Side population in the pancreatic cancer cell lines SW1990 and CFPAC-1 is enriched with cancer stem-like cells

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Abstract. In this study, we first sought to determine the existence of side population (SP) cells in pancreatic cancer cell lines. Furthermore, we compared the biological characteristics of SP and non-SP cells. The presence of side population cells in pancreatic cancer cell lines was detected by Hoechst 33342 staining and FACS analysis. Cell cycle distribution was analyzed using flow cytometry. SP and non-SP cells were exposed to various concentrations of gemcitabine; drug sensitivity was examined using the MTT assay and flow cytometry using Annexin-V and PI staining. To compare the tumorigenic ability in vivo, groups of nude mice were orthotopically inoculated with varying numbers of SP and non-SP cells. The percentages of CD44+CD24+ and CD133+ in SP and non-SP cells were also detected by FACS analysis. The SP fraction was detected in BxPc-3, CFPAC-1, MIA PaCa-2, PANC-1 and SW1990 pancreatic cancer cell lines. Cell cycle analysis revealed that the SP cells contained more cells in the G1 phase and fewer cells in the S phase when compared with the non-SP cells. The SP cells exhibited increased tumorigenic ability following in vitro transplantation into BALB/C nude mice and increased chemoresistance following in vitro exposure to gemcitabine. FACS analysis showed that the SP cells contained more CD44+CD24+ and CD133+ cells than the non-SP cells. In conclusion, these observations suggest that SP cells in the pancreatic cancer cell lines possess the property of cancer stem cells. SP cells may therefore be novel specific targets for the effective treatment of pancreatic cancer.

Introduction

Pancreatic cancer is among the most devastating of human malignancies. Despite recent improvements in surgical and chemotherapeutic approaches, pancreatic cancer patients continue to have a dismal prognosis with an average overall median survival of 4-6 months. The overall 5-year survival is <5% (1). To date, surgical resection is the only potentially curative therapeutic option; however, due to the lack of early symptoms, the vast majority of patients present with metastatic disease, rendering their malignancy inoperable (2). Even when the disease is diagnosed early and surgical resection is performed, nearly all patients develop local recurrence and/or distant metastases following surgery. Additionally, pancreatic cancer exhibits a high resistance to chemotherapy and radiotherapy. Gemcitabine is the current standard chemotherapeutic agent used in patients with pancreatic cancer (3). However, due to a high degree of inherent and acquired chemoresistance the clinical impact of gemcitabine remains modest (4).

Emerging data suggest that malignant tumors are heterogeneous and that tumors are composed of a subpopulation of distinct cells. This subpopulation of cancer stem cells has been demonstrated to be responsible for tumor initiation, propagation, recurrence and resistance to chemotherapy (5-10). The existence of human pancreatic cancer stem cells was reported by Li et al in 2007 (9). Pancreatic cancer stem cells, highly tumorigenic and possessing the abilities to self-renew and produce differentiated progeny, were defined by expression of the cell surface markers CD44, CD24 and ESA. CD133 is also an important cell surface marker for cancer stem cells. Hermann et al found that CD133+ cells in primary pancreatic cancers and pancreatic cancer cell lines also had some characters of cancer stem cells (11).

In addition to the cell surface marker approach, identification of a side population (SP) is also a known method for identifying cancer stem cells. The SP cells, first described by Goodell et al (12), are a small subpopulation of cells with enriched stem cell activity that demonstrate distinctively low levels of Hoechst 33342 dye staining. The existence of SP cells has been shown in many human cancer cell lines (13-19). Among pancreatic cancer cell lines, SP cells have been...
identified in PANC-1 (20,21), PK9 and PK45H (17). However, whether SP cells within pancreatic tumors are enriched with cancer stem cells is still unclear.

Herein, we report the identification of an SP in five human pancreatic cancer cell lines. Furthermore, we compared the biological characteristics of the SP to non-SP cells both in vitro and in vivo.

Materials and methods

Cell culture. The human pancreatic cancer cell lines BxPc-3, CFPAC-1, PANC-1, and SW1990 were purchased from Shanghai Cell Bank (Shanghai, China) and propagated in our laboratory. MIA PaCa-2 was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cell lines were maintained in the culture media recommended by ATCC. Media were supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco). Cells were maintained in a humidified incubator at 37˚C containing 5% CO₂.

Flow cytometry analysis and cell sorting. To identify and isolate SP and non-SP fractions, cells were detached from the culture dish with 0.25% trypsin, washed with phosphate-buffered saline (PBS), and suspended at 1x10⁶ cells/ml in culture dish with 0.25% trypsin, washed with phosphate-buffered saline (PBS), and stained with 20 μg/ml PI and 1 mg/ml RNase and analyzed using flow cytometry. The results are expressed as the percentage of cells in each phase of the cell cycle.

Drug sensitivity assay. Aliquots of 2x10⁴ freshly sorted SP or non-SP SW1990 cells or unsorted SW1990 cells were seeded in 96-well plates with appropriate growth medium at 200 μl per well. After a 12-h recovery period, triplicate wells were exposed to various concentrations of gemcitabine for 72 h. The effects on cell growth were examined by the MTT assay as described previously. The cell survival rate (SR) was calculated using the formula: SR = (mean absorbance of the test well/mean absorbance of the control) x 100%; the inhibition rate (IR) was calculated using the formula: IR = 100% - SR.

To quantify apoptosis, freshly sorted cells were seeded into 6-well plates at a density of 1x10⁵ cells/well. After 12 h of recovery, the cells were exposed to varying concentrations of gemcitabine for 48 h. The cells were then stained with Annexin-V and PI using the Vybrant Apoptosis Assay Kit (Molecular Probes) as per the manufacturer's protocol. Briefly, all cells were harvested by trypsinization and washed twice with cold PBS. The pellets were resuspended in 100 μl of PI at 100 μg/ml was added to each 100 μl of cell suspension. The cells were incubated on ice for 1 h, washed again with cold PBS and resuspended in 300 μl of Annexin-V binding buffer. The stained cells were immediately analyzed by flow cytometry.

Tumorigenicity assay in vivo. Athymic 4- to 5-week-old mice (BALB/c nude mice) were supplied by the Model Animal Research Center of Nanjing University, Jiangsu, China. Mice were housed and maintained in laminar flow cabinets under pathogen-free conditions, and the experiments performed in accordance with regulations and standards for experimental animals of Jiangsu Province, China. Freshly sorted SP and non-SP SW1990 and CFPAC-1 cells were resuspended in PBS at concentrations ranging from 1,000 to 100,000 cells per 50 μl. Groups of mice were orthotopically inoculated with varying numbers of SP and non-SP cells (three mice per group). Tumor growth was monitored weekly. The mice were sacrificed at day 40. The fold difference in tumorigenicity was calculated by the following formula: (minimum number of non-SP cells needed to generate a tumor)/(minimum number of SP cells needed to generate a tumor).

In vitro differentiation study. Freshly sorted SP and non-SP cells were reanalyzed immediately to determine the purity of SP and non-SP cells. The cells were recultured at a density of 1x10⁵ cells/well in a 6-well culture plate in Leibovitz’s L-15 medium with 1% penicillin/streptomycin and 10% FBS and incubated at 37˚C with 5% CO₂. After 7 days, SP- and non-SP-derived cells were reanalyzed for the presence of an SP fraction using the methods described above.

For the cell cycle assay, 5x10⁵ freshly sorted cells were seeded into 6-well plates at a density of 1x10⁵ cells/well. After 12 h of recovery, the cells were exposed to varying concentrations of gemcitabine for 48 h. The cells were then stained with Annexin-V and PI using the Vybrant Apoptosis Assay Kit (Molecular Probes) as per the manufacturer's protocol. Briefly, all cells were harvested by trypsinization and washed twice with cold PBS. The pellets were resuspended in 100 μl of PI at 100 μg/ml was added to each 100 μl of cell suspension. The cells were incubated on ice for 1 h, washed again with cold PBS and resuspended in 300 μl of Annexin-V binding buffer. The stained cells were immediately analyzed by flow cytometry.
Identification of SP cells in pancreatic cancer cell lines. Our experiments confirmed that the human pancreatic cancer cell lines BxPc-3, CFPAC-1, MIA PaCa-2, PANC-1 and SW1990 contained a small subpopulation of SP cells whose proportions were 0.79±0.11, 2.59±0.19, 0.03±0.02, 7.57±1.87 and 4.19±0.98%, respectively, and the SP fraction decreased significantly in the presence of verapamil (Fig. 1).

In vitro growth characteristics of SP and non-SP cells. To determine growth characteristics, we isolated SP and non-SP cells from the SW1990 cell line. Equal numbers of cells from the two populations were cultured in triplicate in 96-well plates. Their growth was measured using the MTT method. The data showed that the proliferation rate of SP cells was not significantly different from non-SP cells (P>0.05) (Table II). Despite the evidence that SP and non-SP cells had similar proliferation rates, the freshly sorted SP cells had a higher proliferation rate compared to the non-SP cells (Table III).

Table II. Difference in proliferation between SP and non-SP cells.

<table>
<thead>
<tr>
<th>Cell proliferative activity (OD, n=3)</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>0.201±0.009</td>
<td>0.396±0.015</td>
<td>0.804±0.013</td>
</tr>
<tr>
<td>Non-SP</td>
<td>0.213±0.022</td>
<td>0.391±0.011</td>
<td>0.865±0.068</td>
</tr>
</tbody>
</table>

Cell proliferation was assessed by MTT assay.

After incubation in the dark for 30 min on ice, the cells were washed twice with PBS and resuspended in 1 ml PBS with 2 μg/ml PI and kept on ice until FACS analysis. Hoechst 33342 was excited by ultraviolet light as previously described. FITC and PE were excited by an argon laser (488 nm), and the emitted fluorescence was detected through the FL1 and FL2 channels, respectively.

Statistical analysis. The data are presented as the mean ± SD. Statistical analyses were performed with SPSS 11.5. The Student's t-test for two samples was performed for the differences between SP and non-SP groups. P<0.05 was considered statistically significant.

Results

SP cells generate both SP and non-SP cells in vitro. To explore whether SP cells can generate both SP and non-SP cells, SP cells and non-SP cells from SW1990 were recultured separately under the same culture condition for 7 days. The cells were then restained with Hoechst 33342 dye and analyzed. The results showed that the SP proportion decreased from 95.91±0.68 to 16.74±4.94% (n=3), whereas the non-SP cells contained only 0.92±0.19% (n=3) SP cells (Fig. 2).

Table I. Primer sequences for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>5'-tgattgcatgttgagaggaca-3'</td>
<td>5'-cagagcgcagcagata-3'</td>
<td>155</td>
</tr>
<tr>
<td>ABCC1</td>
<td>5'-cgcgatgtcctcaacagacg-3'</td>
<td>5'-tcctacaggtgtgccttc-3'</td>
<td>118</td>
</tr>
<tr>
<td>ABCG2</td>
<td>5'-agatgggcttcaaggtct-3'</td>
<td>5'-ccagccccagctagctgacg-3'</td>
<td>91</td>
</tr>
<tr>
<td>HMGA1</td>
<td>5'-ccagccatcatactctc-3'</td>
<td>5'-cctcttgctgctctccac-3'</td>
<td>183</td>
</tr>
<tr>
<td>PLK-1</td>
<td>5'-ccctcaagctccaatc-3'</td>
<td>5'-ttgatgaggtggaggtggagg-3'</td>
<td>244</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ccgacaccctttgtaagc-3'</td>
<td>5'-cgtggagggggagagtc-3'</td>
<td>210</td>
</tr>
</tbody>
</table>

Western blot analysis. The concentration of total protein extracted from SP and non-SP cells was determined by the BCATM Protein Assay Kit (Pierce, USA). Equal amounts of protein were separated by 10% SDS-PAGE and electro-phoretically transferred to PVDF membranes (Millipore, Bedford, MA, USA) using a Mini Trans-blot (Bio-Rad Laboratories, Hercules, CA, USA). Rat anti-human ABCG2 antibody (Abcam, MA, USA) and mouse anti-human HMGA-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect the expression of ABCG2 and HMGA1; β-actin was used as an internal control.

Hoechst 33342 and antibody staining analysis. After Hoechst 33342 staining for 90 min as described above, the cells were centrifuged and resuspended in PBS + 5% FBS at a concentration of 1x10^6 per ml. The cells were then stained with both CD44-FITC and CD24-PE antibodies (both from BD Biosciences) or with CD133-PE (eBioscience) alone.
SP cells have an increased resistance to gemcitabine. SP and non-SP SW1990 cells were treated with varying concentrations of gemcitabine. Inhibition rates are shown in Fig. 3A. The half maximal inhibitory concentration (IC$_{50}$) was calculated. The SP cells demonstrated a higher IC$_{50}$ than the non-SP cells (23.59±9.30 vs. 4.38±1.59; n=3, P=0.024), suggesting that the SP cells were more resistant to gemcitabine.

To evaluate the difference between the SP and non-SP cells in regards to the induction of apoptosis, a cytofluorimetric analysis using PI and Annexin V staining was performed following gemcitabine treatments. Compared to SP cells, non-SP cells showed a high sensitivity to gemcitabine. Non-SP cells treated with 0, 1, and 10 μM gemcitabine were significantly more apoptotic than SP cells receiving the same treatment (3.82±0.411 vs. 2.01±0.065%, 17.19±1.898 vs. 10.21±2.189%, 57.90±8.729 vs. 24.60±1.675%, respectively; Student’s t-test, n=3, P<0.05) (Fig. 3B).

Table III. Cell cycle distribution of SP and non-SP cells.

<table>
<thead>
<tr>
<th></th>
<th>G0-G1 (%)</th>
<th>S (%)</th>
<th>G2-M (%)</th>
</tr>
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<tbody>
<tr>
<td>SP</td>
<td>70.02±2.67</td>
<td>21.63±3.09</td>
<td>8.35±3.91</td>
</tr>
<tr>
<td>non-SP</td>
<td>63.58±1.24</td>
<td>29.22±1.75</td>
<td>7.20±0.84</td>
</tr>
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</table>

*P<0.05, Student’s t-test, n=3

SP cells have an increased resistance to gemcitabine. SP and non-SP SW1990 cells were treated with varying concentrations of gemcitabine.
Higher tumorigenicity of SP cells in vivo. To determine whether the SP cells possess a tumor stem cell phenotype in vivo, we compared the tumorigenicity of SP and non-SP cells from the SW1990 and CFPAC-1 cell lines in nude mice.

For SW1990 inoculation, 1x10⁴ non-SP cells gave rise to new tumors in only one of three mice tested; however, SP cells could form a tumor when only 1x10³ cells were injected (Table IV). This result suggests that SW1990 SP cells are enriched with tumor-initiating cells by at least 100-fold compared with non-SP cells. Furthermore, liver metastasis was detected in two of three mice inoculated with 1x10⁵ SP cells. For CFPAC-1, SP cells gave rise to tumors with as few as 1x10⁴ injected cells (two of three mice), whereas at least 1x10⁵ non-SP cells were needed to form a tumor (one of three mice) (Table IV). Therefore, CFPAC-1 SP cells were significantly enriched with tumorigenic cells by 10-fold compared with non-SP cells (Fig. 4).

The primary cultured cells from the SW1990 SP- and non-SP-derived tumors were reanalyzed using flow cytometry. The results showed that, similar to the SP cells cultured in vitro, the SP cells under in vivo conditions also had the capacity to regenerate both SP and non-SP fractions.

Table IV. Increased tumorigenicity of SP cells in vivo.

<table>
<thead>
<tr>
<th>Cell number for inoculation</th>
<th>SW1990</th>
<th>CFPAC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-SP</td>
<td>0/3⁺</td>
<td>0/3</td>
</tr>
<tr>
<td>SP</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>1x10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10⁴</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>1x10⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10⁶</td>
<td>1/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

⁺The number of mice with a detected tumor; n=3.
In the present study, we demonstrated the presence of an SP fraction in BxPc-3, CFPAC-1, MIA PaCa-2, SW1990, and Panc-1 pancreatic cancer cell lines. We found both in vitro and in vivo evidence for the ability of the SP to regenerate a population of cells comprised of both SP and non-SP, suggesting that SP cells have the properties of self-renewal, producing heterologous descendant cells by asymmetric division. When inoculated into nude mice, SP cells were found to be more tumorigenic than non-SP cells, indicating division. When inoculated into nude mice, SP cells were producing heterologous descendant cells by asymmetric division. This demonstrated that SP cells have the properties of self-renewal, and PANC-1 pancreatic cancer cell lines. We found both SP fraction in BxPc-3, CFPAC-1, MIA PaCa-2, SW1990, and SCC1 pancreatic cancer cell lines. The data showed that expression of ABCB1, ABCG1 and ABCG2 was higher in the SP cells than that in the non-SP cells (Fig. 5). We further examined the mRNA expression level of HMGA1 and PLK-1. The data demonstrated that these genes were also highly expressed in the SP cells (Fig. 5). The results of the Western blot assay were consistent with the mRNA level; expression of ABCG2 and HMGA1 was higher in SP cells (Fig. 5C).

**Discussion**

**Differential gene expression between SP and non-SP cells**. Since the efflux of Hoechst 33342 from the SP cells is mainly through ABC transporters, we first compared the expression status of several ABC transporter family members between SP and non-SP cells derived from the CFPAC-1 and SW1990 cell lines. The data showed that expression of ABCB1, ABCG1 and ABCG2 was higher in the SP cells than that in the non-SP cells (Fig. 5). We further examined the mRNA expression level of HMGA1 and PLK-1. The data demonstrated that these genes were also highly expressed in the SP cells (Fig. 5). The results of the Western blot assay were consistent with the mRNA level; expression of ABCG2 and HMGA1 was higher in SP cells (Fig. 5C).

**SP cells highly express CD44, CD24, and CD133**. To evaluate the possible correlation of pancreatic cancer stem cell markers to the SP phenotype, SW1990 cells were additionally stained with both CD44-FITC and CD24-PE. SP cells contained more CD44+CD24+ cells than non-SP cells (2.35±0.13 vs. 0.56±0.06%; n=3, P<0.001). CD133-PE was also used to restain the Hoechst 33342-stained cells, and the data showed that CD133-positive cells were more abundant in the SP cell population (83.76±2.98 vs. 61.63±1.65%; P<0.001, n=3).

In this study, non-SP cells demonstrated increased S phase cells in the cell cycle distribution, but the proliferation rate of non-SP cells was not significantly different than SP cells. In a drug sensitivity assay, we found that non-SP cells were more apoptotic than SP cells, even when cultured without gemcitabine. This revealed that SP cells are more tolerant to apoptosis than non-SP cells. It has been reported that normal stem/precursor cells of various tissue origins express high levels of anti-apoptotic proteins such as Bcl-2 (22). Moreover, resistance of apoptosis has been suggested as a feature to enrich cancer stem cells from tumor specimens or tumor cell lines (23,24). Taken together, the data suggest that SP cells possess anti-apoptotic characteristics of cancer stem cells.

The SP phenotype is defined by ABC transporter activity. The ABCG2/Bcrp1 transporter, one main member of the ABC transporter family, is most often related to the SP phenotype (25). Consistent with previous studies (13-18), we also found that the expression of ABCG2 mRNA was significantly higher in SP cells from both the SW1990 and CFPAC-1 cell lines. Our results also demonstrated that SP cells had elevated levels of other members of the ABC transporter family, including ABCB1 and ABCB1, which are known to efflux different chemotherapeutic drugs and are associated with drug resistance (26).

In general, pancreatic cancer has an extremely poor prognosis and is resistant to chemotherapy and radiotherapy. Currently, gemcitabine represents the standard first-line chemotherapy for all stages of pancreatic adenocarcinoma (27). Unfortunately, the clinical impact of gemcitabine remains modest, owing to a high degree of inherent and acquired chemoresistance (2,4).

It has been reported that gemcitabine-resistant pancreatic cancer cells have an increased proportion of CD44+CD24+ESA+ cells (28). In this study, we found that not all of the cells in the pancreatic cell line had the same chemoresistance and that SP cells had a higher resistance to gemcitabine than non-SP cells. Furthermore, this study showed that SP cells also contained an increased proportion of CD44+CD24+ or CD133+ cells when compared to non-SP cells. Both of these results suggest that SP cells have the chemoresistance characteristic of cancer stem cells.

Our data showed that SP cells highly expressed HMGA1. Liu et al (29,30) showed that HMGA1 silencing enhanced
chemosensitivity to gemcitabine with an approximately 50% reduction in IC50 in PANC-1 and MIA PaCa-1 cell lines and that HMGAI promotes chemoresistance to gemcitabine through an Akt-dependent mechanism both in vitro and in mouse xenograft models. Akt signaling can control the SP phenotype through the regulation of ABCG2 expression (31). It was reported that the SP proportion was altered by the modulation of Akt signaling in a human hepatic cancer cell line (32) and in human glioma cells (33). High expression of HMGAI presents a possible reason for SP cell resistance to gemcitabine.

Overexpression of PLK-1 can reduce sensitivity to gemcitabine. Our previous research showed that silencing of the PLK-1 gene can enhance chemosensitivity to gemcitabine in pancreatic adenocarcinoma cells (34). Notably, we found that the mRNA level of PLK-1 was also increased in SP cells.

The SP phenotype and SP expression of specific cell surface markers are similar to cancer stem-like cells. Yet, the question regarding the relationship between them still needs clarification. Here, we isolated SP cells from the SW1990 cell line and found that CD133+ and CD44+CD24− cells were enriched in the SP cells. However, non-SP cells also contained many CD44−CD24+ and CD133+ cells, and the percentage of CD44+CD24− and CD133+ cells in the non-SP population showed no significant difference to that of the unsorted SW1990 cells suggesting that the isolated cancer stem-like cells from cell surface marker approach and the SP phenotype may share some common characteristics of cancer stem cells, but they are not equivalent.

In conclusion, our study demonstrated that SP cells from human pancreatic cancer cell lines display characteristics of cancer stem cells. SP cells exhibited properties of pluripotential differentiation both in vitro and in vivo, were more tumorigenic, and exhibited more chemoresistance than non-SP cells. A better understanding of the biological behavior of SP cells may contribute to improvements in therapeutic approaches and outcomes in patients with pancreatic cancer.

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References


