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CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells

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Accumulating evidence suggests that tumors are composed of a heterogeneous cell population with a small subset of cancer stem cells (CSCs) that sustain tumor formation and growth. Recently, there have been efforts to explain drug resistance of cancer cells based on the concept of CSCs having an intrinsic detoxifying mechanism. In the present study, to investigate the role of CSCs in acquiring chemoresistance in pancreatic cancer, gemcitabine-resistant cells were established by exposure to serially escalated doses of gemcitabine in HPAC and CFPAC-1 cells. Gemcitabine-resistant cells were more tumorigenic *in vitro* and *in vivo*, and had greater sphere-forming activity than parental cells. After high-dose gemcitabine treatment to eliminate most of the cells, $CD44^+$ cells proliferated and reconstituted the population of resistant cells. $CD44^+CD24^+ESA^+$ cells remained as a small subset in the resistant cell population. Among ATP-binding cassette (ABC) transporters, which are known as the mechanism of drug resistance in CSCs, ABCB1 (MDR1) was significantly augmented during the acquisition of drug resistance. ABC transporter inhibitor verapamil resensitized the resistant cells to gemcitabine in a dose-dependent manner and RNA interference of CD44 inhibited the clonogenic activity of resistant cells. In human pancreatic cancer samples, CD44 expression was correlated with histologic grade and the patients with CD44-positive tumors showed poor prognosis. These data indicate that cancer stem-like cells were expanded during the acquisition of gemcitabine resistance and in therapeutic application, targeted therapy against the CD44 or ABC transporter inhibitors could be applied to overcome drug resistance in the treatment of pancreatic cancer.
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Key words: pancreatic cancer; drug resistance; cancer stem cell; cell surface marker; ATP-binding cassette transporter

Pancreatic cancer is the fourth leading cause of cancer-related death in Western countries and has a dismal prognosis with a 5-year survival rate of 1–3%.[1] Most patients with pancreatic cancer are diagnosed at an advanced stage, and are therefore usually candidates for chemotherapy.[2] Since the introduction of gemcitabine, a pyrimidine analog, in 1996, it has been used as the first-line agent for the treatment of pancreatic cancer.[3] However, its therapeutic effect seems marginal, and pancreatic cancer cells easily acquire gemcitabine resistance after a few cycles of administration.[4]

Several attempts have been undertaken to elucidate the mechanism of gemcitabine resistance based on its transport or metabolism. Among the genes mediating chemoresistance, those related to nucleoside transport and metabolism or involved in cell cycle regulation, proliferation or apoptosis have been thought to be responsible for gemcitabine resistance in pancreatic cancer. While the nucleoside transporter[5] and M1 or M2 subunit of ribonucleoside reductase[6] belong to the former, genes such as mutated p53,[7] Bcl-xl,[8] c-Src,[9] and focal adhesion kinase[10] belong to the latter.

During the past few years, various studies have suggested that tumors are composed of a heterogeneous cell population having different biologic properties, of which a small population of cancer cells, or "cancer stem cells (CSCs)" sustain tumor formation and growth.[11] In CSCs, the pathway of self-renewal and differentiation are deregulated, resulting in unlimited self-renewal and a subsequent excess of CSCs. In addition, CSCs have aberrant differentiation programs that generate progenitor tumor cells, which then proliferate to form the bulk of the tumor.[12] Evidence of CSCs was first documented in leukemia and myeloma,[13,14] and so far, their existence has been validated in several solid tumors, such as breast,[15] glioblastoma,[16,17] colon,[18] liver[19] and pancreas.[20] In those studies, cell surface markers were used to identify and purify CSCs from tumors. Recently, putative CSCs have been identified in pancreatic cancer based on the expression of the surface markers CD44, CD24, and epithelial specific antigen (ESA).[20] When injected into NOD/SCID mice, as few as 100 pancreatic cancer cells with $CD44^+CD24^+ESA^+$ formed tumors that were histologically indistinguishable from the original tumors. Thus, investigators have suggested that the subpopulation of pancreatic cancer cells showing $CD44^+CD24^+ESA^+$ had stem cell-like properties of self-renewal and the ability to produce differentiated progeny.

The CSC hypothesis offers not only an attractive model of carcinogenesis but also helps to explain the mechanism of drug resistance and tumor recurrence.[11] Based on the CSC model, a tumor contains a heterogeneous population of mature cancer cells and a small number of CSCs. Most conventional therapies have been developed to kill most of the tumor population, however, CSCs, which have intrinsic detoxifying mechanisms, can easily escape conventional treatments. The CSC model explains why standard chemotherapy may result in tumor shrinkage, but most tumors recur and show multidrug resistance. Evidence explaining drug resistance based on the CSC model has been accumulating. In chronic myeloid leukemia, imatinib, an Abl tyrosine kinase inhibitor, dramatically depleted differentiated cells but failed to reduce leukaemic stem cells, which leads to disease progression.[21] About 74% of tumor cells derived from chemotherapy-treated patients with breast cancer consist of $CD44^+CD24^{-/low}$ phenotypic cells, which were known as breast CSCs, compared with 9% of cells from untreated patients.[22] Liu *et al.*[23] demonstrated that $CD133^+$ cells derived from human glioblastoma were significantly resistant to various chemotherapeutic agents compared to $CD133^-$ cells, and CD133 expression was significantly higher in recurrent glioblastoma. These results suggest that CSC-targeted therapy is mandatory for overcoming drug resistance and curing tumors.

Pancreatic cancer is highly resistant to various chemotherapeutic agents. Although CSC markers in pancreatic cancer have recently been proposed,[20] the mechanism of drug resistance based on the CSC model has not been fully elucidated. In the present study, we established gemcitabine-resistant pancreatic cancer cells *in vitro* and evaluated the role of CSCs during the acquisition of gemcitabine resistance. We investigated stem cell-like properties

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in resistant cells and elucidated the mechanism of multidrug resistance based on the CSC model.

Material and methods

Cell lines and culture

Human pancreatic cancer cell lines (HPAC, CFPAC-1, MIAPaCa-2 and PANC-1) were purchased from American Tissue Culture Collection (Manassas, VA). HPAC and CFPAC-1 cells were grown as monolayer cultures in Dulbecco's Modified Eagle's-Ham's F12 Medium (DMEM-F12) and Iscove's Modified Dulbecco's Medium with 10% FBS, penicillin (100 units/mL), amphotericin (2.5 units/mL) and streptomycin (100 g/mL), respectively. MIAPaCa-2 and PANC-1 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with antibiotics. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Drugs

Gemcitabine was supplied by Eli Lilly Korea (Seoul, Korea). Docetaxel and verapamil were purchased from Sigma (St. Louis, MO), dissolved in DMSO as a stock, and stored at −80°C. The gemcitabine and docetaxel solutions were diluted in culture medium immediately before use.

Establishment of gemcitabine-resistant pancreatic cancer cells

Gemcitabine-resistant pancreatic cancer cells were established by escalating doses of gemcitabine serially in HPAC and CFPAC-1 cells.[9] Initially, cells were cultured for 72 hr with IC_{50} of gemcitabine with a defined drug-free interval. As cells adapted to the dose, the gemcitabine concentration was doubled serially. Finally, after cells recovered from 10 uM gemcitabine treatment, 100 uM of the drug was added into medium to delete most of the cell population. The experiments below were performed with the recovered cells.

Drug cytotoxicity assay

The cytotoxicity of gemcitabine in each cell line was assessed with a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma) in accordance with the manufacturer's instructions.[24] Logarithmically growing cells were seeded at 5×10^3 cells/well in 96-well plates and cultured in a CO_2 incubator at 37°C for 24 hr. Various concentrations of gemcitabine and docetaxel were added to the wells and incubated for 72 hr. Treated cells were rinsed twice with PBS, incubated in 10 µl MTT solution for 4 hr at 37°C, and 100 uL DMSO was added to each well. The absorbance of each well was measured at 570 nm using Tilter-Tech 96-well multiscanner (Becton Dickinson, Heidelberg, Germany). The relative number of viable cells compared with the number of cells without drug treatment was expressed as percent cell viability using the following formula: cell viability (%) = A_{570} of treated cells/A_{570} of untreated cells.

Colony formation assay

Colony-forming efficiency was determined using a double-layer soft agar method.[19] A total of 10^4 cells were plated in 0.35% agar over a layer of 0.5% agar containing DMEM and 10% FBS in 6-well plates. Cells were incubated for 21–28 days in a CO_2 incubator, and colonies larger than 50 µm were counted under an Olympus BX51 microscope.

Sphere formation assay

Tumor spheres were made to evaluate sphere-forming activity with modification of previous reports.[17,25] Each cell was diluted to a density of 10^3 cells/mL with serum-free medium (SFM). SFM was DMEM-F12 supplemented with 10 ng/mL fibroblast growth factor (R&D Systems, Minneapolis, MN), 20 ng/mL epidermal growth factor (R&D Systems), and 2.75 ng/mL selenium (insulin-transferrin–selenium solution; Invitrogen, Carlsbad, CA). Then, the 100 µl diluted cell suspension was seeded to each well in 96-well low attached plate with a density of 10^2 cells/well. At day 7, 100 µl of SFM was added on each well. At day 15, spheres larger than 50 µm were counted using an Olympus BX51 microscope.

TABLE I – IC_{50} OF GEMCITABINE IN PANCREATIC CANCER CELL LINES AND ESTABLISHED GEMCITABINE-RESISTANT CELL LINES

Cell line	IC_{50} (nM)
CFPAC-1	33 ± 14
HPAC	92 ± 6
MIAPaCa-2	298 ± 10
PANC-1	1161 ± 85
Gemcitabine-resistant CFPAC-1	447 ± 14*
Gemcitabine-resistant HPAC	1300 ± 323*

*$p < 0.001$ compared to each parental cell.

Fluorescence-activated cell sorting

Cells were grown to 70% confluence, trypsinized and washed with FACS buffer (1× PBS, 2% FBS, 2 mM EDTA), and then resuspended in FACS buffer. Blocking antibody was added and incubated for 1 hr on ice, and the sample was washed with FACS buffer. Primary antibodies were added and incubated for 1 hr on ice. The following antibodies were used: anti-CD44 allophycicyanin, anti-CD24 phycoerythrin (PharMingen, Franklin Lakes, NJ), and anti-ESA-FITC (Biomeda, Foster City, CA).[20] Isotype-matched mouse immunoglobulins (PharMingen) served as controls. Flow cytometry was done using a BD LSRII (BD Biosciences, Franklin Lakes, NJ). Data were analyzed by BD FACSDiva software, which is provided with the system.

Tumor xenografts

Four-week-old male BALB/c nude mice were purchased from the Animal Laboratory Unit of Yonsei University College of Medicine. The mice were maintained under standard conditions and cared for according to the institutional guidelines for animal care. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals of Yonsei University College of Medicine. Initially, to evaluate the capacity to generate tumor nodules, 10^7 CFPAC-1 cells/200 uL PBS were injected subcutaneously in both flanks. Then, parental and resistant CFPAC-1 cells (5×10^5 cells/100 uL PBS) were injected in both flanks. Tumor formation was monitored twice a week by measuring the width and length of the mass, and tumor volume was calculated by the formula v (mm^3) = $(a^2 \times b)/2$, with a as the smallest diameter and b as the largest. Animals with tumor formation were sacrificed at 2 months for histological evaluation.

Quantitative real-time RT-PCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the Superscript II system (Invitrogen) in accordance with the manufacturer's instructions. Quantification of ABCG2, ABCB1 and ABCC1 mRNA was conducted using the SYBR Green RT-PCR kit (Invitrogen) and ABI PRISM 7300 sequence detector (Applied BioSystems, Foster City, CA) according to manufacturers' instructions. In brief, the total volume of the reaction mixture was 25 µl, containing 12.5 µl of SYBR Green qRCR Supermix (Invitrogen), 5 pmol of sense and antisense primer and 5 µl of cDNA. The reaction was run online at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. After real-time RT-PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/minute to construct a melting curve. The results were analyzed with melting curve analysis software (Dissociation Curve 1.0; Applied BioSystems) provided with the ABI PRISM 7300 sequence detector. The expression of mRNA was normalized to that of the reference gene, GAPDH. Relative quantification of mRNA within the samples was examined using the comparative Ct method ($\Delta Ct_{resistantcell} - \Delta Ct_{eachparentalcell} = \Delta\Delta Ct$; relative quantity = $2^{-\Delta\Delta Ct}$).[26] The primers are listed in Table I. Primers for real-time RT-PCR were as

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follows: ABCG2 forward, 5′-TCAAGTGGGGCGATGCTG-3′; ABCG2 reverse, 5′-ATCAGCAGAGGGGGCAGAGA-3′; ABCB1 forward, 5′-GATATGGATTTACGGCTTTGC-3′; ABCB1 reverse, 5′-CGATGCCCTGCTTTACCAA-3′; ABCC1 forward, 5′-GGAA TACCAGCAACCCCGACTT-3′; ABCC1 reverse, 5′-TTTTGGT TTTGTTGAGAGGTGTC-3′; GAPDH forward, 5′-TGGAGGAG CAAAGAAGAAGAAC-3′; and GAPDH reverse, 5′-GCAGCC AAAGTTCCCACCAC-3′.

Rhodamine 123 intracellular uptake assay

In total, 5×10^5 cells were harvested after trypsinization and resuspended in PBS. Rhodamine 123 was added in a final concentration of 1 umol/L, and the cells were incubated in a water bath at 37°C in the dark.[27] After washing with ice-cold PBS twice, flow cytometry was done using a BD LSRII (BD Biosciences). Data were analyzed by BD FACSDiva software, which is provided with the system.

Clonogenic assay

To evaluate the inhibitory effect of verapamil in resistant cells, clonogenic assay was performed.[26] Briefly, 10^3 cells were seeded in 6-well plates and incubated for 72 hr. Then, the cells were incubated with various doses of verapamil and gemcitabine for 72 hr. After incubation for another 7 days, colonies of more than 32 cells were counted under an Olympus BX51 microscope.

Small interfering RNA transfection

Stealth Select RNAi (catalog #1299003) for human CD44 (GenBank Accession Number. NM_001001390.1, NM_001001389.1, NM_001001392.1, NM_000610.3, NM_001001391.1) and Stealth RNAi negative control were purchased from Invitrogen. The oligonucleotide sequences of target sequences for human CD44 are as follows: target 1, GCAAGUCUCAGGAAAUGGUGCAUUU; target 2, GAGCCUGGCGCAGAUCGAUUUGAAU; and target 3, GCUGACCUCUGCAAGGCUUUCAAUA. A total of 10^3 cells were seeded on 6-well plates and incubated for 24 hr in antibiotic-free medium. Then, 60 pmol/L siRNA was added using Lipofectamine RNAiMAX (Invitrogen) as a transfection reagent according to the manufacturer's protocol. After 72 hr of incubation with siRNA, the medium was replaced with fresh one. After incubationfor another 10 days, colonies of more than 32 cells were counted under an Olympus BX51 microscope.

Immunohistochemistry of CD44 using human pancreatic cancer tissues

A total of 53 pancreatic cancer specimens that were surgically resected at Yonsei University Medical Center were used for this study. For histopathological diagnosis, specimens were embedded in paraffin and stained with hematoxylin and eosin (H&E). Clinical data were retrospectively reviewed. The Ethical Committee for Clinical Research of the Institutional Review Board of Yonsei Medical Center, Seoul, Korea, approved this study protocol.

Immunohistochemistry was performed using anti-CD44 antibody (156-3C11, monoclonal, LabVision, Fremont, CA) as previously described.[28] Briefly, after blocking with methanol containing 3% hydrogen peroxide, microwave antigen retrieval was performed in citrate buffer (0.01M, pH 6.0) for 10 min. Sections were incubated sequentially with primary antibody (overnight at 4°C) and secondary antibody (1 hr at room temperature). Slides were developed with DAKO Liquid DAB+ Substrate-Chromogen System (DAKO, Carpinteria, CA) and counterstained with hematoxylin. Normal tonsil tissue was used as a positive control. A negative control was made by applying a secondary antibody without a primary antibody. Samples were evaluated by 2 pathologists who did not have any knowledge of patients' clinical information. We scored the immunoreactivity according to the percentage of CD44-positive tumor cells as follows: <10%, 0; 10–30%, 1+; 30–50%, 2+; and >50%, 3+. For statistical analysis, a score of or above 1 was considered positive.

Statistical analysis

The dose-response curves were analyzed, and IC values were calculated using Prism software (GraphPad Software, San Diego, CA). Statistical significance for the results of colony forming, sphere forming, and clonogenic assay was determined using Mann-Whitney's *U*-test. CD44 expressions of pancreatic cancer were analyzed with Spearman correlation. Survival curve according to CD44 expression was analyzed using Kaplan-Meier method with 95% confidence intervals (95% CIs). The data were expressed as means ± standard deviations. A $p < 0.05$ was considered significant.

Results

Establishment of gemcitabine-resistant pancreatic cancer cells

The responses of CFPAC-1, HPAC, MIAPaCa-2 and PANC-1 cells to gemcitabine treatment were evaluated using MTT assay, and their IC_{50} values were 33 ± 14 nM, 92 ± 6 nM, 298 ± 10 nM, and 1161 ± 85 nM, respectively (Table I). CFPAC-1 and HPAC, showing higher sensitivity to gemcitabine compared with the other cell lines,[29] were selected to be established as gemcitabine-resistant cell lines. Gemcitabine-resistant pancreatic cancer cells were grown by treatment with serially escalated doses of gemcitabine. To determine if CSCs were involved in acquiring chemoresistance, we modulated the protocol of establishing gemcitabine-resistant cell lines.[9] After recovering from 10 uM of gemcitabine, the cells were treated with 100 uM of gemcitabine to delete most of the cell population. After high-dose gemcitabine treatment, a small number of cells survived and slowly repopulated to form colonies. Finally, gemcitabine-resistant pancreatic cancer cells were established, and the IC_{50} of gemcitabine in resistant HPAC (HR) cells and resistant CFPAC-1 (CR) cells was 447 ± 14 nM and 1300 ± 323 nM (Table I; $p < 0.001$, compared with each parental cell).

Evaluation of stemness in gemcitabine-resistant cells

To determine the clonogenecity of resistant cells *in vitro*, we compared their clonal ability to parental cells using soft agar colony formation assay.[19] Compared to parental HPAC (HP) cells, HR cells were able to form more colonies (Fig. 1*a*, right panel; HP *vs.* HR, 133 ± 71 *vs.* 372 ± 76; $p < 0.05$). CR cells also formed greater numbers of colonies than parental CFPAC-1 (CP) cells (Fig. 1*a*, left panel; CP *vs.* CR, 101 ± 23 *vs.* 237 ± 70; $p < 0.05$).

It has been well documented that neural CSCs could be expanded as neurospheres,[17] and it was recently demonstrated that pancreatic CSCs could form tumor spheres.[25] Therefore, we investigated sphere-forming activity of both parental and resistant cells. Compared with HP cells, HR cells were able to form more tumor spheres in SFM (Fig. 1*b*, right panel; HP *vs.* HR, 47 ± 6 *vs.* 253 ± 8/ 10^3 cells; $p < 0.05$). Although CP cells had lower sphere-forming activity compared with HP cells, CR cells formed significantly greater numbers of tumor spheres than CP cells (Fig. 1*b*, left panel; CP *vs.* CR, 16 ± 3 *vs.* 115.5 ± 8/10^3 cells; $p < 0.01$).

To determine whether resistant cells were more tumorigenic *in vivo* than parental cells, we compared the tumor formation capacity between CP and CR cells in BALB/c nude mice. When we subcutaneously injected 10^7 CP cells into mice ($n = 2$), CP cells induced tumor nodules. The tumor volume was 1080 mm^3 after 2 months. Subsequently, we injected 5×10^5 cells of CP and CR cells into mice ($n = 8$ each). Tumor nodules were formed in all mice (8/8) injected with 5×10^5 CR cells but no tumor formation was observed in any mice (8/8) injected with 5×10^5 CP cells until 6 months (0/8; Fig. 1*c*). CR cells formed tumor nodules as early as 1 week after injection, and the tumor volume was 1803 ± 655 mm^3 at 2 months (Fig. 1*d*). The tumors, which were formed by 5×10^5 CR cells, were histologically similar to the tumors by 10^7 CP cells (Supporting Information).

According to these data, resistant cells were more tumorigenic *in vitro* and *in vivo* and had greater sphere-forming activity than

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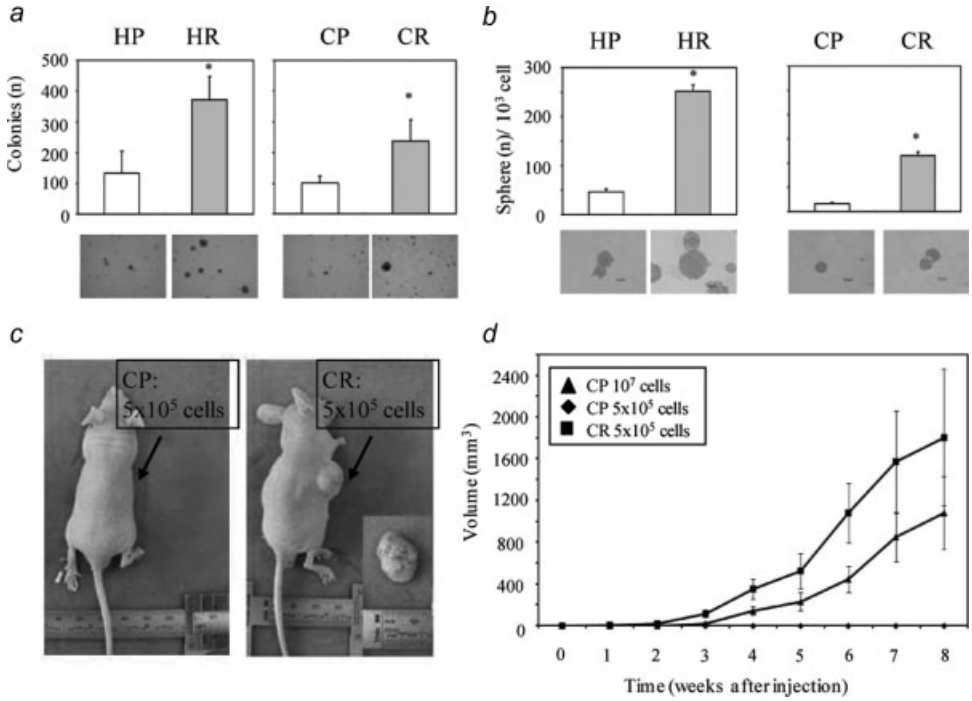

FIGURE 1 – Establishment of gemcitabine-resistant pancreatic cancer cells and evaluation of stemness. (*a*) Colony formation assay. A total of 10^4 cells were plated in 0.35% agar over a layer of 0.5% agar and incubated for 15–21 days. Colonies larger than 50 μm were counted. Compared with parental cells, the number of colonies were increased in resistant cells (original magnification, 40×). (*b*) Sphere formation assay. Cells (10^3/ml) were seeded onto 96-well low attachment plates with serum-free medium. After incubation for 15 days, spheres larger than 50 μm were counted. Resistant cells showed more sphere-forming activity than parental cells (original magnification, 100×). (*c*) Tumor formation in BALB/c nude mice injected with 5×10^5 cells of CP and CR cells. CR cells formed tumor nodules in all injected mice (8/8), whereas CP cells did not induce any tumor nodules until 6 months (left). (*d*) Growth curve of xenograft tumors in BALB/c nude mice. Data are shown as mean ± standard deviation. $*p < 0.05$.

parental cells, which are characteristic of CSCs. It suggests that expansion of cancer stem-like cells might have an important role for the acquisition of gemcitabine resistance.

Expansion of CD44$^+$ cells during acquisition of gemcitabine resistance

Phenotypic characteristics were widely used to identify the existence of CSCs and isolate them. We hypothesized that the phenotypes of parental cells would be changed during the acquisition of gemcitabine resistance. To evaluate the phenotypic changes, we carried out FACS analysis for CD24, CD44, and ESA, which were reported as putative markers of CSCs in pancreatic cancer.[20] The results showed that CD44$^+$CD24$^+$ESA$^+$ cells retained a small proportion in recovered cells from high-dose gemcitabine treatment (HP *vs.* HR, 0.1 ± 0.1% *vs.* 1.9 ± 0.1%; CP *vs.* CR, 0.1 ± 0% *vs.* 1.3 ± 0.2%). Interestingly, the CD44$^+$ subfraction was dramatically increased in resistant cells compared with parental cells (HP *vs.* HR, 3.6 ± 0.2% *vs.* 74.7 ± 6.4%; CP *vs.* CR, 3.6 ± 0.1% *vs.* 81.9 ± 2.5%; Fig. 1*a*). However, the CD24$^+$ cells were unchanged, and the ESA$^+$ cells were decreased in resistant cells compared with parental cells (Fig. 2*a*). According to the CSC model, cells in recurred tumors would be a heterogeneous population, comprised of predominant resistant cells and small numbers of CSCs.[11] Thus, our results suggest that most of the repopulated cells after surviving high-dose gemcitabine are CD44+, and they reconstituted the resistant cell population. The response of resistant cells to gemcitabine was re-evaluated with MTT assay after culturing them in drug-free medium (DFM). After subculturing in DFM for a extended period, the CD44$^+$ subfraction of HR cells decreased from 69.2 to 24.7%, whereas the CD44$^+$ subfraction of CR cells decreased from 81.3 to 60.0% (Fig. 2*b*). As the proportion of CD44+ cells decreased, HR cells were resensitized to gemcitabine treatment (Fig. 2*c* upper panel; $p < 0.05$). However, CR cells were able to sustain gemcitabine resistance (Fig. 2*c*; lower panel).

Responsibility of ABC transporters for acquisition of multidrug resistance

To evaluate whether gemcitabine-resistant cells acquired multidrug resistance, MTT assay was performed with docetaxel. Docetaxel stabilizes microtubule assembly and induces apoptosis, which is a different mechanism from gemcitabine.[30] MTT assay showed that gemcitabine-induced resistant cells were also resistant to docetaxel, meaning that they acquired multidrug resistance (IC_{50} of HP *vs.* HR, 65 nM *vs.* 139 nM; IC_{50} of CP *vs.* CR, 66 nM *vs.* 206 nM; $p < 0.01$).

The mechanism of multidrug resistance in CSCs are poorly understood, but ABC transporters such as ABCG2, ABCB1 and ABCC1 are thought to be responsible.[11] Hence, mRNA expression levels of ABCG2, ABCB1 and ABCC1 were measured by real-time RT-PCR. The relative expressions of ABCG2 mRNA were augmented in resistant cells (HP *vs.* HR, 1.01 ± 0.19 *vs.* 3.01 ± 1.47; CP *vs.* CR, 1.01 ± 0.17 *vs.* 1.80 ± 0.70; Fig. 3*a*, right panel; $p < 0.05$). Though the mRNA of ABCB1 (MDR1) was hardly detected in parental cells, it was robustly expressed in resistant cells (HP *vs.* HR, 3.49 ± 4.73 *vs.* 793.24 ± 303.01; CP *vs.* CR, 3.42 ± 4.63 *vs.* 875.99 ± 213.72; Fig. 3*a*, middle panel; $p < 0.05$). The expression of ABCC1, however, did not change in resistant cells (HP *vs.* HR, 1.0.1 ± 0.15 *vs.* 1.00 ± 0.09; CP *vs.* CR, 1.00 ± 0.13 *vs.* 1.02 ± 0.1; Fig. 3*a*, right panel; $p > 0.05$). Additionally, we evaluated rhodamine 123 intracellular uptake assay to investigate the functional activity of ABCB1 between parental and resistant cells. The mean value of rhodamine 123 was significantly decreased in resistant cells compared with parental cells by as much as 38% in HR cells and 45% in CR cells (Fig. 3*b*, $p < 0.05$).

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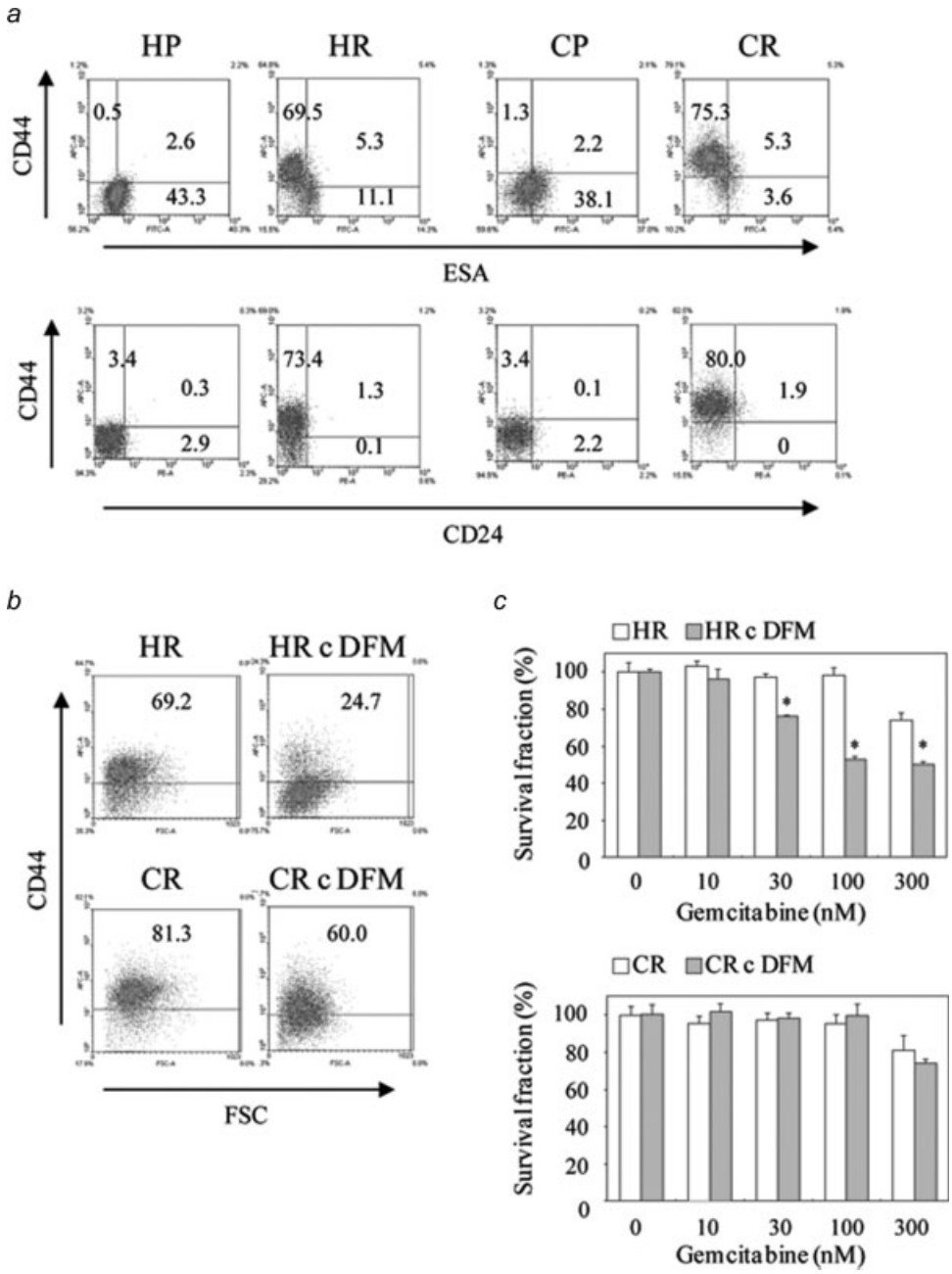

FIGURE 2 – Expansion of the subfraction of CD44+ cells during acquisition of gemcitabine resistance. (*a*) FACS analysis of parental and resistant cells stained with CD24, CD44 and ESA. Plots are representative examples of patterns of CD44 and ESA staining (top) and CD44 and CD24 staining (bottom). $CD44^{+}$ cells increased in resistant cell population. (*b*) FACS analysis of cells cultured in DFM stained with CD44. When cultured in DFM, the subpopulation of CD44+ cells decreased in HR cells whereas it was maintained in CR cells. (*c*) Dose response of resistant cells cultured in DFM to gemcitabine. When cultured in DFM, HR cells resensitized to gemcitabine treatment, whereas CR cells showed similar resistance to gemcitabine compared to resistant cells. $*p < 0.05$.

These data suggest that upregulation of ABC transporters is one of the mechanisms leading to multidrug resistance in gemcitabine-resistant pancreatic cancer cells.

Therapeutic implications of ABC transporter inhibitor and anti-CD44 siRNA

On the basis of these data, we hypothesized that the inhibitor of ABC transporters might reverse gemcitabine resistance in resistant cells. To test this hypothesis, clonogenic assay with gemcitabine plus verapamil was performed. Verapamil has been shown to be an inhibitor of ABC transporters.[11] Verapamil treatment resensitized resistant cells to gemcitabine treatment in a dose-dependent manner (Fig. 4*a*). We also investigated whether CD44 knock-down by antisense RNA would inhibit proliferative activity of resistant cells by clonogenic assay. Anti-CD44 siRNA decreased the number of colonies by as much as 44% in HR cells and 77% in CR cells compared to controls (Fig. 4*b*, $p < 0.05$). Anti-CD44 siRNA also had antiproliferative activity against parental cells (decrease in the number of colonies in HP

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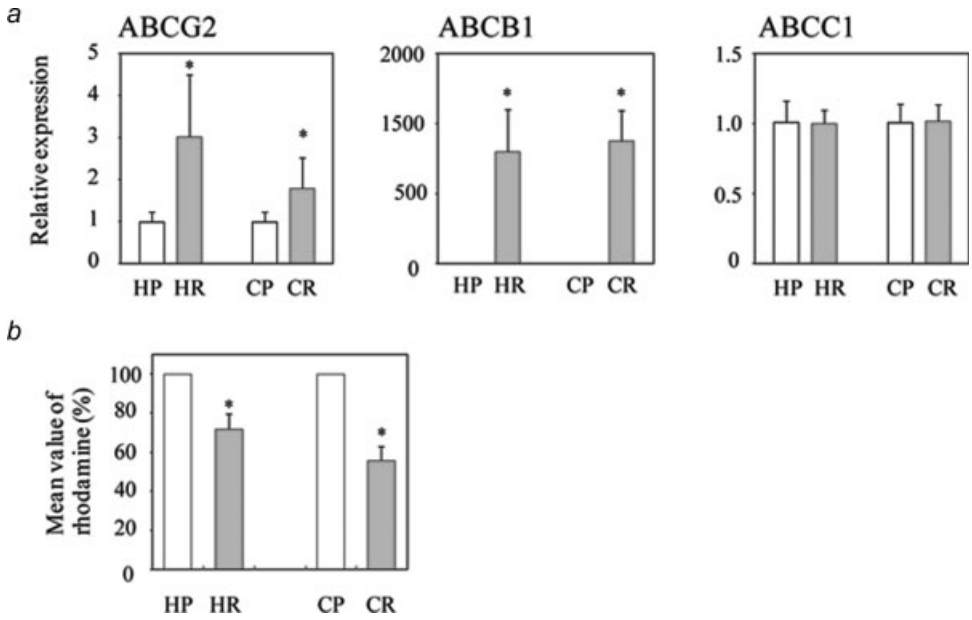

FIGURE 3 – Expressions of ABC transporters in parental and resistant cells. (*a*) The mRNA expression levels of ABCG2, ABCB1 and ABCC1 were measured by real-time RT-PCR. Expression of mRNA was normalized to that of the reference gene, GAPDH. Relative quantification of mRNA within the samples was examined using the comparative Ct method ($\Delta Ct_{resistantcell} - \Delta Ct_{eachparentalcell} = \Delta\Delta Ct$; relative quantity $= 2^{-\Delta\Delta Ct}$). Among them, the relative expression of ABCG2 and ABCB1 were augmented in resistant cells. (*b*) Rhodamine 123 intracellular uptake test in parental and resistant cells. A total of 5×10^5 cells were incubated with rhodamine 123 for 1 hr in the dark, and flow cytometry was done. The mean value of intracellular rhodamine was significantly decreased in resistant cells compared with parental cells. $^*p < 0.05$.

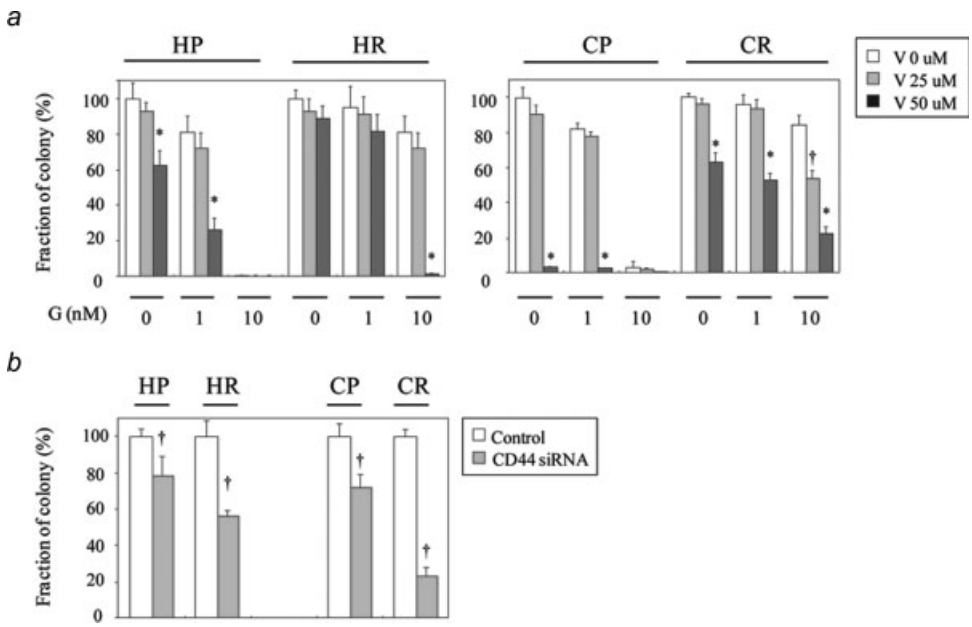

FIGURE 4 – Treatment of ABC transporter inhibitors and anti-CD44 siRNA in resistant cells. (*a*) Clonogenic assay with verapamil treatment in resistant cells; 10^3 cells were seeded and incubated with 0 μM (white bar), 25 μM (gray bar) and 50 μM (black bar) of verapamil and gemcitabine. After verapamil treatment, resistant cells resensitized to gemcitabine in a dose-dependent manner. (*b*) Clonogenic assay with anti-CD44 siRNA treatment in resistant cells; 10^3 cells were seeded and incubated with negative control (white bar) and 60 pmol/l of siRNA (gray bar). Clonogenic activity of resistant cells was decreased after anti-CD44 siRNA treatment. $^*p < 0.05$.

and CP, 22% and 28%; Fig. 4*b*; $p < 0.05$). These data suggest that the inhibitor of ABC transporters or anti-CD44-targeted therapy can be applied to overcome drug resistance in pancreatic cancer.

Evaluation of CD44 expression in human pancreatic cancer tissues

Our data suggest that CD44-positive cells might be responsible for multidrug resistance. Therefore, we hypothesized that pancre-

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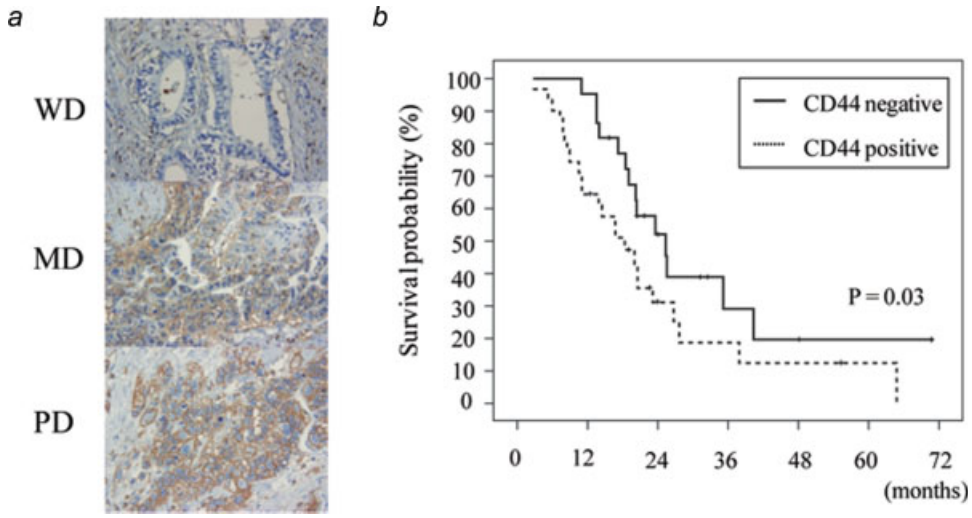

FIGURE 5 – Expression of CD44 in pancreatic cancer. (*a*) CD44 expression pattern in pancreatic cancer. Paraffin samples of 53 pancreatic cancers were stained with anti-CD44 antibody. CD44 was stained in the membrane of cancer cells, and its expression was correlated with tumor grade (original magnification, 400×). (*b*) Kaplan–Meyer survival curve according to CD44 expression. Patients with CD44-expressed pancreatic cancers showed poor prognosis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

atic cancer with positive expression of CD44 might show poor prognosis. To test this hypothesis, immunohistochemical staining with anti-CD44 antibody was performed (Fig. 5*a*). Among the 53 pancreatic cancer patients who underwent radical operation, 30 (50.6%) showed positive staining of CD44, and its expression was correlated with histologic grade. CD44 expression was positive in 1 of 6 (16.7%), 22 of 41 (56.4%) and 7 of 8 (87.4%) in well-, moderate- and poorly differentiated adenocarcinomas, respectively (Fig. 5*a*; $p < 0.001$). The median survival time of all patients was 20.3 months (95% CI: 18.2–22.4 months). The median survival was significantly longer in patients with CD44-negative tumors (25.3 months, 95% CI: 19.1-31.4 months) than CD44-positive tumors (16.9 months, 95% CI: 10.4-23.4 months). Kaplan-Meyer survival curve showed that CD44 expression was a poor prognostic factor in pancreatic cancer ($p < 0.05$).

Discussion

In this study, we have demonstrated that cancer stem-like cells were expanded during the acquisition of gemcitabine resistance. After exposure to high-dose gemcitabine, CD44-positive cells reproliferated and reconstituted the resistant cell population. Among the ABC transporters, the expression of ABCB1 (MDR1) was significantly augmented in resistant cells. We showed that verapamil and anti-CD44 treatment could overcome multidrug resistance of pancreatic cancer.

CD44 is well known as an adhesion molecule and membrane receptor for hyaluronan, and is involved in cell motility and metastases.[31] The gene encoding CD44 generates a variety of isoforms by alternative splicing, which predominantly affects the extracellular membrane-proximal structure of CD44 proteins. The expression of CD44 variants was significantly correlated with poor prognosis in colon and pancreatic cancer.[32,33] Recently, CD44 has been evaluated as a CSC marker in solid tumors. Actually, CD44 alone served as a CSC marker in head and neck carcinoma.[34] Prince *et al*. demonstrated that $CD44^+$ cells isolated from primary head and neck carcinoma samples have the ability to self-renew and differentiate in an *in vivo* mouse model, and the $CD44^+$ subpopulation in primary tumors vary from 0.1–42%. However, in other tumors, multiple cell surface markers have been used to purify CSCs, and CD44 has been used as one of the CSC markers. CSCs could be characterized by $CD44^+CD24^{-/low}$ in breast cancer[15] and $EpCAM^{hi}/CD44^+$ in colon cancer.[35] In pancreatic cancer, although $CD44^+$ cells themselves were more tumorigenic than $CD44^-$ cells, $CD44^+CD24^+ESA^+$ cells had more stem cell-like characteristics than $CD44^-CD24^-ESA^-$ cells.[20] When patients' samples were sorted by those markers, 2–9% of cells expressed CD44, whereas only 0.2–0.8% of cells were $CD44^+CD24^+ESA^+$. Although CD44 itself is not sufficient to define all of the phenotypes of CSCs, it seems obvious that CD44 is one of the important CSC markers in pancreatic cancer. On the basis of the CSC model of drug resistance, after treatment with high-dose gemcitabine, surviving CSCs might differentiate into progenitor cells, and these cells would proliferate and differentiate to generate recurred tumors.[11] This data show that $CD44^+$ cells are mainly responsible for this process. There is no consensus about the proportion of CSCs in primary or recurred tumors; however, it has been accepted that CSCs might represent a small proportion of heterogeneous tumor cells.[11] Our data also showed that CSCs confined to a small subset in recurred and resistant cell population.

CSCs are naturally resistant to chemotherapy through their quiescence, capacity for DNA repair and ABC transporter expression.[11] Among the ABC transporters, ABCG2 and ABCB1 have been most extensively studied in stem cells. Because cells harboring ABCG2 (BCRP1) efflux the fluorescent dye Hoechst 33,342, these cells have been referred to as side population (SP) cells,[36] and SP cells have been used to identify and purify CSCs in tumors.[26] It seems that ABCG2 is exclusively expressed in stem cells, in which expression is turned off in most committed progenitor and mature blood cells.[37] This is why its expression was slightly augmented during the acquisition of multidrug resistance in our experiments. In this study, we showed that the expression of ABCB1 (MDR1) was gradually increased along with the proliferation of $CD44^+$ cells. A series of studies have suggested a relationship between CD44 and ABCB1. Miletti-Gonzalez *et al*.[38] showed that P-glycoprotein, a product of the ABCB1 gene, was highly expressed in drug-resistant cells and had a positive correlation with the level of CD44 expression. In a recent investigation on the interaction between CD44 and MDR1 expression based on CSCs,[39] interaction between hyaluronan (HA) and CD44 promoted Nanog expression, followed by the expression of stem cell regulators such as Rex1 and Sox2 in breast and ovarian tumor

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cells. Nanog formed a complex with Stat-3-activated MDR1 expression, and HA-CD44 interaction also activated MDR1 by ankyrin, resulting in multidrug resistance. This explains why ABCB1 was strongly augmented in resistant cells as $CD44^+$ cells proliferated. For these reasons, the inhibitors of ABC transporters have been studied in the treatment of cancers,[40] and our data also suggest that they could reverse chemoresistance in pancreatic cancer.

In this study, we revealed that CD44-targeted therapy is a possible option for reversing chemoresistance of pancreatic cancer cells. In acute myeloid leukemia, administration of monoclonal antibody against CD44 markedly reduced the repopulation of leukemic stem cells *in vivo*, however, this effect was due to the inhibition of proper homing of leukemic stem cells to microenvironmental niches.[41] A recent study showed that CD90 is a potential marker for liver CSCs, and $CD90^+CD44^+$ cells had a more aggressive phenotype than $CD90^+CD44^-$ cells.[42] The study demonstrated that blockage of CD44 activity inhibited the survival of $CD90^+$ cells *in vitro* and prevented tumor engraftment *in vivo*. Because our data showed that $CD44^+$ cells reconstitute the resistant cell population, CD44 could be a therapeutic target to overcome drug resistance and cure the disease.

Although we demonstrated the role of CD44 in this study, a recent report identified CD133 as a CSC marker in pancreatic cancer,[43] which also has been used to identify CSC populations in brain and colon cancers.[16,18] The study showed that CD133+ cells were resistant to gemcitabine. This suggests that not 1 single marker but a set of surface markers would denote pancreatic CSCs.[25]

In conclusion, cancer stem-like cells play a pivotal role in acquiring multidrug resistance in pancreatic cancer and in particular, $CD44^+$ cells, which repopulate after chemotherapy, were responsible for chemoresistance mediated by ABCB1. In therapeutic application, targeted therapy against CD44 or ABC transporter inhibitors could be applied to overcome drug resistance and might be beneficial in the treatment of pancreatic cancer.

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