Intrinsic gemcitabine resistance in a novel pancreatic cancer cell line is associated with cancer stem cell-like phenotype

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Abstract. Pancreatic ductal adenocarcinoma (PDA) remains one of the most lethal malignancies in the world, often diagnosed at an advanced stage, resistant to conventional chemotherapy and having high invasive and metastatic potential. The mechanism of drug resistance of PDA is still not clear. In the present study, we established two novel pancreatic cancer cell lines PAXC-002 and PAXC-003 from human primary xenograft models. The cell lines were characterized by morphology, karyotype, pancreatic cancer marker and short tandem repeat (STR) analysis, and growth kinetics and tumorigenicity. The *in vitro* anti-proliferation test revealed that PAXC-002 cell was intrinsically resistant to the standard of care chemotherapy-gemcitabine, compared with that of PAXC-003 and other widely used pancreatic cancer cell lines. Interestingly, the gemcitabine resistant PAXC-002 cell line was more potent in forming colonies in 3-Dimensional matrigel culture conditions and had a higher percentage of CD133 positive cells, which is recognized as a cancer stem cell marker, compared to the gemcitabine-sensitive PAXC-003 cell line. In this study, we present two novel pancreatic cancer cell lines which could be used for gemcitabine resistance investigation, mechanism identification of pancreatic cancer and anticancer drug screening. The preliminary data indicate that the drug resistance of pancreatic carcinoma cells is associated with a cancer stem cell-like phenotype.

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Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most intractable human malignancies, which has an extremely poor prognosis (a 5-year overall survival of <5%). It is the fourth most common cause of cancer death yearly in the United States. Furthermore, the incidence of pancreatic cancer in China increased about four-fold during the past 20 years, and now it is the ninth cancer death cause (2.62 died in 100,000 people in 2005) (1).

Despite the huge effort on research and development of chemotherapies for pancreatic cancer, these are only a few treatment options approved in clinic. Gemcitabine, a novel pyrimidine nucleoside analogue, has become the standard-of-care therapy used in patients with pancreatic cancer, which showed advantage over the previously used 5-fluorouracil (2,3). However, no more than 25% of patients benefit from this therapy, and the palliative treatment with gemcitabine could only prolong patient survival from maximum 4 months to about 6-7 months, with improved life quality (4,5). Moreover, a substantial number of potential drug combinations have been tested clinically, but no convincing results have been obtained (6-9). The drug resistance of pancreatic cancers is still under wide investigation.

There are many different mechanisms involved in gemcitabine resistance. The drug transporters and metabolic enzymes of gemcitabine were extensively studied (10). The expression of human equilibrative nucleoside transporter-1 (hENT-1), which plays a key role in gemcitabine intracellular uptake, was found decreased in the acquired resistant cell line (11), and hENT expression could significantly influence the clinical survival (12,13). The downstream converting enzymes, such as deoxycytidine kinase (dCK), the ribonucleotide reductase M1 (RRM1) and M2 (RRM2) were also found associated with the acquired gemcitabine resistance (14). In addition, the apoptosis-regulation proteins, such as Bcl-2, Bax and Bak were deregulated in pancreatic cancers (15). The multidrug resistance-associated protein (MRP) (16), focal adhesion kinase (FAK) phosphorylation (17), and others were also found involved in drug resistance. However, these studies were mainly conducted on the acquired resistant cell lines, and the intrinsically resistant cell models are rare.

The cancer stem cells (CSC) have advanced the research of pancreatic cancer resistance. Several groups identified in 2007 the pancreatic cancer stem cells with CD24, CD44, epithelial specific antigen (ESA) triple positive markers or CD133 positive marker, which had increased tumorigenic potential and metastatic activity compared with non-CSC bulk tumor cells (18,19). Moreover, Hermann *et al* also found that the pancreatic cancer stem cells were resistant to the gemcitabine induced apoptosis (18). Later, Shah *et al* and Du *et al* established the acquired gemcitabine-resistant pancreatic cancer cell lines and they found the resistant cells had more cancer stem cell-like phenotypes compared with their parental cells (20,21). Recently, new insights were presented indicating that the resistance of pancreatic cancer was partly due to the pancreatic desmoplastic stroma and poor vascularization in mouse transgenic models, which impaired the drug delivery *in vivo* (22-24).

In the present study, we established two novel human pancreatic cancer cells lines PAXC-002 and PAXC-003, and both cell lines were well characterized. These low passage tumor cells maintained their clinical and pathological characteristics, which facilitated the translation of clinical knowledge. PAXC-002 cell line was intrinsically resistant to gemcitabine compared with PAXC-003 and other widely used pancreatic cancer cell lines. Furthermore, the gemcitabine resistant PAXC-002 cells had more *in vitro* colony formation ability and CD133 positive cancer stem-like cells. These results indicated that the pancreatic cancer cell resistance may associate with cancer stem cell phenotype. The two cell lines will be useful for pancreatic cancer research and anticancer drug screening.

Materials and methods

Patient tumors. Human pancreatic ductal adenocarcinoma (PDA) samples were obtained from Shanghai Changhai Hospital in accordance with protocols approved by the Institutional Ethics Committee. The tumor samples were placed in pre-cooled sterile 'collecting saline' (HBSS containing antibiotic-antimycotic) (Invitrogen, USA) and transported on ice to specific pathogen-free (SPF) animal facility of Shanghai ChemPartner Co. Ltd. for human primary tumor establishment, which is accredited by AAALAC.

Generation of human primary xenografts. Six to eight weeks-old female SCID mice (Beijing Vital River, China) were used for implantation of patient tumor fragments. The mice were bred in SPF animal facilities for at least three days before experiments. Their care, housing and experiments were in accordance with ChemPartner's IACUC guidelines. The tumor samples were washed twice with fresh pre-cooled collecting saline and then cut with a sterile scalpel blade into 2x2 mm pieces on ice. Two to three tumor pieces were implanted subcutaneously (s.c.) into the right flank of the mice. The mice were monitored for tumor growth and body weight for up to 10 weeks. The tumor length (L) and width (W) were measured by digital caliper and the tumor volume (TV) was calculated by the following formula: $TV = 1/2 \times L \times W^2$.

Establishment of cell lines. When the tumors grew to 500-700 mm^3, the mice were euthanized and the tumors were removed in sterile condition and were used for *in vitro* primary culture. The tumors were washed twice with sterile collecting saline, and the necrosis and connective tissue were carefully removed. The tumor tissue was minced finely using sterile scalpel blade and, after extensively washing with culture medium, transferred into a T-25 culture flasks and incubated at 37˚C, 100% humidity with 5% CO_2. The culture medium was RPMI-1640 medium supplemented with 10% heat-inactived fetal bovine serum (FBS) (Invitrogen), 10 μg/ml human recombinated insulin (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen). Controlled trypsinizations were done to preferentially remove the contaminating fibroblasts. The cultures were fed twice a week and subcultured when the cells grew to 70-80% confluence. The mycoplasma contamination was analyzed by PCR detection (MycoScan™ Mycoplasma Detection Kit, HDB Biosciences, China). The exponentially growing cells were used for future experiments after passage 20. These cell lines were stocked at China Center for Typical Culture Collection (CCTCC).

Cell culture. AsPC-1, BxPC-3, MIA PaCa-2, PANC-1 and Capan-1 cell lines were obtained from ATCC and maintained according to the instruction. AsPC-1 and BxPC-3 cells were maintained in RPMI-1640 + 10% FBS, Capan-1 cells were cultured in IMDM (Invitrogen) + 20% FBS, PANC-1 cells were cultured in DMEM (Invitrogen) + 10% FBS, MIA PaCa-2 cells were cultured in DMEM + 10% FBS + 2.5% horse serum (Invitrogen).

Morphologic analysis. Exponentially growing cells were observed via inverted phase-contrast microscope (Olympus, Japan). Digital pictures were taken from a camera mounted to a microscope (Olympus). Tumor cells were harvested and cultured as a monolayer on sterile chamber slides. Then they were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized in PBS containing 0.1% Triton X-100 (Sigma, USA), incubated in 0.3% H_2O_2 solution to quench endogenous peroxidase activity, and blocked with 4% goat serum (Invitrogen) in PBS. Fixed cells were incubated with anti-cytokeratin (Santa Cruz), anti-CA19-9 (Santa Cruz) and anti-CEA (Santa Cruz) antibodies. Then the slides were developed by DAB methods (Maxim, China) and mounted. The slides were observed and visualized by Eclipse Motorized Advanced Research Microscope (Nikon, Japan).

Chromosome analysis. Exponentially growing cells were seeded in new T-75 flasks. When the cells grew to 50-60% confluence, the cultures were fed with fresh medium containing 0.2 μg/ml colchicines (Sigma) and incubate for another 4-6 h. The M phase cells were harvest by gently taping and resuspended carefully in pre-warmed 0.075 mol/l KCl hypotonic solution and then incubate at 37˚C for 10 min. The cells were fixed in the fix solution (methanol:glacial acetic acid = 3:1, freshly prepared) several times. Then the cells were spread on the pre-cooled slide evenly and stained by Giemsa solution (Invitrogen). The chromosome numbers were counted under the microscope (Nikon, Japan) and the chromosome frequency of each cell line was analyzed by Origin software.

STR profiling. Tumor cells were harvested and washed in sterile PBS solution. The genomic DNA was extracted by

AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, USA) and the STR repeats were analyzed by AmpF/STR® Identifiler® PCR Amplification Kit (ABI, USA). All manipulations followed the vendors' instructions.

Population doubling time. Tumor cells were trypsinized and inoculated into the 96-well plates (poly-D-lysine coated, blank wall and clear bottoms) (Becton-Dickinson, USA) at the density of 2000 cells/well. The plates were fixed in Prefer Fix solution (Anatech, USA) at different time-points (12, 24, 36, 48, 72 and 96 h after inoculation). Then the plates were washed with PBS solution, permeabilized in 0.1% Triton X-100 solution, and stained with 1.5 μg/ml propidium iodide and 100 μg/ml RNase solution. The plates were analyzed by Acumen eX3 instrument (TTP, UK) and the cell numbers in each well were counted. The cell population doubling times (DT) were linearly fitted and calculated by following formula: DT = log2/S (S means the slope of the linear fitted curve of the log cell growth).

In vivo tumorigenicity. Six to eight week-old female SCID mice (Beijing Vital River, China) were bred in the SPF animal facilities and used for the xenograft tumor generation. Tumor cells were expanded and harvested, washed in pre-cooled serum-free 1640 medium and the cell concentration were adjusted to $5.0x10^7$/ml and placed on ice. The cell suspension was mixed with the matrigel (Becton-Dickinson) at a ratio of 1:1. The cell mixture was inoculated at the right flank of the mice s.c. ($5.0x10^6$ cells/mouse, 10 mice for each line). The tumor growth and mouse body weights were monitored twice a week. When the tumor grew to ~1500 mm^3, the mice were euthanized and the tumors were collected. The viable tumor tissues were fixed in 4% formaldehyde, paraffin-embedded and diagnosed (H&E staining).

In vitro anti-proliferation test. Tumor cells were seeded into 96-well tissue culture plates (Corning, USA) (the optimal cell density were determined by cell growth curve study and the control cells were still exponentially growing at the assay endpoint) in 150 μl culture medium. Gemcitabine (Chemiceutical, USA) was added into triplicate wells at the time of cell inoculation (gemcitabine was dissolved in DMSO, started from 100 μM, 1:5 serially diluted, 10 points, and the final DMSO concentration in medium was 0.5%). After a 96-h incubation, the cell viability in treated and control wells were measured by CellTiter Glo method (Promega, USA) according to the manufacturer's instructions. Drug effects were presented as IC_{50} drug concentration and inhibition index (IC_{50} was the concentration which resulted in 50% inhibition and was determined by XLFit software, equation 205. Inhibition index was the sum of inhibition rates at each tested drug concentration). AsPC-1, BxPC-3, MIA PaCa-2, PANC-1, Capan-1 cell lines were tested against gemcitabine according to the same protocol.

In vitro 3-Dimension colony formation assay. The 96-well tissue culture plates were coated with 50 μl of 0.6% agarose (Takara, Japan) in PBS and then solidified at 4˚C overnight. Tumor cells which grew as a 2-D monolayer were trypsinized and seeded into each well (2,000 cells in 100 μl medium-matrigel mixture). Plates were incubated at 37˚C overnight and 100 μl fresh culture medium was added into each well the next day. After a 6-day incubation, the colony formation were counted under the microscope and measured by Alamar Blue method (Invitrogen).

Table I. STR profile of the two pancreatic cancer cell lines.

STR locus	PAXC-002	PAXC-003
Amelogenin	x	x, y
THO1	7, 9	9
TPOX	8, 11	8, 11
D13S317	11, 12	10
vWA	16, 18	16
D16S539	9	12, 13
D5S818	12	12, 13
CSF1PO	12	10, 12
D7S820	8, 9	11, 12

Cancer stem cell marker expression detection. Cells were harvested, washed in pre-cooled serum-free 1640 medium and then stained with PE conjugated anti-CD133 antibody (Miltenyi Biotec, Germany) on ice for 1 h and washed three times with PBS. Flow cytometry was done using a BD FACSCalibur flow cytometer.

Statistical analysis. Data were expressed as the mean ± SD and the *in vivo* tumor growth was expressed as the mean ± SEM. Statistically significant differences were determined by the Student's t-test, where appropriate, and defined as $P<0.05$.

Results

Establishment of PDA cell lines and characterization. Clinical resected pancreatic tumor samples were subcutaneously implanted into the SCID mice, and tumors developed successfully after about two months. The human primary tumors were removed and placed in culture as described in Materials and methods. After several weeks of cultivation, outgrowth of both epithelioid cells and fibroblast-like cells were observed from the explanted pieces of tumor tissue. To separate the presumed epithelioid tumor cells from the fibroblast-like cells, controlled trypsinizations were optimized and adopted. The cells were digested and split into new tissue culture flasks when they grew to ~80% confluence. After several passages in culture, the contaminated fibroblasts were removed completely. These tumor cells could be subcultured continuously *in vitro* (>50 passages in our lab) and were designated as PAXC-002 and PAXC-003, respectively. Both cell lines were free of mycoplasma contamination and deposited at China Center for Typical Culture Collection for public research (CCTCC, the deposit number of PAXC-002 is C201007 and PAXC-003's no. is C201010).

The tumor cell lines were banked at the low passages in liquid nitrogen tanks and characterized after passage 20 when the growth kinetics became stable. Both the cell lines grew with typical slabstone-like epithelioid morphology on plastic surfaces (Fig. 1). The PAXC-002 cells showed obvious clonal shape while the PAXC-003 cells grew more evenly.

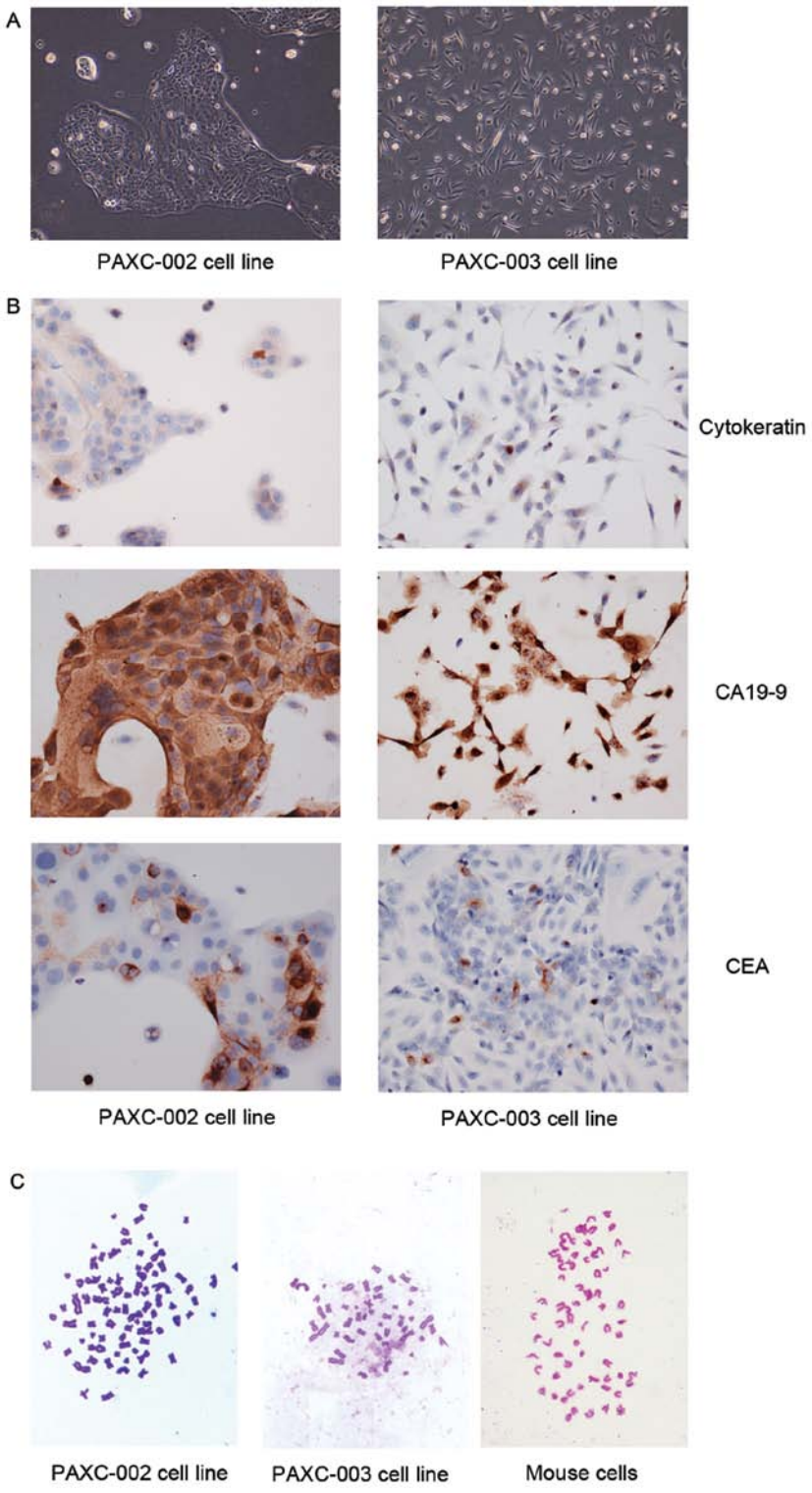

Figure 1. Morphological studies of the novel pancreatic cancer cell lines. (A) PAXC-002 and PAXC-003 cell lines were observed under an inverted microscope. Magnification x100. (B) Identification of pancreatic cancer markers, including cytokeratin, CA19-9 and CEA. Tumor cells growing on chamber slide were fixed and stained with antibody against cytokeratin, CA19-9 and CEA. Antigen presences were visualized by DAB methods. Magnification x200. (C) Chromosome analysis of PAXC-002, PAXC-003 and mouse cells. Human cell chromosomes had median centromere, but the mouse cells had telocentric chromosome. Magnification x1000.

In order to confirm the human origin and identity of these cell lines, studies were made of chromosome and STR analysis, and immunocytochemistry staining. The results demonstrated that both cell lines had obvious median centromere in all chromosomes to indicate their human cell origin (25) distinguished from the mouse telocentric chromosome (26) (Fig. 1). PAXC-002 cells had a modal chromosome number of 80±4, and the modal number of PAXC-003 was 43±3. The aberrant chromosome number suggested the malignant phenotype of these cells. In addition, the STR profiling of each cell line were tested to avoid the possibility of cell line cross-contamination (27,28). The data of the 8 core loci (Table I) were searched

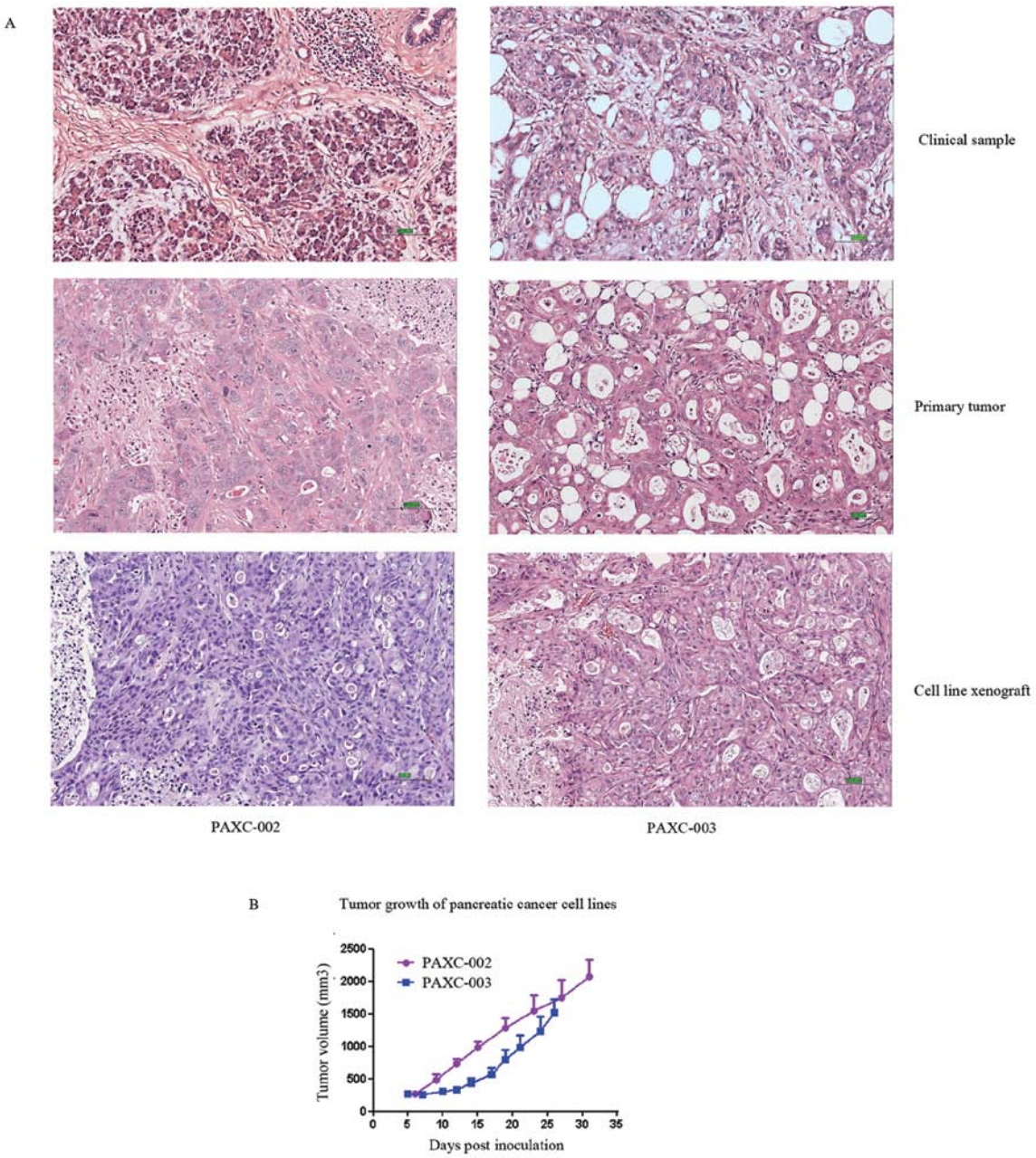

Figure 2. *In vivo* tumorigenicity test of the pancreatic cancer cell lines. (A) H&E staining of the clinical samples, primary tumors generated in mice and the cell line xenograft tumors of the pancreatic cancer cell lines. Magnification x200. (B) Tumor growth were monitored twice a week and the tumor volume was measured by a digital caliper.

in ATCC and DSMZ STR databank and did not return any similar results. Furthermore, as seen in Fig. 1, these cell lines are positive to the pancreatic cancer markers, cytokeratin, CA19-9 and carcinoembryonic antigen (CEA). All these data indicated they were novel human pancreatic cancer cell lines.

PAXC-002 cells were routinely subcultured at a ratio of 1:2-1:3 twice a week with a population doubling time of ~48 h in log growth phase. PAXC-003 cells could be split at a ratio of 1:3-1:4 twice a week with a population doubling time of ~34 h.

In vivo tumorigenicity test and pathological diagnosis. PAXC-002 and PAXC-003 pancreatic cancer cells were harvested and injected into the SCID mice subcutaneously with matrigel. Tumors developed successfully in all mice. When the tumors reached ~1,500 mm^3, the mice were euthanized and the tumor tissue was fixed, paraffin embedded and pathologically diagnosed. As shown in Fig. 2, the H&E staining of the cell line xenograft tumors were compared with their parent clinical tumor samples and the corresponding human primary tumors generated in mice, and they preserved similar histological characteristics and differentiation (Fig. 2 and Table II). H&E staining showed that PAXC-002 tumors were composed of a mixture of densely packed, small irregular glands as well as solid tumor cell sheets and nests, and the tumor cells had large neclei with marked pleomorphism. On the other hand, PAXC-003 tumors showed a mixture of medium-sized, incompletely duct-like and tubular structures of variable shapes. In summary,

Table II. Pathological characterization of the pancreatic tumors.

Pathological diagnosis	PAXC-002	PAXC-003
Clinical samples	Head of pancreas: poorly to moderately differentiated PDA	Uncinate process of the pancreas: moderately differentiated PDA
Human primary tumors generated in mice	Pancreas: poorly to moderately differentiated PDA	Pancreas: moderately differentiated PDA
Cell line xenograft tumors	Pancreas: poorly to moderately differentiated PDA	Pancreas: moderately differentiated PDA

When tumors grew to appropriate volume, the viable tumor tissues were fixed, paraffin-embedded, stained with H&E and diagnosed, as shown in Fig. 2.

Table III. *In vitro* growth inhibition of gemcitabine on pancreatic tumors.

Cell line	IC_{50} (nM)	Inhibition index
PAXC-002	>100000	99.006
PAXC-003	14.564	517.114
AsPC-1	568.354	324.102
BxPC-3	13.683	525.234
Capan-1	6.614	462.709
MIA PaCa-2	7.734	545.304
PANC-1	136.786	324.602

IC_{50} was the concentration which resulted in 50% inhibition and inhibition index is the sum of inhibition rates at each test drug concentration (Fig. 3).

PAXC-002 is a poorly to moderately differentiated pancreatic ductal adenocarcinoma (PDA) cell line, and PAXC-003 is a moderately differentiated PDA cell line.

In vitro growth inhibition assays. Gemcitabine is the standard-of-care chemotherapy of pancreatic cancer and it is very potent in *in vitro* growth inhibition assays with IC_{50}s between 2 and 20 nM on most pancreatic cancer cell lines (29,30). Exponentially growing PAXC-002, PAXC-003, AsPC-1, BxPC-3, MIA PaCa-2, PANC-1 and Capan-1 cells were exposed to serially diluted gemcitabine for 96 h and the cell viability was measured by using CellTiter Glo method and the IC_{50} of gemcitabine in each cell line were determined by XLFit software. PAXC-003, BxPC-3, MIA PaCa-2, PANC-1 and Capan-1 cells were very sensitive to gemcitabine which IC_{50}s ranged from 1 to 20 nM. AsPC-1 cell line moderately tolerated gemcitabine with an IC_{50} concentration of 568 nM. However, the IC_{50} of PAXC-002 cell line was more than 100 μM and the PAXC-002 cell viability remains >50% even at 100 μM of gemcitabine, which meant this cell line was intrinsically resistant to gemcitabine (the results are summarized in Fig. 3 and Table III).

In vitro 3-D colony formation assay. The *in vitro* 3-D culture model mimics the *in vivo* physiological property. After a 6-day

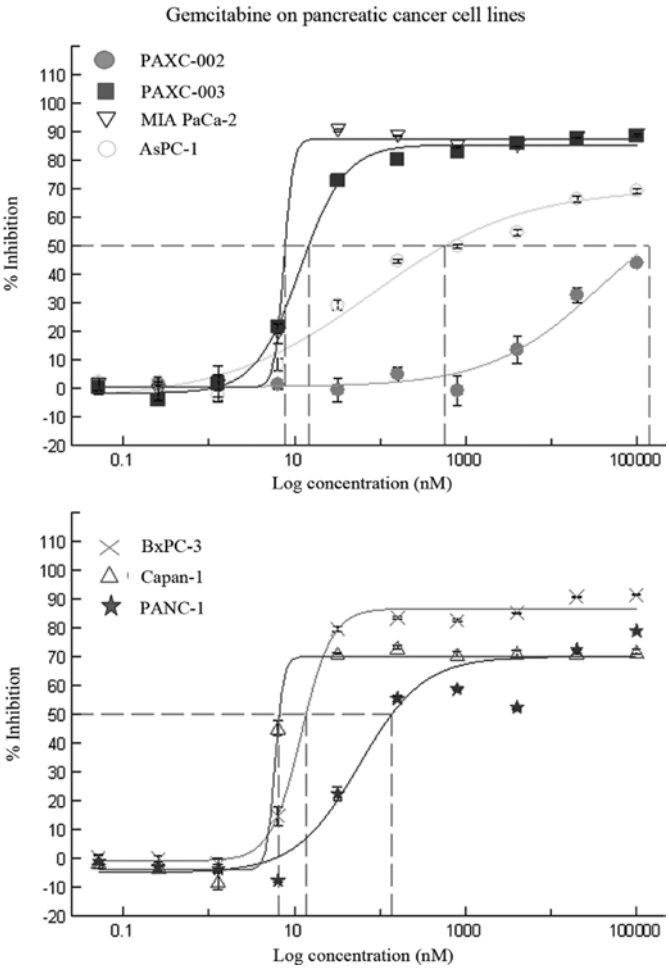

Figure 3. The *in vitro* anti-proliferation test of gemcitabine on pancreatic cancer cell lines. Gemcitabine concentration was started from 100 μM and 1:5 serial diluted (10 points). After 96-h drug exposure, cell viabilities were measured by CellTiter Glo methods and IC_{50} were determined by XLFit software.

incubation, both cancer cell lines formed colonies in matrix. The gemcitabine-resistant PAXC-002 colonies were significantly more abundant than drug-sensitive PAXC-003 line (Fig. 4). In addition, the colony viability measured by Alamar blue method confirmed the increased colony formation ability of PAXC-002 cells (Fig. 4). This result demonstrated that gemcitabine-resistant

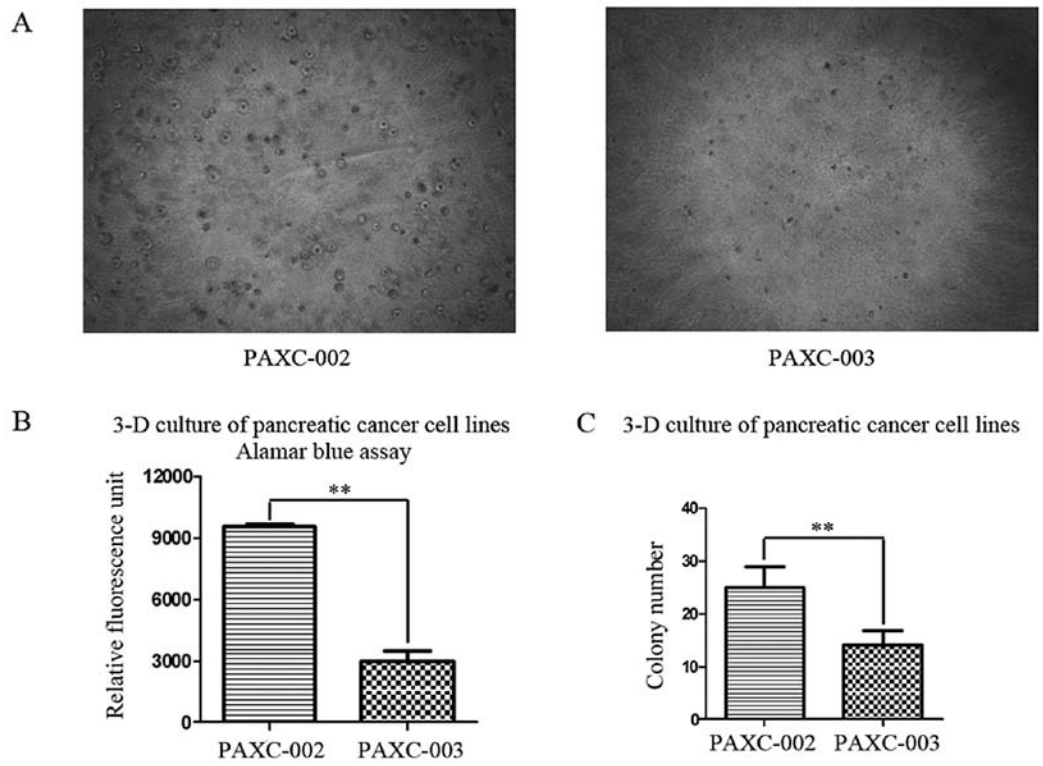

Figure 4. *In vitro* 3-D colony formation assay. (A) PAXC-002 and PAXC-003 cells were seeded in matrigel and grew in 3-D culture conditions. Magnification x40. (B) Colony viabilities were measured by Alamar blue method. (C) Tumor cell colonies were counted under an inverted microscope. **P<0.001.

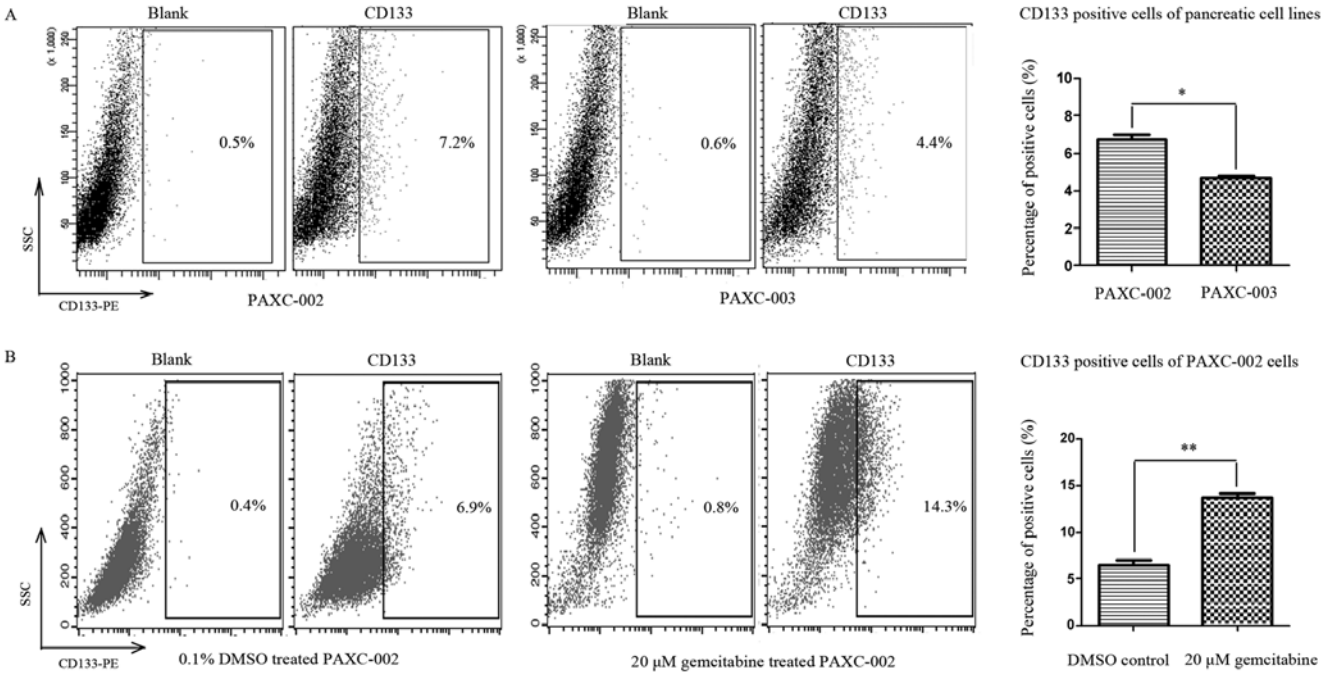

Figure 5. CD133 was used as the cancer stem cell marker in pancreatic cancer cells. (A) PAXC-002 and PAXC-003 were stained with CD133-PE antibody. (B) PAXC-002 cells were treated with 0.1% DMSO or 20 μM gemcitabine, and then the viable cells were stained with CD133-PE. Plots are representative examples of CD133 staining from three individual experiments, with the frequency of the CD133 positive population as a percentage of cancer cells in the specimen. *P<0.05; **P<0.001.

PAXC-002 cell line had more *in vitro* tumorigenic capability indicating the cancer stem cell phenotype.

Cancer stem cell marker expression. The expression of CD133, a cancer stem cell marker which had been reported in pancreatic

carcinoma (18,31), was examined in these two cancer cell lines. As demonstrated in Fig. 5, there were 6.7±0.2% cells positive of CD133 in the gemcitabine-resistant PAXC-002 cell population, whereas only 4.7±0.1% cells of drug sensitive PAXC-003 were CD133 positive ($P<0.05$). This suggested there were an increased percentage of cancer stem-like cells in gemcitabine-resistant PAXC-002 cell line.

Gemcitabine could only inhibit ~50% of PAXC-002 cell growth even at 100 μM after a 96-h exposure (Fig. 4) and the CD133 expression were measured between the vehicle control (0.1% DMSO) and gemcitabine treated PAXC-002 cells. The percentage of CD133 positive PAXC-002 cells was elevated after 20 μM gemcitabine treatment (6.5 vs. 13.8%, Fig. 5), which suggested the correlation of drug resistance and CD133 positive stem-like phenotype.

Discussion

The present report describes the establishment and characterization of the novel pancreatic cancer cell lines PAXC-002 and PAXC-003, derived from human pancreatic ductal adenocarcinomas. It was found that PAXC-002 cells were intrinsically resistant to gemcitabine. Interestingly, the drug resistant cells had more cancer stem cell-like property, which indicated the correlation of gemcitabine resistance and cancer stem cell phenotype.

In this study, both cell lines were established from the *in vivo* passaged human pancreatic cancer xenografts, which facilitated the human tumor cell growth *in vitro*. Although there was the possibility of mouse fibroblast contamination in the primary culture, the mouse cells were eliminated by control trypsinizations after several passages (32), and the purity of human cell origin was confirmed by their epithelioid morphology and median centromere-chromosome. The STR profile also showed that they were novel cell lines and free from cell line cross-contamination. The expression of cytokeratin, CA19-9 and CEA (33-35), and tumorigenicity in mice indicated that they were pancreatic cancer cells. Moreover, the corresponding cell line xenograft tumors preserved similar pathological profiles and differentiation compared with their clinical samples and human primary tumors, which suggested that these low passage tumor cells maintained the clinical characteristics of pancreatic carcinoma, which could represent pancreatic tumor models both *in vitro* and *in vivo*, and help in translation of clinical information.

Gemcitabine has become the first-line chemotherapy of pancreatic cancer prolonging patient survival and improving the quality of life. However, patients usually have limited response to this therapy, even in combinations, mainly due to the drug resistance of pancreatic carcinomas. Cellular resistance to gemcitabine can be intrinsic or acquired during long-term drug treatment, which is under wide investigation. In this study, PAXC-002 cell line was found innate resistant to gemcitabine compared with PAXC-003 and other widely used pancreatic cancer cell lines (AsPC-1, BxPC-3, MIA PaCa-2, PANC-1 and Capan-1). Unlike some previous studies (17,36), gemcitabine exerted potent growth inhibition effect on these pancreatic cell lines (the IC_{50} concentration of these lines mainly ranged from tens to hundreds of nano-molar) in our assay format, and these data emphasized the significance of the intrinsically resistant PAXC-002 cell line, which IC_{50} was more than 100 μM. It is of interest to find out how PAXC-002 cells were resistant to gemcitabine.

The limited drug efficacy is due to many different mechanisms, including abnormal membrane receptor transport, inefficient metabolic drug conversion or increased metabolite inactivation, enhanced DNA repair and alterations in the apoptotic pathways, and even the tumor microenvironment (10). Recently, the study of cancer stem cells gave new insight into drug resistance. Cancer stem cells represent only a small fraction of tumors, and they possess the self-renewal capability to regenerate a tumor *in vivo* and *in vitro*. They are relative quiescent, resistant to drugs and toxins, tolerate apoptosis and have activated DNA-repair mechanism (37,38). The *in vitro* 3-D colony formation assay is used to measure the self-renewal ability, in which tumor cells could represent *in vivo* physiological properties (38). In our study, PAXC-002 cells formed more colonies in the matrix, which indicated the drug resistant PAXC-002 cells had more tumorigenic cells compared with the drug sensitive PAXC-003 cells. Furthermore, previous studies used $CD133^+$, or $CD24^+/CD44^+/ESA^+$ or ALDH to identify the pancreatic cancer stem cells (18,19,39,40). Here we used CD133 to identify the cancer stem cells, and the CD133-positive stem-like cells were more abundant in drug-resistant PAXC-002 cells than that in the sensitive PAXC-003 cells. Furthermore, the CD133 positive PAXC-002 cells increased markedly after gemcitabine treatment. It suggested that the CD133 positive cells could survive the drug exposure, or the drug treatment would stimulate CD133 expression and stem cell phenotype. All these results indicated that CD133-positive stem-like cells would be responsible for the resistance.

In conclusion, we established the novel human pancreatic carcinoma cell lines PAXC-002 and PAXC-003, and PAXC-002 is intrinsically resistant to gemcitabine. Furthermore, we find its drug resistance may associate with cancer stem cell-like phenotype. The molecular mechanisms of the drug resistance need to be further investigated. Moreover, the novel cell lines of PAXC-002 and PAXC-003 could be useful in cancer research and anticancer drug screening.

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