Isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma

Peng Huang, Chun-You Wang, Shan-Miao Gou, He-Shui Wu, Tao Liu, Jiang-Xin Xiong

Abstract
AIM: To explore the method of isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma cell line PANC-1.

METHODS: The PANC-1 cells were cultured in Dulbecco modified eagle medium F12 (1:1 volume) (DMEM-F12) supplemented with 20% fetal bovine serum (FBS). Subpopulation cells with properties of tumor stem cells were isolated from pancreatic adenocarcinoma cell line PANC-1 according to the cell surface markers CD44 and CD24 by flow cytometry. The proliferative capability of these cells in vitro were estimated by 3-(4,5-dimehyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method. And the tumor growth of different subpopulation cells which were injected into the hypodermis of nude mice was studied, and expression of CD44 and CD24 of the CD44+CD24+ cell-formed nodules and PANC-1 cells were detected by avidin-biotin-peroxidase complex (ABC) immunohistochemical staining.

RESULTS: The 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, 57.8% -70.1% expressed CD24, only 2.1%-3.5% of cells were CD44+CD24- cells. Compared with CD44+CD24+ cells, CD44+CD24- cells had a lower growth rate in vitro. Implantation of 107 CD44 CD24+ cells in nude mice showed no evident tumor growth at wk 12. In contrast, large tumors were found in nude mice implanted with 107 CD44+CD24+ cells at wk 4 (2/8), a 20-fold increase in tumorigenic potential (P < 0.05 or P < 0.01). There was no obvious histological difference between the cells of the CD44+CD24+ cell-formed nodules and PANC-1 cells.

CONCLUSION: CD44+CD24+ may be used as the cell surface markers for isolation of pancreatic cancer stem cells from pancreatic adenocarcinoma cell line PANC-1. Subpopulation cells CD44+CD24+ have properties of tumor stem cells. Because cancer stem cells are thought to be responsible for tumor initiation and its recurrence after an initial response to chemotherapy, it may be a very promising target for new drug development.

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Key words: Pancreatic tumor; Stem cells; Tumor stem cells; Isolation; Identification

INTRODUCTION
Pancreatic carcinoma is an obstinate disease that is difficult to deal with. Though pancreatic cancer accounts for only 2%-3% of all cancers, it is the fourth most frequent cause of cancer death in industrialized countries[3]. It is estimated in the United States in 1998 that at least 29,000 new cases of pancreatic cancer will be diagnosed[3]. Unfortunately, only 18% will survive one year after diagnosis, the five-year survival rate is 4%. This is because by the time a patient exhibits symptoms, and the cancer is diagnosed, it is no longer in its early stage[13]. The main conventional treatments for pancreatic cancer are surgery, radiation therapy and chemotherapy. Despite advances in surgical and...
Medical therapy, little effect has been made on the mortality rate of this disease. According to Bjerkvig et al.8, the capacity of a tumor to grow and propagate is dependent on a small subset of cells (so-called tumor stem cells), tumor stem cells are immature cells that can replicate or self-renew, and are able to differentiate or grow into all the cells that an organism or particular organ system need. It has profound implications to understand how tumors evolve and how we treat tumors. If we can destroy these tumor stem cells, it will be possible to treat the patients successfully. However, it is difficult to purify tumor stem cells because of lack of specific cell surface markers in solid tumors. Recently, it was reported that cancer stem cells existed in some solid malignancies, including breast9, brain10, prostate10, and lung cancers11. Thus, we deduced that pancreatic cancer might contain its own stem cells responsible for its metastasis and recurrence. To prove this hypothesis, we isolated subpopulation cells that have characteristics of tumor stem cells according to markers CD44 and CD24 by flow cytometry from pancreatic adenocarcinoma cell line PANC-1, and explore their biological characteristics. This study was to identify the method of isolation of pancreatic tumor stem cells and the ability of propagation of the tumor stem cells in vitro and in vivo.

MATERIALS AND METHODS

Experimental materials
Male nude mice, aged 6-8 wk and weighing 20 ± 2 g, were provided by the Experimental Animal Center, Hubei Center for Disease Control and Prevention, China. The nude mice were caged individually under specific pathogen free (SPF) conditions. Human pancreatic adenocarcinoma cell line PANC-1 was obtained from American Type Culture Collection, Manassas, Virginia, the Dulbeccoo modified eagle medium F12 (1:1 volume) (DMEM-F12) from Hyelone, Wuhan, China, the fetal bovine serum from Sijiqing, Hangzhou, China, trypsin from Sigma-Aldrich, Shanghai, China, the epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), insulin-transferrin-selenium solution (ITS) and trypsin from Sigma-Aldrich, Shanghai, Hangzhou, China and the fetal bovine serum from Sijiqing, Wuhan, China, the epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), insulin-transferrin-selenium solution (ITS) and trypsin from Sigma-Aldrich, Shanghai, Hangzhou, China and the fetal bovine serum from Sijiqing, Wuhan, China, the epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), insulin-transferrin-selenium solution (ITS) and trypsin from Sigma-Aldrich, Shanghai, Hangzhou, China and the fetal bovine serum from Sijiqing, Wuhan, China.

Cell culture
The cells were cultured in incubator filled with 5% CO₂ at 37°C. The PANC-1 cells were cultured in DMEM-F12 (1:1 volume) supplemented with 20% fetal bovine serum (FBS), penicillin (1 × 10⁵ U/L) and streptomycin (100 mg/L).

Flow cytometric analysis
Cells were dissociated by trypsin-EDTA solution (trypsin, 0.25%; EDTA, 0.02%) for 2-5 min at 37°C, transferred to a 5-mL tube, washed twice with PBS with 2% heat-inactivated calf serum (HICS; 5 min at 1000 r/min), resuspended in 100 μL (per 10⁶ cells) of PBS, then were counted. PE anti-human CD44 and (or) FITC anti-human CD24 (appropriate dilution per antibody) were added and incubated for 30 min at 4°C, and then washed twice with PBS. Flow cytometry was performed on a FACS, and data were analyzed with the Cell Quest software (BD, America). Using forward and side scatter profile, debris and dead cells were gated out. Cells were routinely sorted twice, and reanalyzed for purity. Then CD44⁺, CD44⁻ cells, CD44⁺CD24⁻ and CD44⁺CD24⁺ and unsorted cells were obtained.

Estimation of proliferative capability of cells in vitro
The CD44⁺CD24⁻, CD44⁺CD24⁺ and unsorted cells were diluted to a density of about 10⁵ cells/mL with serum-free medium (SFM), a mixture of DMEM-F12 containing 10 ng/mL fibroblast and 20 ng/mL epidermal growth factors, 5 kg/mL insulin, 2.75 mg/mL transferrin, 2.75 mg/mL selenium (insulin-transferrin-selenium solution), penicillin (1 × 10⁵ U/L) and streptomycin (100 mg/L). The 200-μL well diluted cell suspension was plated to 96-well culture dishes. The wells with 2 × 10⁵ cells were observed everyday under an Olympus CKX41 microscope; the images were captured using an Olympus C5050Z camera. Each group was set up with five duplicate holes. Their OD values were measured with spectrophotometer at 490 nm by 3-[4,5-dimehyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method, and a 96-well plate was determined every 24h. The mean value was obtained and a growth curve was drawn.

Transplantation of cells into nude mice
After resuspension, CD44⁺, CD44⁻, CD24⁻, CD24⁺, CD44⁺CD24⁻, CD44⁺CD24⁺ and unsorted cells were diluted to a density of about 5 × 10⁵ to 5 × 10⁶ cells/mL with SCM. The cells (0.1 mL) were injected into the hypodermis of right and left armpit of nude mice. The mice were maintained in a specific pathogen-free room under constant temperature and humidity.

Immunohistochemical staining of CD44 and CD24
All samples of the CD44⁺CD24⁻ cell-formed nodules were placed into 10% formalin immediately, processed with routine histological procedures, and embedded in paraffin. Serial sections were cut 5 μm thick, and parts of them were stained with hematoxylin and eosin for routine histological observation under light microscope. The others were used for immunohistochemical examination for the CD44 and CD24. After deparaffinization (hydration), sections were treated sequentially with normal goat serum, anti-human CD44 polyclonal antibody (1:200) or anti-human CD24 polyclonal antibody (1:200), biotin-labeled goat anti-mouse IgG, and avidin-biotin-peroxidase complex (ABC). The sites of peroxidase binding were demonstrated by the dianimobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. Similar procedures were done for the PANC-1 cells. The numbers and areas of CD44⁺ and CD24⁺positive foci > 0.2 mm in diameter and the total areas of the examined sections were measured using a Olympus C5050Z digital camera, Adobe Photoshop version 7.0, and Image-Pro Plus version 6.0.
As shown in Figure 1, 5.1% of Lineage tumorigenic cells generated tumor growth was found at wk 12 while 10\(^{5}\) cells had a lower growth rate and longer doubling time in vitro. Compared with CD44 positive cells, CD44 negative cells were seen under light microscope. The total CD44 positive and CD24 positive cells had large tumors at wk 4 (2 of 8), a 20-fold increase in tumorigenic potential compared with marker negative cells (P = 0.029). Similar results were obtained with CD24. Injection of CD44 CD24 cells resulted in an enhanced tumorigenic potential compared with single marker sorted cells. More tumors formed with injection of as few as 10\(^{3}\) cells, and no tumor formed in marker-negative cells until at least 5 \(\times 10^{3}\) cells were injected (1 of 10 animals).

There was no obvious histological difference between the cell-formed nodules and PANC-1 cells.

**DISCUSSION**

The theory of tumor stem cells indicates that tumor cells have heterogeneity, i.e., the majority of cells in the injected, one of six mice developed tumors. For cancer cells sorted for the markers CD44 and CD24, expression of individual markers identified cell populations with enhanced tumorigenic potential. For example, injection of 5 \(\times 10^{3}\) CD44\(^{+}\) cells would occasionally form a tumor (1 of 6 animals), whereas no tumor was observed with CD44\(^{-}\) cells until at least 5 \(\times 10^{3}\) cells were injected (1 of 10 animals). Six of 10 animals developed tumors when injected with 5 \(\times 10^{4}\) CD44\(^{+}\) cells, representing a 10-fold increase in tumorigenic potential compared with marker negative cells (P = 0.029). Similar results were obtained with CD24. Injection of CD44 CD24\(^{-}\) cells resulted in an enhanced tumorigenic potential compared with single marker sorted cells. More tumors formed with injection of as few as 10\(^{3}\) cells, and no tumor formed in marker-negative cells until at least 5 \(\times 10^{3}\) cells were injected. The sorted cell population with the highest tumorigenic potential was those expressing CD44 and CD24. For example, injection of 10\(^{5}\) CD44\(^{-}\) CD24\(^{-}\) cells in nude mice found no tumor growth at wk 12. In contrast, nude mice injected with 10\(^{3}\) CD44\(^{-}\) CD24\(^{-}\) cells had large tumors at wk 4 (2 of 8), a 20-fold increase in tumorigenic potential (P < 0.05 or P < 0.01) (Table 1).

There was no obvious histological difference between the CD44\(^{+}\)CD24\(^{-}\) cell-formed nodules and PANC-1 cells.
tumor have lost the growth potential, only a small subset of cells have the capability of the infinite proliferation, the differentiation and the formation of cloning in vitro. The initial isolation and identification of tumor stem cells was first proved in hematological malignancies. The CD34+CD38- phenotype cells (5% of the cancer cells) with obvious proliferation, differentiation and self-renewal ability were purified from the blood of the patients with acute myeloid leukemia. In 2003, researchers found that only a small subset of human breast cancer cells, with the phenotype CD44+CD24-, formed new tumors in NOD/SCID mice. These breast cancer-initiating cells can be isolated and propagated in vitro as extensively proliferating, clonal, nonadherent spherical clusters are able to differentiate along different mammary epithelial lineages. A small population of cancer-initiating cells (also called cancer stem cells) was later found in several malignancies, including brain, prostate, liver, lung, melanoma, and colon tumors.

Although there is increasing evidence that a rare population of undifferentiated cells is responsible for tumor formation and maintenance, little work has been done on the identification of pancreatic cancer special surface markers or on isolation of pancreatic tumor-initiating cells. Based on studies in breast cancer and pancreatic adenocarcinoma, we identified cells with the characteristics of tumor stem cells according to the cell surface markers CD44 and CD24 by flow cytometry from pancreatic adenocarcinoma cell line PANC-1. Tumor stem cells have the capability to maintain themselves in culture in an undifferentiated state, initiate tumor growth after xenotransplantation in mice, and differentiate into cancers that are phenotypically indistinguishable from the original tumor. We found that 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, 57.8%-70.1% expressed CD24, and only 2.1%-3.5% of cells were CD44+CD24+. To take a small subset of cells and put it in the organism and see if it regenerates the original tissues is the classic definition of a stem cell. We injected cells into the hypodermis of the right and left armpit of nude mice to test the capability of tumor initiation. When $5 \times 10^3$ unsorted PANC-1 cells were injected into nude mice, no tumor grew at wk 12 unless at least $10^5$ cells were injected. For cancer cells sorted for the markers CD44 and CD24, injection of $5 \times 10^3$ CD44+ cells would form a tumor, whereas no tumor was observed with CD44+ cells until at least $5 \times 10^5$ cells were injected. Similar results were obtained with CD24+. The sorted cell population with the highest tumorigenic potential was those cells expressing CD44 and CD24. For instance, injection of $10^3$ CD44+CD24+ cells into nude mice, no tumor growth was evident at wk 12. In contrast, nude mice injected with $10^3$ CD44+CD24- cells had large tumors at wk 4. Moreover, the CD44+CD24- cells maintained the ability to engraft and reproduce the same histological and antigenic pattern of the PANC-1. In addition, compared with CD44+CD24- cells in vitro, CD44+CD24- cells had a lower growth rate. The reason is that tumor stem cells are similar to stem cells, which is in relatively static group of cells, and besides other primates, the stem cell pool proliferates once a year. For the CD44+CD24- cells, there were biological behaviors of the lower proliferative index and the faster tumor growth rate in vivo. It is self-contradictory. The reason awaits further studies. In addition, there was no obvious histological difference between the CD44+CD24- cell-formed nodules and PANC-1 cells.

The above results showed that CD44 and CD24 may be used as markers for isolation of pancreatic cancer stem cells from pancreatic adenocarcinoma cell line PANC-1, subpopulation cells CD44+CD24+ have the characteristics of tumor stem cells. The purification and

### Table 1: Tumor formation ability of sorted pancreatic cancer cells using surface markers (number of tumors formed/number of injections)

| Groups       | $5 \times 10^3$ | $10^3$ | $5 \times 10^4$ | $10^4$ | $5 \times 10^5$ | $10^5$ | $10^6$ |
|--------------|----------------|--------|----------------|--------|----------------|--------|--------|
| Unsorted     | 6/6            | 5/6    | 3/6            | 1/6    | 0/6            | 0/0    | 0/0    |
| CD44+        | 0/0            | 9/10   | 6/10           | 3/10   | 1/6            | 0/4    | 0/0    |
| CD24+        | 0/0            | 2/10   | 1/10           | 0/4    | 0/0            | 0/0    | 0/0    |
| P            | 0.0027         | 0.0286 | 0.3297         | 0.0007 | 0.0051         | 0.0035 | 0.0385 |
| CD44+CD24+   | 0/0            | 7/8    | 4/8            | 3/8    | 0/4            | 0/0    | 0/0    |
| CD44CD24+    | 0/0            | 2/8    | 1/8            | 0/8    | 0/0            | 0/0    | 0/0    |
| P            | 0.0203         | 0.1410 | 0.1000         | 0.0007 | 0.0051         | 0.0035 | 0.2333 |

Compared with results from marker-negative cells.
other biological behaviors of pancreatic adenocarcinoma stem cells need to be further studied in the future.

**COMMENTS**

**Background**

Pancreatic carcinoma is an obstinate disease that is difficult to deal with. Though pancreatic cancer accounts for only 2%-3% of all cancers, it is the fourth most frequent cause of cancer deaths in industrialized countries. Unfortunately, only 18% will survive one year after diagnosis, the five-year survival rate is only 4%. Conventional main treatments for pancreatic cancer are surgery, radiation therapy and chemotherapy. Despite advances in surgical and medical therapy, little effect has been achieved on the mortality rate of this disease.

**Research frontiers**

The initial isolation and identification of tumor stem cells was first proved in hematological malignancies. The CD34⁺CD38⁻ phenotype cells (5% of the cancer cells) with obvious proliferation, differentiation and self-renewal ability had been purified from the blood of the patients with acute myeloid leukemia. Researchers have discovered a small population of cancer-initiating cells (also called cancer stem cells) in several malignancies, including brain, prostate, liver, lung, melanoma, and colon tumors.

**Innovations and breakthroughs**

The authors isolated pancreatic adenocarcinoma cell line Panc-1 according to the cell surface markers CD44 and CD24 by flow cytometry, obtained subpopulation cells which have properties of tumor stem cells, and identified the ability of propagation of the tumor stem cells in vitro and in vivo.

**Applications**

Because cancer stem cells are thought to be responsible for tumor initiation and its recurrence after an initial response to chemotherapy, it may be a very promising target for new drug development.

**Peer review**

This study corroborates a recent publication in the pancreas reporting that a subpopulation of Panc1 cells can propagate to form spheres and that these cells express stem cell markers such as CD44. The study is very interesting.

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**REFERENCES**

1. Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. *Nat Rev Cancer* 2002; 2: 897-909
2. Murphy SL. Deaths: final data for 1998. *Natl Vital Stat Rep* 2000; 48: 1-105
3. Cameron JL, Crist DW, Sitzmann JV, Hruban RH, Boitnott JK, Seidler AJ, Coleman J. Factors influencing survival after pancreaticoduodenectomy for pancreatic cancer. *Am J Surg* 1991; 161: 120-124; discussion 124-125
4. Niederhuber JE. Brennan MF, Menck HR. The National Cancer Data Base report on pancreatic cancer. *Cancer* 1995; 76: 1671-1677
5. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin* 2002; 52: 23-47
6. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer* 2005; 5: 899-904
7. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; 100: 3983-3988
8. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; 63: 5821-5828
9. Galli R, Binda E, Orfaneli U, Gipelletti B, Gritti A, De Vitis S, Fiseco R, Foroni C, Dimeco F, Vescovi A. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004; 64: 7011-7021
10. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; 65: 10946-10951
11. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronrt RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005; 121: 823-835
12. Litvinov SV, Velders MP, Bakker HA, Fleuren GJ, Warnaar SO. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol* 1994; 125: 437-446
13. Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Chinnathambi S, Alexandrunas D, Brennan MF, Menck HR. The National Cancer Data Base report on pancreatic cancer. *Clin Cancer Res* 2000; 6: 120-124; discussion 124-125
14. Weichert W, Denkert C, Burkhardt M, Ganssukh T, Bellach J, Altevogt P, Dietel M, Kristiansen G. Cytoplasmic CD24 expression in colorectal cancer independently correlates with shortened patient survival. *Clin Cancer Res* 2005; 11: 6574-6581
15. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer* 2005; 5: 899-904
16. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; 67: 1030-1037
17. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; 3: 730-737
18. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Camens-Cortes J, Minden M, Paterson B, Calliguri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; 367: 645-648
19. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005; 65: 5506-5511
20. Chiha T, Kita K, Zheng YW, Yokusouka O, Saisho H, Iwama A, Nakauchi H, Taniguchi H. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. *Hepatology* 2006; 44: 240-251
21. Suetsugu A, Naganik M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of CD133⁺ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* 2006; 351: 820-824
22. Dome B, Timar J, Dobos J, Meszaros L, Raso E, Paku S, Kennesy I, Ostos G, Magyar M, Ladanyi A, Bogos K, Tovari J. Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. *Cancer Res* 2006; 66: 7341-7347
23. Grichnik JM, Burch JA, Schultz RD, Shao S, Liu J, Darrow TL, Vervaet CE, Seigler HF. Melanoma, a tumor based on a mutant stem cell? *J Invest Dermatol* 2006; 126: 142-153
24. O’Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; 445: 106-110
25. Ricci-Vitiani L, Lombardi DG, Pilotto E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; 445: 111-115
26. Dunnwald M, Chinnathambi S, Alexandrunas D, Bickenbach JR. Mouse epithelial stem cells proceed through the cell cycle. *J Cell Physiol* 2003; 195: 194-201

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