Human Equilibrative Nucleoside Transporter 1, as a Predictor of 5-Fluorouracil Resistance in Human Pancreatic Cancer

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Abstract. *Background: The purpose of this study was to find a novel biomarker to predict 5-fluorouracil (5-FU) or gemcitabine (2',2'-difluoro-deoxycytidine) sensitivity in pancreatic cancer. Materials and Methods: The relationship between 5-FU and gemcitabine sensitivity and the mRNA levels of human equilibrative nucleoside transporter 1 (hENT1), thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) was investigated using seven types of human pancreatic carcinoma cell line (AsPC1, BxPC3, MiaPaCa-2, PSN1, Panc1, PCI6, and KMP-4). Quantitative mRNA expression was measured by LightCycler. A [^{3}H] gemcitabine cellular uptake assay was performed to examine the inhibition of hENT1 by nitrobenzylmercaptoprine ribonucleoside (NBMPR). Results: The expression levels of hENT1 mRNA significantly correlated with the IC_{50} value of 5-FU in all seven lines and also correlated with gemcitabine resistance in six lines (except AsPC1). No significant association was observed between TS or DPD mRNA levels and 5-FU sensitivity. In the PSN1 cells, [^{3}H] gemcitabine uptake via hENT1 was significantly inhibited by NBMPR, and 5-FU sensitivity was significantly increased when the cells were pretreated with NBMPR. Conclusion: Our results suggest that hENT1 plays an important role in 5-FU resistance and that hENT1 mRNA levels might be a useful marker to predict5-FU sensitivity in pancreatic cancer.*

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Pancreatic cancer is one of the most lethal of all the common gastrointestinal malignancies. This disease carries a dismal prognosis with a 5-year survival rate of less than 5% (1). In advanced disease where surgery is not an option, other therapeutic options including 5-fluorouracil (5-FU) chemoradiation and gemcitabine (2',2'-difluoro-deoxycytidine) chemotherapy are considered (2). 5-FU, first synthesized by Heidelberger *et al.* in 1957 (3), is one of the most commonly used chemotherapeutic reagents in digestive carcinoma including pancreatic cancer. According to several randomized trials, 5-FU chemotherapy combined with external beam radiation therapy has become a frequently employed therapy for patients with locally advanced pancreatic carcinomas (1). Gemcitabine, a novel pyrimidine nucleoside analogue, has been reported to improve the survival and clinical benefit responses compared to 5-FU in patients with advanced, symptomatic pancreatic cancer (4). Although single-agent gemcitabine is currently the standard first-line treatment for the patients with metastatic pancreatic cancer (2), it provides only limited benefit because of the endogenous or acquired resistance of tumor cells, and 5-FU or its derivatives still play a key role in combination with gemcitabine (5, 6).

Cells can synthesize nucleotides not only through the *de novo* synthesis pathway but also the salvage pathway. In the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by nucleoside transporter (NT) proteins. In addition to nucleosides, nucleoside analogues, such as gemcitabine, are also taken up into the cell *via* these specific transporters (7). Gemcitabine is a substrate for five of the NTs found in humans (8). Human equilibrative nucleoside transporter 1 (hENT1) is one of those NTs, and gemcitabine is transported into cells mostly by hENT1 (8). It has been reported that the sensitivity to nucleoside analogues correlates with the expression of hENT1, and that cells lacking hENT1 are highly resistant to gemcitabine (8). On the other hand, 5-FU

is known as a *de novo* synthesis inhibitor, and there is a possibility that the cellular uptake and supply of nucleosides and nucleobases through hENT1 (*via* the salvage pathway) would interfere with the effect of 5-FU in the cells. Recently, Kubota reported that high mRNA expression of hENT1 might result in low sensitivity to 5-FU in colorectal cancer (9). However, it remains to be elucidated whether or not the expression level of hENT1 influences the sensitivity to 5-FU or gemcitabine in human pancreatic cancer.

5-FU is one of the thymidylate synthase (TS) inhibitors (10), and multiple clinical investigations have suggested that high TS expression resulted in 5-FU resistance in colorectal and gastric cancers (11-13). The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU) (14). High levels of DPD mRNA expression in colorectal tumors have been shown to correlate with resistance to 5-FU (15). However, in pancreatic cancer, the relationship between expression levels of TS or DPD and 5-FU resistance is still obscure.

Understanding the mechanism by which pancreatic carcinomas become resistant to chemotherapy is an essential step towards predicting or overcoming that resistance. Although several molecular markers have been reported to determine the sensitivity/resistance to 5-FU or gemcitabine in human pancreatic cancer (16-26), these markers have not yet been useful in clinical settings. In this study, in order to find a novel biomarker to predict chemosensitivity, the mRNA expression of the genes related to metabolism and nucleoside transport which may affect the efficacy of 5-FU or gemcitabine were focused upon.

Materials and Methods

Cell cultures. Seven types of human pancreatic carcinoma cell line were used in the present study. The AsPC1, MiaPaCa-2 and PSN1 cell lines were obtained from the Japanese Collection of Research Bioresources (JCRB, Japan). The BxPC3 and Panc1 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The PCI6 cell line was a gift from Dr. H. Ishikawa (Hokkaido University, Sapporo, Japan) and the KMP-4 cell line was a gift from Professor Imamura (Kyoto University, Kyoto, Japan). All the cell lines were cultured at 37˚C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Nikken Biomedical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Rockville, MD, USA) and 100 units/ml each of penicillin and streptomycin.

Chemicals. 5-FU was purchased from Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan). Gemcitabine was kindly provided by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). [^{3}H] gemcitabine was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). Nitrobenzylmercaptopurine ribonucleoside (NBMPR) was purchased from Sigma (St. Louis, MO, USA). 5-FU, gemcitabine, and NBMPR were dissolved in distilled water and applied to the cells at a volume less than 0.1% of the medium volume.

Table I. *Pairs of primers for detecting hENT1, TS, or DPD gene by (quantitative) RT-PCR.*

Gene		Primers
hENT1	Forward	5'-AATATCTTTGACTGGTTGGG-3'
	Reverse	5'-CAGCCATGAAGAAAATGAAC-3'
TS	Forward	5'-TCTGCTGACAACCAAACGTG-3'
	Reverse	5'-GTTCACCACATAGAACTGGC-3'
DPD	Forward	5'-GAGAAGCAATGAGATGCCTG-3'
	Reverse	5'-CAGCCGGAACTGAGGAATTT-3'

RT-PCR, reverse transcription-polymerase chain reaction; hENT1, human equilibrative nucleoside transporter 1; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction was performed with Trizol Reagent (Life Technologies, Inc., Grand Island, NY, USA) in a single-step method and complementary DNA (cDNA) was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). PCR was performed in a 25 μl reaction mixture containing 2 μl of cDNA template, 1x Perkin Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.5 pmol of primers, and 1 unit of Taq DNA Polymerase (AmpliTaq Gold, Roche Molecular Systems Inc., Branchburg, NJ, USA). The PCR primers used for detection of hENT1, TS and DPD are shown in Table I. The primers for porphobilinogen deaminase (PBGD) were synthesized as described previously (27). Following PCR (hENT1, 35 cycles of 94˚C for 1 min, 62˚C for 1 min, 72˚C for 1 min; TS, DPD and PBGD, 35 cycles of 94˚C for 1 min, 58˚C for 1 min, 72˚C for 1 min), the products were run on 2 % agarose gel and visualized by ethidium bromide staining.

Quantification of hENT1, TS, and DPD gene expression. A quantitative gene expression assay was performed using a LightCycler (Idaho Technology, USA), as described previously (28). The PCR primers used for detection of hENT1, TS and DPD were the same as used for RT-PCR. The PCR conditions were set up as follows: hENT1, one cycle of denaturing at 95˚C for 2 min, followed by 40 cycles of 95˚C for 30 sec, 62˚C for 30 sec and 72˚C for 30 sec; TS, DPD and PBGD, one cycle of denaturing at 95˚C for 2 min, followed by 40 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec. In each assay, a house-keeping gene, PBGD was employed as an internal standard. The quantification data from each sample were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. The relative gene expression levels were shown as the quantified gene expression divided by the quantified PBGD levels.

Growth inhibitory effect of 5-FU, gemcitabine or combination of NBMPR and 5-FU. The cell viability was determined by MTT (3- [4,5-dimethylthiazol-2-yl] -2,5- diphenyltetrazolium bromide) assay (Sigma). The cells were seeded in 96-well culture plates in culture medium at an optimal density. After 24 h, the medium was exchanged with fresh culture medium containing either vehicle only or the indicated dose of the test chemicals. To assess the effect of NBMPR on the 5-FU sensitivity, the cells were treated with 1 μM NBMPR for 3 min before and during 5-FU exposure. After a 2-day

Table II. *IC_{50} values for 5-FU and gemcitabine in seven types of human pancreatic cancer cell line.*

	IC_{50}	
	5-FU (μg/ml)	Gemcitabine (ng/ml)
AsPC1	33.2±2.4	129.0±10.4
BxPC3	1.7±0.2	50.0±7.1
MiaPaCa-2	2.3±0.2	27.1±4.3
PSN1	1.7±0.3	1.4±0.2
Panc1	3.7±0.4	178.3±23.6
PCI6	6.3±0.3	217.4±20.1
KMP-4	2.0±0.3	32.2±3.5

5-FU: 5-fluorouracil.

incubation, the MTT assay was performed according to the instructions provided by the manufacturer. Absorbance was measured at 560 nm using a microtiter plate reader (Wako, Osaka, Japan). With this method, the results only correlated with the viable cell number. The IC_{50} values for each chemical were estimated by plotting the rate of growth inhibition *versus* the drug concentration.

[^{3}H] gemcitabine cellular uptake assay. To examine the inhibition of hENT1 function by NBMPR, a [^{3}H] gemcitabine cellular uptake assay was performed as described previously (29). The cells were seeded in a flat-bottomed 24-well microplate ($1x10^4$ / well) and incubated for 24 h. The cells were treated with 1 μM NBMPR for 3 min, and then exposed to [^{3}H] gemcitabine at a concentration of 23.9 ng/ml (1.0 μCi/ml). After 1 h exposure, the cells were washed three times in 1 ml of ice cold phosphate-buffered saline (PBS). The cells were then re-suspended in 0.5 ml of 0.5% Triton X-100, and 0.4 ml aliquots were sampled for radioactivity counting. Aliquots of 20 μl were also sampled for protein determination. The uptake level of [^{3}H] gemcitabine was expressed as the radioactivity level divided by protein concentration measured by the Bradford method (Bio-Rad Laboratories, Madrid, Spain).

Statistical analysis. All data were expressed as mean ± SD. The differences between groups were examined using the Student's *t*-test or Mann-Whitney *U*-test. Correlation between the level of gene expression and extent of sensitivity to each reagent was examined by Pearson's correlation analysis. A *p*-value less than 0.05 denoted the presence of a statistically significant difference.

Results

Chemosensitivity of human pancreatic cancer cells to 5-FU and gemctabine. The IC_{50} values for 5-FU and gemcitabine in the seven types of human pancreatic cancer cell line are shown in Table II. The AsPC1 cells (IC_{50}: 33.2 mg/ml) were much more resistant to 5-FU than the other six lines (range of IC_{50}: 1.7-6.3 mg/ml). The IC_{50} values for gemcitabine were spread from the lowest at 1.43 ng/ml to the highest at 217.4 ng/ml.

Expression of hENT1, TS and DPD in human pancreatic cancer cells. The expression of hENT1, TS and DPD mRNAs were detected in all seven cell lines tested by RT-PCR analysis (Figure 1).

Correlation between hENT1 levels and the sensitivity to 5-FU or gemcitabine. As shown in Figure 2A, there was a significant correlation between hENT1 mRNA level and the sensitivity to 5-FU, with a coefficient of correlation of 0.989, which was statistically significant at $p<0.001$. That is, the expression of hENT1 was higher in the cells which were less sensitive to 5-FU. Because the AsPC1 cells showed much higher values in both hENT1 mRNA level and IC_{50} of 5-FU than the other six cell lines, the coefficient of correlation was re-analyzed using the cell lines except AsPC1, giving a value of 0.872 which was again statistically significant at $p<0.03$ (Figure 2B). Although no significant association was observed between hENT1 mRNA level and sensitivity to gemcitabine in the 7 lines ($R=0.414$, $p=0.3787$, Figure 3A), a strong correlation was observed between hENT1 mRNA level and the IC_{50} value of gemcitabine ($R=0.877$, $p<0.02$, Figure 3B) when the data for AsPC1 were excluded because the hENT1 expression of this cell line was much higher than that of the other lines.

Correlation between TS or DPD levels and the sensitivity to 5-FU. The relationships between the TS and DPD mRNA expression level and sensitivity to 5-FU are shown in Figures 4 and 5. No statistically significant trend was observed between TS and DPD mRNA expression levels and response to 5-FU in the seven types of human pancreatic cancer cell line (Figures 4A and 5A). Only when the data of the AsPC1 cells were excluded because of its extremely low sensitivity to 5-FU compared with the other six lines, was a moderate correlation coefficient observed between the IC_{50} value of 5-FU and TS or DPD expression levels (0.637 or 0.600, respectively) (Figures 4B and 5B).

Enhanced growth inhibitory effect of 5-FU by nucleoside transporter inhibitor. We hypothesized that the uptake of nucleosides or nucleobases through hENT1 might prevent 5-FU from inhibiting *de novo* DNA synthesis in pancreatic cancer cells. To clarify this hypothesis, we examined whether the inhibition of hENT1 could enhance the 5-FU cytotoxicity *in vitro*. NBMPR, known as an hENT1 inhibitor, was used for the inhibition of hENT1.

Firstly, in order to examine the effect of single-agent NBMPR in pancreatic cancer cells, PSN1 cells, which showed high sensitivity to 5-FU and gemcitabine, were treated with increasing doses of NBMPR in the range of 0.1-10 μM. No growth inhibition was observed in PSN1 cells treated with NBMPR alone up to 10 μM (data not shown). However, the uptake of [^{3}H] gemcitabine was significantly

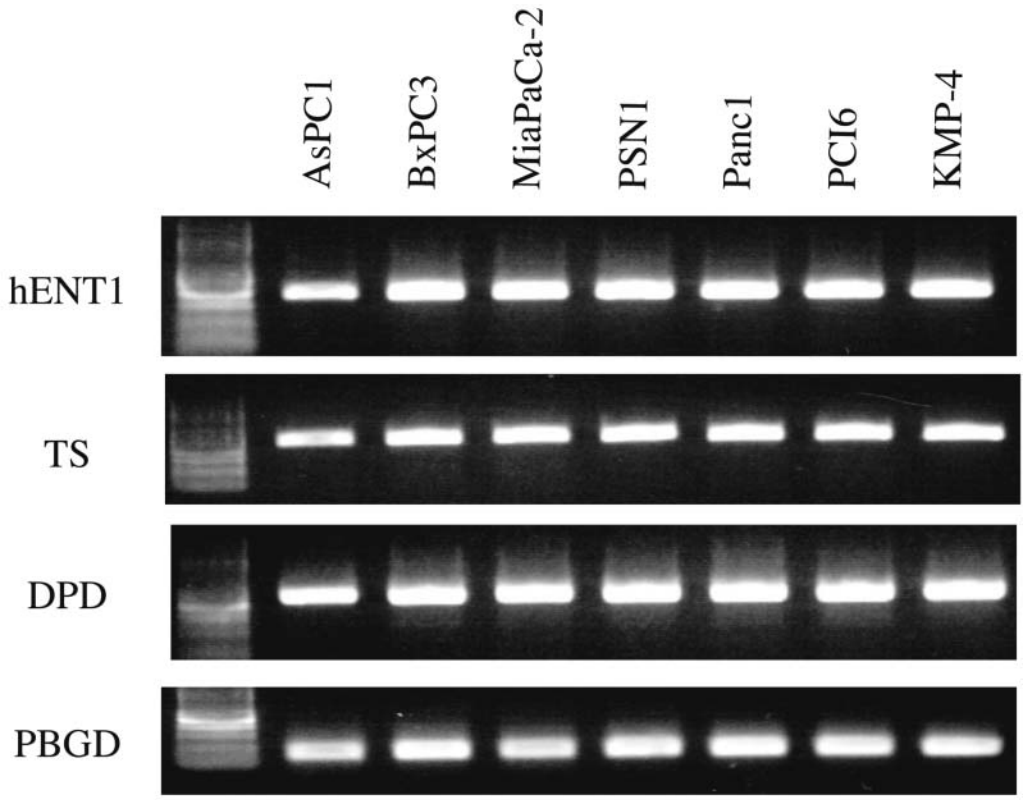

Figure 1. *Expression of hENT1, TS and DPD mRNA in seven human pancreatic cancer cell lines.*

inhibited when the cells were treated with 1 μM NBMPR ($p<0.05$, Figure 6).

To examine whether the hENT1 inhibitor enhanced the cytotoxic effect of 5-FU in pancreatic cancer cells, PSN1 cells were treated with a combination of NBMPR and 5-FU. As shown in Figure 7, the IC_{50} value of 5-FU in the cells pretreated with 1 μM NBMPR was significantly lower than that in the control cells pretreated with PBS ($p<0.05$). The IC_{50} values of NBMPR-pretreated cells and PBS-pretreated cells were 0.91±0.12 and 1.88±0.18, respectively.

Discussion

In this study, the basal mRNA level of hENT1 expression significantly correlated with the IC_{50} value of 5-FU in human pancreatic cancer cell lines. That is, the cells with higher mRNA expression of hENT1 were less sensitive to 5-FU. 5-FU, one of the TS inhibitors, is known to block *de novo* DNA synthesis and deplete intracellular nucleotide pools so that cells depend on the salvage pathway to get preformed nucleosides from the extracellular fluid. The lower sensitivity to 5-FU in the cells with higher hENT1 expression might be due to the better supplies of preformed nucleosides *via* hENT1. To prove this hypothesis, we next examined the 5-FU sensitivity in pancreatic cancer cells pretreated with NBMPR, which is a specific inhibitor of hENT1. Treatment with 1 μM NBMPR resulted in significant inhibition of the uptake of nucleoside analogues, but single-agent NBMPR (up to 10 μM) did not show any growth inhibitory effect on the pancreatic cancer cells tested. The sensitivity to 5-FU was significantly increased (IC_{50} value was decreased 52±11%) when the cells were pretreated with 1 μM NBMPR. Our results suggest that hENT1 plays an important role in regulating the mechanism of 5-FU resistance in human pancreatic cancer cells.

Recently, Kubota *et al.* analyzed the relationship between the mRNA levels of several types of genes including hENT1, TS and DPD and chemosensitivity to 5-FU using surgically obtained colorectal cancer specimens and reported that the quantified gene expression levels correlated with the sensitivity to 5-FU (9). Both TS and DPD are known to be key enzymes for treatment with 5-FU (14). Several studies using human tumor cell lines and clinical samples have reported that TS and DPD mRNA levels are correlated with the response to 5-FU in several types of carcinoma (11-15). In our study using pancreatic cancer cell lines, however, the correlation between TS and DPD mRNA levels and 5-FU antitumor activity was not evident. Although showing very

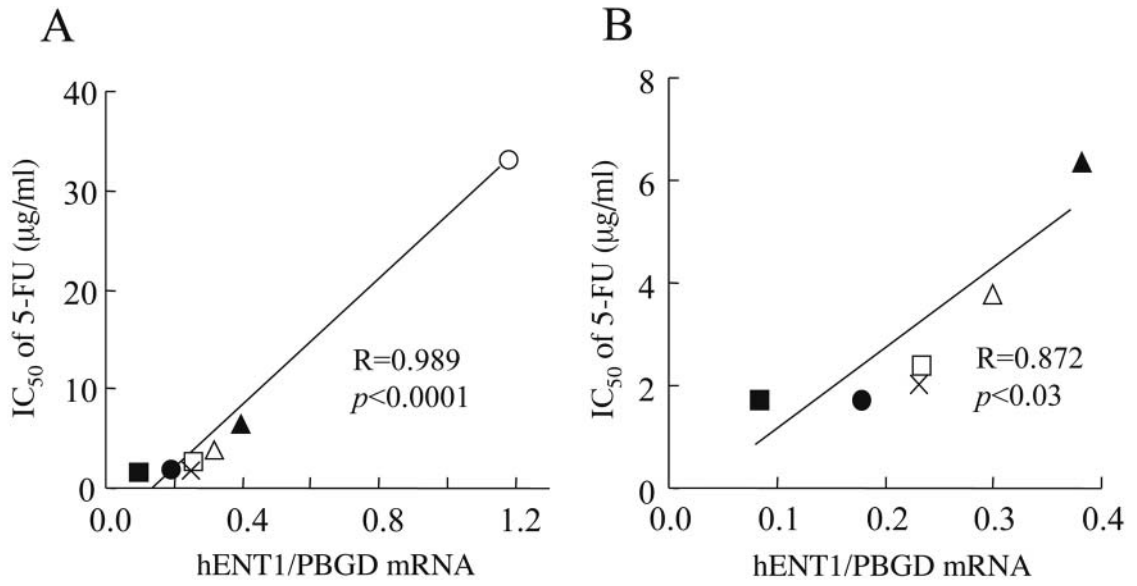

Figure 2. *Correlation between hENT1 mRNA levels and IC_{50} values of 5-FU in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). hENT1 mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

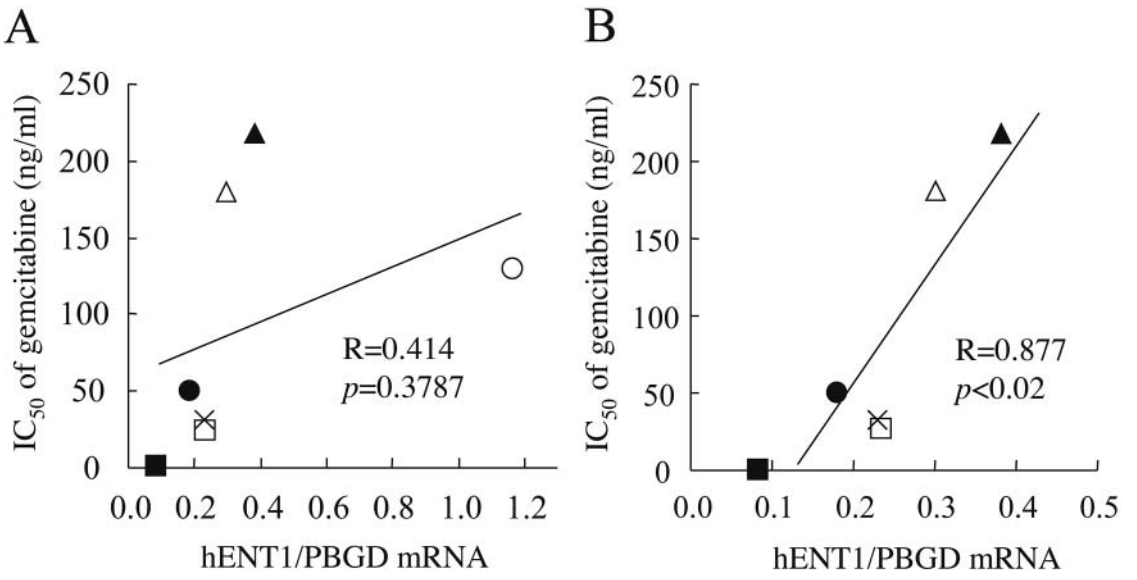

Figure 3. *Correlation between hENT1 mRNA levels and IC_{50} values of gemcitabine in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). hENT1 mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

low mRNA expression of TS and DPD, the AsPC1 cells were the most resistant to 5-FU of the seven lines tested. Interestingly, the highest expression of hENT1 was observed in the AsPC1 cells. There is a possibility that better supplies of nucleosides and nucleobases through highly-expressed hENT1 might interfere with the 5-FU function that blocks *de novo* DNA synthesis. The re-analysis of the correlation coefficient using six lines (excluding AsPC1) still showed only moderate correlations between IC_{50} values of 5-FU and TS or DPD expression, and they were not statistically

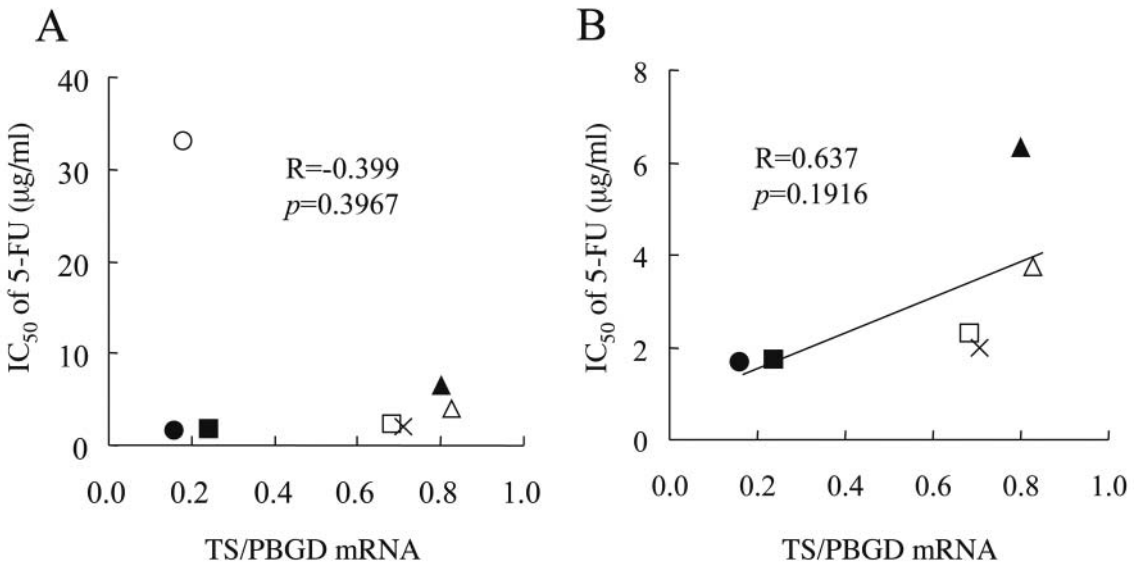

Figure 4. *Correlation between TS mRNA levels and IC_{50} value of 5-FU in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). TS mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

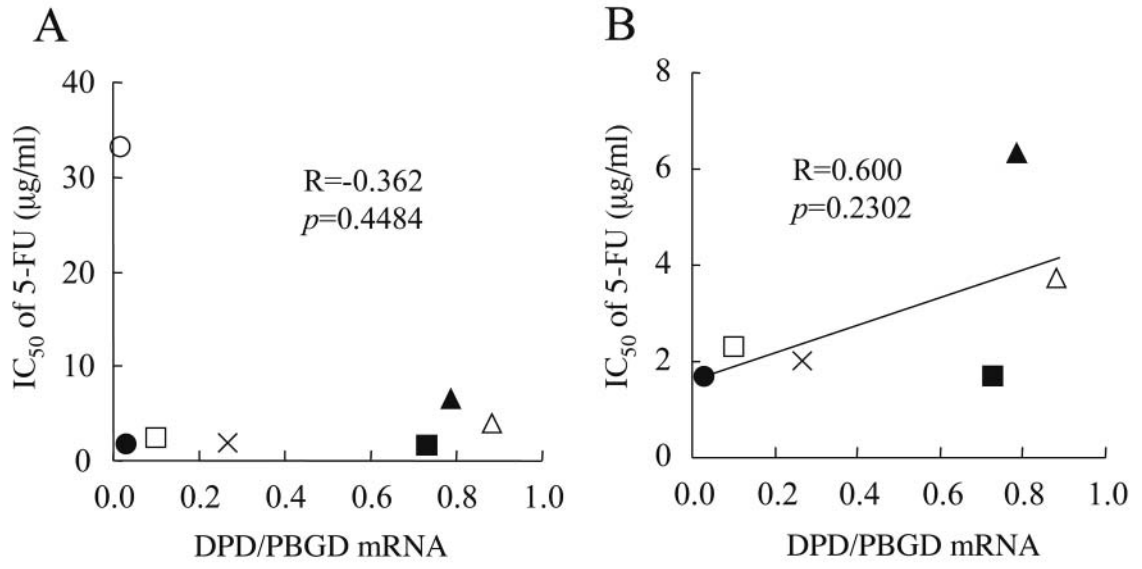

Figure 5. *Correlation between DPD mRNA levels and IC50 value of 5-FU in seven human pancreatic cancer cell lines (A) or in six cell lines, excluding AsPC1 (B). DPD mRNA levels are expressed as ratios to PBGD, the internal control. (○) AsPC1, (●) BxPC3, (□) MiaPaCa-2, (■) PSN1, (△) Panc1, (▲) PCI6, (x) KMP-4. Correlation coefficient (R) and p-value are shown.*

significant. These results suggested that the contribution of TS and DPD mRNA levels to 5-FU sensitivity might not be essential in human pancreatic cancer.

Gemcitabine is a cell cycle-dependent (S-phase specific) deoxycytidine analogue of the antimetabolite class. It has also been reported that the expression levels of hENT1 mRNA were significantly correlated with IC_{50} values for gemcitabine in human non-small cell cancer *in vitro* (30). Moreover, the expression of hENT1 is also reported to be associated with survival in patients with gemcitabine-treated pancreatic cancer (31, 32). Based on those reports, higher expression of hENT1 should result in higher

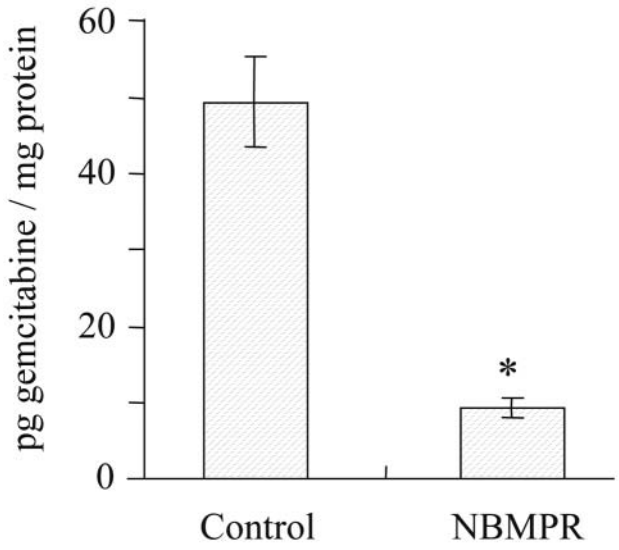

Figure 6. *Effect of NBMPR on the uptake of gemcitabine in PSN1 cells. PSN1 cells were treated with NBMPR or control PBS, and then exposed to [3H] gemcitabine. The uptake level of [^{3}H] gemcitabine was expressed as radioactivity levels divided by protein concentrations. Columns, mean of results in triplicate; bars, SD; *p<0.05.*

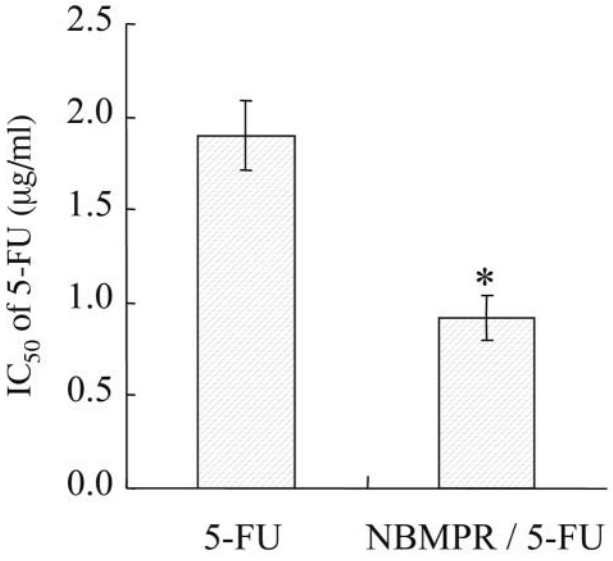

Figure 7. *Effect of NBMPR on the antitumor effect of 5-FU in PSN1 cells. PSN1 cells were pretreated with NBMPR or PBS (control), and then treated with 5-FU. Columns, mean of results in three independent experiments; bars, SD; *p<0.05.*

sensitivity to gemcitabine. In our current study, however, the expression levels of hENT1 mRNA were higher in the cell lines which were less sensitive to gemcitabine when the data of the AsPC1 cell line were excluded. Nakano *et al.* have reported that the expression of hENT1 mRNA was increased in the development of gemcitabine resistance, and they did not find any correlations between IC_{50} value of gemcitabine and hENT1 expression in pancreatic cancer cell lines (33). Once transported inside the cell, gemcitabine is converted by deoxycytidine kinase (dCK) to its triphosphate, the active form, which is incorporated into DNA and causes apoptosis. Deficiency in dCK activity has been considered to be one of the main mechanisms responsible for the development of gemcitabine resistance. Moreover, ribonucleotide reductase (RR) is also reported to be another factor in gemcitabine resistance (34-36). Therefore, although hENT1 plays an important role in gemcitabine uptake and subsequent cytotoxicity (37), other factors (such as dCK, RR, or apoptosis-related genes) or the balance between hENT1 and those factors might be more important in determining gemcitabine sensitivity in pancreatic cancer cells (18, 19, 38, 39).

In conclusion, in the present study, it was demonstrated that poor 5-FU sensitivity might be strongly influenced by the hENT1 mRNA level in human pancreatic cancer cells. The measurement of mRNA expression level using the quantitative RT-PCR method could easily be applied to clinical specimens. Further studies are needed to determine whether hENT1 would be useful as a predictive marker of resistance to 5-FU in pancreatic cancer patients.

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