSC-like cells with capacities for tumorigenesis, asymmetric division, self-renewal, and proliferation. Some studies have isolated and characterized CSCs with specific biomarkers, including CD44, CD133, CD166, and epithelial-specific antigen [18, 19]. Up to now, however, no cell markers have been identified that could be used to isolate CSCs from different malignant tumors.

We showed that SP cells could be reliably detected under the experimental conditions used in this study. SP cells were more tumorigenic than non-SP cells when injected into NOD/SCID mice, despite being less plentiful, indicating a significant enrichment of tumor-initiating cells in this small SP subpopulation. This fact is potentially significant because effective curative therapy depends on the successful eradication of these cancer-initiating cells. It is likely that a population of stem-like cells exists within the PANC-1 pancreatic carcinoma cell line that is involved in resistance to chemotherapeutic agents. Studies have shown that ABCG2 is a molecular determinant of the

SP phenotype, and expression of ABCG2 mRNA was markedly elevated in SP cells in this study. Our results also revealed that SP cells expressed elevated levels of CD133 and Notch1, a stem cell membrane marker and a protein of the cellular signal transduction pathway. Several studies have shown that $CD133^+$ cells have CSC-like properties in hepatic carcinomas [3, 4], colon cancer [2, 20], and glial brain tumors [21].

The expression levels of CD133, ABCG2 and Notch1 were significantly higher in SP cells than in non-SP cells in our study. We also found more CD133+ cells in the xenografted tumors treated with gemcitabine than in the control ones. The pancreatic carcinoma xenografts were all derived from injected SP cells, suggesting that the SP cells were enriched with CD133+ cells. Furthermore, these cells had high levels of ABCG2 expression, so potentially increasing their ability to resist chemotherapeutic drugs, which might therefore act only against conventional cancer cells, and not against CSCs. Our study also showed that SP cells were more resistant than non-SP cells when they were incubated with gemcitabine, as found for other CSCs [22].

SP cells appeared smaller than non-SP cells and conventional cancer cells. They were almost round or oval in shape, with a single, large nucleus. According to Jordan and Soltysova's description, CSCs are defined as having the capacity for self-renewal, the potential to develop into any cell type in the overall tumor population, and the proliferative ability required to drive continued expansion of the population of malignant cells [1, 23]. We can conclude that the SP of PANC-1 pancreatic carcinoma cells are enriched with CSCs. This method for SP analysis may provide an effective method for the detection and isolation of other CSCs from various malignant tumors. It could also aid in the development of effective methods of treating malignant tumors through research into the drug resistance of CSCs.

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