Swiss Medical Weekly
Formerly: Schweizerische Medizinische Wochenschrift
The European Journal of Medical Sciences

Basic science in medicine | Published 31 May 2011, doi:10.4414/smw.2011.13208
Cite this as: Swiss Med Wkly. 2011;141:w13208

Cyclopamine reverts acquired chemoresistance and down-regulates cancer stem cell markers in pancreatic cancer cell lines

Jie Yao[a, 1], *Yong An*[b, 1], *Ji-shu Wei*[b], *Zhen-ling Ji*[c], *Zi-peng Lu*[b], *Jun-li Wu*[b], *Kui-rong Jiang*[b], *Ping Chen*[a], *Ze-kuan Xu*[b], *Yi Miao*[b]

[a] Department of General Surgery, The First Affiliated Hospital of Yangzhou University, Yangzhou 225001, P. R China
[b] Centre for Pancreatic Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210009, P. R China
[c] Department of General Surgery, Zhongda Hospital, Southeast University, Nanjing, 210009, P. R China
[1] These authors contributed equally to the study

Summary

BACKGROUND: The hedgehog (Hh) pathway has been implicated in the pathogenesis of cancer including pancreatic ductal adenocarcinoma (PDAC). Recent studies have suggested that Hh plays an important role in maintaining the cancer stem cell (CSCs) pool. Gemcitabine-resistant pancreatic cancer cells highly express some of the CSCs markers. However, the expression level of Hh members in gemcitabine-resistant pancreatic cancer cells remains unknown. The aim of this study was to verify the expression of HH members, such as Shh, Ptc, SMO and Gli-1 in gemcitabine-resistant PDAC cell lines, and to explore a new strategy to overcome chemoresistance in PDAC.

MATERIAL AND METHODS: Quantitative real-time RT-PCR (Q-PCR) and western blot were used to evaluate the relative expression level of HH members in SW1990, CFPAC-1 cells and gemcitabine-resistant SW1990, CFPAC-1 cells. The change of cancer stem cell markers and the expression level of HH members before and after cyclopamine treatment was evaluated using flow cytometry and Q-PCR, western blot, respectively. Cell apoptosis after cyclopamine treatment was measured by flow cytometry.

RESULTS: CD44, CD133 and the expression level of HH members, including Shh, SMO, Gli-1, were found to be highly expressed in gemcitabine-resistant cells, which were significantly down-regulated by cyclopamine treatment. Flow cytometry analysis showed increased cell apoptosis after cyclopamine treatment.

CONCLUSION: Gemcitabine-resistant pancreatic cancer cells highly express CSCs markers and some of the HH members, and inhibition of HH by cyclopamine is an effective method of reversing gemcitabine resistance in pancreatic cancer.

Key words: *cancer stem cells; hedgehog signaling pathway; gemcitabine; drug resistance; cyclopamine; pancreatic cancer; chemoresistance*

Introduction

Pancreatic cancer is among the most devastating of human malignancies. Despite recent improvements in surgical and chemotherapeutic approaches, pancreatic cancer continues to have a dismal prognosis, with an average overall median survival of 4–6 months. Overall 5-year survival is less than 5% [1]. Currently, gemcitabine is the standard chemotherapeutic agent used in patients with pancreatic cancer [2]. However, the clinical impact of gemcitabine remains modest [3]. This limitation in conventional treatments is mainly due to the profound resistance of cancer cells to anti-cancer drugs, which can be inherent and/or acquired [4, 5]. To study acquired chemoresistance in pancreatic cancer, several drug-resistant pancreatic cancer cell lines have been established. In the research of Shah [6] and Hong [7], pancreatic cancer stem cells (CSCs) had been found to be enriched in gemcitabine-resistant pancreatic cancer cell lines. The CSC hypothesis offers new insight into the mechanism of drug resistance [8]. Most conventional therapies kill most of the tumour population, but CSCs, which have intrinsic detoxifying mechanisms, can easily elude conventional therapies. The CSCs model also explains why standard chemotherapy may result in tumour shrinkage but is then followed by tumour recurrence and multidrug resistance. Therapies targeting cancer stem cells may contribute a new strategy for overcoming drug resistance.

Pancreatic cancer stem cells are highly tumorigenic and possess the abilities to self-renew and produce differentiated progeny. They are defined by the expression of the cell surface markers CD44, CD24 and ESA [9] and CD133. [10, 11]. Cancer stem cells are different from cancer cells in many ways. Not only are cancer stem cells more resistant to standard chemotherapy drugs, they also employ different signalling pathways [12]. Misregulated Hedgehog (HH) signalling, which is normally an essential pathway during embryonic pancreatic development, has been implicated in several forms of cancer, including human pancreatic carcinoma [13–15]. Activation of HH signalling is typically initiated by the binding of hedgehog ligands (Son-

ic, India, Desert hedgehog) to a 12-transmembrane protein receptor patched (Ptc). Binding of HH to Ptc relieves Smoothened (SMO), a 7-transmembrane protein, from the inhibitory effect of Ptc, and activated SMO in turn triggers a series of intracellular events, resulting in the regulation of downstream target genes through the GLI transcriptional effectors Gli-1, Gli-2 and Gli-3 [16, 17]. Blockage of HH signalling has been shown to inhibit pancreatic cancer cell growth [15], invasion, and metastases [18]. Recent studies demonstrated that a HH inhibitor can restore drug resistance in CD34+ leukaemic cells [19]. The combined blockade of Hh and mTOR signalling together with chemotherapy is capable of eliminating pancreatic CSCs [11]. Lee et al. showed that the HH signalling pathway is overexpressed in CD44+CD24+ESA+ pancreatic cancer stem cells [9]. Gemcitabine-resistant pancreatic cells are enriched with cancer stem cells, but whether these cells overexpress the HH signalling pathway remains unknown.

In a previous study we established gemcitabine-resistant pancreatic cell lines CFPAC-1/res and SW1990/res, and we also found that CD44, ABCG2 and ABCB1 were highly expressed in gemcitabine-resistant cells, as reported by Hong [7]. In the present study we evaluated the expression of the HH signalling pathway in drug-resistant pancreatic cell lines as compared to parental cells. We also blocked the HH signalling pathway with cyclopamine to determine whether ABCG2, ABCB1, CD44, and CD133 can be down-regulated in gemcitabine-resistant cells and thus whether acquired gemcitabine-resistance can be reversed by blockade of the HH signalling pathway.

Materials and methods

Cell culture

The human pancreatic cancer cell lines CFPAC-1 and SW1990 were purchased from Shanghai Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) at 37ºC with 5% CO_2, supplemented by 1% penicillin/streptomycin.

Establishment of gemcitabine-resistant pancreatic cancer cells

Pancreatic CFPAC-1 and SW1990 cell lines were established from spleen metastases and liver metastases of PDAC respectively. Resistant cells were obtained by culturing parental CFPAC-1 and SW1990 cells in serially increasing concentrations of gemcitabine. In brief, the cells were first cultured in medium with increasing concentrations of gemcitabine, starting at the IC_{50}, for 3 days, followed by recovery periods in drug-free medium until the cells regained exponential growth. The new IC_{50} of gemcitabine-treated cells was then evaluated by MTT assay. The concentration of gemcitabine was then increased to the new IC_{50} to kill half of the cells. Gemcitabine was purchased from the Lilly Company (Lilly France).

Drug sensitivity assay

To detect the IC_{50} of both pancreatic cancer cell lines, aliquots of 2×10^3 CFPAC-1 and 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. A 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to examine the effects on cell growth. In brief, 20 µL MTT solution (5 mg/ mL) (Sigma) was added into each well and incubated at 37 ºC for 4 hr. A 150-µL aliquot of DMSO was then added and absorbance was measured by a microplate reader (Multiskan MK3, Thermo Labsystems, USA) at a wavelength of

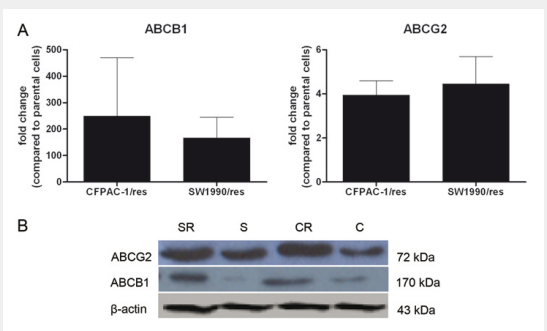

Figure 1

Gemcitabine-resistant pancreatic cancer cells highly express ABCB1 and ABCG2. A. Quantitative RT-PCR was used to evaluate the relative expression level of ABCB1 and ABCG2 gene expression in gemcitabine-resistant cells when compared to their parental cells. Fold change was analysed by the $2^{-\Delta\Delta Ct}$ method, while the expression of β-actin was used to normalise the relative expression of each gene within each cell line. B. The results of the western blot analysis were consistent with the mRNA expression. Values represent mean ± S.D. of three independent experiments.

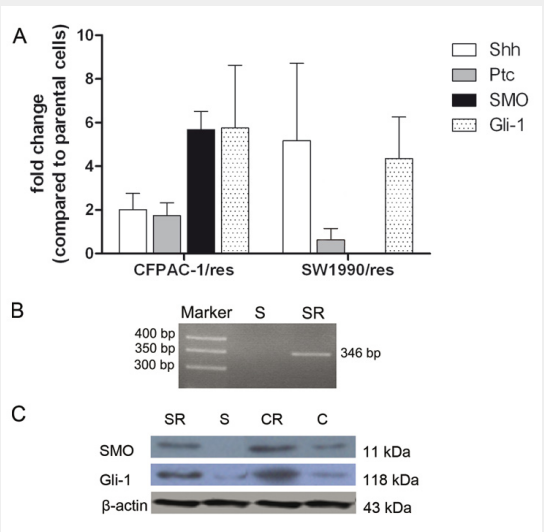

Figure 2

Gemcitabine-resistant pancreatic cell lines highly express hedgehog members. A, Quantitative RT-PCR was used to evaluate the relative expression level of Shh, Ptc, SMO, and Gli-1 in gemcitabine-resistant cells in comparison to their parental cells. Fold change was analyzed using the $2^{-\Delta\Delta Ct}$ method, while the expression of β-actin was used to normalise the relative expression of each gene within each cell line. Values represent mean ± S.D. of three independent experiments. B, mRNA expression of SMO can be detected in SW1990/res cells but not parental cells. C, The different protein expression levels of SMO and Gli-1 are consistent with mRNA expression.

490 nm. The cell survival rate (SR) was calculated using the formula SR = (mean absorbance of the test well/mean absorbance of the control) × 100%; the inhibition rate (IR) was calculated using the formula (IR) = 100% - SR. The IC_{50} of each cell line was calculated.

Flow cytometry analysis

After detachment from the culture dish with 0.25% trypsin, the cells were washed with phosphate-buffered saline (PBS) and suspended in PBS at a concentration of 1×10^6 per mL. The cells were then stained with CD44-FITC and CD24-PE (BD Bioscience) or with CD133-PE (Ebioscience) alone. After incubation on ice in the dark for 30 min, the cells were washed twice with PBS and resuspended in 1 mL PBS. The cells were kept on ice until flow cytometry analysis.

Quantitative real-time RT-PCR and RT-PCR

Total RNA was extracted from gemcitabine-resistant cells and parental cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was reverse transcribed into cDNA using the Promega AMV reverse transcription system (Promega, Madison WI, USA). Quantitative RT-PCR was performed with SYBR Green master mix real-time core reagents on an ABI 7500 (Applied Biosystems) according to the manufacturer's instructions. Primers for quantitative PCR were as follows: ABCB1 sense: 5'-TGATTGCATTTGGAGGACAA-3', ABCB1 anti-sense: 5'-CCAGAAGGCCAGAGCATAAG-3'. ABCG2 sense: 5'-AGATGGGTTTCCAAGCGTTCAT-3', ABCG2 anti-sense, 5'-CCAGTCCCAGTACGACTGTGACA-3'. Shh sense: 5'-GTGTACTACGAGTCCAAGGCAC-3', Shh anti-sense: 5'-AGGAAGTCGCTGTAGAGCAGC-3'. Ptc sense: 5'-TCCCAAGCAAATGTACGAGCA-3'. Ptc Anti-sense: 5'-TGAGTGGAGTTCTGTGCGACAC-3'. SMO sense: 5'-CTGGTACGAGGACGTGGAGG-3', SMO anti-sense: 5'-AGGGTGAAGAGCGTGCAGAG-3'. Gli-1 sense: 5'-CTCCCGAAGGACAGGTATGTAAC-3', Gli-1 anti-sense: 5'-CCCTACTCTTTAGGCACTAGAGTTG-3'. β-actin sense: 5' -AGAAAATCTGGCACCACACC-3'. β-actin anti-sense: 5' -TAGCACAGCCTGGATAGCAA-3'. The expression of mRNA was normalised to that of the reference gene, β-actin. Relative quantification of mRNA within the samples was examined using the $2^{-\Delta\Delta Ct}$ method. The primers for SMO used for SW1990 were designed by Karhadkar.(20)

Western blot

The concentration of total protein extracted from parental and gemcitabine-resistant cells was determined with a BCA Protein Assay Kit (Pierce, USA). Equal amounts of protein were separated by 10% SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Bedford, USA) using a mini trans-blot (Bio-Rad laboratories, Hercules, CA, USA). Rat anti-human SMO, Gli-1(Millipore, Bedford, USA), ABCB1, and ABCG2 (Abcam, MA, USA) were used to detect the expression of homologous proteins. β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as an internal control. Electrochemiluminescence was performed with a Chemilmager 5500 imaging system (Alpha Innotech Co., San Leandro, CA, USA), according to the manufacturer's instructions.

Cyclopamine treatment

CFPAC-1/res and SW1990/res cells were seeded in 6-well plates (2×10^5 cells per well) for 12 hrs, and then gemcitabine-free culture media containing 2 μM or 5 μM cyclopamine were added. After 12 h, 24 h or 48 h, the cells were harvested. The expression of SMO, Gli1, ABCG2 and ABCB1 before and after cyclopamine treatment was examined using RT-PCR and western blotting. DMSO (vehicle) was used as a negative control.

To detect CSC marker changes, gemcitabine-resistant cells were treated with 2 μM or 5 μM cyclopamine in gemcitabine-free culture media for 7 days, then the cells were collected and labelled with flow cytometry antibodies as described above. The cells cultured in cyclopamine-free, gemcitabine-free medium were used as a control group.

Cell death and apoptosis assays

After treatment with cyclopamine (2 μM and 5.0 μM) for 24 h, CFPAC-1/res and SW1990/res cells were exposed to gemcitabine-containing culture media at final gemcitabine concentrations of 50 μM and 100 μM respectively. After 24 hrs, the cells were harvested and stained with Annexin-V and PI using the Vybrant Apoptosis Assay Kit (Molecular Probes) per the manufacturer's protocol. Briefly, all cells were harvested by trypsinisation and washed twice with cold PBS. The pellets were resuspended in 100 μL 1× Annexin binding buffer and 5 μl fluorescein isothiocyanate (FITC)–Annexin-V (component A). A 1-μL working solution of PI at 100 μg/mL was added to each 100 μL of cell suspension. The cells were incubated on ice for 1 hr, washed again with cold PBS and re-suspended in 300 μL 1×Annexin-binding buffer. The stained cells were immediately analysed by flow cytometry.

Results

In vitro establishment of gemcitabine-resistant pancreatic cancer cells CFPAC-1/res and SW1990/res

After more than 5 months of repetitive exposure to gemcitabine, both cell lines demonstrated a permanent acquired chemoresistance. CFPAC-1/res and SW1990/res cells were able to propagate and be passaged in 50 μM and 100 μM gemcitabine-containing culture medium respectively. As detected by the MTT assay, the IC_{50} of CFPAC-1/res and SW1990/res cells were 68.3 ± 4.5 μM and 306.8 ± 12.3 μM respectively (table 1). Also, the stability of the resistant phenotype in the absence of gemcitabine was examined in both CFPAC-1/res and SW1990/res cells. When the cells were passaged in gemcitabine-free media for 2 weeks, the IC_{50} remained stable.

CFPAC-1/res and SW1990/res highly express CD44, CD133 and ABC transporters

We carried out FACS analysis for CD44 and CD24 or CD133 alone to detect the phenotypic difference between

the gemcitabine-resistant cells and their parental cells. The CD44+ subfraction was dramatically increased in gemcitabine-resistant cells compared with their parental cells (CFPAC-1, 5.64 ± 0.82% vs. 85.07 ± 2.59%; SW1990, 29.45 ± 1.99% vs. 93.16 ± 2.46%). CD133 was also found to be highly expressed in both CFPAC-1/res and SW1990/res cells (CFPAC-1, 28.94 ± 0.5% vs. 75.4 ± 2.25%; SW1990, 59.37 ± 1.69% vs. 95.88 ± 1.47%).

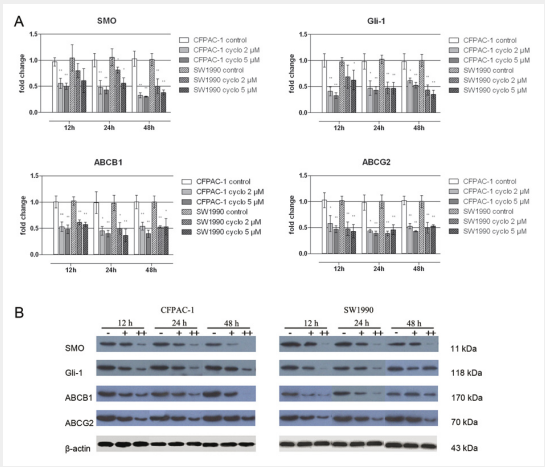

Figure 3

Effects of cyclopamine on the expression of SMO, Gli-1, ABCB1 and ABCG2 in gemcitabine-resistant pancreatic cell lines. A, Relative mRNA level changes were evaluated using quantitative RT-PCR, and the expression of β-actin was used as a control to normalise the relative expression of each gene before and after cyclopamine treatment. Fold change was analysed using the $2^{-\Delta\Delta Ct}$ method. Values are expressed as means ± SD. B, Changes in protein level before and after cyclopamine treatment were tracked by western blot assay. Treatment with 2 μM (+) and 5 μM (++) cyclopamine down-regulated protein levels of SMO, Gli-1, ABCB1 and ABCG2 in gemcitabine-resistant pancreatic cancer cell lines, compared with DMSO group (-). Membranes were reprobed with a β-actin antibody to verify equal loading and transfer. The molecular size markers (in kDa) are indicated on the right.

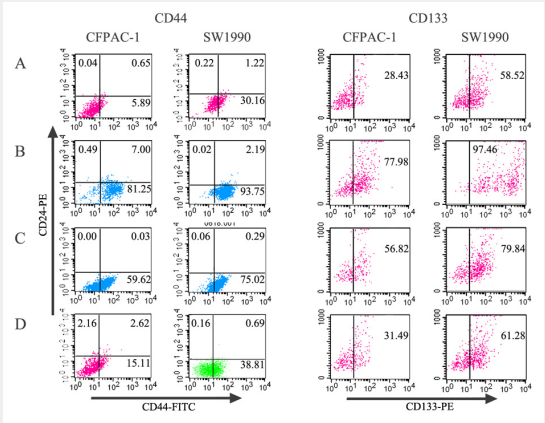

Figure 4

Cyclopamine down-regulates CSC markers in gemcitabine-resistant pancreatic cell lines. Parental CFPAC-1 and SW1990 cells and their gemcitabine-resistant counterparts were double-labelled with CD44-FITC and CD24-PE or CD133-PE alone. The percentages of CD44+ and CD133+ cells increased in gemcitabine-resistant cells (b), compared to parental cells (a). After treatment with 2 μM cyclopamine for 3 (c) and 7 (d) days, the percentage of CD44+ and CD133+ cells decreased to close to parental levels.

ABC transporter family member expression is also characteristic of CSCs, which are responsible for the side population (SP) subfraction [21]. We measured the mRNA expression levels of ABCG2 and ABCB1 in gemcitabine-resistant cells and their parental cells. The relative ABCG2 mRNA expression in CFPAC-1/res and SW1990/res cells were increased 3.94 ± 0.65 and 4.44 ± 1.25 fold when compared to their parental cells. The relative ABCB1 mRNA expression levels in CFPAC-1/res and SW1990/res cells increased by 249.65 ± 220.66- and 166.5 ± 78.24-fold, respectively (fig. 1). The results of the western blot analysis were consistent with the mRNA expression.

CFPAC-1/res and SW1990/res highly express Hedgehog signalling members

Since gemcitabine-resistant cells highly expressed some of the markers of pancreatic cancer stem cells, we hypothesised that they might also highly express some cancer stem cell-related signalling pathways, such as the HH signalling pathway, that have been reported to be highly expressed in CD44+CD24+ESA+ pancreatic cancer stem cells. To test this hypothesis, the mRNA expression levels of Shh, ptch, SMO, and Gli-1 were compared in gemcitabine-resistant cells and parental cells by quantitative real-time PCR, and the protein levels of SMO and Gli-1 were detected by western blot. The relative expression levels of Shh mRNA were increased 2.01 ± 0.74- and 5.19 ± 3.54-fold in CFPAC-1/res and SW1990/res cells, respectively. The mRNA levels of Ptch were found to be 1.73 ± 0.59- and 2.79 ± 0.53-fold increased in CFPAC-1/res and SW1990/res cells respectively (fig. 2a). In CFPAC-1 cells, SMO increased 5.68 ± 0.83-fold when compared to parental cells. Interestingly, we did not detect any mRNA expression of SMO in parental SW1990 cells, although it can definitely be detected in SW1990/GZ cells (fig. 2b). The expression of Gli-1 increased 5.75 ± 2.88- and 4.35 ± 1.90-fold in CFPAC-1/res and SW1990/res cells respectively.

Inhibition of Hedgehog signalling pathway by cyclopamine inhibits mRNA and protein levels of SMO, Gli-1, ABCG2 and ABCB1

Because cyclopamine had been shown to inhibit HH signalling by interfering with the activation of SMO [22, 23], we treated CFPAC-1/res and SW1990/res with two different concentrations (2 μM and 5 μM) of cyclopamine. To determine the effect of cyclopamine on the expression of SMO, Gli-1, ABCG2 and ABCB1 in gemcitabine-resistant cells, the mRNA and protein expression levels before and after cyclopamine treatment were compared using quantitative RT-PCR and western blotting respectively. Our data showed that treatment with cyclopamine results in decreases in SMO, Gli-1, ABCB1 and ABCG2 in both gemcitabine-resistant cell lines. Similar results were obtained for protein levels. Western blot analysis showed that the protein level of SMO was markedly down-regulated by 5 μM cyclopamine at 24–48 hrs after administration of cyclopamine (fig. 3b).

Cyclopamine treatment down-regulates CSC markers

After treatment of gemcitabine-resistant cells with 2 μM or 5 μM cyclopamine for 7 days in gemcitabine-free culture

medium, the cells cultured in 5 μM cyclopamine underwent significant cell death and apoptosis, so that the number of cells became too small for further experiment. The cells cultured in 2 μM cyclopamine were used to follow the change in CSC markers. Our data show that the percentage of CD44+ cells in CFPAC-1/res decreased from 85.31 ± 2.59% to 57.81 ± 1.99% after 3 days and to 17.1 ± 0.55% after 7 days. The percentage in SW1990/res cells decreased from 93.16 ± 2.46% to 74.05 ± 1.52% after 3 days and to 36.68 ± 2.44% after 7 days. The percentage of CD133+ cells decreased from 75.4 ± 2.24% to 55.20 ± 1.77% after 3 days and to 31.38 ± 1.99% after 7 days in CFPAC-1/res cells and from 95.87 ± 1.47% to 76.52 ± 2.97% after 3 days and to 62.76 ± 1.28% after 7 days in SW1990/res cells. However, after culture in gemcitabine-free and cyclopamine-free medium, the cells in the control group displayed no significant differences in the percentages of CD44+ and CD133+ cells.

Cyclopamine restores gemcitabine sensitivity in gemcitabine-resistant cells

After treatment with 2 μM cyclopamine for 24 h, significantly decreased expression of SMO, Gli-1, ABCG2 and ABCB1 was detected. We then re-exposed gemcitabine-resistant cells to gemcitabine-containing cyclopamine-free culture medium for another 24 h. As shown in figure 4, pretreatment with 2 μM or 5 μM cyclopamine significantly reduced cell survival in gemcitabine-containing culture medium. For SW1990/res, 53.68 ± 5.24% and 69.99 ± 3.16% cells underwent cell death when treated with 2 μM and 5 μM cyclopamine respectively, when combined with 100 μM gemcitabine. For CFPAC-1/res cells, 63.60 ± 7.26% and 76.96 ± 4.28% cells were killed in 2 μM and 5 μM cyclopamine, respectively, when combined with 50 μM gemcitabine.

Discussion

Chemoresistance is a major cause of gemcitabine treatment failure in pancreatic adenocarcinoma. After consecutive treatments, the majority of patients with gemcitabine-treated pancreatic adenocarcinoma become resistant and fail to derive benefit from chemotherapy. Hence it is extremely important to understand the mechanism behind chemoresistance and to identify predictive markers of inherent and acquired chemoresistance to gemcitabine to improve the treatment of these patients.

The cancer stem cell hypothesis provides a new insight into chemoresistance. Standard chemotherapy can greatly reduce tumour bulk but may be less effective on cancer stem cells. In previous studies [6, 7], tumour stem cells were found to be enriched in established pancreatic gemcitabine-resistant cells. After exposure to high-dose gemcitabine, most of the repopulated cells are CD44+, and they reconstituted the resistant cell population. We also carried out FACS analysis for CD24 and ESA in resistant cells, which were reported as putative markers of CSCs in pancreatic cancer. However, the percentage of CD24+ and ESA+ cells showed no significant difference. RNA interference of CD44 inhibited the clonogenic activity of gemcitabine-resistant cells [7]. CD133 is also one of the important cancer stem cell markers [10, 24–26]. In hepatic cancer, CD133+ cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway [27]. In the present study we found that the CD133+ subfraction in SW1990/res and CFPAC-1/res was also increased significantly when compared to their parental cells.

When cultured in gemcitabine-free media, a significant decrease in CD44 and CD133 expression in gemcitabine-resistant cells was observed after 1 week of treatment with 2 μM cyclopamine. At this concentration of cyclopamine, resistant cells can propagate and be passaged without increased cell death and apoptosis. In Mueller's research [11], treatment with cyclopamine combined with rapamycin can abolish CD133+ and CD44+ cell populations in both primary pancreatic cells and the L3.6pl cell line. Our data suggest that blockage of the HH signalling pathway by cyclopamine can also inhibit CSCs markers in gemcitabine-resistant cells.

Sustained HH signalling activity was detected in pancreatic adenocarcinoma cell lines isolated from both primary and metastatic tumours. Inhibition of the HH signalling pathway by cyclopamine in Cfpac-1 cells can reduce cell proliferation and survival through down-regulation of Gli-1 activity [13]. In our study we evaluated the different mRNA and protein levels of HH signalling molecules between the gemcitabine-resistant pancreatic cancer cells and their parental cell lines. We found upregulation of Shh, SMO and Gli-1 in gemcitabine-resistant cells. Interestingly, SMO mRNA and protein expression could not be detected in the parental SW1990 cell line, which is consistent with Gao's study [28]. Cyclopamine can block pancreatic cancer cell growth when SMO mRNA expression is below the level of detection by QRT-PCR [15]. We therefore suggest that SMO might be present in parental SW1990 cells, but at levels too low to be detected by RT-

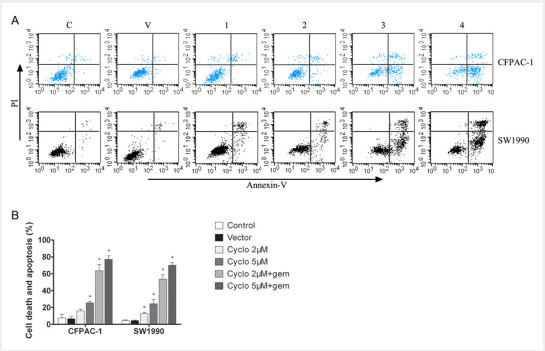

Figure 5

Cyclopamine restored gemcitabine sensitivity in gemcitabine-resistant cells as assessed by annexin V and PI staining. CFPAC-1/res and SW1990/res cells were treated with gemcitabine (mock control) (c), or DMSO (vector control) (v), or treated with 2 μM cyclopamine for 48 h (1) or 5 μM cyclopamine for 48 h (2), pretreated by 2 μM cyclopamine for 24 h and then given gemcitabine-containing culture medium for another 24 h (3), or given 5 μM cyclopamine pretreatment and then gemcitabine treatment (4). The concentration of gemcitabine was 50 μM for CFPAC-1/res and 100 μM for SW1990/res. The cells were then harvested and stained using a Vybrant Apoptosis Assay Kit, as described in the materials and methods section. Values are expressed as means ±SD. *P <0.05.

PCR. After HH signalling molecules had been enriched in gemcitabine-resistant SW1990 cells, SMO was detectable.

Since SMO was upregulated in both the Cfpac-1/res and the SW1990/res cells, we treated gemcitabine-resistant cells with cyclopamine, which has been reported to inhibit HH signaling through inactivation of SMO [22, 23]. Resistant cells treated with 2 μM cyclopamine showed no significant difference in cell death and apoptosis, while treatment with 5 μM cyclopamine caused a slight increase in cell death and apoptosis. However, when gemcitabine-resistant cells were treated with 2 μM or 5 μM cyclopamine in combination with the previous concentration of gemcitabine (at the gemcitabine concentration in which the resistant cells can proliferate and be passaged), the percentage of the population undergoing cell death and apoptosis was significantly increased. This result suggests that the HH pathway may play a crucial role in maintenance cell proliferation and survival in high-dose gemcitabine-containing culture media. Once the increased HH pathway activity had been inhibited by cyclopamine, increased cell death and apoptosis was observed in resistant cells.

It has been reported that combined targeting of histone deacetylases and HH signalling enhances gemcitabine cytotoxicity in PANC-1 and BxPC3 pancreatic cancer cells. Cell death was associated with nuclear localisation of survivin, increased bax expression, and activation of caspases 3 and 7 [29]. These findings support our finding that inhibition of HH signalling pathway induced cell death and apoptosis in gemcitabine-resistant cells and changed the cell phenotype. However, according to Thayer and Feldmann's research [13, 18], both PANC-1 and BxPC3 cells are HH-independent cells, and treatment of parental BxPC3 and PANC-1 cells with cyclopamine did not result in a significant inhibition of cell proliferation. Together with our study, we suggest that these data indicate that a HH inhibitor combined with another signalling pathway inhibitor may be necessary to overcome gemcitabine resistance in HH-independent pancreatic cancer cell lines. In HH-dependent pancreatic cancer cell lines, including CFPAC-1 in this study [13], a single HH inhibitor still had a persistent function in inhibiting gemcitabine-resistant cell proliferation.

Treatment of the human pancreatic cell line E3LZ10.7 with cyclopamine causes growth inhibition in vitro and a significant down-regulation of the HH target gene Gli-1 [18]. Treatment of L3.6pl cells with cyclopamine can also inhibit the downstream HH target Gli-1 [11]. In the present study, after treatment of gemcitabine-resistant cells with cyclopamine, down-regulation of Gli-1, ABCB1 and ABCG2 was observed. The expression of ABC transporters has been correlated with clinical chemoresistance through increasing drug efflux [30, 31]. The combined use of chemotherapy drugs and ABC transporter inhibitors such as verapamil could be employed to kill cancer stem cells selectively and more efficiently [32]. However, this may be difficult to accomplish in vivo, because both ABCB1 and ABCG2 play an important role in maintaining the blood-brain barrier [8]. In oesophageal adenocarcinomas, ABC transporter proteins can be down-regulated by treatment of cancer cells with Gli-1-specific small interfering RNA or treatment with cyclopamine [33], although the detailed mechanism remained unknown. Our data demonstrate that blockade of HH signalling in pancreatic drug resistant cells by cyclopamine results in a decrease in Gli-1 expression. This result may correspond to the decreased expression of ABCB1 and ABCG2, suggesting a different and more feasible approach to overcoming acquired gemcitabine resistance in pancreatic cancer cells.

In conclusion, the HH signalling pathway plays a pivotal role in maintaining the gemcitabine resistance of pancreatic cancer cells. Inhibition of the HH pathway by cyclopamine represents an effective method of reversing gemcitabine resistance in HH-dependent pancreatic cancer cells. In therapeutic application, a targeted therapy against HH signalling pathway could be applied to overcoming gemcitabine resistance and may be beneficial in the treatment of pancreatic cancer.

Funding / potential competing interests: This work was supported by the Postgraduate Innovation Project of Jiangsu province, China, (No. CX08B-175Z, 2008), the Research Fund for the Doctoral Programme of Higher Education, China, (No. 20060312008) and National Natural Science Foundation of China（No.30972912).

Correspondence: *Prof. Yi Miao, Ph D, Centre for Pancreatic Surgery, The First Affiliated Hospital of Nanjing Medical University, CN - Nanjing 210009, China, miaoyi@njmu.edu.cn*

References

1 Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008;58:71–96.

2 Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997;15:2403–13.

3 Carmichael J, Fink U, Russell RC, Spittle MF, Harris AL, Spiessi G, et al. Phase II study of gemcitabine in patients with advanced pancreatic cancer. Br J Cancer. 1996;73:101–5.

4 Muerkoster S, Wegehenkel K, Arlt A, Witt M, Sipos B, Kruse ML, et al. Tumor stroma interactions induce chemoresistance in pancreatic ductal carcinoma cells involving increased secretion and paracrine effects of nitric oxide and interleukin-1beta. Cancer Res. 2004;64:1331–7.

5 Banerjee D, Mayer-Kuckuk P, Capiaux G, Budak-Alpdogan T, Gorlick R, Bertino JR. Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. Biochim Biophys Acta. 2002;1587:164–73.

6 Shah AN, Summy JM, Zhang J, Park SI, Parikh NU, Gallick GE. Development and characterization of gemcitabine-resistant pancreatic tumor cells. Ann Surg Oncol. 2007;14:3629–37.

7 Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. Int J Cancer. 2009;125:2323–31.

8 Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5:275–84.

9 Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res. 2007; 67:1030–7.

10 Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell. 2007;1:313–23.

11 Mueller MT, Hermann PC, Witthauer J, Rubio-Viqueira B, Leicht SF, Huber S, et al. Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. Gastroenterology. 2009;137:1102–13.

12 Tanaka H, Nakamura M, Kameda C, Kubo M, Sato N, Kuroki S, et al. The Hedgehog signaling pathway plays an essential role in maintaining the CD44+CD24-/low subpopulation and the side population of breast cancer cells. Anticancer Res. 2009;29:2147–57.

13 Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature. 2003;425:851–6.

14 Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature. 2003;425:846–51.

15 Kayed H, Kleeff J, Keleg S, Guo J, Ketterer K, Berberat PO, et al. Indian hedgehog signaling pathway: expression and regulation in pancreatic cancer. Int J Cancer. 2004;110:668–76.

16 Kalderon D. Transducing the hedgehog signal. Cell. 2000;103:371–4.

17 McMahon AP. More surprises in the Hedgehog signaling pathway. Cell. 2000;100:185–8.

18 Feldmann G, Dhara S, Fendrich V, Bedja D, Beaty R, Mullendore M, et al. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. Cancer Res. 2007;67:2187–96.

19 Kobune M, Takimoto R, Murase K, Iyama S, Sato T, Kikuchi S, et al. Drug resistance is dramatically restored by hedgehog inhibitors in CD34+ leukemic cells. Cancer Sci. 2009;100:948–55.

20 Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature. 2004;431:707–12.

21 Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. 2001;7:1028–34.

22 Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 2002;16:2743–8.

23 Sims-Mourtada J, Izzo JG, Apisarnthanarax S, Wu TT, Malhotra U, Luthra R, et al. Hedgehog: an attribute to tumor regrowth after chemoradiotherapy and a target to improve radiation response. Clin Cancer Res. 2006;12:6565–72.

24 Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A. 2003;100:15178–83.

25 Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res. 2003;63:5821–8.

26 Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature. 2007;445:111–5.

27 Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene. 2008;27:1749–58.

28 Gao J, Li Z, Chen Z, Shao J, Zhang L, Xu G, et al. Antisense Smo under the control of the PTCH1 promoter delivered by an adenoviral vector inhibits the growth of human pancreatic cancer. Gene Ther. 2006;13:1587–94.

29 Chun SG, Zhou W, Yee NS. Combined targeting of histone deacetylases and hedgehog signaling enhances cytoxicity in pancreatic cancer. Cancer Biol Ther. 2009;8:1328–39.

30 Takara K, Sakaeda T, Okumura K. An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. Curr Pharm Des. 2006;12:273–86.

31 Perez-Tomas R. Multidrug resistance: retrospect and prospects in anticancer drug treatment. Curr Med Chem. 2006;13:1859–76.

32 Dean M. Cancer stem cells: redefining the paradigm of cancer treatment strategies. Mol Interv. 2006;6:140–8.

33 Sims-Mourtada J, Izzo JG, Ajani J, Chao KS. Sonic Hedgehog promotes multiple drug resistance by regulation of drug transport. Oncogene. 2007;26:5674–9.