

Is the resistance of gemcitabine for pancreatic cancer settled only by overexpression of deoxycytidine kinase?

NAOTAKE FUNAMIZU^{1,2}, AIKOU OKAMOTO⁴, YUKO KAMATA³, TAKEYUKI MISAWA¹,
TADASHI UWAGAWA¹, TAKESHI GOCHO¹, KATSUHIKO YANAGA¹ and YOSHINOBU MANOME²

¹Department of Surgery, ²Department of Molecular Cell Biology and ³Oncology, Institute of DNA Medicine,

⁴Department of Gynecology, School of Medicine, The Jikei University School of Medicine,
3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan

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Abstract. The prognosis of pancreatic cancer remains poor, and the standard first-line chemotherapy with gemcitabine (GEM) has a response rate of less than 20%. Since expression of deoxycytidine kinase (dCK) seems important for improvement of GEM sensitivity, overexpression of dCK was investigated using pancreatic cancer cell lines (Panc-1, MIAPaCa-2 and BxPC-3). dCK gene was introduced into the cell lines by retrovirus and changes in IC₅₀ were examined. Sensitivity of two pancreatic cancer cell lines to GEM elevated dramatically in comparison with control cells, but change of sensitivity remained at 1.8 times in BxPC-3. Since addition of tetrahydro uridine (THU), an inhibitor of deoxycytidine deaminase (CDA), increased the sensitivity 54-fold, overexpression of CDA seems to be the mechanism for improvement of the sensitivity. In conclusion, dCK is a key enzyme of GEM, but resistance of GEM is not improved in all pancreatic cancer cells by overexpression of dCK. Combination treatment based on expression of GEM metabolism-related gene may become an effective therapy in the future.

Introduction

The prognosis of pancreatic cancer remains poor, for which surgery remains the only potentially curative treatment. This poor prognosis relates to the advanced disease stage at the time of diagnosis and to its profound resistance to existing therapies. Gemcitabine (GEM) is a cytotoxic pyrimidine

deoxynucleoside analogue, with activity against several solid tumors such as cancers of the pancreas, lung, breast and ovary. GEM is transported into the cell mostly by human equilibrative and concentrative nucleoside transporters (hENT and hCNT, respectively). Cells deficient in hENT1 are highly resistant to GEM (1), while hCNT1 transfection increases GEM sensitivity in pancreatic cancer cell lines (2). This drug is activated by deoxycytidine kinase (dCK), and phosphorylates a monophosphate, diphosphate and triphosphate (dFdCTP). dCK is the rate-limiting enzyme in the transformation of nucleoside analogs, and the increase in dCK activity may improve the efficacy of GEM (3,4). dFdCTP is mainly incorporated into DNA leading to masked chain termination. In addition, its active metabolite can inhibit ribonucleotide reductase (RR), resulting in a decrease in deoxynucleoside triphosphate (dNTP) pools that are required for DNA repair and synthesis as well as inhibition of DNA polymerase. GEM is inactivated by deamination and catalyzed by deoxycytidine deaminase (CDA) (Fig. 1). Mechanism of intrinsic resistance to GEM in carcinomas remains unclear although that of acquired resistance to the drug in pancreatic carcinoma has been intensively studied. Some of these studies have disclosed a decrease in dCK activity (2), increased activity of CDA (5), and increased RR activity (6,7). The aim of this study was to prove whether overexpression of dCK by retrovirus vector improves the therapeutic efficacy of GEM for pancreatic cancer.

Materials and methods

Reagent and chemicals. GEM (difluorodeoxycytidine, dFdC) was a generous gift of Eli Lilly (Indianapolis, IN, USA). Drugs were dissolved in sterile distilled water and diluted in culture medium immediately before use. Tetrahydrouridine (THU) was purchased from Calbiochem (La Jolla, CA, USA) as a sterile white powder in glass vials and stored at -20°C.

Retroviral vector. Retroviral vector and a wild-type dCK cDNA have been described previously (8). The retroviral vector pMV-7 and pMV-7-dCK are transcribed from the murine Molony leukemia virus long terminal repeat (LTR). The neomycin resistance gene is transcribed from a thymidine kinase promoter (tk).

Correspondence to: Dr Naotake Funamizu, Department of Surgery, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan
E-mail: funamizu@jikei.ac.jp

Abbreviations: GEM, gemcitabine; dCK, deoxycytidine kinase; THU, tetra-hydro uridine; CDA, deoxycytidine deaminase

Key words: gemcitabine, pancreatic cancer, deoxycytidine kinase, deoxycytidine deaminase, tetra-hydro uridine

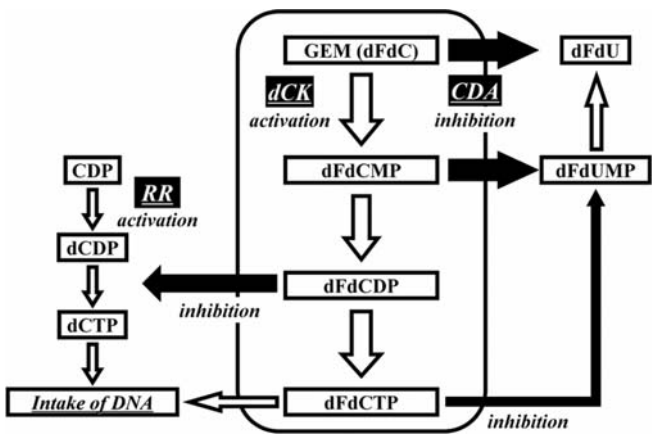


Figure 1. Metabolic pathway of gemcitabine (GEM). GEM is taken in DNA through phosphorylation by dCK. On the contrary, CDA inactivates GEM by deamination.

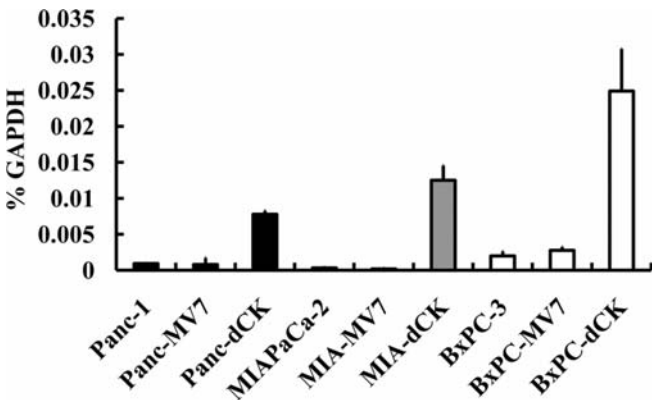


Figure 2. Expression of dCK mRNA of cell lines transfected by a retrovirus vector.

Cell lines and retroviral infection. The following human pancreatic adenocarcinoma cell lines were used in this study: Panc-1, MIA-PaCa-2 and BxPC-3, which were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The amphotropic PA317 retrovirus packaging cell line was purchased from the same manufacturer. Panc-1 and PA317 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), and MIA-PaCa-2 was grown in DMEM medium supplemented with 15% FBS with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B). BxPC-3 cells were cultivated in RPMI-1640 in the presence of 10% FBS and the same antibiotics. All cell lines were routinely maintained as monolayer cultures at 37°C and 5% CO₂ atmosphere. Cells were harvested with 0.05% EDTA and 0.5% trypsin.

Construction of dCK expressing retroviral vectors and stable transfectants. Three different cell lines were transduced by the pMV-7-dCK or control pMV-7 using viral supernatant from the respective PA317 producer cell lines. The tissue culture medium was filtered, and the viral supernatant was added in the pancreatic cancer cell lines. The cells were incubated for 48 h, and then polyclonal selection of stable cell lines integrated by retrovirus was performed by adding 100 µg/ml geneticin sulfate (Gibco BRL, Gaithersburg, MD, USA) to the culture media and cultured for 4 weeks. Respective stable cell lines (Panc-MV-7, Panc-dCK, MIA-MV-7, MIA-dCK, BxPC-MV-7, BxPC-dCK) transduced by

pMV-7 or pMV-7-dCK were characterized for dCK expression by real-time PCR analysis.

RNA preparation and real-time polymerase chain reaction (real-time PCR). The isolation of RNA was performed with RNazol as previously described (9). Cell pellets were suspended in an aliquot of 1 ml of RNazol per 3 cm dish. Six micrograms of the isolated RNA was used for reverse transcription into cDNA (GE Health Care, Buckinghamshire, UK). Random hexamers were used as primers according to the manufacturer's instructions. After incubation at 65°C for 10 min to remove secondary structures, samples were quickly cooled down on ice and annealing of the hexamers also took place on ice. The reaction was terminated by heating at 37°C for 60 min. cDNA samples were diluted and stored at -20°C until further use. The design of the dCK primers is shown in Table I, which were selected requiring an optimal annealing temperature of 58°C, absence of hairpins and no predictable stable primer-dimer formations. Real-time PCR was performed in triplicates using the ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) with the SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK). The PCR conditions were as follows: one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 58°C for 30 sec, and 72°C for 30 sec. After amplification, dissociation curves were drawn to ensure that a single PCR product had been amplified. Products were also analyzed by gel electrophoresis and sequencing on first

Table I. Blue print of primer used with RT-PCR.

Gene	Forward primer	Reverse primer	Conditions
dCK	ggaagtgggtcctgaacctgttg	ctctgcattcttgagcttgcc	58°C 33 cycle
CDA	gaagcgctcctgcctgca	ctggaccgtcatgacaatatagc	58°C 33 cycle
GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	58°C 28 cycle

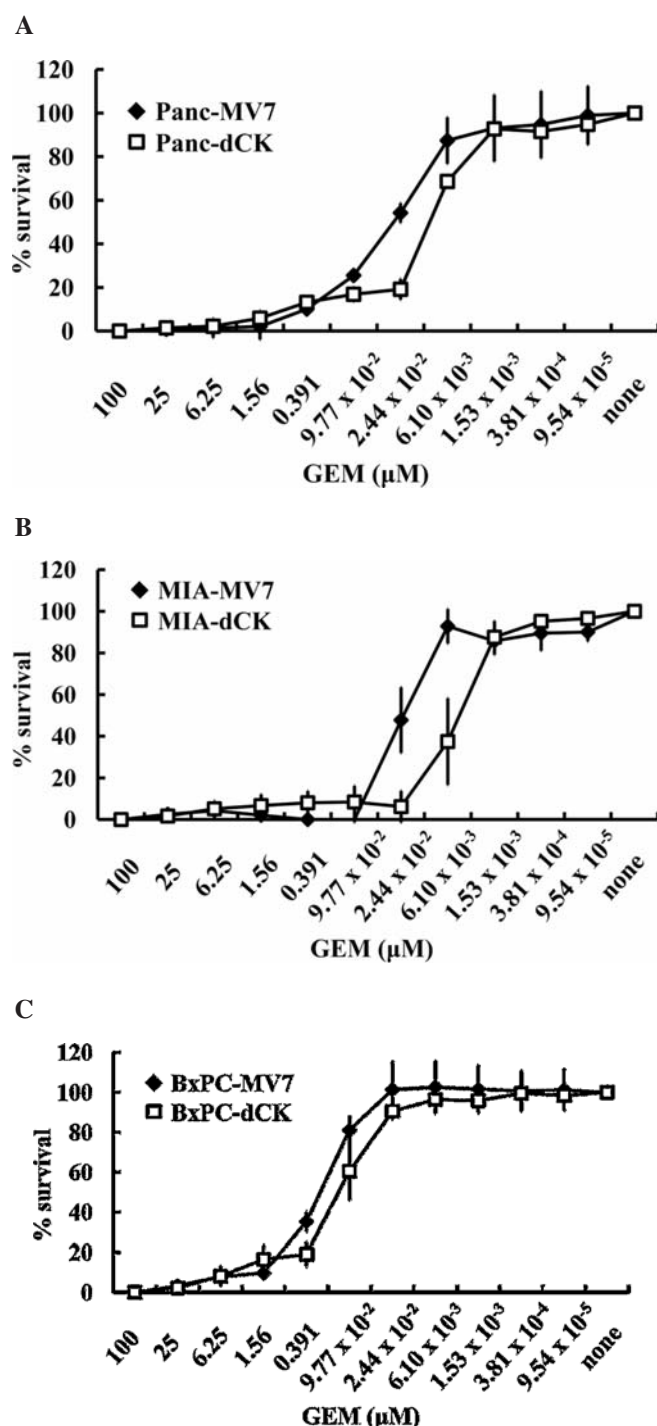


Figure 3. Sensitivity for GEM increased by transduction of dCK gene. Sensitivity of GEM by transduction of dCK gene increased in each cell line, 3.19 times for Panc-1, 5.18 times for MIA-PaCa-2 and 1.85 times for BxPC-3.

primer pair usage to ensure that the correct gene fragment was amplified. All the reactions were performed in triplicates, and the standard method was used for the quantification of the expression for each segment, by use of *GAPDH* as a normalization control gene.

Cytotoxic assay. Cells were seeded in 96-well sterile plastic plates (Costar) at 5000 cells/well. After 12 h, cells were treated with stepwise 2-fold dilution of GEM ($\geq 100 \mu\text{M}$) to assess cell viability, and incubated at 37°C for 96 h. The

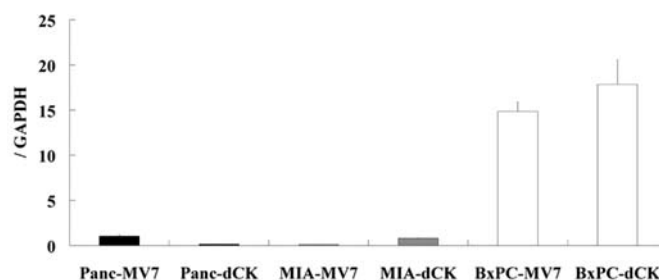


Figure 4. Expression of CDA mRNA level was examined by real-time PCR. BxPC-3 cell lines showed poor sensitivity to GEM.

cells were fixed at 25% glutaraldehyde after drug exposure for 30 min at room temperature and then viable cells were stained with $200 \mu\text{l}$ of 0.05% methylene blue for 20 min to evaluate the cytotoxicity of GEM. The dye was eluted with 0.33 M HCl for 20 min with agitation. Absorbance was measured in a microplate reader (model 3550, Bio-Rad, Tokyo, Japan) at 598 nm. Mean values were calculated from three different wells in triplicates. Chemosensitivity was evaluated by IC₅₀.

Results

dCK stable cell lines (Panc-dCK, MIA-dCK and BxPC-dCK) and control cell lines (Panc-MV-7, MIA-MV-7 and BxPC-MV-7) were established to estimate a change of GEM sensitivity by overexpression of dCK. pMV-7 or pMV-dCK vector was transfected in retroviral packaging cells (PA317). After two days, the supernatant was collected and cells were precipitated with centrifuge at 3000 rpm and assumed as the stock of virus liquid. Cell lines infected with retrovirus were selected by G418 for 3 weeks. Expression level of dCK of each cell line was measured by real-time PCR. The expression level of Panc-dCK, MIA-dCK and BxPC-dCK were 9.02, 9.69 and 71.4 times, respectively, higher than their controls (Panc-MV7, MIA-MV7 and BxPC-MV7) respectively (Fig. 2). IC₅₀ of control cell lines (Panc-MV7, MIA-MV7 and BxPC-MV7) to GEM was 29.77 ± 4.11 , 23.26 ± 6.87 and 256.12 ± 17.4 nM after 96 h of exposure, respectively. The sensitivity of BxPC-MV-7 to GEM was >8 times lower than the other cell lines (Panc-MV-7, MIA-MV-7 cells). Similarly GEM was administered with a dCK induction cell to examine change in IC₅₀, transducing of dCK gene increased the sensitivity to GEM by 3.19, 5.18 and 1.85 times, respectively (Fig. 3). Though the expression of dCK increased >70 times in BxPC-dCK cells, IC₅₀ did not change much in comparison with other cell lines. mRNA expression of metabolism-related gene was examined by RT-PCR. Expression of CDA, dCMPDA, TK2, 5-NT, p53, hENT-1, RRM1 and RRM2 was confirmed with all cell lines. The expression of other mRNA was almost at the same level (data not shown), but only CDA mRNA level showed significant difference (Fig. 4). Expression of CDA developed >14 times in BxPC-dCK in comparison with Panc-dCK or MIA-dCK. When THU, an inhibitor of CDA with GEM, was given, only the sensitivity of BxPC-dCK cell line increased by as high as

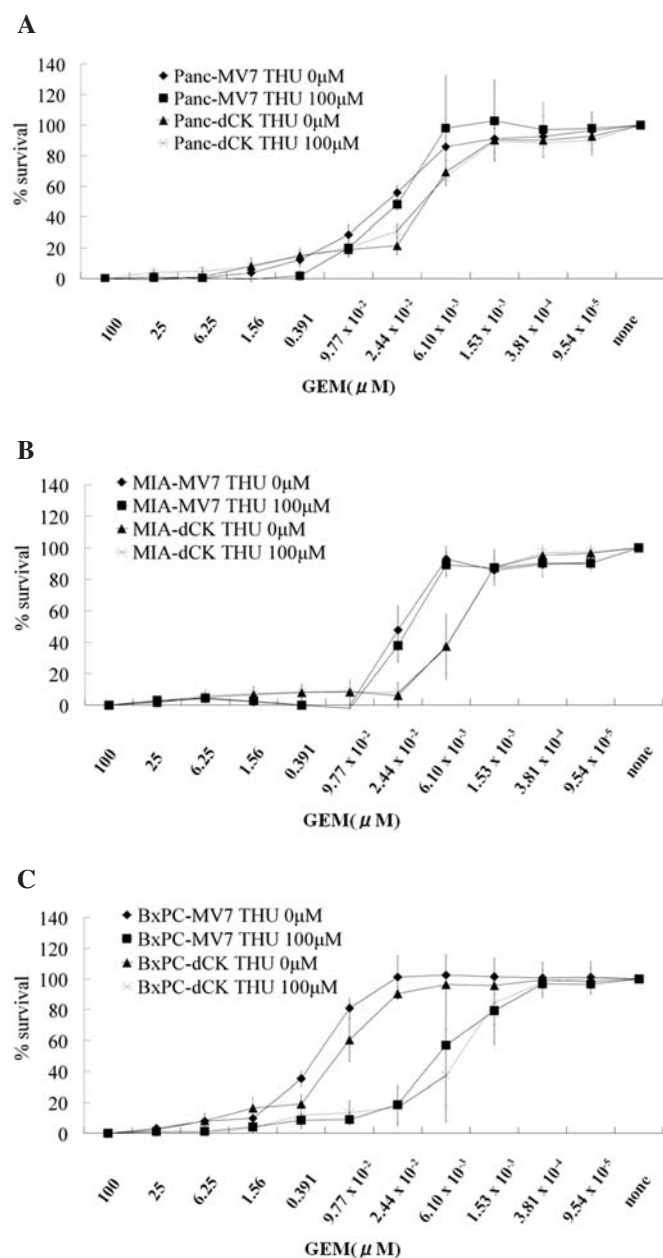


Figure 5. In the cytotoxic assay, the addition of THU, an inhibitor of CDA, increased the sensitivity of BxPC-3 to GEM.

54 times (Fig. 5). Panc-dCK, MIA-dCK-cell lines showed minimal change in IC50.

Discussion

The effectivity of gene therapy by single gene induction or anticancer drug is limited for treatment of pancreatic cancer, because the chemosensitivity to GEM differs depending on genetic expression of the pancreatic cancer. The combination treatment that matched with genetic expression is required to treat pancreatic cancer of variable characteristics. Our study suggested that the combination of dCK and THU may be an effective treatment for pancreatic cancer.

Various studies have reported on mechanism of GEM-resistance in pancreatic cancer, especially on dCK (3,4,10-12), CDA (5,13,14), RR (6,7,15) and hENT (1,2,16-20). dCK

gene, a key enzyme of GEM metabolism, was transduced by retrovirus into three cell lines, Panc-1, MIAPaCa-2 and BxPC-3. Expression level of dCK mRNA in the cell lines transduced with dCK gene were examined and compared with control cells by RT-PCR. The sensitivity to GEM was not in proportion to expression of dCK in our study. Therefore, overexpression of dCK was not effective in all pancreatic cancer cell lines.

As to the cause, overexpression of CDA was suggested, since CDA metabolizes GEM rapidly to its inactive metabolite, and CDA is known to be associated with GEM resistance.

dCK is a key enzyme, but is not necessarily effective in all pancreatic cancer cell lines. Treatment in accordance with the characteristics of the pancreatic cancer cells is needed. Many studies have reported the effectiveness of combination treatment of anticancer drug and inhibitor or gene transduction (22-32). There is no treatment that is effective for all carcinomas with various characteristics. These regimens may be more effective if the regimen is chosen depending on gene expression of pancreatic cancer.

In conclusion, THU is effective for pancreatic cancer when CDA is expressed at high level, and GEM sensitivity increased by transducing the dCK gene. Therefore, combination treatment depending on gene expression could be useful.

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