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Original Article

Establishment of pancreatic cancer stem cells by flow cytometry and their biological characteristics

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Abstract: To investigate the method of separating human pancreatic cancer stem cells by Hoechst 33342 labeled flow cytometry and to analyze the biological properties of pancreatic cancer stem cells. The human pancreatic cancer cell line PC-3 was divided into SP and non-SP cells by flow cytometry. The number of two cell clone spheres and nude mice tumor formation rates were compared by cultivating in serum-free medium; The expression of CD133, Nestin mRNA and protein was analyzed by real-time fluorescence quantitative PCR and Western blot; The expression of two cell drug resistance genes (MDR1, ABCG2, ABCA2 and MRP1) was analyzed by real time fluorescent quantitative PCR. The number of the cloned spheres in SP cells in serum-free medium was significantly higher than that of non-SP cells ($P<0.05$). The incidence of SP cells in the tumor of immunodeficiency nude mice was significantly higher than that of non-SP cells, and the difference was statistically significant ($P<0.05$). Real-time fluorescence quantitative PCR analysis showed that the expression of CD133 and Nestin mRNA in SP cells was significantly higher than those of non-SP cells, and the expression of CD133 and Nestin protein in SP cells was also significantly higher than those of non-SP cells ($P<0.05$). In conclusion, SP side population pancreatic cancer cells by Hoechst 33342 separation have the stem cell characteristics, higher tumor formation rate and higher drug resistance, which may be related to chemotherapy resistance.

Keywords: Pancreatic cancer stem cells, flow cytometry, SP cells, non-SP cells

Introduction

Pancreatic cancer is a common malignant tumor with extremely high malignant degree, difficult diagnosis and treatment. Its mortality and incidence are high, and it has become the focus of clinical treatment [1, 2]. Chemotherapy is one of the main methods to treat pancreatic cancer. Pancreatic cancer has a very high chemotherapy resistance and recurrence rate in the long-term clinical practice. However, the mechanism is not entirely clear [3, 4]. In recent years, studies have shown that tumor stem cells may be the leading cause of recurrence and drug resistance. Cancer stem cells are a kind of cells with self-renewal, proliferation and differentiation abilities, which can produce daughter-cells identical with themselves, express some specific genes at the same time and maintain the characteristics of their own stem cells. These cells increased the difficulty to treat tumors [5, 6]. The tumor stem cells were mainly separated by stem cell specific Marker. Studies showed that this kind of cells had the characteristics of the stem cells [7, 8]. The tumor stem cells were successively separated from pancreatic cancer cells using Hoechst33342 dye. The analysis showed that it had the characteristics of stem cells and was correlated with chemotherapy resistance. The successful establishment of the method will provide another pathway for the study on pancreatic cancer stem cells.

Materials and methods

Tumor stem cell separation

Pancreatic cancer cell line SHG44 (ATCC, Manassas, USA) was cultured to the exponential growth phase and digested by trypsin with DMEM medium (Gibco, Grand Island, NY, USA). The cell density was adjusted to 1×10^6/ml. Two tubes were prepared. Verapamil (40 μg/ml)

Table 1. Primers used in this study

Primers	Upstream (5'-3')	Downstream (5'-3')
CD133	TCTTGACCGACTGAGACCCAAC	ACTTGATGGATGCACCAAGCAC
Nestin	GACGGAGGAGGTAGCCCGCA	GCCTCCACAGCCAGCTGGAACT
MDR1	ATATCAGCAGCCCACATCAT	GAAGCACTGGGATGTCCGGT
ABCG2	GGCTTTCTACCTGCACGAAAACCAGTTGAG	ATGGCGTTGAGACCAG
ABCA2	CCACTGGGCAGCGAGAAGTTGTC	GAAGCTGGAGTTCTGGCGGATCT
MRP1	TGTGAGCTGGTCTCTGCCATA	CTGGCTCATGCCTGGACTCT
GAPDH	AAGGACTCATGACCACAGTCCAT	CCATCACGCCACAGTTTCC

(Sigma, St. Louis, MO, USA) and Hoechst33342 (5 μg/ml) *(Sigma, St. Louis, MO, USA)* were added in the one tube, Hoechst33342 (5 μg/ml) was added in the other tube. They were cultured in 37°C 5% CO_2 culture box for 90 min, rotated and mixed every 15 min, washed twice with pre-cooling PBS, 5 min/time. The cells were resuspended in 400 μl PBS, detected and separated with BD flow cytometer (BD Biosciences, New Jersey, USA). SP cells and non-SP cells were collected respectively.

Cloning experiment

The SP cells and non-SP cells were separated and added in 96-well plate containing 100 μl DMEN/F12 medium (Gibco, Grand Island, NY, USA) (5 μg/ml Bfgf, 5 μg/ml EGF and 10 μg/ml LIF), cultured in 37°C, 5% CO_2 incubator for ten days. Then the number of clone spheres was calculated under microscope (Olympus AX80, Olympus, Tokyo, Japan). The average value was calculated.

Nude mouse tumor formation experiment

The Balb/c mice were purchased from Silaike experiment animal Co., Ltd., Shanghai, China, 7-8 w old, body weight 20 g. The cell densities were adjusted to 1000/ml and 10000/ml respectively after SP cells and non-SP cells were separated with flow cytometry. Then 100 μl cells were inoculated in the fat pad of the left ribs near the armpit in nude mice. The mice were fed under the normal conditions. The tumor formation was observed in 30 days. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Eighth Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Huaihe Hospital of Henan University.

Real-time fluorescence quantitative PCR

In this study, the expressions of Marker and drug resistance genes in SP cells and non-SP cells were analyzed by real-time fluorescence quantitative PCR. The SP cells and non SP cells were separated with flow cytometry. The mRNAs of the two kinds of cells were extracted with RNA extraction kit (TaKaRa, Dalian, China), then reversely transcribed into cDNA with the reverse transcription kit (TaKaRa, Dalian, China). The concentration of cDNA was determined as the template of PCR. The RT-PCR primers (Sangon, Shanghai, China) were designed according to the sequences of CD133, Nestin, MDR1, ABCG2, ABCA2 and MRP1 in **Table 1**. The primer concentration was diluted to 10 mM after the annealing temperature of the primer was optimized. The following reaction systems were prepared: 10 μl 2* SYBR Green universal qPCR Master Mix (Roche, Basel, Switzerland), 1 μl upstream/downstream primers (10 μmmol•L-1), 1 μl cDNA, The double distilled water was supplemented to a final volume of 20 μl. PCR was performed according to the following reaction conditions: pre-denaturation: 95°C for 30 s, denaturation: 95°C for 3 s, annealing: 58°C for 30 s, extension: 72°C for 40 s. The solubility curve was constructed. Finally, the data was read directly from the real-time PCR instrument *(Applied Biosystems,* Foster City, CA, *USA)*.

Western blot

SP cells and non-SP cells were treated using cell lysis buffer for 30 min and centrifuged for 10 min by 15, 000 g. 5* Loading buffer was added in the supernatant and boiled in the boiling water bath for 10 min. The electrophoresis was conducted with 12% SDS-PAGE gel. The protein was transferred to PVDF membrane, closed for 30 min with 5% skim milk powder, coated overnight with CD133 mouse monoclonal antibody (Pierce, Rockford, IL, USA) (catalog no. MA5-15875; dilution, 1:2000) and Nestin mouse monoclonal antibody (Santa-Cruz, CA, USA) (catalog no. sc-101541; dilution, 1:1000) at 4°C, washed for 3 times with PBST, 5 min/once, then incubated with HRP conjugated goat anti mouse secondary antibody (ZSGB-BIO,

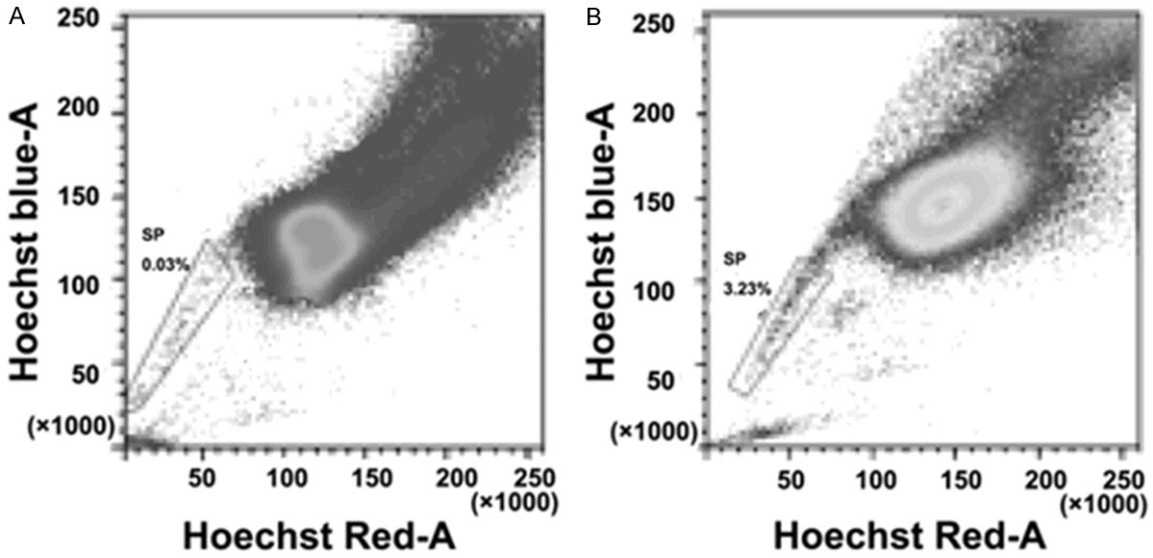

Figure 1. SP cells of pancreatic cancer were analyzed by flow cytometry. A. Control cells with treatment by Verapamil; B. Cells without treatment by Verapamil.

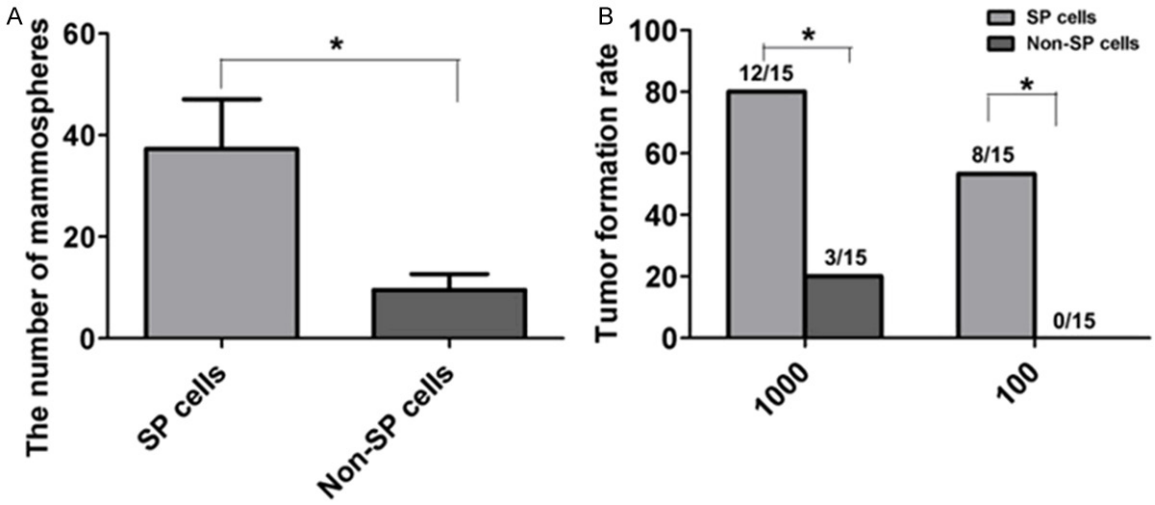

Figure 2. Comparison of tumor spheres and tumors incidence in SP cells and non-SP cells. A. Tumor spheres formation assay; B. Tumors incidence assay.

Beijing, China) (catalog No. SP-9002; dilution, 1:500) at 37°C for 1 h. The luminous liquid (Wako, Osaka, Japan) was coated on the film for direct imaging.

Statistical analysis

All data were analyzed using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL, USA). The measurement data were shown by X ± S. The enumeration data was shown by chi square test. The measurement data were compared with t test. $P<0.05$ indicated the difference had statistical significance.

Results

Establishment of pancreatic cancer stem cells by flow cytometry

Verapamil and Hoechst33342 treated cells were taken as the control in the experiment. Because verapamil can inhibit pancreatic cancer to excrete Hoechst 33342 dye from the cells, the proportion of cells in p2 area was only 0.03% in **Figure 1A**; If the cells were not treated by verapamil, some cells will excrete Hoechst33342 dye from the cells. These cells were side population cells, abbreviated as SP cells.

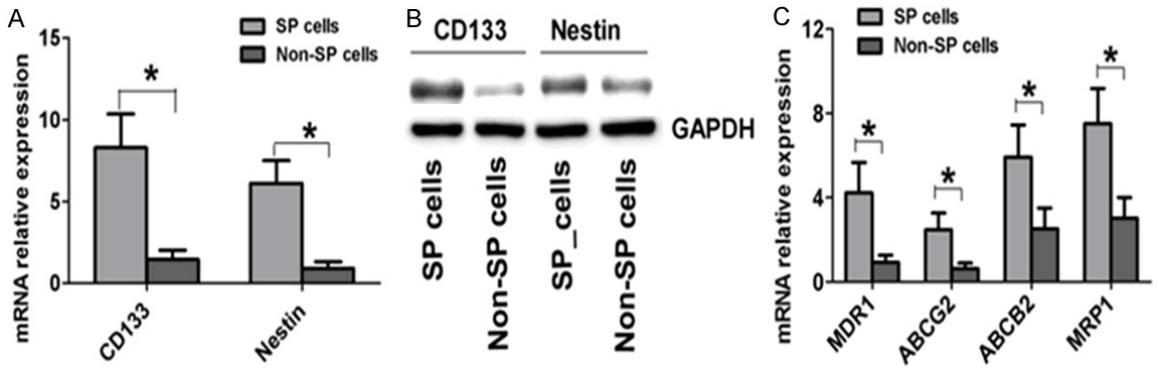

Figure 3. Expression analysis of stem cell specific gene and drug resistance gene in SP cells and non-SP cells. A. CD133 and Nestin mRNA level in SP cells and non-SP cells; B. CD133 and Nestin protein expression were analyzed by Western blot; C. Expression of drug resistance gene in SP cells and non-SP cells.

The other cells were non-SP cells. The proportion of pancreatic cancer cell line SHG44 was 3.23% (**Figure 1B**).

Analysis of SP cells and non-SP cells clone spheres

The stem cell properties of SP cells were further analyzed. The cells were accurately counted with flow cytometry. 100 SP cells and non-SP cells were inoculated in serum-free DMEM/F12 culture medium. The number of clone balls were counted under the microscope10 days later (**Figure 2A**). The number of SP cell clones (37.3 ± 9.8) was significantly higher than that of non-SP cells (8.45 ± 4.16), and the difference was statistically significant ($P<0.05$).

Analysis of SP cells and non-SP cells tumor formation in vivo

100 and 1000 SP cells and non-SP cells were inoculated respectively in the fat pad of left ribs close to the armpit. The proportion of tumor formation was observed. The results showed that the tumor formation rates were 80% (12/15) and 53.33% (8/15) after 100 and 1000 SP cells were inoculated in **Figure 2B**. The tumor formation rates were only 20% (3/15) and 0% (0/15) after 100 and 1000 non-SP cells were inoculated. The tumor formation rate of SP cells was significantly higher than that of non-SP cells, and the difference was statistically significant ($P<0.05$).

Expression of SP cell and non-SP cell markers

Real-time fluorescence quantitative PCR assay showed that the expression of CD133 and Nestin mRNA in SP cells was significantly higher than those of non-SP cells ($P<0.05$) (**Figure 3A**). Western blot showed the protein levels in **Figure 3B**. The expression of CD133 and Nestin protein in SP cells was significantly higher than those of non-SP cells, and the difference was statistically significant ($P<0.05$).

Drug resistance gene analysis of SP cells and non-SP cells

Tumor stem cells had the higher drug resistance to chemotherapy drugs. The expression of SP cells and chemotherapy resistance-related genes was further analyzed. The results showed that the levels of MDR1, ABCG2, ABCA2 and MRP1 drug resistance related gene mRNA in SP cells were significantly higher than those of non-SP cells. And the difference was statistically significant ($P<0.05$) (**Figure 3C**).

Discussion

Tumor tissue is heterogeneous. The tumor microenvironment is formed by different cells, maintaining tumor proliferation, invasion and metastasis. A few cells among them can be acted as the stem cells. They have the abilities of self-renewal, unlimited proliferation and multi-directional differentiation, and exhibit strong chemotherapy resistance [9, 10]. Although the tumor stem cells occupy a small proportion in tumor tissue, they play a role in the occurrence, development, invasion, metastasis and recurrence of tumor. So the tumor stem cells are the focus of the research at present [11]. Pancreatic cancer is a common malignant tumor in digestive tract. Study showed that

there were also tumor stem cells in pancreatic cancer [12]. How to obtain the tumor stem cells for research is the main problem. At present, pancreatic cancer stem cells were mainly separated by some stem cells specific markers (CD133 and Nestin etc.) by flow cytometry. CD133 is a recognized tumor cell marker, but strictly speaking, it is not pancreatic cancer stem cell specific marker. Therefore, seeking a more reliable method to obtain pancreatic cancer stem cells is the study basis [13, 14].

SP cells were firstly proposed in the analysis of bone marrow stem cells by Goodell et al [15]. The method has been widely used in the analysis of tumor stem cells [15]. At present, SP cells were successfully separated in many tumors such as lung cancer and breast cancer using flow cytometry after Hoechst33342 staining. And it was confirmed that the cells had the characteristics of the stem cells [16, 17]. The principle of this method was that ABCG2 gene expressed by stem cells could excrete Hoechst33342 dye from the cells, so these cells might have the characteristics of the stem cells. At present, it was seldom reported that pancreatic cancer cells were separated by Hoechst33342 staining flow cytometry. In this study, the method of separating SP cells by Hoechst33342 staining flow cytometry was established and the biological properties of SP cells were analyzed.

The results showed that after SP cells and non-SP cells were inoculated into the culture medium and cultured for a certain time, there was a suspended tumor sphere in SP culture medium. The suspended growth is one of the main biological characteristics of the stem cells [18, 19]. The self-renewal and proliferation abilities of tumor stem cells are the second biological properties of tumor stem cells [20]. A little SP cells and non-SP cells were inoculated in immunodeficient nude mice. The results showed that SP cells tumor formation rate was significantly higher than that of non-SP cells, indicating that these SP cells had self-renewal and proliferation abilities. The levels of CD133, Nestin mRNA and protein in SP cells were significantly higher than those of non-SP cells by cell specific markers analysis, real-time fluorescence quantitative PCR and Western blot analysis. The experiment further confirmed that SP cells obtained were pancreatic cancer stem cells. In addition, the study showed that SP cells had high drug resistance, because it could excrete the drug from the cells. Our results showed that drug excretion related genes in SP cells were highly expressed. This result also explained the reasons for drug resistance to chemotherapy.

In conclusion, pancreatic cancer SP cells separated after Hoechst33342 staining have the characteristics of the stem cells, and can be used as the basic materials for pancreatic cancer stem cells.

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Disclosure of conflict of interest

None.

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