Acquiring gemcitabine chemoresistance is associated with cancer stem cell expansion in pancreatic cancer cells

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Acquiring gemcitabine chemoresistance is associated with cancer stem cell expansion in pancreatic cancer cells

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Abstract

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Pancreatic cancer is the fourth leading cause of cancer-related death in Western countries and shows dismal therapeutic response to the chemotherapy. Although gemcitabine is the only effective anticancer agent, pancreatic cancer either harbors intrinsic resistance to gemcitabine or rapidly acquires it during chemotherapy. Though recent observation has suggested that cancer stem cells which have intrinsic detoxifying mechanism play a role in chemoresistance, the scientific evidence remains elusive. In the present study, the role of cancer stem cells in acquiring chemoresistance was evaluated in pancreatic cancer cells. To evaluate the dynamic mechanisms of drug resistance, gemcitabine-resistant pancreatic cancer cell lines were established by exposure to serially-escalated doses of gemcitabine in HPAC and CFPAC-1 cells. MTT assay was performed to define EC_{50} of each cell.

To evaluate the biological changes occurring during the acquisition of chemoresistance, colony forming assay, sphere forming assay, and FACS analysis with putative stem cell markers in pancreatic cancer were performed in both parental and resistant cells. Real-time RT PCR and Western blot were carried out to elucidate the mechanism of drug resistance in these cells. The EC_{50} of resistant cells was significantly increased comparing to that of parental cells (HPAC parental vs. resistant cells, 81 ± 8 nM vs. 447.2 ± 14 nM; CFPAC-1 parental vs. resistant cells, 24 ± 3 nM vs. 1300 ± 323 nM; $P < 0.01$). The colony forming assay and sphere forming assay showed that the proportion of cancer stem cells was increased in resistant cells. Interestingly, among the putative markers of cancer stem cell in pancreatic cancer, $CD44^+$ cell population dramatically expanded during the process of acquiring multi-drug resistance (HPAC parental vs. resistant cells, 4.9 ± 4.7 nM vs. 49.4 ± 19.6 uM; CFPAC-1 parental vs. resistant cells, 5.8 ± 0.4 nM vs. 72.6 ± 8.2 uM; $P < 0.01$). The real-time RT-PCR and Western blot showed that ABC transporters, especially ABCG2 and ABCB1, seem to be one of the main mechanisms of drug resistance in pancreatic cancer stem cells. Moreover the verapamil, an inhibitor of ABC transporter, could resensitize the resistant cells to gemcitabine. According to the

present study, cancer stem like cells, especially $CD44^+$ cells, play an important role in acquiring gemcitabine chemoresistance in pancreatic cancer. In therapeutic implication, targeted therapy against $CD44^+$ cells or ABC transporter inhibitor could be applied to overcome the drug resistance of pancreatic cancer.

Keywords; pancreatic cancer, drug resistance, cancer stem cell, ABC transporter

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I. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in western countries and has a dismal prognosis[1]. In Korea, pancreatic cancer accounts for the 8th highest cancer incidence and the 5th highest cancer-related mortality[2]. Surgical resection is the only potentially curative therapy for pancreatic cancer, but only 10% to 20% of these patients are candidates for curative surgical resection at diagnosis[3]. Even after surgery, 50% to 80% of these patients develop a local or distant recurrence. Furthermore, pancreatic cancer is generally highly resistant to the various chemotherapeutic agents[4]. Since the introduction of gemcitabine, a pyrimidine analogue in 1996, it has been used for the treatment of pancreatic cancer[5]. However, its therapeutic effect seems marginal, and new chemotherapeutic agents against pancreatic cancer are under development[6].

Cancer cells can acquire drug resistance by a variety of mechanisms, including the mutation of the drug target and inactivation or elimination of the drug from the cancer cell[7]. In the conventional view, a few cancer cells acquire multi-drug resistance by a series of genetic changes during chemotherapy, then repopulate, and finally develop recurrence of the tumor. There have been many efforts to elucidate the mechanism of drug resistance and to explore molecular candidates that can be targeted to overcome drug resistance. Among the genes mediating chemoresistance, genes related to nucleoside transport and metabolism, or involved in cell cycle regulation, proliferation or apoptosis, have been thought to be responsible for gemcitabine chemoresistance in pancreatic cancer. While nucleoside transporter[8] and M1 or M2 subunit of ribonucleoside reductase[9, 10] belong to the former, genes, such as mutated p53[11], Bcl-xl[12], c-Src[13], and focal adhesion kinase[14] belong to the latter. A recent study showed that gemcitabine sensitivity could be predicted according to the expression level of genes involved in gemcitabine transport and metabolism[15], and the patients with low mRNA level of ribonucleotide reductase subunit M2 showed good response after gemcitabine treatment[16].

During the last few years, a variety of studies has suggested that tumors are composed of heterogeneous cell population having different biologic

properties, and among them, a small population of cancer cells so called cancer stem cells (CSCs) sustain tumor formation and growth[7]. This CSCs theory was based on the concept of organ stem cells. Normal pluripotent stem cells possess both the self-renewal capabilities and the ability of differentiation to clonally reconstitute mature cells within a tissue[17]. A previous report has demonstrated that human mammary epithelial cells derived from reduction mammoplasties, when cultured to form non-adherent spheroids (mammospheres), differentiated into distinctive three mammary cell lineages[18]. In CSCs, however, the pathway of self-renewal and differentiation are deregulated, which are resulting in unlimited self-renewal and 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[19]. The evidence of CSCs was first documented in leukemia and myeloma[20, 21], and so far, their existence has been validated in several solid tumors, such as breast cancer[22], glioblastoma[23, 24], melanoma[25], prostate[26], lung[27], colon[28], liver[29], and pancreatic cancer[30]. In those studies, cell surface markers were used to identify and purify CSCs in tumors. Recently, CSCs were identified in pancreatic cancer using CD44, CD24, and epithelial specific antigen (ESA)[30]. 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 suggested that subpopulation of pancreatic cancer cells showing $CD44^+CD24^+ESA^+$ had the stem cell properties of self-renewal and the ability to produce differentiated progeny.

The CSC hypothesis not only offers an attractive model of carcinogenesis, but also helps to explain resistance to therapeutic agents and tumor recurrence[7]. Based on the CSC model, the tumor contains heterogenous population of mature cancer cells and small number of CSCs. Most conventional therapies have been developed to kill most of the tumor population, however, the CSCs, which have intrinsic detoxifying mechanisms, can easily escape the conventional treatments. These CSCs model explains why standard chemotherapy may result in tumor shrinkage, but most tumors recur and show multi-drug resistance. Liu et al. 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[31]. It means that CSCs-targeted therapy is mandatory for overcoming drug resistance and curing the tumors.

Although pancreatic cancer is highly resistant to various chemotherapeutic agents, the CSC markers have been recently proposed[30] and the mechanism of

drug resistance, on the basis of CSC model, has not been fully elucidated. In the present study, gemcitabine-resistant pancreatic cancer cells were established and the role of CSCs in acquiring drug resistance was validated.

II. Materials and Methods

1. Cell lines and culture

The human pancreatic cancer cell lines (HPAC and CFPAC-1) were purchased from American Tissue Culture Collection (Manassas, VA, U.S.A). HPAC and CFPAC-1 cells were grown as monolayer cultures in Dulbecco's modified Eagle's-Ham's F12 medium 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, at 37^{o}C in a humidified atmosphere with 5% CO_2.

2. Drugs

Gemcitabine was supplied by Eli Lilly Korea (Seoul, Korea). Docetaxel and verapamil were purchased from Sigma (St. Louis, MO, U.S.A), dissolved in DMSO as a stock and stored at -80^{o}C. The gemcitabine and docetaxel solution were diluted in culture medium immediately before use.

3. 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. Initially cells were cultured for 72 hours with EC_{50} of gemcitabine with defined drug free interval.

As the cells adapted to the drug dose, the gemcitabine concentration was doubled serially. The gemcitabine-resistant cell lines were established after 12 months.

4. 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 St. Louis, MO, U.S.A) in accordance with the manufacturer's instructions. Logarithmically growing cells were seeded at 5 X 10^3 cells/well in 96-well plates and cultured in a CO_2 incubator at 37 ^oC for 24 hours. Various concentrations of gemcitabine and docetaxel were added to the well and incubated for 72 hours, respectively. Treated cells were rinsed twice with PBS, incubated in 10 $\mu\ell$ MTT solution for 4 hours at 37 ^oC, 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 (Beckton and Dickenson, Heidelberg, Germany). Relative number of viable cells as compared to 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. The dose response curves were analyzed and the EC values were calculated using Prism software (GraphPad Software, San Diego, CA,

U.S.A).

5. Colony forming assay

Colony-forming efficiency was determined using a double-layer soft-agar method. 10^4 cells were plated in 0.35% agar over a layer of 0.5% agar containing DMEM and 10% FBS in six well plates. Cells were incubated for 15-21 days in a CO_2 incubator and colonies larger than 50 ㎛ were counted under Olympus BX51 microscope.

6. Sphere forming assay

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, 20 ng/mL epidermal growth factor, and 2.75 ng/mL selenium (insulin-trasferin-selenium solution). Then, the 100 ㎕ of diluted cell suspension was seeded to each well in 96-well low attached plate. At day 7, 100 ㎕ of SFM was added on each well. At day 15, larger than 50 ㎛ spheres were counted using an Olympus BX51 microscope.

7. Clonogenic assay

To evaluate the inhibitory effect of verapamil in resistant cells, clonogenic

assay was performed. Briefly, 10^3 cells were seeded in 6 well plate and incubated for 24 hours. Then, the cells were incubated with various doses of verapamil and gemcitabine for 72 hours. After incubating for another 7 days, colonies, more than 32 cells, were counted under an Olympus BX51 microscope.

8. Fluorescence-Activated Cell Sorting (FACS)

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

9. 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, Carlsbad, CA, U.S.A) 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, U.S.A) according to manufacturers' instructions. In brief, the total volume of the reaction mixture was 25 $\mu\ell$, containing 12.5 $\mu\ell$ of SYBR Green qRCR Supermix (Invitrogen), 5 pmol of sense and antisense primer, and 5 $\mu\ell$ 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 s and 60 °C for 60 s. After real-time RT PCR, the temperature was increased from 60 to 95 °C at a rate of 2 °C/min to construct a melting curve. The Results were analyzed with the 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 ($\Delta Ct_{sample} - \Delta Ct_{calibrator} = \Delta\Delta Ct$; relative quantity = $2^{-\Delta\Delta Ct}$) [21]. The primers were listed in Table 1.

Table 1. Primers for Real-time RT-PCR

Primer		Sequence
ABCG2	Forward	5’-TCAAGTGGGGCGATGCTG-3’
	Reverse	5’-ATCAGCAGAGGGGGCAGAGA-3’
ABCB1	Forward	5’-GATATGGATTTACGGCTTTGC-3’
	Reverse	5’-CGATGCCCTGCTTTACCAA-3’
ABCC1	Forward	5’-GGAATACCAGCAACCCCGACTT-3’
	Reverse	5’-TTTTGGTTTTGTTGAGAGGTGTC-3’
GAPDH	Forward	5’-TGGAGGAGCAAAGAAGAAGAAC-3’
	Reverse	5’-GCAGCCAAAGTTCCCACCAC-3’

10. Western blot

The proteins were extracted from parental cells and resistant cells, and evaluated for ABCG2 expression. Samples were size-separated on 6 % polyacrylamide gels and transferred overnight to nitrocellulose membrane. Membranes were blocked for 2 hours with 4% nonfat milk and hybridized for 3 hours with mouse anti-ABCG2 monoclonal antibody (PharMingen, San Diego, CA, U.S.A; 1:1000) or rabbit anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A; 1:1000). Membranes were then incubated with anti-mouse and anti-rabbit horseradish peroxidase conjugated

antibody. Complexes were visualized by enhanced chemiluminescence autoradiography (Amersham Life Science, Chicago, IL, U.S.A).

11. Statistical analysis

MTT assay was analyzed using Prism software (GraphPad Software, San Diego, CA, U.S.A). Statistical significance for the results of colony forming assay, sphere forming assay, and clonogenic assay was determined using Mann-Whitney's *U*-test. The data were expressed as means ± standard deviations. $P < 0.05$ were considered significant.

III. Results

1. Establishment of gemcitabine-resistant cell lines

To determine EC_{50} of each cell, MTT assay was performed. The EC_{50} of gemcitabine in parental HPAC (HP) and CFPAC-1 (CP) were 81 ± 8 nM and 24 ± 3 nM, respectively. To establish gemcitabine-resistant pancreatic cancer cells, initially EC_{50} of gemcitabine was administrated on parental cells for 72 hours. Once surviving cells reached 80% confluence, they were subcultured twice with fresh medium and double dose of EC_{50} was administrated. The dose of gemcitabine was serially escalated upto 100 uM over a period of 12 months. Finally, gemcitabine-resistant pancreatic cells were established and the EC_{50} of gemcitabine in resistant HPAC cells (HR) and resistant CFPAC-1 cells (CR) were 447.2 ± 14 nM and 1300 ± 323 nM, respectively (Figure 1, Table 2). As shown in Figure 2, the HR and CR were morphologically different from their representative parental cells. Compared to parental cells, the resistant cells lost cell to cell adhesion, had spindle shaped morphology, and more often formed pseudopodia, which are the characteristics of mesenchymal phenotypes. These morphological changes have been maintained through more than 20 passages.

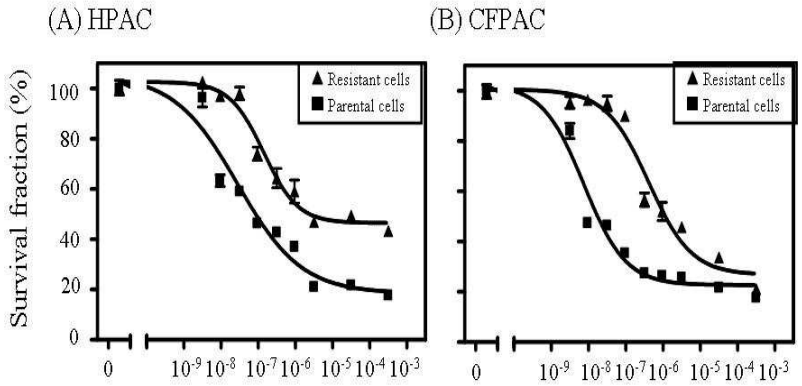

Figure 1. Dose-response curve of gemcitabine in parental and resistant cells of HPAC and CFPAC-1. MTT assay was done in parental cells and resistant cells with various dose of gemcitabine. Compared to parental cells, each resistant cell acquired gemcitabine resistance.

Table 2. EC_{50} of gemcitabine in HPAC and CFPAC-1 cells

Cells	EC_{50} (nM)	P
HPAC		< 0.001
Parental	81 ± 8	
Resistant	447.2 ± 14	
CFPAC-1		< 0.001
Parental	24 ± 3	
Resistant	1300 ± 323	

Each value was expressed as means ± standard deviations.

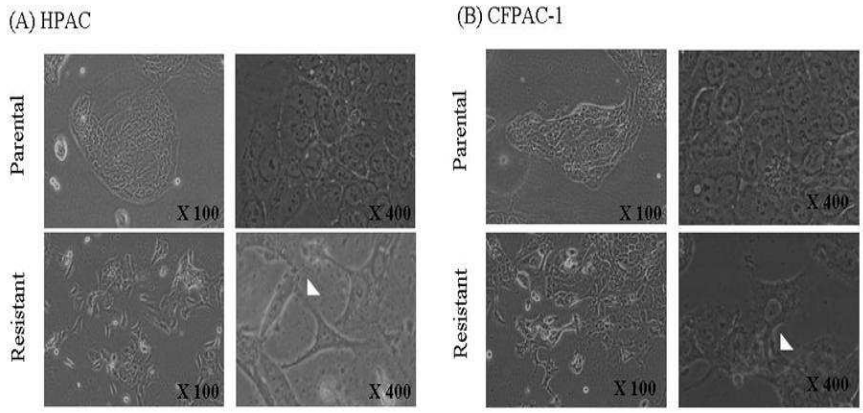

Figure 2. Morphological changes in resistant cells of HPAC and CFPAC-1. The cells were captured using an Olympus BX51 microscope. The resistant cells lost cell to cell adhesion, had spindle shaped morphology, and more often formed pseudopodia (white arrow).

2. Gemcitabine-resistant cells show higher tumorigenic activity in vitro

To compare the tumorigenic activity between parental cells and resistant cells, colony forming assay was performed. Colonies, larger than 50 um, were counted. Compared with parental cells, gemcitabine resistant cells formed more colonies (HP versus HR, 133 ± 71 versus 372 ± 76; CP versus CR, 101 ± 23 versus 237 ± 70, respectively; $P<0.01$, Figure 3). Furthermore, the colonies, which were formed by resistant cells, were larger than those by parent cells.

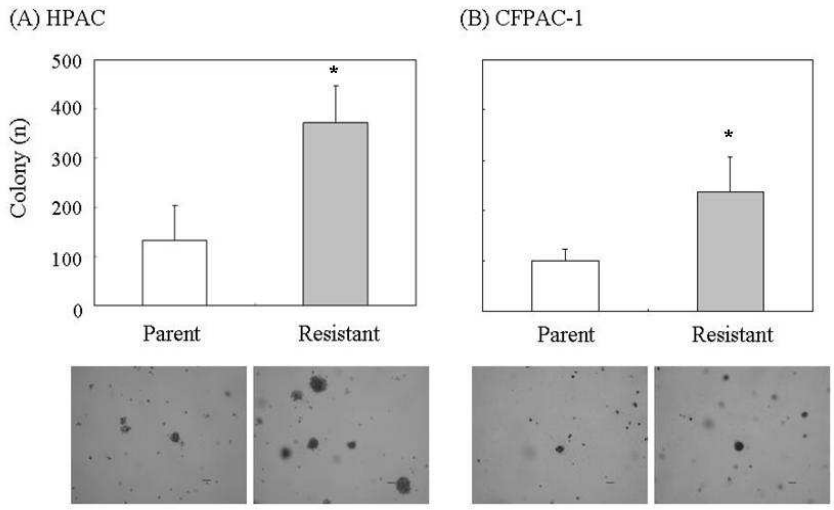

Figure 3. Colony forming assay in HPAC and CFPAC-1 cells. 10^4 cells were plated in 0.35% agar over a layer of 0.5% agar. After incubation for 15-21 days, colonies larger than 50 ㎛ were counted. Compared to the parental cells, the number and the size of colonies were increased in resistant cells. The experiments were performed three times and each value was expressed as means ± standard deviations. *P < 0.01.

3. Gemcitabine-resistant cells show higher sphere forming activity

To compare the stem-like characteristics between parental cells and resistant cells, sphere forming assay was performed. Gemcitabine-resistant cells made more spheres, which were larger than 50 um, than parental cells (HP versus

HR, 47 ± 6 versus 253 ± 8/ 10^3 cells; CP versus CR, 16 ± 3 versus 115.5 ± 8/ 10^3 cells, respectively; $P < 0.01$, Figure 4). The spheres, which were formed by resistant cells, were larger than those by parental cells. According to the results of colony forming assay and sphere forming assay, it was suggested that the proportion of cancer stem like cells were increased in resistant cells compared to parental cells.

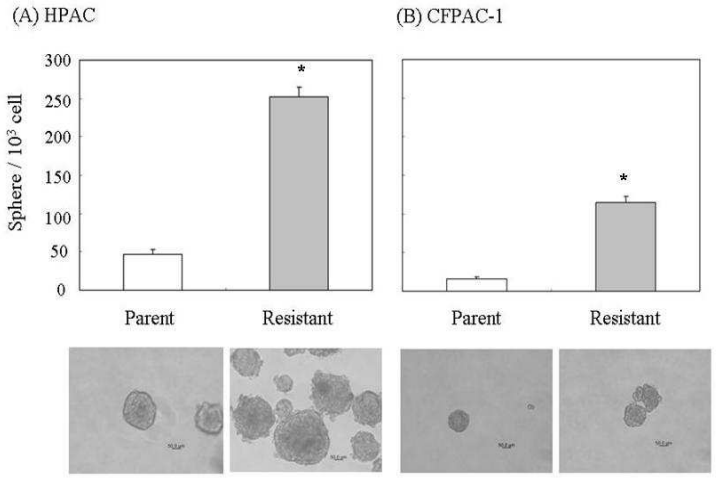

Figure 4. Sphere forming assay in HPAC and CFPAC-1 cells. Cells (10^3/ml) were seeded onto 96 well low attachment plate with serum free medium. After incubation for 15 days, the spheres, larger than 50 um, were counted. The number and the size of sphere in resistant cells were increased compared to parental cells. The experiments were performed three times and each value was expressed as means ± standard deviations. *$P < 0.01$.

4. Subfraction of CD44 positive cells expand during the acquisition of gemcitabine resistance.

To evaluate the changes of stem-like cell proportion in resistant cells, FACS analysis was performed with CD24, CD44, and ESA, which were reported as putative markers of cancer stem cell in pancreatic cancer[30]. Compared to parental cells, $CD44^+$ subfraction was dramatically increased in resistant cells (HP versus HR, 4.9 ± 4.7 % versus 49.4 ± 19.6 %; CP versus CR, 5.8 ± 0.4 % versus 72.6 ± 8.2 %, respectively; Figure 5, Table 3). However the subpulation of $CD24^+$ or ESA^+ were not definitely changed or slightly decreased in resistant cells.

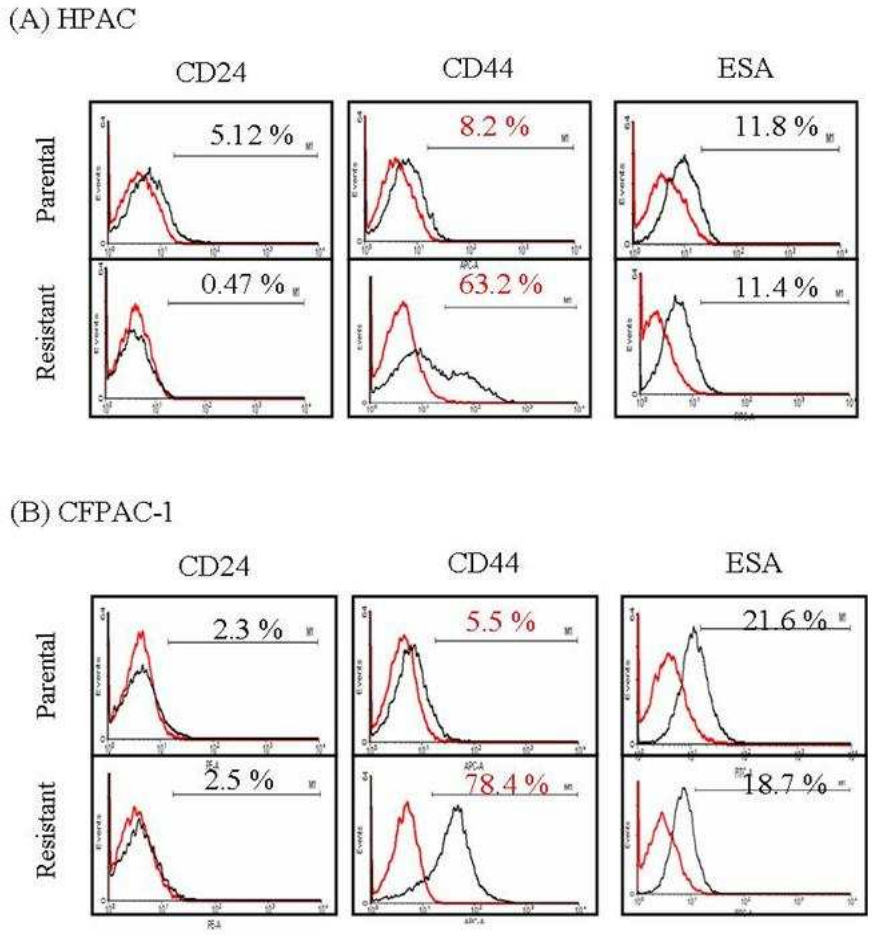

Figure 5. Example of FACS analysis with CD24, CD44, and ESA in HPAC and CFPAC-1 cells. The subfraction of $CD44^+$ cells in resistant cells was significantly increased compared to parental cells. $CD24^+$ or ESA^+ subfraction were not definitely changed or slightly decreased in resistant cells compared to parental cells.

Table 3. Summary of FACS analysis in HPAC and CFPAC-1 cells.

	HPAC (%)		CFPAC-1 (%)	
	Parent	Resistant	Parent	Resistant
CD24	5.0 ± 0.2	0.1 ± 1.1	2.2 ± 0.1	1.6 ± 1.3
CD44	4.9 ± 4.7	49.4 ± 19.6	5.8 ± 0.4	72.6 ± 8.2
ESA	13.3 ± 2.1	11.3 ± 0.1	18.4 ± 4.5	20.7 ± 2.8

The experiments were performed three times.

Each value was expressed as means ± standard deviations.

5. Gemcitabine-resistant cells acquire multi-drug resistance

To evaluate whether gemcitabine resistant cells acquired the multi-drug resistance, MTT assay was performed with docetaxel. Docetaxel stabilizes microtubule assembly and induces apoptosis, which is different mechanism from gemcitabine. MTT assay showed that gemcitabine-induced resistant cells were also resistant to docetaxel, which means that resistant cells acquired multi-drug resistance (EC_{50} of HP versus HR, 65 nM versus 139 nM; EC_{50} of CP versus CR, 66 nM versus 206 nM, respectively; $P < 0.01$, Table 4).

Table 4. EC_{50} of docetaxel in HPAC and CFPAC-1 cells

Cells	EC_{50} (nM)	P
HPAC		< 0.001
Parent	65	
Resistant	139	
CFPAC-1		< 0.001
Parent	66	
Resistant	206	

The experiments were performed three times.

The data was expressed as mean ± standard deviation.

6. ABC transporter is one of the main mechanisms of multi-drug resistance in pancreatic cancer stem like cells

To evaluate the mechanism of multi-drug resistance, the mRNA expression level of ABC transporters (ABCG2, ABCB1, and ABCC1) was measured by real-time RT-PCR. ABC transporters, especially ABCG2 and ABCB1 have been considered as the main mechanism of chemoresistance in cancer stem cells. The relative expressions of ABCG2 and ABCB1 mRNA were increased in resistant cells compared to parental cells (Figure 6, Figure 7). However, the relative expression of ABCC1 mRNA in resistant cells was not definitely

different from that in parental cells (Figure 7). Western blot analysis of ABCG2 showed consistent result to that of real-time RT-PCR.

To confirm the mechanism of ABC transporter in gemcitabine-resistant cells, clonogenic assay with gemcitabine plus verapamil was performed. Verapamil has been known as an inhibitor of ABC transporters. The results of clonogenic assay showed that verapamil resensitized the resistant cells to gemcitabine with dose dependant manner (Figure 8). It demonstrated that ABCG2 and ABCB1 were one of the main mechanisms in acquiring multi-drug resistance in cancer stem like cells in pancreatic cancer.

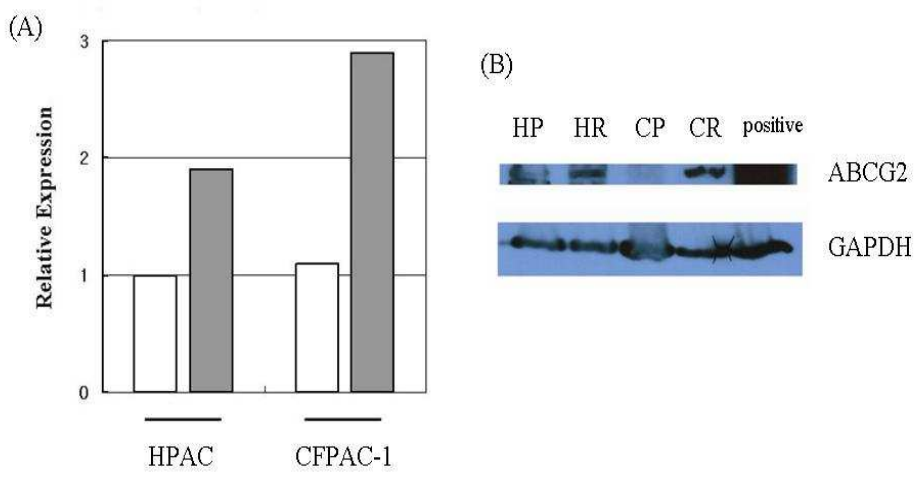

Figure 6. ABCG2 expression of HPAC and CFPAC-1 cells. (A) mRNA expression of ABCG2 was increased in resistant cells compared to parental cells. Relative quantification of mRNA within the samples was examined using the comparative Ct method. (B) The result of Western blot was consistent to that of Real-time RT-PCR. HP, HPAC parental cell; HR, HPAC resistant cell; CP, CFPAC-1 parental cell; CR, CFPAC-1 resistant cell.

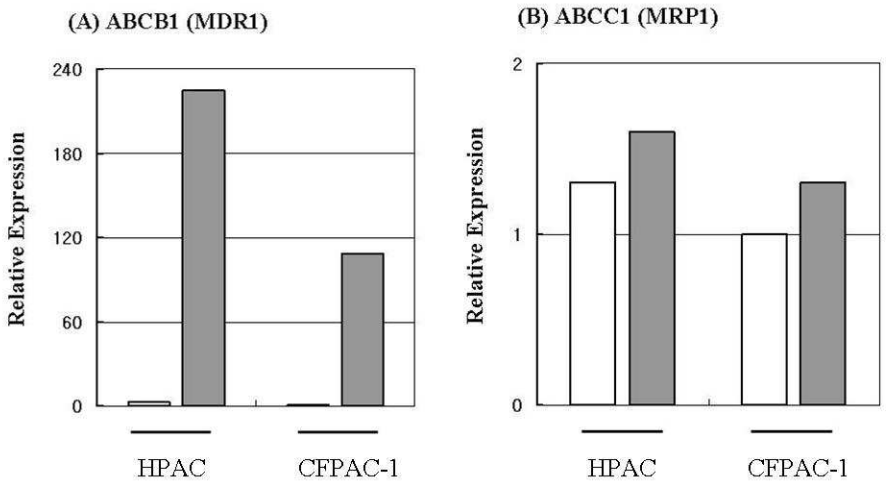

Figure 7. Real-time RT-PCR of ABCB1 and ABCC1 in HPAC and CFPAC-1 cells. (A) The mRNA expressions of ABCB1 were markedly increased in resistant cells compared to parental cells. (B) However, the mRNA expression of ABCC1 showed no definite change. Relative quantification of mRNA within the samples was examined using the comparative Ct method.

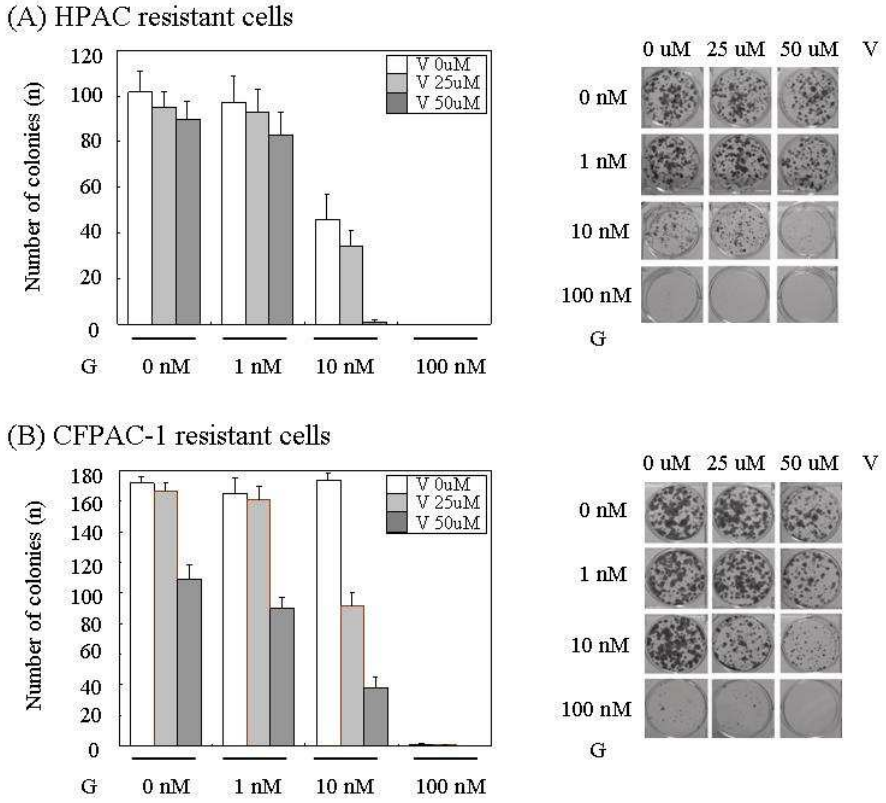

Figure 8. Clonogenic assay of HPAC and CFPAC-1 resistant cells with gemcitabine and verapamil. 10^3 cells were seeded on 6 well plate and gemcitabine (0 nM, 1 nM, 10 nM, and 100 nM) and verapamil (25 uM and 50 uM) were added. Verapamil, an ABC transporters inhibitor, resensitized the resistant cells to gemcitabine with dose-dependant manner. G, gemcitabine; V, verapamil.

IV. Discussion

Cancer cells can acquire drug resistance during chemotherapy, and then it manifests clinically tumor progression or recurrence. Typically, tumors that have progressed after an initial chemotherapy are resistant to multiple drugs (multi-drug resistance)[7]. Recently CSCs hypothesis has been introduced to explain the mechanism of drug resistance. CSCs are naturally resistant to chemotherapy through their quiescence, their capacity for DNA repair, and ABC-transporter expression. As a result, CSCs can survive chemotherapy and support regrowth of the tumor[7]. In the present study, to evaluate the dynamic mechanism of drug resistance on the view point of CSCs model, gemcitabine induced resistant pancreatic cancer cells were established.

After long-term gemcitabine exposure, the resistant cells have undergone distinct morphologic changes. Compared with parental cells, resistant cells showed spindle-shaped morphology, abundant pseudopodia, and loss of adhesion characteristics, which are hallmark of epithelial-to-mesenchymal transition (EMT). Shah et al. reported that these morphological changes after gemcitabine exposure were related to increase of vimentin and β-catenin nuclear traslocation, and decrease of E-cadherin in resistant cells[32]. The investigators also suggested that these changes in resistant cells might be related to increased number of CSCs.

In the present study, colony forming assay and sphere forming assay were performed to evaluate the biological properties, especially tumorigenic activity and stemness of resistant cells. Sphere forming assay has been recently used to identify and separate CSCs from heterogenous tumors and cell lines[33]. The CSCs as well as normal stem cells, such as neural stem cells and mammary gland stem cells, can form floating aggregates (sphere) and be enriched in the spheres when cultured in serum-free medium with growth factor on low-attachment plates. Recently Gou et al. demonstrated that spheres derived from PANC-1 pancreatic cancer cells were about 20-fold capacity for tumor formation in nude mice and excluded Hoechst 33342 dye, which is a hallmark of stem cells[34]. In the present study, after incubation with SFM during 14 days, not only the number of spheres but also the size of spheres was dramatically increased in resistant cells compared to parental cells. The in-vitro tumorigenic activity of resistant cells was also increased, which was consistent to the results of sphere forming assay. These results mean that the proportion of the stem like cells was increased during acquiring drug resistance.

Although recent studies proposed CD24, CD44, and ESA as putative stem cell markers in pancreatic cancer[30], cell surface markers, which have been used to identify and purify stem cells, are various according to the type of

solid tumors. While $CD44^+CD24^-ESA^+$ cancer cells were responsible for the propagation of breast cancers[22], $CD133^+$ subpopulations were regarded as cancer stem cells in brain tumors[24]. CD133 was also served as CSCs marker in other tumors, such as colon[28], liver[29], and prostate[26]. Interestingly, FACS analysis showed that $CD44^+$ subfraction was dramatically increased in resistant cells, however $CD24^+$ or ESA^+ cells were not definitely changed or decreased. According to the CSCs model, surviving CSCs repopulate after chemotherapy, then the recurred tumors harbor a small population of CSCs and a large number of undifferentiated resistant cells, which are derived from CSCs. Because more than 95% of cells underwent apoptotic process after administration of double dose of gemcitabine, the repopulated cells might include a small number of CSCs and a large number of CSCs-derived resistant cells. The present study showed that CSCs-derived resistant cells expressed CD44 cell surface antigen. MTT data showed that resistant cells of CFPAC-1 were more resistant than that of HPAC and it could be explained that resistant cells of CFPAC-1 harbored more CD44+ cells. Actually, CD44 was widely used as stem cell marker in other tumors, such as breast[22], bone[35], and prostate[26]. In pancreatic cancer, it was demonstrated that mRNA expression of CD44 was increased in sphere derived from PANC-1 cells[34] and CD44 was strongly correlated to the expression of P-glycoprotein, which was the product

of MDR1 (ABCB1), and one of the main mechanisms of multi-drug resistance[36]. Because CSCs are suggested to play an important role in drug resistance and tumor recurrence, it is essential to validate cancer stem like cell markers in tumors, which might be applied to CSC-targeted therapy to overcome the resistance for the conventional therapy. Our result means that CD44 targeted therapy might be beneficial to overcome drug resistance and reduce recurrence in pancreatic cancer.

CSCs are regarded as being resistant to chemotherapy through their quiescence, their capacity for DNA repair, and ABC transporter expression[7]. ABC proteins are a large family of integral membrane proteins, which contains two nucleotide-binding folds and two transmembrane domain encoded by a single polypeptide[37]. These ABC transporters have been regarded as one of the main mechanisms of drug resistance. Among these ABC transporters, ABCB1 (MDR1) and ABCG2 (BCRP1) have been proposed to play an important role in the drug resistance in CSCs[38]. In the present study, ABCB1 and ABCG2 were significantly increased in resistant cells compared to parental cells, while ABCC1 (MRP1) was not definitely changed. Previous report also showed that ABCC1 was not related to the CSCs[39]. We demonstrated that verapamil, ABC transporter inhibitor, resensitized resistant cells to gemcitabine. It means that ABCB1 and ABCG2

were important mechanism of multi-drug resistance in pancreatic cancer stem like cells and inhibitor of ABC transporter might have therapeutic effect on pancreatic cancer.

Conclusion

1. Gemcitabine-resistant pancreatic cancer cells were established (EC50 of HP and HR, 81 ± 8 nM and 447.2 ± 14 nM; EC50 of CP and CR, 24 ± 3 nM and 1300 ± 323 nM; $P < 0.01$).
2. The results of colony forming assay demonstrated that resistant cells had more tumorigenic activity than parental cells in (HP versus HR, 133 ± 71 versus 372 ± 76; CP versus CR, 101 ± 23 versus 237 ± 70; $P < 0.05$).
3. The results of sphere forming assay showed that resistant cells had more stem-cell like activity than parental cells (HP versus HR, 47 ± 6 versus 253 ± 8/ 10^3 cells; CP versus CR, 16 ± 3 versus 115.5 ± 8/ 10^3 cells).
4. FACS analysis demonstrated that CD44 positive stem-like cells were markedly increased in resistant cells compared to parental cells (HP versus HR, 4.9 ± 4.7% versus 49.4 ± 19.6%; CP versus CR, 5.8 ± 0.4% versus 72.6 ± 8.2%).
5. ABCG2 and ABCB1 might play an important role in the multi-drug resistance mechanism in pancreatic cancer cells, and verapamil, an inhibitor of ABC transporter, resensitized resistant cells to gemcitabine.

The present study demonstrated that CSCs play a pivotal role in acquiring multi-drug resistance in pancreatic cancer and especially $CD44^+$ cancer stem like cells were dominantly involved in these process. This multi-drug resistance was mediated by ABC transporters, ABCB1 and ABCG2 and ABC transporter inhibitor could reverse the resistance. In therapeutic implication, targeted therapy against CD44 or ABC transporter inhibitor could be applied to overcome the drug resistance, and might be beneficial in the treatment of pancreatic cancer.

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Abstract (in Korean)

췌장암세포에서 종양줄기세포 증대에 의한

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췌장암은 서구에서 암 사망률 4위에 해당하며 항암약물 치료에 효과가 없는 등 불량한 예후를 보인다. 비록 젬싸이타빈이 일부 치료 효과를 보이기도 하나 췌장암은 선천적으로 혹은 치료 중 빠르게 젬싸이타빈에 대한 내성을 획득한다. 비록 선천적으로 해독 능력을 보유한 종양줄기세포로 종양의 항암제 내성 기전을 설명하려는 노력이 있으나 아직 연구는 부족한 실정이다. 이에 본 연구에서는 췌장암 세포주에서 항암제 내성 기전 획득에 췌장암 종양줄기세포의 역할에 대하여 알아보고자 하였다. 약제 내성 기전을 연구하기 위하여 HPAC과

CFPAC-1 세포에 젬싸이타빈 농도를 순차적으로 높여 내성 세포주를 확립하였다. 각 세포주에서 중간유효농도 (EC_{50})를 평가하기 위하여 MTT 분석을 시행하였다. 내성을 획득하는 동안 발생하는 생물학적 변화를 평가하기 위하여 부모 세포주와 내성 세포주에서 colony forming assay, sphere forming assay 및 췌장암 종양줄기세포 표지자를 이용하여 유세포 분석을 시행하였다. 항암제 내성 기전을 밝히기 위하여 다약제 내성 유전자에 대한 real-time RT-PCR과 Western blot을 시행하였다. 부모 세포주에 비하여 내성 세포주에서 젬싸이타빈에 대한 EC_{50}가 증가함을 확인하였다 (HPAC parental vs. resistant cells, 81 ± 8 nM vs. 447.2 ± 14 nM; CFPAC-1 parental vs. resistant cells, 24 ± 3 nM vs. 1.3 ± 0.3 uM; $P < 0.01$). Colony forming assay와 sphere forming assay 결과에 의하면 내성 세포주에서 종양줄기세포의 비율이 증가하였다. 흥미롭게도 기존에 알려진 췌장암 종양줄기세포 표지자 중

CD44 양성 세포가 내성 세포주에서 급격히 증가하였다 (HPAC parental vs. resistant cells, 4.9 ± 4.7 nM vs. 49.4 ± 19.6 uM; CFPAC-1 parental vs. resistant cells, 5.8 ± 0.4 nM vs. 72.6 ± 8.2 uM; P < 0.01). Real time RT-PCR과 Western blot 결과에 의하면 다약제 내성 유전자인 ABC 수송체 중 ABCG2와 ABCB1이 췌장암 종양줄기세포의 주요한 약제 내성 기전 중 하나로 보인다. 또한 ABC 수송체 억제제인 verapamil을 내성 세포주에 투여하였을 때 젬싸이타빈에 대한 내성이 약화됨을 확인하였다. 이상의 결과에 의하면 췌장암에서 항암제 내성 기전의 획득에 CD44 양성 췌장암 종양줄기세포가 중요한 역할을 함을 알 수 있었다. 치료적 입장에서 보면 CD44 양성 세포 타겟 치료나 ABC transporter 억제제 등이 췌장암의 항암제 내성을 극복하고 치료하는데 도움이 될 것으로 기대된다.

핵심되는 말: 췌장암, 약제 내성, 종양줄기세포, ABC 수송체