Down-regulation of Deoxycytidine Kinase Enhances Acquired Resistance to Gemcitabine in Pancreatic Cancer

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Abstract. *Background: The functional roles of deoxycytidine kinase (dCK) in acquired resistance to gemcitabine remain unknown in pancreatic cancer. Here, the functional involvement of dCK in gemcitabine-resistance of pancreatic cancer was investigated. Materials and Methods: The levels of the dCK gene as well as other gemcitabine-related genes (hENT1, RRM1 and RRM2) were analyzed in gemcitabine-resistant pancreatic cancer cells (GR cells) using quantitative real-time reverse transcription polymerase chain reaction. The effects of inhibition of these genes on sensitivity to gemcitabine were evaluated. Results: In GR cells, expression of dCK was significantly reduced compared with that of parental cells ($p<0.05$). The dCK-targeting siRNA significantly reduced gemcitabine sensitivity ($p<0.01$) without affecting cell proliferation. The RRM1- and RRM2-targeting siRNAs increased gemcitabine sensitivity ($p<0.05$) and reduced cell proliferation even without gemcitabine treatment. The hENT-targeting siRNA did not affect gemcitabine sensitivity or cell proliferation. Conclusion: Down-regulation of dCK specifically enhanced acquired resistance to gemcitabine in pancreatic cancer cells without affecting their proliferation.*

Pancreatic cancer is one of the most aggressive malignancies and has 5-year survival rates of 1-4% (1). Surgery is the only curative treatment for patients with pancreatic cancer. However, only approximately 10-20% of patients have surgically resectable disease at presentation, and even in these cases, the 5-year survival rate is only 20% (1). This situation probably arises due to the difficulties associated with diagnosis at early stages of the disease, high incidence of metastasis and lack of effective drugs.

Gemcitabine is a deoxycytidine analogue with antitumor activity that bears a resemblance, both structurally and metabolically, to arabinosyl cytosine (AraC) (2). Gemcitabine is widely accepted as the first-line treatment for patients with advanced pancreatic cancer. Although gemcitabine produces a significant clinical benefit in patients with advanced pancreatic cancer, the median overall survival of gemcitabine-treated patients is still only 5 months and their 1 year-survival rate is only 15% (3).

Resistance to gemcitabine is a major cause of unsatisfactory improvement during pancreatic cancer treatment. Understanding the mechanism of resistance to gemcitabine should be useful for identifying novel therapeutic target genes in order to enhance the efficacy of gemcitabine treatment. Several studies regarding the mechanism of resistance to gemcitabine have been reported and several genes have been shown to be correlated with resistance to gemcitabine (4-7). Deoxycytidine kinase (dCK) phosphorylates gemcitabine to gemcitabine diphosphate and gemcitabine triphosphate in a rate-limiting step. Gemcitabine triphosphate is incorporated into DNA, where it leads to masked chain termination. Increased expression of dCK in colon carcinoma cells results in enhanced gemcitabine triphosphate accumulation, prolonged elimination kinetics and ultimately a potentiated *in vivo* tumor response to gemcitabine (8). Overexpression of dCK increases gemcitabine sensitivity in colon carcinoma cells, breast carcinoma cells and small cell lung adenocarcinoma cells (9). Immunohistochemical studies revealed that the levels of dCK protein expression in a panel of human pancreatic cancer tissues were correlated with overall survival following gemcitabine treatment (10). The data from these functional analyses in several other types of cancer and expression analyses in pancreatic cancer suggest that dCK plays an important role in gemcitabine resistance of pancreatic cancer. However, there are no reports regarding the functional involvement of dCK in gemcitabine resistance of pancreatic cancer.

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Expression of human equilibrative nucleoside transporter-1 (hENT1), which transports gemcitabine into cells (4), was reported to be correlated with survival in pancreatic cancer patients treated with gemcitabine (11). Ribonucleotide reductase (RR) is a dimeric enzyme comprising M1 and M2 subunits. RRM2 modulates the enzymatic activity of RR, while RRM1 has a key role in gemcitabine treatment (12, 13). *In vivo*, RRM1 is involved in acquired resistance to gemcitabine (6, 14), while RRM2 gene silencing by short interfering RNAs (siRNAs) is an effective therapeutic adjunct to gemcitabine treatment (7, 15). These data suggest that *hENT, RRM1* and *RRM2* may be involved in the mechanism of resistance to gemcitabine. However, the issue of whether the functions of these genes are involved in acquired resistance to gemcitabine in pancreatic cancer remains to be investigated.

In the present study, we established gemcitabine-resistant pancreatic cancer cells, and investigated the expression levels of the above-mentioned gemcitabine-related genes using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). In addition, the roles of these gemcitabine-related genes in acquired resistance to gemcitabine were evaluated by inhibiting their expression levels with specific siRNAs and examining the sensitivities of the siRNA-transfected pancreatic cancer cells to gemcitabine.

Materials and Methods

Cell lines and establishment of gemcitabine-resistant cells. Human pancreatic cancer cell lines were used in the present study. SUIT2 and PANC1 were generously provided by Dr. H. Iguchi (National Shikoku Cancer Center, Matsuyama, Japan), while MiaPaCa2 was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Gemcitabine-resistant cells were generated from SUIT2 cells by exposure to gradually increasing concentrations of gemcitabine. The initial concentration of gemcitabine was 10 nM, which did not seem to affect the proliferation of SUIT2 cells. When the cells had adapted to the drug, the concentration of gemcitabine was gradually increased by 10-20 nM per week to a final concentration of 200 nM. Gemcitabine was dissolved in phosphate-buffered saline (PBS) and added to the media of cell cultures.

Propidium iodide (PI) assay. Cell proliferation was evaluated by measuring the fluorescence intensity of PI as described elsewhere (16). Briefly, pancreatic cancer cells were plated at 2×10^4 cells/well in 24-well tissue culture plates (Becton Dickinson Labware, Bedford, MA, USA) and cultured for 24 h. Several different concentrations of gemcitabine were used, and the cells were incubated for a further 72 h. PI (30 μM) and digitonin (600 μM) were added to each well to ensure that all nuclei were labeled with PI. The fluorescence intensities corresponding to the total cells were measured using a CytoFluor multiwell plate reader (PerSeptive Biosystems, Framingham, MA, USA) with 530-nm excitation and 645-nm emission filters. The results were converted to percentage survival rates by comparing treated cells with untreated cells.

Analysis by qRT-PCR. Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) with DNase (Roche) treatment according to the manufacturer's instructions. We designed specific forward and reverse primers for *hENT1* (forward, 5'-TCTTCTTCATGGCTGCCTTT-3'; reverse, 5'-CCTCAGCTGGCTTCACTTTC-3'), *dCK* (forward, 5'-GCTGC AGGGAAGTCAACATT-3'; reverse, 5'-TCAGGAACCACTTCC CAATC-3'), *RRM1* (forward, 5'-GGCACCCCGTATATGCTCTA-3'; reverse, 5'-CCAGGGAAGCCAAATTACAA-3'), *RRM2* (forward, 5'-GGCTCAAGAAACGAGGACTG-3'; reverse, 5'-TCAGGCAAG CAAAATCACAG-3') and 18S rRNA (forward, 5'-GTAACCCGTT GAACCCCATT-3'; reverse, 5'-CCATCCAATCGG TAGTAGCG-3') and performed BLAST searches to ensure the primer specificities. We performed qRT-PCR with a QuantiTect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The mRNA expression for each gene was calculated by reference to a standard curve constructed from values for total RNA from SUIT2 pancreatic cancer cells. The mRNA expression of each gene was normalized by the corresponding expression of 18S rRNA.

Inhibition of dCK, RRM1, RRM2 and hENT1 mRNA expressions by siRNAs. Inhibition of *dCK, RRM1, RRM2* and *hENT1* expressions was achieved by RNA interference. SUIT2 cells were transfected with specific siRNAs targeting *dCK, RRM1, RRM2* and *hENT1* (B-Bridge International, Mountain View, CA, USA). We also used a control siRNA provided by Qiagen. SUIT2 cells were transfected with the individual siRNAs using Nucleofector (Amaxa Biosystems GmbH, Koln, Germany) according to the manufacturer's instructions. Cells were harvested at 24 h after transfection for PI assays or RNA extraction. The expression levels of the target mRNAs were evaluated by qRT-PCR.

Statistical analysis. Values are expressed as the mean±SD. Comparisons among all groups were carried out by one-way ANOVA, while comparisons between two groups were carried out using Student's *t*-test. The level of statistical significance was set at $p<0.05$. To confirm the induction results, the experiments were repeated at least three times.

Results

Gemcitabine sensitivities and expression levels of gemcitabine transporter and metabolism-related genes in pancreatic cancer cell lines. All the cells were highly sensitive to gemcitabine in the decreasing order of PANC1, SUIT2 and MiaPaCa2 cells. Specifically, the IC_{50} of PANC1 cells was 98.3±21.9 nM, that of SUIT2 cells was 200.9±24.7 nM and that of MiaPaCa2 cells was 930.4±114.2 nM (Figure 1A).

To examine the relationships between the expression levels of gemcitabine transporter or metabolism-related genes and the gemcitabine sensitivities of pancreatic cancer cells, the expression of each gene in SUIT2, MiaPaCa2 and PANC1 cells was measured by qRT-PCR. MiaPaCa2 cells with low sensitivity to gemcitabine expressed significantly lower levels of *dCK* mRNA than PANC1 and SUIT2 cells with higher sensitivities to gemcitabine (Figure 1B-a). Various levels of *hENT1* mRNA expression were detected in the three pancreatic cancer cell lines, but were not correlated

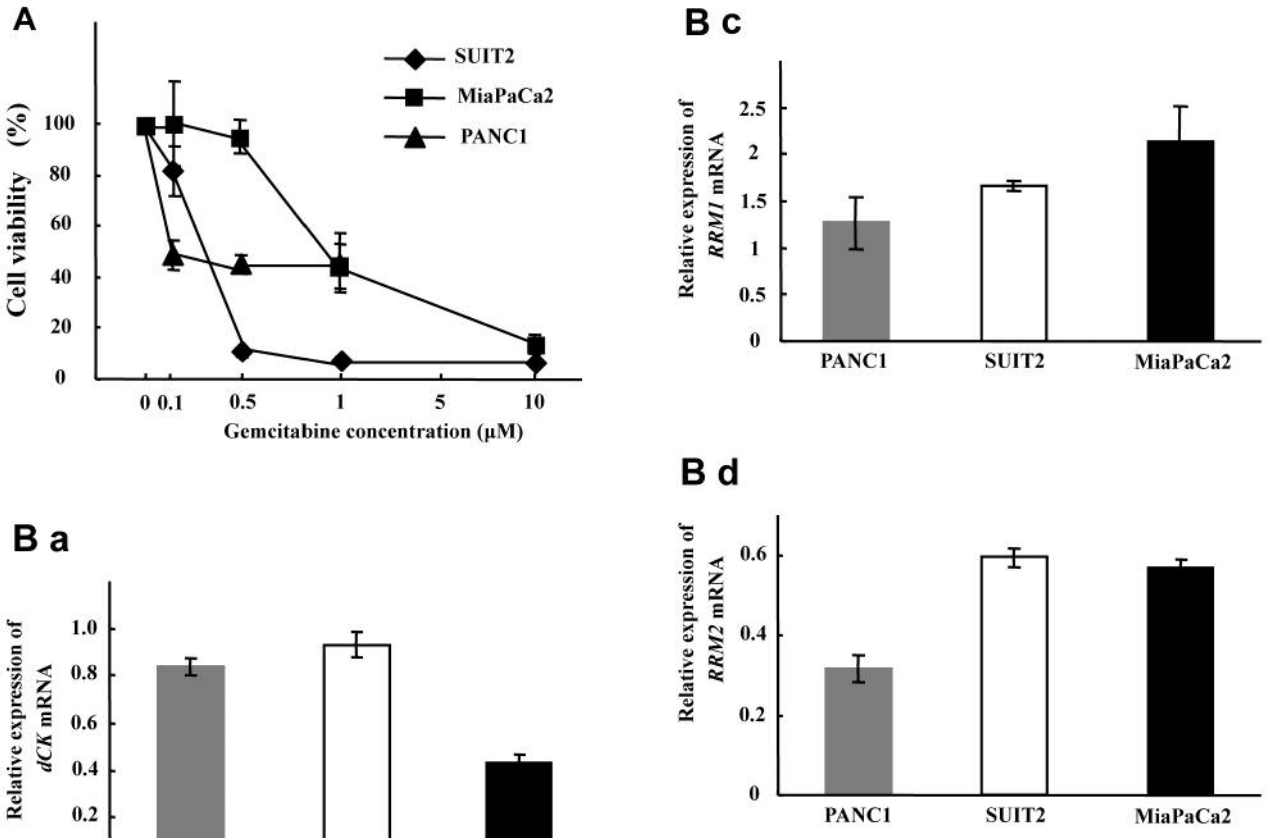

Figure 1. *Gemcitabine sensitivities and expressions of gemcitabine transporter and metabolism-related genes in pancreatic cancer cell lines. A, Gemcitabine sensitivities of pancreatic cancer cell lines. The IC_{50} of PANC1 cells was 98.3±21.9 nM, that of SUIT2 cells was 200.9±24.7 nM and that of MiaPaCa2 cells was 930.4±114.2 nM. B-a, MiaPaCa2 cells with low sensitivity to gemcitabine express significantly lower levels of dCK mRNA than PANC1 and SUIT2 cells with higher sensitivities to gemcitabine. B-b, Different levels of hENT1 mRNA expression were detected in the three pancreatic cancer cell lines, but are not correlated with their sensitivities to gemcitabine. B-c and B-d, There were no significant differences in the RRM1 and RRM2 expression levels among the three pancreatic cancer cell lines.*

with their sensitivities to gemcitabine (Figure 1B-b). No significant differences in the *RRM1* and *RRM2* expression levels were found among the three pancreatic cancer cell lines (Figure 1B-c and B-d).

Establishment of gemcitabine-resistant pancreatic cancer cells. Gemcitabine-resistant SUIT2 cells were generated by exposure to gradually increasing concentrations of gemcitabine. The final concentration of gemcitabine was 200 nM. The viability of parental SUIT2 cells significantly decreased to 68% after treatment with 100 nM gemcitabine (Figure 2A). However, the viability of gemcitabine-resistant SUIT2 cells remained unchanged after treatment with 100 or 200 nM gemcitabine. After treatment with 500 nM gemcitabine, the viabilities of the parental and gemcitabine-resistant SUIT2 cells were 20% and 43%, respectively (Figure 2A). Using these data, we calculated that the IC_{50} of the parental SUIT2 cells was 216.8±38.4 nM, while that of the gemcitabine-resistant SUIT2 cells was 433.9±20.8 nM. The difference between these two IC_{50} values was significant ($p<0.01$).

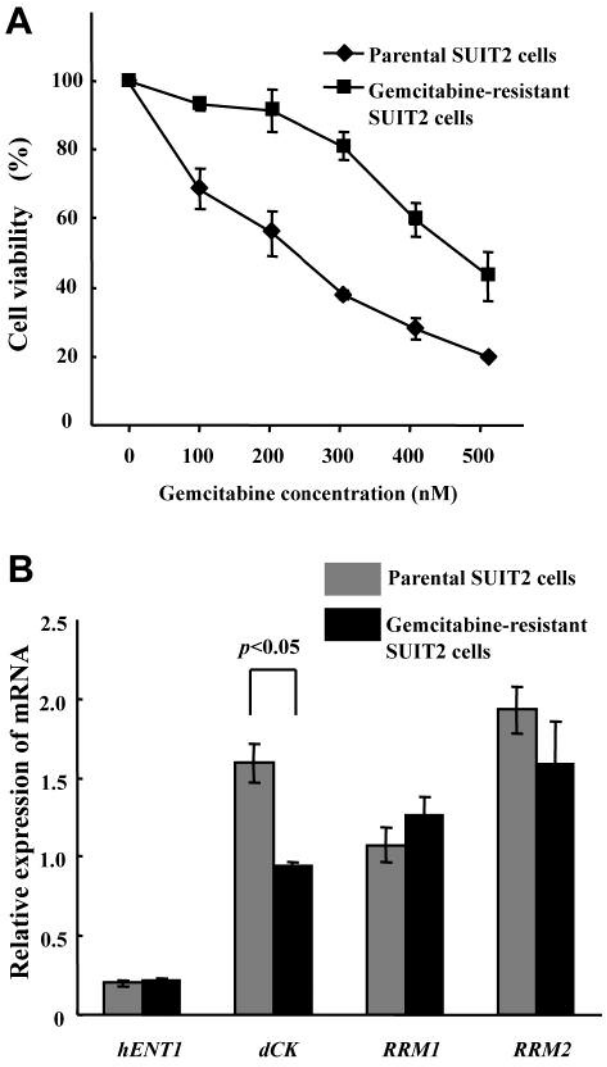

Figure 2. *Cell viabilities and expressions of gemcitabine transporter and metabolism-related genes in parental and gemcitabine-resistant SUIT2 cells. A, The changes in viability of parental and gemcitabine-resistant SUIT2 cells were evaluated by PI assays and are shown as gemcitabine dose-response curves. The IC_{50} of parental SUIT2 cells was 216.8±38.4 nM while that of gemcitabine-resistant SUIT2 cells was 433.9±20.8 nM, showing a significant difference ($p<0.01$). B, The changes in the expression levels of gemcitabine transporter and metabolism-related genes (hENT1, dCK, RRM1 and RRM2) in parental and gemcitabine-resistant SUIT2 cells are shown as the relative expression of each gene after normalization by the corresponding level of 18s rRNA. Expression of dCK significantly decreased in gemcitabine-resistant SUIT2 cells compared with parental SUIT2 cells ($p<0.05$), whereas the expression levels of hENT1, RRM1 and RRM2 remained unchanged (hENT1, p=0.23; RRM1, p=0.10; RRM2, p=0.16).*

We also tried to establish gemcitabine-resistant cells from PANC1 and MiaPaCa2 cells. However, we were unable to establish such resistant cells because we could not obtain proliferating cells after long-term culture, even with low concentrations of gemcitabine.

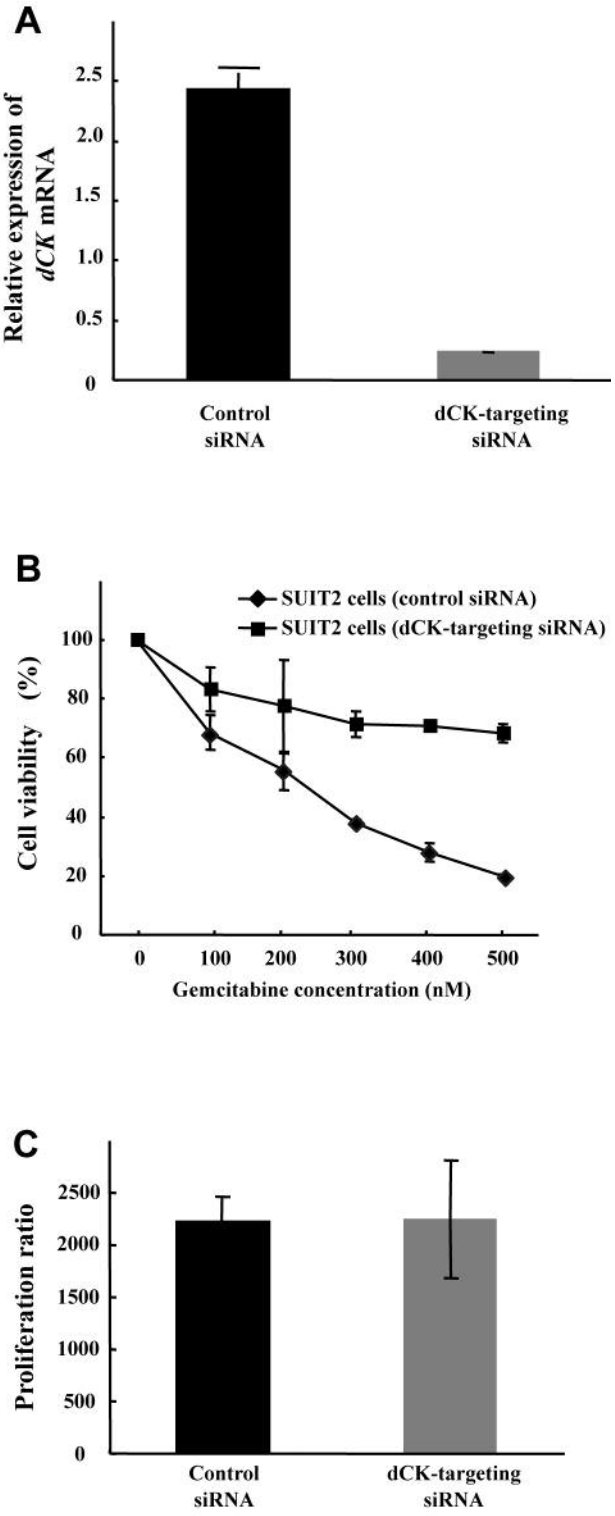

Figure 3. *Inhibition of dCK expression in SUIT2 cells by a dCK-targeting siRNA. A, Expression of dCK is suppressed to less than 10% by the dCK-targeting siRNA. B, The dCK-targeting siRNA significantly reduced the gemcitabine sensitivity of SUIT2 cells ($p<0.01$ for 300, 400 and 500 nM gemcitabine). C, The dCK-targeting siRNA did not affect the proliferation of SUIT2 cells without gemcitabine treatment (p=0.93).*

Changes in the expression levels of gemcitabine-related genes in gemcitabine-resistant cells. To examine the relationships between gemcitabine resistance and these gemcitabine-related genes, we compared their levels of expression in parental and gemcitabine-resistant SUIT2 cells. The expression levels of *hENT1, dCK, RRM1* and *RRM2* were normalized by the corresponding levels of 18S rRNA. Expression of *dCK* significantly decreased in gemcitabine-resistant SUIT2 cells compared with parental SUIT2 cells (Figure 2B; *dCK*, $p<0.05$), whereas the expression levels of *hENT1, RRM1* and *RRM2* remained unchanged (Figure 2B; *hENT1*, $p=0.23$; *RRM1*, $p=0.10$; *RRM2*, $p=0.16$).

Effect of inhibition of dCK expression on gemcitabine sensitivity in pancreatic cancer cells. Based on the estimated functional roles of the above-mentioned genes, down-regulation of *hENT1* and *dCK* or up-regulation of *RRM1* and *RRM2* would be expected to increase gemcitabine resistance. In the present study, we observed down-regulation of *dCK* in acquired resistance to gemcitabine. Therefore, to investigate the functional role of *dCK* in pancreatic cancer cells with respect to gemcitabine sensitivity, we investigated the effects of a dCK-targeting siRNA on cell proliferation and gemcitabine sensitivity. To confirm that the siRNA inhibited *dCK* expression, SUIT2 cells were harvested at 24 h after transfection of the siRNA and analyzed for their expression of *dCK* mRNA by qRT-PCR. Expression of *dCK* mRNA was suppressed to less than 10% by the dCK-targeting siRNA (Figure 3A).

The dCK-targeting siRNA significantly reduced gemcitabine sensitivity (Figure 3B; $p<0.01$ for 300, 400 and 500 nM gemcitabine). When cells were cultured without gemcitabine treatment, the dCK-targeting siRNA did not affect their proliferation (Figure 3C). These data suggest that dCK is a gemcitabine treatment-specific enhancer that only has an inhibitory effect on cell proliferation in the presence of gemcitabine.

Effects of inhibition of RRM1, RRM2 and hENT expressions on gemcitabine sensitivity. Several researchers have reported that other gemcitabine-related genes, such as *RRM1*, *RRM2* and *hENT*, also affect cellular sensitivity to gemcitabine in several cancers (6, 7, 17). Therefore, we investigated the effects of *RRM1*-, *RRM2*- and *hENT1*-targeting siRNAs on the sensitivity of pancreatic cancer cells to gemcitabine. The mRNA expression levels of *RRM1* (Figure 4A), *RRM2* (Figure 5A) and *hENT1* (Figure 6A) were suppressed to 65%, 33% and 16% by their specific siRNAs, respectively. Inhibition of *RRM1* increased cellular sensitivity to gemcitabine (Figure 4B; $p=0.01$ for 100 nM gemcitabine) and decreased cell proliferation by 40%, even without gemcitabine treatment (Figure 4C). Similarly, inhibition of *RRM2* increased cellular sensitivity to gemcitabine (Figure 5B; $p=0.01$ for 100 and 200 nM gemcitabine) and decreased

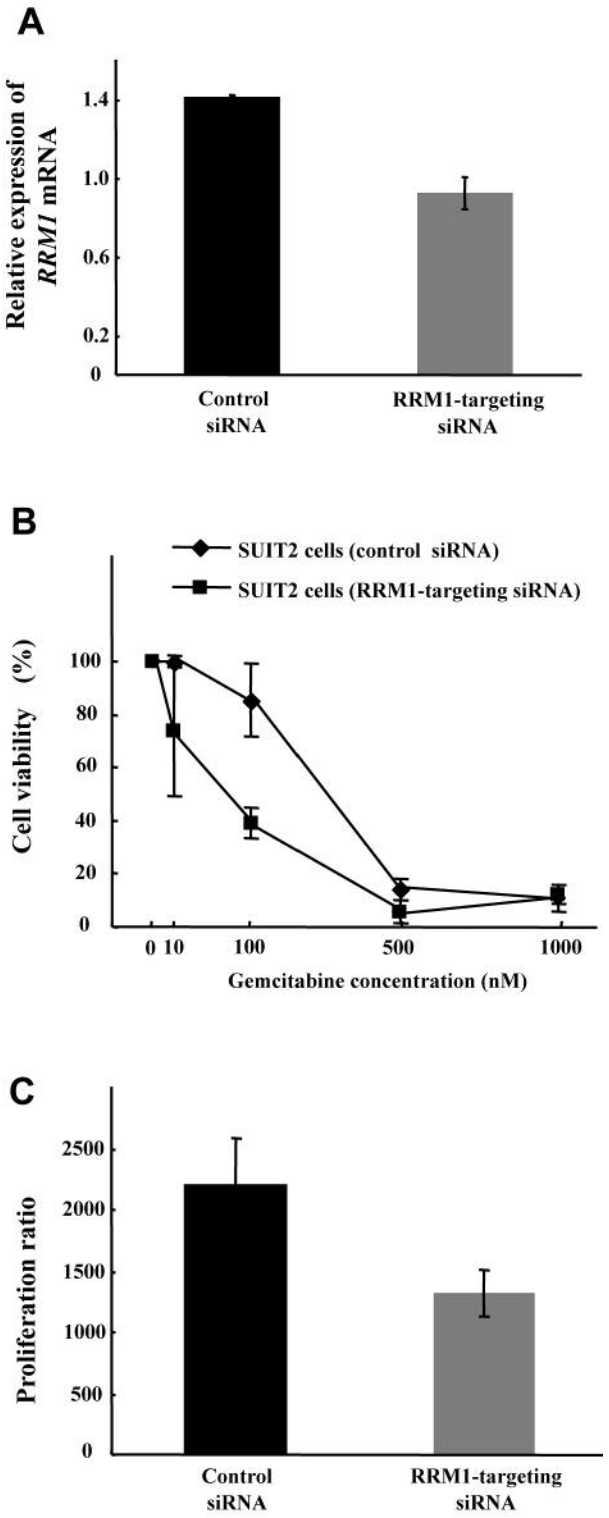

Figure 4. *Inhibition of RRM1 expression in SUIT2 cells by an RRM1-targeting siRNA. A, Expression of RRM1 was suppressed to 65% by the RRM1-targeting siRNA. B, The RRM1-targeting siRNA increase the gemcitabine sensitivity of SUIT2 cells (p=0.01 for 100 nM gemcitabine). C, The RRM1-targeting siRNA reduced the proliferation of SUIT2 cells by 40%, even without gemcitabine treatment (p<0.05).*

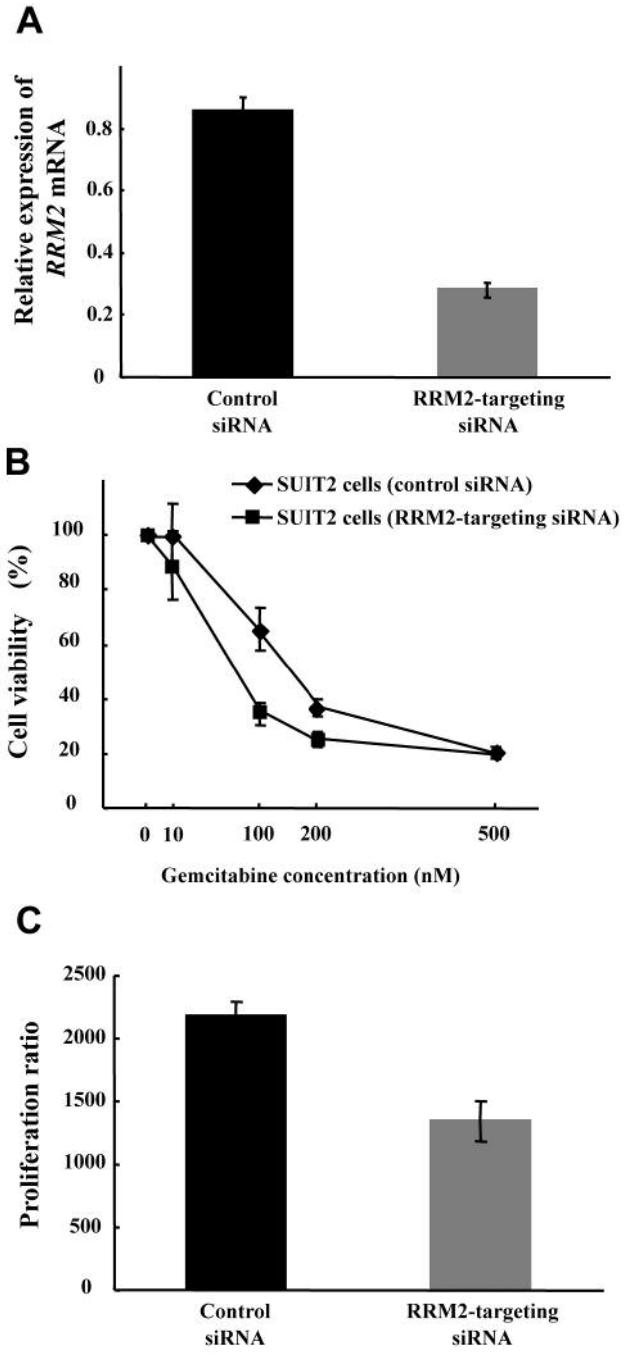

Figure 5. *Inhibition of RRM2 expression in SUIT2 cells by an RRM2-targeting siRNA. A, Expression of RRM2 was suppressed to 33% by the RRM2-targeting siRNA. B, The RRM2-targeting siRNA increase the gemcitabine sensitivity of SUIT2 cells ($p<0.01$ for 100 and 200 nM gemcitabine). C, The RRM2-targeting siRNA reduced the proliferation of SUIT2 cells by 39%, even without gemcitabine treatment ($p<0.01$).*

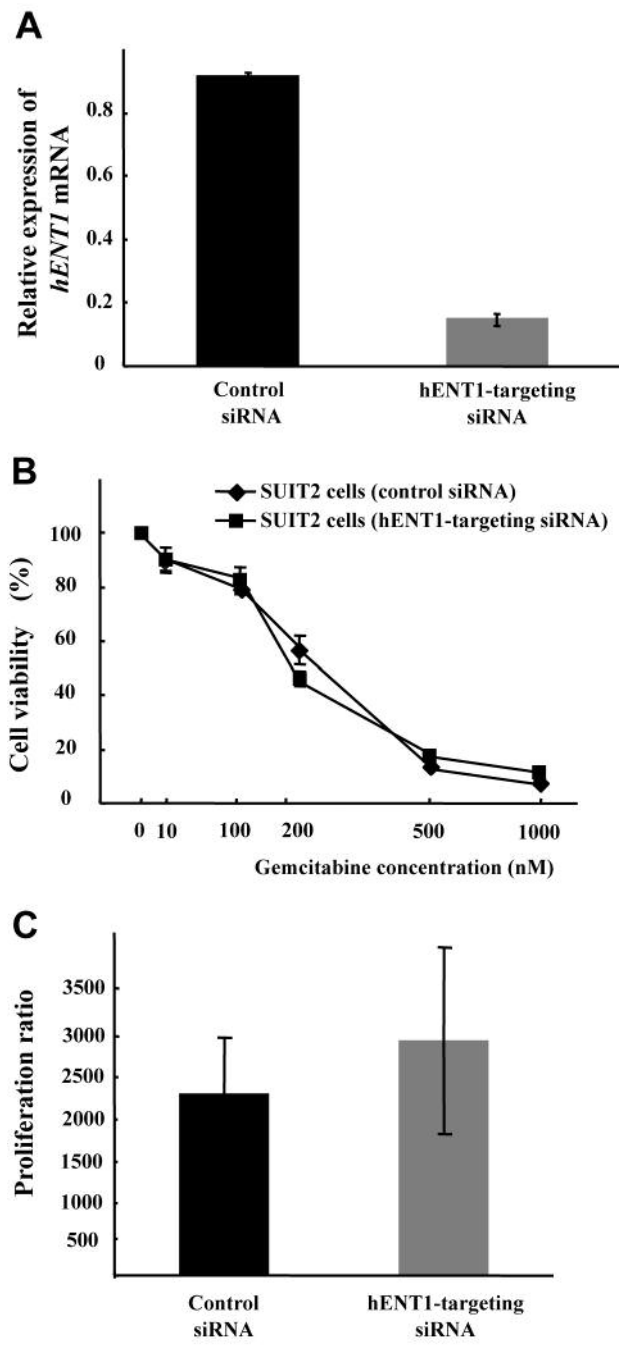

Figure 6. *Inhibition of hENT1 expression in SUIT2 cells by a hENT1-targeting siRNA. A, Expression of hENT1 was suppressed to 85% by the hENT1-targeting siRNA. B, The hENT1-targeting siRNA did not change the gemcitabine sensitivity of SUIT2 cells ($p>0.05$ for 10, 100 and 200 nM gemcitabine). C, The hENT1-targeting siRNA did not affect the proliferation of SUIT2 cells without gemcitabine treatment ($p=0.45$).*

cell proliferation by 39%, even without gemcitabine treatment (Figure 5C). In contrast, inhibition of *hENT1* did not alter the cellular sensitivity to gemcitabine (Figure 6B; $p>0.05$ for 10, 100 and 200 nM gemcitabine) or cell proliferation (Figure 6C; $p=0.45$). These data suggest that *RRM1* and *RRM2* affect cell proliferation in a manner unrelated to gemcitabine treatment and that *hENT1* does not influence cell proliferation or sensitivity to gemcitabine.

Discussion

The present study represents the first report of a functional analysis of *dCK* in pancreatic cancer. We found a significant decrease in *dCK* expression in newly established gemcitabine-resistant pancreatic cancer cells, whereas the expression levels of *hENT1, RRM1* and *RRM2* remained unchanged. We further found that inhibition of *dCK* expression decreased gemcitabine sensitivity without influencing cell viability in the absence of gemcitabine. Inhibition of *RRM1* or *RRM2* increased cellular sensitivity to gemcitabine and decreased cell proliferation even without gemcitabine treatment. In contrast, inhibition of *hENT1* did not alter the cellular sensitivity to gemcitabine or cell proliferation. These data suggest that the combination of a gene therapy targeting *dCK* and gemcitabine would reinforce the specific effects of gemcitabine on gemcitabine-resistant cells, while *RRM1* and *RRM2* affect cell proliferation in a manner unrelated to gemcitabine treatment and *hENT1* does not influence cell proliferation or sensitivity to gemcitabine.

It has been reported that overexpression of *dCK* increased gemcitabine sensitivity in colon carcinoma, breast carcinoma and small cell lung adenocarcinoma cells (9) and that *dCK* expression decreased in gemcitabine-resistant ovarian cancer cells (18). Furthermore, *in vivo* experiments revealed that *dCK* gene transfer enhanced the cytotoxic effects of gemcitabine (8). These results are consistent with the present results regarding pancreatic cancer. Taken together, these data suggest that *dCK* plays an important role in resistance to gemcitabine in several types of cancer, including pancreatic cancer.

Previously, the level of *hENT1* expression was found to be correlated with survival in pancreatic cancer patients treated with gemcitabine (11, 19). Moreover, *hENT1* was reported to be a predictive marker for the efficacy of gemcitabine therapy in pancreatic cancer patients (20). These data were obtained on the basis of expression analyses. However, in the present functional analyses, we found no change in *hENT1* expression in gemcitabine-resistant pancreatic cancer cells, and no change in cellular gemcitabine sensitivity after inhibition of *hENT1* expression. The reason for this inconsistency between the studies remains to be clarified. Previously, reduced expression of hENT1 protein was found in human lymphoid cells showing resistance to AraC, a nucleotide analogue, although the *hENT1* mRNA level remained unchanged (21). Therefore, it is possible that the level of *hENT1* mRNA expression is not directly correlated with the functional level of hENT1. To investigate the relationship between the expression and function of hENT1, further studies are required.

Positive roles for *RRM1* and *RRM2* in gemcitabine resistance were previously reported for pancreatic cancer (6, 7, 14). However, in the present study, we found no significant differences in the expression levels of *RRM1* and *RRM2* between parental and gemcitabine-resistant SUIT2 cells, although the cellular sensitivities to gemcitabine were increased after inhibition of *RRM1* or *RRM2* expression. We further found that inhibition of *RRM1* or *RRM2* strongly suppressed cell proliferation even without gemcitabine treatment. These data suggest that *RRM1* and *RRM2* are not specific enhancers of gemcitabine treatment.

In conclusion, the present data revealed that down-regulation of *dCK* expression is strongly correlated with acquired resistance to gemcitabine in pancreatic cancer. These results suggest that *dCK* is a promising specific enhancer to increase the sensitivity of pancreatic cancer to gemcitabine without any non-specific cytotoxic effects.

Acknowledgements

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