difficult to eradicate pancreatic tumors with adenovirus-mediated gene therapy alone (19, 20). There is also concern that the dose of adenovirus necessary to achieve therapeutic effectiveness may have some significant toxicity. Therefore, adenovirus alone may not be an effective treatment for cancer, and it may be necessary to combine adenovirus-mediated gene therapies with conventional treatments to maximize the antitumor effects for pancreatic cancer.

Recently, the combination of radiotherapy and adenovirus-mediated gene therapy has been reported to be effective for cancer treatment. Shi et al. (21) reported that adenovirus-mediated gene therapy targeting endostatin enhanced the antitumor effect of radiation therapy in colorectal cancer. Similarly, Geoerger et al. (22), Portella et al. (23), and Rogulski et al. (24) reported that ONYX-015, an E1B-55-kDa gene–deleted adenovirus that replicates selectively in and lyses tumor cells with abnormalities in p53 function, combined with radiation therapy is a promising strategy for treatment of gliomas and thyroid cancers and that there are synergistic effects with such combination therapies. Previously, we reported that gene therapy with an adenovirus vector expressing NK4 (Ad-NK4), which acts as an HGF antagonist, could be a viable option for treatment of pancreatic cancer (25–27). More recently, we reported that radiation therapy enhances the invasiveness of pancreatic cancer cells via the activation of the HGF receptor c-Met and that NK4 inhibits this radiation-enhanced invasiveness (28, 29), suggesting that a combination of radiation therapy and NK4 gene therapy may be a viable strategy for treatment of pancreatic cancer. However, the effects of radiation on features of adenovirus-mediated gene therapies, such as adenovirus uptake and efficiency of target gene expression, have remained unknown.

In the present study, to investigate the effect of radiation on the efficiency of transfer and expression of a target gene, we examined the effect of radiation on NK4 expression by an adenovirus-based vector (Ad-NK4) as a representative gene therapy. We found that radiation increased expression of NK4 via enhanced activation of the cytomegalovirus (CMV) promoter, which is commonly used as the target gene promoter in adenovirus vectors, and we also found that radiation enhanced

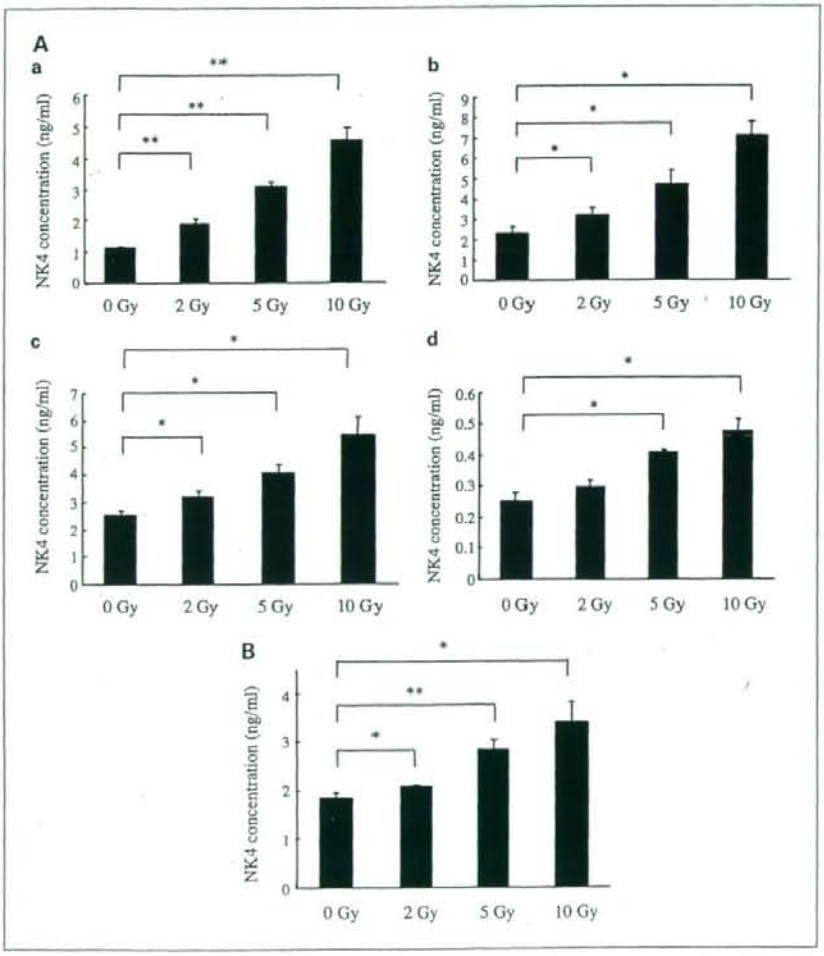

Fig. 1. Radiation significantly increases NK4 expression in Ad-NK4–infected cells. *A*, pancreatic cancer cells were irradiated and infected with Ad-NK4 at MOI of 10 at 24 h after radiation. NK4 levels in culture media were measured by ELISA on postinfection day 2. *a*, SUIT-2; *b*, KP-1N; *c*, PANC-1; and *d*, AsPC-1. Columns, mean of three independent samples; bars, SD. **, $P < 0.01$; *, $P < 0.05$. *B*, proteins were isolated from irradiated SUIT-2 cells infected with Ad-NK4 as described above on postinfection day 2, and NK4 concentrations were determined by ELISA.

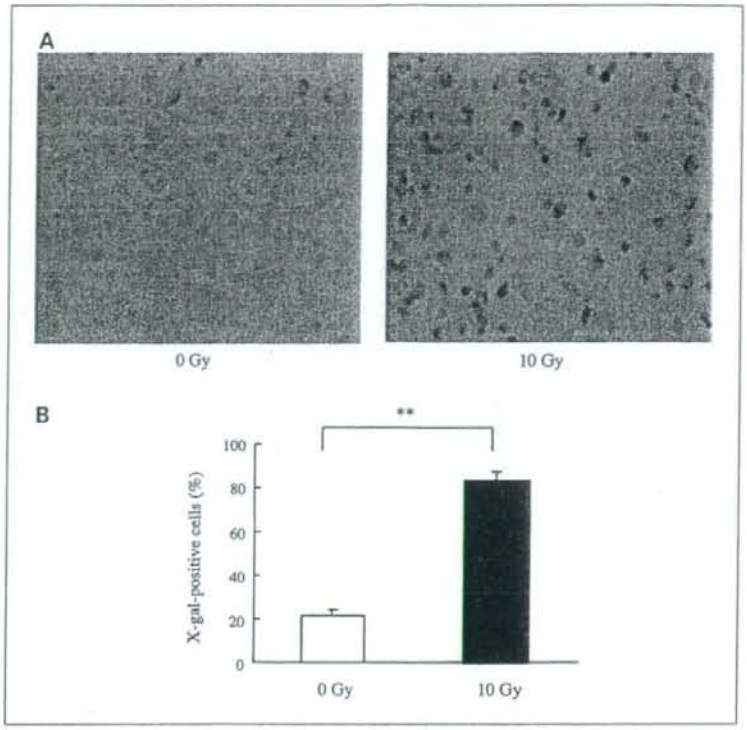

Fig. 2. Radiation significantly increases β-galactosidase expression by Ad-lacZ – infected cells. SUIT-2 cells were irradiated with 10 Gy and then infected with Ad-lacZ at MOI of 10 at 24 h after radiation. β-Galactosidase activity was assessed with X-gal staining at 48 h after infection. *A*, photomicrographs of X-gal – stained nonirradiated or irradiated cultures; magnification, 100×. *B*, percentage of β-galactosidase – positive nonirradiated or irradiated cells. Columns, mean of five independent fields; bars, SD; **, $P < 0.01$.

uptake of the adenovirus vector. The present data also suggest that activation of p38 mitogen-activated protein kinase (MAPK) and up-regulation of dynamin 2 may be involved in the radiation-enhanced activation of CMV promoter and adenovirus uptake, respectively.

Materials and Methods

Cells and reagents. Human pancreatic cancer cell lines SUIT-2, AsPC-1, PANC-1, and KP-1N were generously donated by Dr. H. Iguchi (National Shikoku Cancer Center) and cultured in DMEM supplemented with streptomycin, penicillin, and 10% fetal bovine serum at 37°C in 5% CO_2. The p38 inhibitor SB203580 was purchased from Calbiochem. Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells expressing human HGF cDNA (30, 31).

Construction of recombinant adenovirus. A recombinant Ad-NK4 was constructed as described previously (32). In brief, Ad-NK4 was generated by homologous recombination of the pJM17 plasmid (33) and the shuttle plasmid vector pSV2+ (34) containing an expression cassette and the CMV early promoter/enhancer followed by human *NK4* cDNA (35) and a polyadenylation signal. A control vector expressing the bacterial β-galactosidase gene (*lacZ*) was constructed by the same procedure with pJM17 and pCA17, which contains the *lacZ* gene. Recombinant Ad-NK4 and Ad-lacZ were propagated in HEK293 cells.

Radiation treatment. Cells were irradiated with a dose of 2, 5, or 10 Gy at room temperature with a ^{137}Cs source (Gamma Cell 40; Atomic Energy of Canada, Ltd.) with a delivery rate of 1.0 Gy/min.

Adenovirus infection of cells. Cells (5×10^4) were seeded in six-well plates and cultured in DMEM supplemented with 10% fetal bovine serum for 24 h. Cells were treated with or without radiation and then infected with Ad-NK4 or Ad-lacZ at multiplicities of infection (MOI) of 10 or MOI of 50 at 24 h after radiation treatment. The culture medium was replaced with fresh medium 1.5 h after transfection.

Extraction of proteins from cells infected with Ad-NK4. SUIT-2 cells were irradiated and infected with Ad-NK4, as described above. Two days after infection with Ad-NK4, the cells were lysed in 500 μL ice-cold lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L EDTA, 5 μg/mL leupeptin, 1 mmol/L phenylmethyl sulfonyl fluoride, and 0.5% (v/v) Triton X-100]. Cell debris was removed by centrifugation at 14,000 × *g* for 20 min at 4°C, supernatants were collected, and the protein concentrations were measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) at absorbances of 280 nm and adjusted to 2.0 mg/mL with lysis buffer.

Electroporation. pcDNA3-NK4 (2.5 μg; NK4-expressing plasmid) or pcDNA3 (2.5 μg; empty vector) was mixed with 5×10^6 SUIT-2 cells and electroporated with a Nucleofector (Amaxa Biosystems GmbH) according to the manufacturer's instructions. The cells were immediately transferred to complete medium prewarmed to 37°C and allowed to recover for 24 h.

NK4 expression by Ad-NK4 – infected or NK4-expressing plasmid – transfected cancer cells. After infection of SUIT-2, AsPC-1, PANC-1, and KP-1N cells with Ad-NK4 or transfection of SUIT-2 with NK4-expressing plasmid, the medium was changed every 24 h. Conditioned media were collected on posttransduction days 1, 2, 3, and 4. The NK4 concentration in the media was measured by ELISA with a human HGF ELISA kit (Immunis HGF EIA, Institute of Immunology) according to the manufacturer's protocol.

Invasion assay. Invasiveness of pancreatic cancer cells was quantified as the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson) as described previously (36). In brief, transwell inserts with 8-μm pores were coated with Matrigel (20 μg/well;

Becton Dickinson). SUIT-2 cells (1×10^7) were untreated or irradiated with 10 Gy and then allowed to recover for 24 h. These cells were infected with Ad-lacZ or Ad-NK4 at MOI of 50, and culture media were collected on postinfection day 3. New untreated SUIT-2 cells were seeded in 24-well plates at a density of $1 \times 10^5/cm^2$ in the upper chamber in 250 μL of DMEM supplemented with 10% fetal bovine serum and cultured with 750 μL of conditioned media from the irradiated or untreated SUIT-2 cells infected with Ad-lacZ or Ad-NK4. After 24 h of incubation in the presence of 3 ng/mL HGF, cells that had invaded to the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with H&E, and counted in five randomly selected fields under a light microscope.

Western blotting of phosphorylated p38 MAPK. Untreated or irradiated SUIT-2 cells were lysed in ice-cold lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L DTT, 1 mmol/L orthovanadate, 1 mmol/L phenylmethyl sulfonylfluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin (pH 7.4)]. The lysates were boiled for 10 min, and the proteins were fractionated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated overnight at 4°C with rabbit polyclonal antiphosphorylated p38 MAPK antibody (1:1,000; Cell Signaling Technology) and then probed with antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology).

Assessment of transgene distribution by evaluation of β-galactosidase expression. At 48 h after adenovirus infection, SUIT-2 cells were rinsed twice with PBS and fixed with 0.25% glutaraldehyde in PBS for 15 min at 4°C. β-Galactosidase activity was detected by immersing cells into 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) staining solution (5 mmol/L K_4FeCN, 5 mmol/L K_3FeCN, and 2 mmol/L $MgCl_2$ containing 1 mg/mL X-gal) for 6 h at 37°C.

Real-time PCR and reverse transcription–PCR assays. Ad-lacZ DNA content of infected cells was determined by real-time PCR analysis as described previously (37) with primers for the β-galactosidase gene (5′-CACGGCAGATACACTTGCTG-3′ and 3′-ATCGCCATTTGACCACTACC-5′; ref. 38). The number of copies of viral DNA was calculated from a standard curve of purified adenovirus vector (CMV-β-galactosidase) and was further adjusted to the protein concentration of each lysate. *Dynamin 2* mRNA levels were quantified with 100 ng of total RNA by real-time reverse transcription-PCR assay with a QuantiTect SYBR Green reverse transcription-PCR kit (Qiagen) and primers specific for *dynamin 2* (5′-AGGAGTACTGGTTTGTGCTGACTG-3′ and 3′-GTGCATGATGGTCTTTGGCATGAG-5′; ref. 38). Levels of *dynamin 2* mRNA were normalized to those of *18S* rRNA amplified with specific primers (5′-GTAACCCGTTGAACCCCATT and 3′-GCGATGATGGCTAACCTACC; ref. 39) and expressed as a ratio compared with nonirradiated controls.

Inhibition of dynamin 2 in cells by RNA interference. SUIT-2 cells were transfected with *dynamin 2*–specific short interfering RNA (siRNA; Dharmacon) or control siRNA provided by Qiagen with a Nucleofector (Amaxa) and plated at 1×10^6 cells per well in six-well plates. At 24 h after transfection, cells were irradiated or left untreated. At 48 h after transfection, the cells were infected with Ad-lacZ at MOI of 10, as described above. Viral gene uptake in *dynamin 2*–specific siRNA-transfected cells is expressed as a ratio compared with that in control siRNA-transfected cells.

Evaluation of radiation-induced expression of NK4 in vivo in xenografts in nude mice. Six-week-old female nude mice (BALB/c nu/nu) were obtained from Japan SLC. To investigate the radiation-induced therapeutic effect of Ad-NK4 *in vivo*, 10 subcutaneous tumors were preestablished in five nude mice by injection of 5×10^6 SUIT-2 cells into both flanks. Seven days later, six tumors in three mice were irradiated with 10 Gy and four tumors in two mice were untreated, and at 24 h after radiation, 5×10^7 plaque-forming units of Ad-NK4 (100 μL) were injected into the tumors with a 26-gauge needle. Tumors were irradiated locally with animals restrained in a custom lead block. To examine expression of NK4 protein in subcutaneous tumors, mice were killed 48 h after administration of Ad-NK4 and tumors were excised. The samples were immediately washed once in PBS and homogenized with (300 μL/tumor) protein lysis buffer [50 mmol/L NaCl, 30 mmol/L sodium PPi, 50 mmol/L NaF, 5 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride,

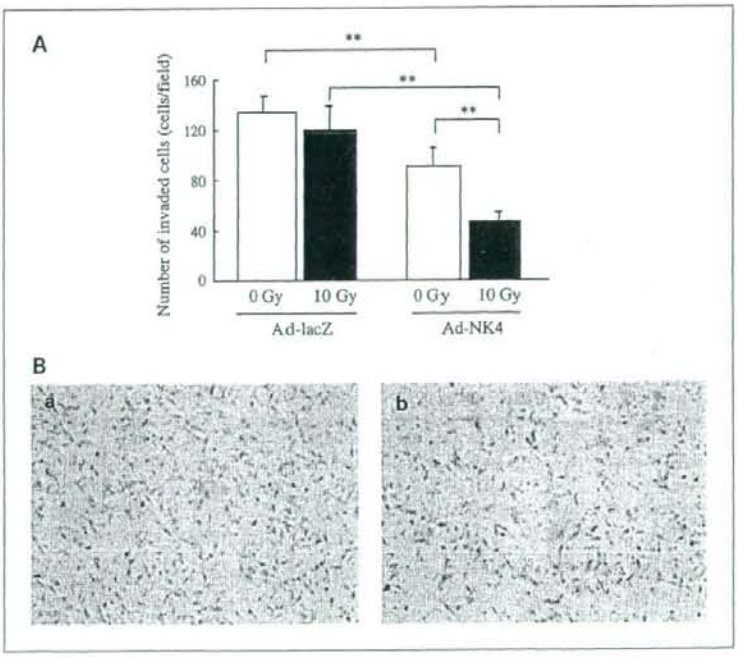

Fig. 3. Radiation significantly enhances Ad-NK4–mediated inhibition of HGF-induced invasion. SUIT-2 cells (1×10^7) were nonirradiated or irradiated with 10 Gy and then allowed to recover for 24 h. Cells were then infected with Ad-lacZ or Ad-NK4 at MOI of 50, and culture media were collected on postinfection day 3. Untreated new SUIT-2 cells were seeded in the upper chambers of 24-well plates and then exposed to one of the four different conditioned media in the presence of 3 ng/mL HGF for 24 h. *A*, number of cells that invaded to the lower surface of the Matrigel-coated membrane. Columns, mean of five randomly selected fields; bars, SD. **, $P < 0.01$. *B, a*, photomicrographs of *in vitro* invasion assay with SUIT-2 cells cultured with conditioned media from SUIT-2 cells infected with Ad-NK4 at MOI of 50 without radiation. *b*, photomicrographs of SUIT-2 cells cultured with conditioned media from SUIT-2 cells infected with Ad-NK4 after radiation treatment (10 Gy). H&E stain. Magnification, 100×.

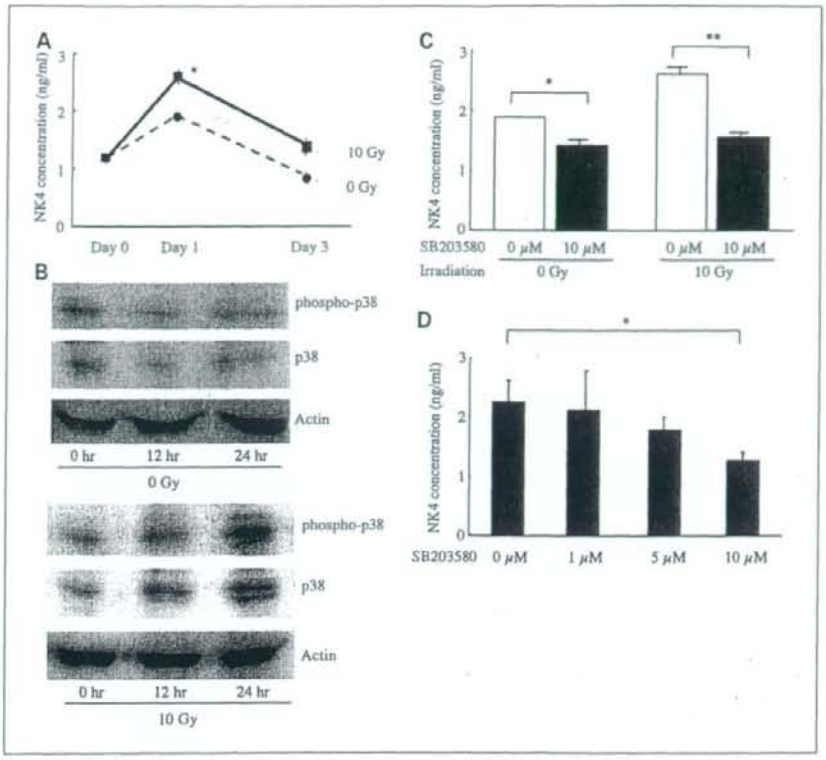

Fig. 4. Radiation enhances CMV promoter activity via p38 MAPK activation. *A*, NK4 expression by cells transfected with NK4-expressing plasmid with or without radiation. SUIT-2 cells were transfected with NK4-expressing plasmid and then irradiated with 10 Gy at 24 h after transfection. NK4 concentrations in culture media were measured on postradiation days 0, 1, and 3. Points, mean of three independent samples; bars, SD. *, $P < 0.05$. *B*, response of phosphorylated p38 to radiation (10 Gy). SUIT-2 cells were nonirradiated or irradiated with 10 Gy, and proteins were isolated at 12 and 24 h after radiation and subjected to Western blot analysis with antibodies that specifically recognize activated phosphorylated p38 or the indicated proteins. *C*, effect of p38 MAPK inhibitor SB203580 on NK4 expression by NK4-expressing plasmid – transfected cells with or without radiation. SUIT-2 cells transfected with NK4-expressing plasmid were incubated in the presence or absence of p38 inhibitor (10 μmol/L) and irradiated with 10 Gy or nonirradiated at 24 h after transfection. NK4 concentrations in culture media were measured on postirradiation day 1. The culture medium and p38 inhibitor were replaced every 24 h. Columns, mean of three independent samples; bars, SD. **, $P < 0.01$; *, $P < 0.05$. *D*, SUIT-2 cells transfected with NK4-expressing plasmid were incubated in the presence or absence of p38 inhibitor SB203580 (0, 1, 5, or 10 μmol/L) and irradiated with 10 Gy. NK4 concentrations in culture media were measured on postirradiation day 1. The medium and p38 inhibitor were replaced every 24 h.

and 0.1% bovine albumin], and protein concentrations were measured as described above and adjusted to 10.0 mg/mL with lysis buffer. The NK4 concentration in the extract was analyzed by ELISA (Immunis HGF EIA).

Statistical analysis. Values are expressed as mean ± SD. Comparisons between all groups were analyzed by one-way ANOVA and Student's *t* test for comparison between the two groups. The level of statistical significance was set at $P < 0.01$ or $P < 0.05$. To confirm the induction results, experiments were repeated at least thrice.

Results

Effect of radiation on expression of target genes delivered by adenoviral vector. To investigate the effect of radiation on the expression of a target gene delivered with an adenoviral vector, we measured expression of NK4 in culture media of pancreatic cancer cells infected with Ad-NK4 with or without radiation. SUIT-2, KP-1N, PANC-1, and AsPC-1 cells (2×10^5 each cell line) were irradiated with 2, 5, or 10 Gy and allowed to recover for 24 hours. Cells were then infected with Ad-NK4 at MOI of 10. Culture media were collected on postinfection days 1, 2, and 3. NK4 expression by Ad-NK4–infected cells peaked on day 2 after transfection (data not shown). As shown in Fig. 1A, radiation significantly increased NK4 expression in all cell lines in a dose-dependent manner (SUIT-2, $P < 0.01$; KP-N1, PANC-1, and AsPC-1, $P < 0.05$). NK4 expression was not detected in cells that were not infected with Ad-NK4 (data not shown). To investigate intracellular NK4 protein levels, we extracted proteins from irradiated SUIT-2 cells infected with Ad-NK4, as described above, and measured levels of NK4. As shown in Fig. 1B, radiation also significantly increased intracellular NK4 protein levels in a dose-dependent manner. These data suggest that radiation enhances expression of a target gene delivered by adenovirus vector.

Effect of radiation on β-galactosidase expression by Ad-lacZ–infected cells. To investigate the effect of radiation on the expression of another gene delivered with an adenoviral vector, we used Ad-lacZ instead of Ad-NK4 and examined expression of β-galactosidase by transfected cells. SUIT-2 cells (2×10^5) were irradiated with 10 Gy, allowed to recover for 24 hours, and then infected with Ad-lacZ at MOI of 10. At 48 hours after infection, cells were stained for β-galactosidase. As shown in Fig. 2A, a large number of irradiated cells showed the characteristic blue staining indicative of β-galactosidase activity, but only a small number of nonirradiated cells were positive for β-galactosidase. The numbers of β-galactosidase–positive cells in five independent fields were counted, and the percentage of β-galactosidase–positive irradiated cells was significantly larger than that of nonirradiated cells ($P < 0.01$; Fig. 2B). These data are consistent with those of our Ad-NK4 experiments.

Effect of radiation on Ad-NK4–mediated inhibition of HGF-induced invasion of pancreatic cancer cells. We previously reported that NK4 inhibits HGF-induced invasion of pancreatic cancer cells (25, 26). In the present study, we tested the effect of radiation on Ad-NK4–induced inhibition of invasion of pancreatic cancer cells. SUIT-2 cells (1×10^7) were treated with

or without 10 Gy and infected with Ad-lacZ or Ad-NK4 at MOI of 50 at 24 hours after radiation. The culture media were collected on postinfection day 3. We used an *in vitro* invasion assay to examine inhibition of HGF (3 ng/mL)-induced invasiveness of nonirradiated pancreatic cancer cells cultured with each of the four different conditioned media described above (0 Gy with Ad-lacZ, 10 Gy with Ad-lacZ, 0 Gy with Ad-NK4, or 10 Gy with Ad-NK4). The number of invading cells cultured in conditioned media from SUIT-2 cells infected with Ad-NK4 was less than that of cells infected with Ad-lacZ ($P < 0.01$). These data are consistent with those of previous reports (26). We also found that conditioned medium of SUIT-2 cells irradiated with 10 Gy before infection with Ad-NK4 significantly inhibited invasiveness of pancreatic cancer cells compared with that of nonirradiated cells ($P < 0.01$; Fig. 3A and B). These data suggest that radiation-enhanced adenovirus gene transfer improved efficiency of NK4-induced inhibition of invasion for treatment of pancreatic cancer.

Effect of radiation on NK4 expression by pancreatic cancer cells transfected with NK4-expressing plasmid. Ad-NK4 and Ad-lacZ use the CMV promoter to drive expression of the target gene. To evaluate the effect of radiation on the CMV promoter, we transfected SUIT-2 cells with a plasmid that expresses NK4 under the control of the CMV promoter before radiation treatment and measured NK4 expression after radiation. SUIT-2 cells (5×10^6) were transfected with NK4-expressing plasmid or empty vector. Cells were incubated for 24 hours and then irradiated with 10 Gy. Culture media were collected on post-radiation days 0, 1, and 3. NK4 expression in NK4-expressing vector-transfected cells is shown in Fig. 4A. Irradiated cells expressed significantly higher levels of NK4 than did nonirradiated cells ($P < 0.05$). NK4 expression was not detected in cells transfected with empty vector (data not shown). These data suggest that radiation increases the activity of the CMV promoter.

We next tested the effect of radiation on p38 MAPK and interaction between p38 MAPK and the CMV promoter. It has been reported that activation of p38 MAPK increases expression of transgenes driven by the CMV promoter (40, 41). Therefore, we tested whether radiation activates p38 MAPK and found that radiation increased expression of phosphorylated p38 MAPK (Fig. 4B). We also investigated the role of p38 MAPK in activation of the CMV promoter using the p38 inhibitor SB203580. SUIT-2 cells transfected with NK4-expressing vector were treated with or without SB203580 (10 μmol/L). Cells were then irradiated with 10 Gy, and the medium was replaced 24 hours after transfection. Culture media were collected on postradiation day 1. As shown in Fig. 4C, SB203580 reduced NK4 expression significantly in both irradiated cells and nonirradiated cells (irradiated cells, $P < 0.01$; nonirradiated cells, $P < 0.05$). NK4 expression in SUIT-2 cells treated with p38 inhibitor (0, 1, 5, or 10 μmol/L SB203580) on postradiation day 1 is shown in Fig. 4D. p38 inhibitor reduced radiation-enhanced NK4 expression in a dose-dependent manner. These data suggest that phosphorylation of p38 MAPK is involved in radiation-induced activation of the CMV promoter.

It is also possible that radiation activates the CMV promoter via activation of nuclear factor-κB. It has been reported that radiation activates nuclear factor-κB (42) and that the nuclear factor-κB pathway increases CMV promoter activity (43). Therefore, we examined the effect of radiation on activation of nuclear factor-κB in SUIT-2 cells and found that activation of nuclear factor-κB was not affected by radiation in the pancreatic cancer cell lines examined here (data not shown).

Effect of radiation on adenoviral gene uptake. We next investigated the effect of radiation on adenoviral gene uptake by pancreatic cancer cells. SUIT-2 cells were irradiated with 2, 5, or 10 Gy, allowed to recover for 24 hours, and then infected with Ad-lacZ at MOI of 10. At 24 hours after infection, the viral DNA content was quantified by real-time PCR. As shown in Fig. 5, the viral DNA content of cells at 24 hours after radiation was significantly higher than that of nonirradiated cells ($P < 0.01$). These data suggest that radiation increases viral gene uptake in a dose-dependent manner.

Effect of radiation on viral infection and expression of dynamin 2. Endocytosis of adenovirus mediated by clathrin-coated vesicles (44, 45) requires the action of the large GTPase dynamin as a constrictase (46). It was recently reported that radiation induces adenovirus infection via dynamin 2 in colon cancer, brain cancer, and breast cancer (37, 38). To investigate the effect of radiation on expression of dynamin 2 by pancreatic cancer cells, we quantified *dynamin 2* mRNA levels in SUIT-2 cells by real-time reverse transcription-PCR. We found that *dynamin 2* mRNA expression was significantly higher in irradiated cells ($P < 0.05$ at 12 hours after radiation) than in nonirradiated cells (Fig. 6A). We next used siRNA to inhibit dynamin 2 expression to determine whether dynamin 2 affects radiation-induced viral infection. We transfected SUIT-2 cells with a *dynamin 2*-specific siRNA or control siRNA and confirmed that dynamin 2 expression was significantly lower in cells transfected with the specific siRNA than in those transfected with control siRNA (Fig. 6B). Radiation enhanced Ad-lacZ uptake in the control siRNA-treated cells as much as 6.3 ± 0.8-fold, whereas Ad-lacZ uptake was significantly impaired in cells transfected with siRNA targeting *dynamin 2* ($P < 0.05$; Fig. 6C). These data suggest that increased expression of dynamin 2 is involved in radiation-induced increase of adenovirus uptake. We also examined expression of *clathrin* mRNA after radiation and found that *clathrin* did not respond to radiation (data not shown). Although cell surface adenovirus receptors, such as coxsackie and adenovirus receptor and

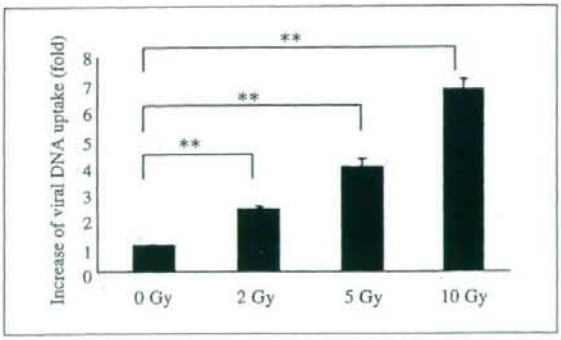

Fig. 5. Radiation increases adenovirus infection. SUIT-2 cultures were irradiated and infected with Ad-lacZ at MOI of 10 at 24 h after radiation. DNA was extracted at 24 h after infection. Viral DNA content was quantified by real-time PCR and expressed as fold-increase compared with nonirradiated cells. Columns, mean of triplicate measurements; bars, SD. **, $P < 0.01$.

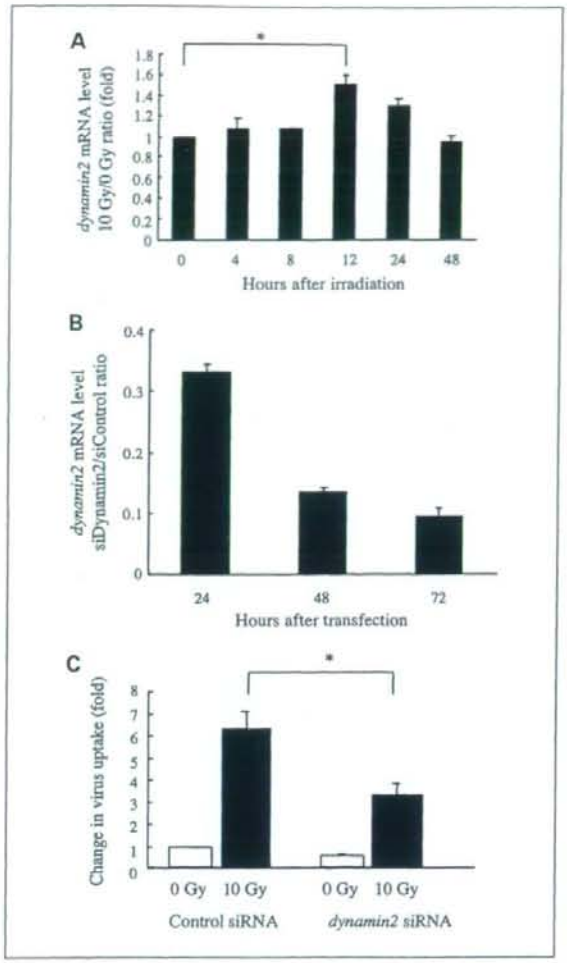

Fig. 6. Radiation-induced viral infection is mediated by dynamin 2. *A*, radiation-induced dynamin 2 expression. *Dynamin 2* mRNA was quantified by real-time reverse transcription – PCR from total RNA of irradiated (10 Gy) or nonirradiated SUIT-2 cells at 4, 8, 12, 24, and 48 h after radiation and expressed as fold-increase compared with nonirradiated cells. *B* and *C*, reduction of dynamin 2 by siRNA inhibited radiation-induced adenovirus infection. *, $P < 0.05$. *B*, *dynamin 2* mRNA was quantified by real-time reverse transcription – PCR from total cellular RNA of *dynamin 2* – specific siRNA-transfected cells (*siDynamin2*) or control siRNA-transfected cells (*siControl*) at 24, 48, and 72 h after transfection and expressed as fold-decrease compared with control siRNA-transfected cells. *C*, SUIT-2 cultures were transfected with *dynamin 2* – specific siRNA or control siRNA, irradiated (10 Gy) or nonirradiated at 24 h after transfection, and infected with Ad-lacZ at MOI of 10 at 24 h after radiation. Cellular DNA was isolated 48 h after infection. Viral DNA content was quantified by real-time PCR and expressed as fold-increase or fold-decrease compared with control siRNA-transfected and nonirradiated cells. Columns, mean of triplicate measurements; bars, SD. *, $P < 0.05$.

α_v integrin receptor, may affect the efficiency of adenovirus infection, expression of the mRNAs for these receptors was not changed by irradiation (data not shown).

Effect of radiation on expression of NK4 delivered by Ad-NK4 in nude mice xenografts. To evaluate the effect of radiation on NK4 expression of pancreatic cancers treated with Ad-NK4 *in vivo*, we established six irradiated tumors and four untreated tumors. At 24 hours after radiation, 5×10^7 plaque-forming units of Ad-NK4 (100 μL) were injected into each tumor. To examine the expression of NK4 protein in the subcutaneous tumors, mice were killed 48 hours after administration of Ad-NK4, and the NK4 concentrations in the tumor lysates were measured by ELISA. Irradiated tumors expressed 5.8 ± 3.5–fold ($P = 0.017$) higher levels of NK4 than did nonirradiated tumors (Fig. 7). This result is consistent with our *in vitro* data and suggests that radiation can enhance expression of a target gene delivered by adenovirus vector *in vivo*.

Discussion

In the present study, we found that radiation enhances expression of target genes delivered to pancreatic cancer cells by an adenovirus-based vector in a dose-dependent manner. Furthermore, we investigated the mechanisms that underlie the radiation-enhanced target gene transfer and found that radiation increased activation of the CMV promoter through phosphorylation of p38 MAPK and increased adenoviral uptake through increased expression of dynamin 2.

Despite previous reports describing the combination of radiation therapy and adenovirus-mediated gene therapy, the mechanism by which radiation enhances the expression of adenovirus-mediated gene has remained unknown. In the present study, we found that radiation enhances expression of target gene via activation of the CMV promoter, which is commonly used to drive expression of target genes by adenovirus vectors. Breuning et al. (40) and Chen et al. (41) reported that activation of p38 MAPK increases expression of transgenes under control of the CMV promoter. We previously showed that radiation increases p42 and p44 MAPK activity under specific conditions (47). Our present data indicate that radiation also increases phosphorylation of p38 MAPK leading to

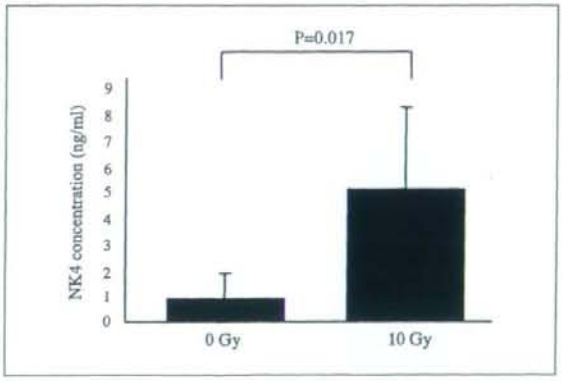

Fig. 7. Radiation significantly increases NK4 expression in subcutaneous tumor in nude mice. Ten subcutaneous tumors were established with 5×10^6 SUIT-2 cells in the flank on day 0. Seven days later, six tumors were irradiated with 10 Gy and four tumors were left untreated. At 24 h after radiation, 5×10^7 plaque-forming units of Ad-NK4 (100 μL) were injected into each tumor. Mice were killed 48 h after administration of Ad-NK4, and the protein lysates of tumors were each adjusted to 10 mg/mL with lysis buffer. NK4 concentrations in the tumor extract were analyzed by ELISA. Irradiated tumors expressed 5.8 ± 3.5–fold higher NK4 than did nonirradiated tumors ($P = 0.017$). Columns, mean of the NK4 expression in six irradiated and four untreated tumors, respectively; bars, SD.

activation of the CMV promoter, suggesting that radiation-induced phosphorylation of p38 MAPK is involved in radiation-enhanced expression of target genes.

In the present study, we also found that adenovirus-specific gene uptake by pancreatic cancer cells increases after radiation, suggesting that radiation enhances infection by adenovirus. Zhang et al. (37) reported that radiation improves gene transfer efficiency in human colon cancer, breast cancer, and brain cancer cells. These data suggest that radiation may improve the efficiency of gene therapy not only for pancreatic cancer but also many cancers. It has been reported that dynamin 2 and clathrin are involved in virus infection (48, 49). We found that *dynamin 2* mRNA levels increase after radiation, whereas *clathrin* expression is not affected by radiation. Although cell surface adenovirus receptors, such as coxsackie and adenovirus receptor and α_v integrin receptor, are key factors in adenovirus infection, we have found that expression of mRNAs for these molecules is not altered by radiation (data not shown). Qian et al. (38) reported that dynamin 2 mediates radiation-induced adenovirus infection in colon, brain, and breast cancer cell lines. In the present study, inhibition of dynamin 2 production by RNA interference significantly reduced Ad-lacZ uptake. These data suggest that dynamin 2 might be involved in radiation-induced adenovirus infection in pancreatic cancer cells.

The mortality rate of pancreatic cancer remains highest among cancers (10). Gene therapy with adenovirus vector is a promising strategy for treatment of cancer, but the antitumor effect of a single dose of adenovirus-mediated gene therapy is often insufficient in clinics (6, 19, 20), possibly due to limited transduction efficiency of adenovirus vectors. In the present study, we found that radiation dramatically enhanced adenovirus-mediated gene expression, and we clarified the mechanism of this phenomenon. In conclusion, the present data suggest that regional radiation may significantly improve adenovirus-mediated gene transfer efficiency in pancreatic tumors and probably contributes to decreasing the dose of adenovirus required for gene transfer and controlling side effects of adenovirus infection in nonirradiated normal tissue.

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ORIGINAL ARTICLE

Midkine mRNA Is Overexpressed in Pancreatic Cancer

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Abstract *Purpose Midkine* (*MK*) has been reported to be a possible molecular marker for the diagnosis of pancreatic cancer. We investigated the feasibility of quantitative analysis of *MK* mRNA by quantitative real-time RT-PCR (qRT-PCR) as a promising tool for the diagnosis of pancreatic cancer. *Results* We found that pancreatic cancer tissues expressed significantly higher levels of *MK* mRNA than intraductal pancreatic mucinous neoplasm (IPMN) and non-neoplastic pancreatic tissues ($P < 0.05$); in contrast, we did not find any differences in *MK* mRNA expression between IPMN and non-neoplastic pancreatic tissues. Additionally, we observed that poorly differentiated carcinoma samples expressed higher levels of *MK* mRNA than well-differentiated carcinoma samples, although a significant difference was not observed. *Conclusions* The present data suggests that quantitative analysis of *MK* mRNA provides an objective and sensitive evaluation and may be a promising modality for the diagnosis of pancreatic cancer and the prediction of its prognosis.

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Keywords Midkine · Pancreatic cancer · Quantitative real-time RT-PCR

Introduction

Pancreatic cancer is one of the most aggressive malignancies and has a 5-year survival rate 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, a high incidence of metastasis, and a lack of effective drugs.

Midkine (*MK*) is a heparin-binding growth and differentiation factor that was identified as a product of a retinoic acid-responsive gene [2]. *MK* has mitogenic effects on fibroblasts and neurotrophic activities [3], and promotes the survival of retinal cells in constant light-induced retinal degeneration [4]. Moreover, *MK* has been reported to be an angiogenic, fibrinolytic, and antiapoptotic factor in carcinoma cell lines [5–8] and is related to carcinogenesis and tumor progression [5, 9]. The overexpression of *MK* has been reported in a number of malignant tumor, including Wilms tumor, breast cancer, hepatocellular carcinoma, gastrointestinal cancer, colorectal cancer, and bladder cancer [10–14]. RT-PCR analyses revealed that *MK* is expressed in various normal tissues, such as lung, stomach, colon, and spleen, but not normal pancreatic tissue. On the other hand, it has been reported that *MK* is expressed in pancreatic cancer tissue [15]. Immunohistochemical expression of *MK* is significantly correlated with venous invasion, microvessel density, liver metastasis and prognosis in pancreatic head

carcinoma [16]. These data suggest that *MK* is a possible molecular marker for the diagnosis and therapy of pancreatic cancer.

However, it is sometimes difficult to perform objective evaluation using immunohistochemical staining of clinical samples. In particular, immunohistochemistry of *MK* protein needs to be performed under stringent conditions. Therefore, in clinics, it is difficult to use immunohistochemical staining of *MK* as a modality for cancer diagnosis or prediction of the prognosis and the effect of treatment. In general, quantitative analysis of mRNA provides an objective evaluation compared with immunohistochemical staining, and is a promising modality for the diagnosis of pancreatic cancer and the prediction of its prognosis or treatment effect.

In the present study, to investigate the feasibility of quantitative analysis of *MK* mRNA with quantitative real-time RT-PCR (qRT-PCR) as a promising tool for the diagnosis of pancreatic cancer, we examined the expression of *MK* mRNA in bulk pancreatic tissues as well as in 15 pancreatic cancer cell lines by qRT-PCR.

Materials and Methods

Cell Lines and Pancreatic Tissues

The following human pancreatic cancer cell lines were used: ASPC-1, BxPC-3, KP-1N, KP-2, KP-3 PANC1, and SUIT2 (Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan); MiaPaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan); Capan-1, Capan-2, H48N, CFPAC-1, SW1990, and HS766T (American Type Culture Collection, Manassas, VA, USA), and NOR-P1 (established in our laboratory). Cells were maintained in DMEM (Sigma Chemical Co., St. Louis, MA) supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C in a humidified atmosphere with 10% CO_2. Tissue samples were obtained during surgery at Kyushu University Hospital (Fukuoka, Japan). Eleven cancer tissue samples and 18 intraductal papillary mucinous neoplasm (IPMN) samples were obtained from the primary tumor of each resected pancreas, and 16 normal tissue samples were taken from peripheral soft tissue away from the tumors. The tissue samples were removed as soon as possible after resection and stored at −80°C until use. Tissue adjacent to the specimen was examined histologically and the diagnosis was confirmed. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Quantitative Assessment of *MK* mRNA Levels by Real-Time RT-PCR

Total RNA was extracted from cultured cells and bulk tissues with a PicoPure RNA Isolation kit (Arcturus, Mountain View, CA) or a High Pure RNA kit (Roche Diagnostics, Mannheim, Germany) with DNase I treatment (Roche Diagnostics) according to the manufacturer's instructions. We designed the following specific primers: *MK* forward, ccaagaaagggaagggaaag and reverse, acgagcagacagaaggcact; and *18S* rRNA forward, gtaacccgttgaaccccatt and reverse, ccatccaatcggtagtagcg. Quantitative real-time RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) with a LightCycler Quick System 350S (Roche Diagnostics) as described previously [17]

Statistical Analyses

Data were analyzed using the Mann–Whitney *U* test because the data did not follow a normal distribution. Statistical significance was set at $P < 0.05$.

Results

Quantitative Analysis of *MK* mRNA in Human Pancreatic Cancer Cell Lines

We measured the expression of *MK* mRNA in 15 pancreatic cancer cell lines. To normalize *MK* mRNA expression, we used *18S* rRNA as a reference gene. We detected significant expression of *MK* mRNA in all pancreatic cancer cell lines (Fig. 1). Several pancreatic cancer cell lines originating from metastatic lesions, such as KP-3, AsPC-1, SUIT2, and CFPAC1, showed especially high levels of *MK* mRNA expression compared to pancreatic cancer cell lines originating from primary lesions, although a significant difference was not observed.

We also investigated the correlation between the levels of *MK* mRNA in pancreatic cancer cell lines and the histological grade of differentiation in pathological examination of original pancreatic tumor and found no correlation between them.

Quantitative Analysis of *MK* mRNA Levels in Human Pancreatic Cancer, IPMN, and Non-Neoplastic Bulk Pancreatic Tissues

We measured the levels of *MK* mRNA expression in pancreatic cancer and non-neoplastic pancreatic tissues. We also measured the levels of *MK* mRNA in IPMN, which was reported to be a precursor lesion of a subset of pancreatic

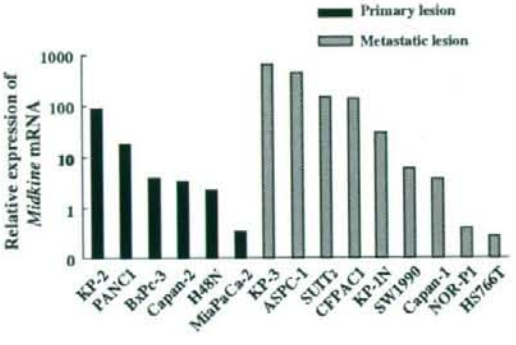

Fig. 1 Quantitative analysis of *MK* mRNA in human pancreatic cancer cell lines. Several cell lines originating from metastatic lesions showed especially high expression of *MK* compared to cell lines originating from primary lesions. Cell lines originating from primary lesions (*black bar*). Cell lines originating from metastatic lesions (*gray bar*)

cancer. Previously, we investigated the expression of several candidates of reference genes and reported that *18S* rRNA is a promising reference gene with which to normalize gene expression in bulk pancreatic tissues. Therefore, in the present study, to normalize *MK* mRNA expression, we used *18S* rRNA as the reference gene. The results revealed that pancreatic cancer tissues expressed significantly higher levels of *MK* mRNA than IPMN and non-neoplastic pancreatic tissues (Fig. 2a, $P < 0.05$). The median value of *MK* mRNA expression in pancreatic cancer tissue was 5.4-fold higher than those of pancreatic non-neoplastic tissues. The median value of *MK* mRNA expression was 4.6-fold higher in pancreatic cancer tissues than in IPMN tissues. *MK* mRNA expression did not differ significantly between IPMN and pancreatic non-neoplastic tissues ($P = 0.32$).

In addition, the relationship between *MK* mRNA expression and pathological differentiation was examined. Eleven samples of pancreatic cancer bulk tissues, consisting of ten conventional invasive ductal pancreatic carcinomas and one mucinous cystadenocarcinoma, were studied. Of the ten pancreatic invasive ductal carcinoma samples, two, expressing the highest levels of *MK* mRNA, were moderately to poorly differentiated carcinomas according to the final pathological examination. The three samples expressing the next highest levels of *MK* mRNA were moderately differentiated carcinomas and the other five samples, expressing low levels of *MK* mRNA, were well to moderately differentiated carcinomas, although we did not find any statistical correlation between the expression level of *MK* mRNA and pathological differentiation (Fig. 2b).

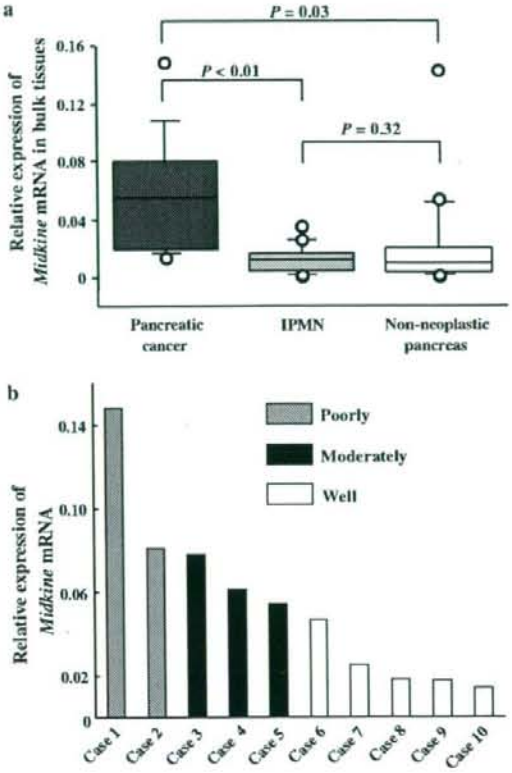

Fig. 2 **a** Quantitative analysis of *MK* mRNA levels in human pancreatic cancer, IPMN, and non-neoplastic bulk pancreatic tissues. Pancreatic cancer tissues expressed significantly higher levels of *MK* than IPMN and non-neoplastic pancreatic tissues ($P < 0.05$). However, *MK* expression was not significantly different between IPMN and normal tissues. **b** The relation between *MK* mRNA expression and pathological differentiation. Of ten pancreatic invasive ductal carcinoma samples, two invasive ductal carcinoma samples, expressing the highest levels of *MK* mRNA, were moderately to poorly differentiated carcinomas according to the final pathological examination. The three samples expressing the next highest levels of *MK* mRNA were moderately differentiated carcinomas and the other five samples, expressing low levels of *MK* mRNA, were well to moderately differentiated carcinomas

Discussion

In the present study, we examined *MK* mRNA expression in pancreatic tissues and pancreatic cancer cell lines by qRT-PCR. In the cell-line analyses, all pancreatic cancer cell lines expressed significant levels of *MK* mRNA. In particular, several cell lines of metastatic origin showed remarkably high expression of *MK* mRNA compared with those with primary origin. In the bulk tissue analyses, *MK* mRNA expression was significantly higher in pancreatic

cancer tissues than in IPMN tissue, a precursor lesion of a subset of pancreatic cancer, and in non-neoplastic tissues, whereas there was no difference in *MK* mRNA expression between IPMN and non-neoplastic tissues.

It has been reported that *MK* protein expression is involved in carcinogenesis and the progression of cancers [9]. Moreover, *MK* protein expression has been reported to be increased in cases with poor prognosis or metastasis of cancer in pancreatic head carcinoma and oral squamous cell carcinoma [16, 18]. Therefore, *MK* may be a molecular marker of diagnosis or a predictive marker of prognosis for pancreatic cancers. It has also been reported that there is a significant correlation between the levels of *MK* mRNA and protein expression during the carcinogenesis of cervical cancer [19]. In the present study, we also found that pancreatic cancer tissues expressed high levels of *MK* mRNA as well as *MK* protein. These data suggest that a quantitative analysis of *MK* mRNA expression can be a useful tool for the diagnosis of pancreatic cancer or the prediction of its prognosis, as an objective evaluation method, instead of the evaluation of protein levels by immunohistochemical analysis.

Since *MK* is a secreted protein, it can be detected in the blood. Shimada et al. [20], and Obata et al. [21] reported that the *MK* level in blood is increased in patients with advanced esophageal and gastric cancer, and that it may be a promising marker of prognosis. However, despite reports regarding an increase in *MK* protein level in blood in patients with advanced cancer, there has been no report showing that measurement of *MK* level in blood is useful for early diagnosis. There is a possibility that these modalities do not have enough sensitivity to detect a slight increase in *MK* protein level in early-stage cancers. We previously reported that quantitative analysis of *MUC1* and *MUC5AC* mRNA levels in pancreatic juice is a promising approach for the preoperative diagnosis of pancreatic cancer, and possibly for the diagnosis of early pancreatic cancer [22]. Although pancreatic juice includes only a small number of target cells, it often includes premalignant or non-invasive cancer cells, which are important for the early diagnosis of pancreatic cancer. In general, PCR-based analyses have high sensitivity to detect target gene expression. Therefore, it may be possible to measure *MK* mRNA in cells from the pancreatic juice for the early diagnosis of pancreatic cancer, although further examination is needed.

In clinics, it is often difficult to distinguish malignant IPMN from benign IPMN [23]. In the present study, there was a significant difference in *MK* mRNA expression between pancreatic cancer and non-malignant IPMN. Therefore, analysis of *MK* mRNA expression in pancreatic juice may be useful to distinguish malignant IPMN from benign IPMN.

In the present study, we found that pancreatic cancers with poorly differentiated carcinomas expressed higher levels of *MK* mRNA than those without poorly differentiated carcinomas. Tumor differentiation is a major prognostic factor, and poorly differentiated tumors are highly aggressive with extremely poor prognosis [24, 25]. These data suggest that *MK* mRNA may be a possible predictive marker of prognosis in patients with pancreatic cancer.

In conclusion, the present data suggest that quantitative analysis of *MK* mRNA provides an objective and sensitive evaluation and may be a promising modality for the diagnosis of pancreatic cancer and the prediction of its prognosis or treatment effect.

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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

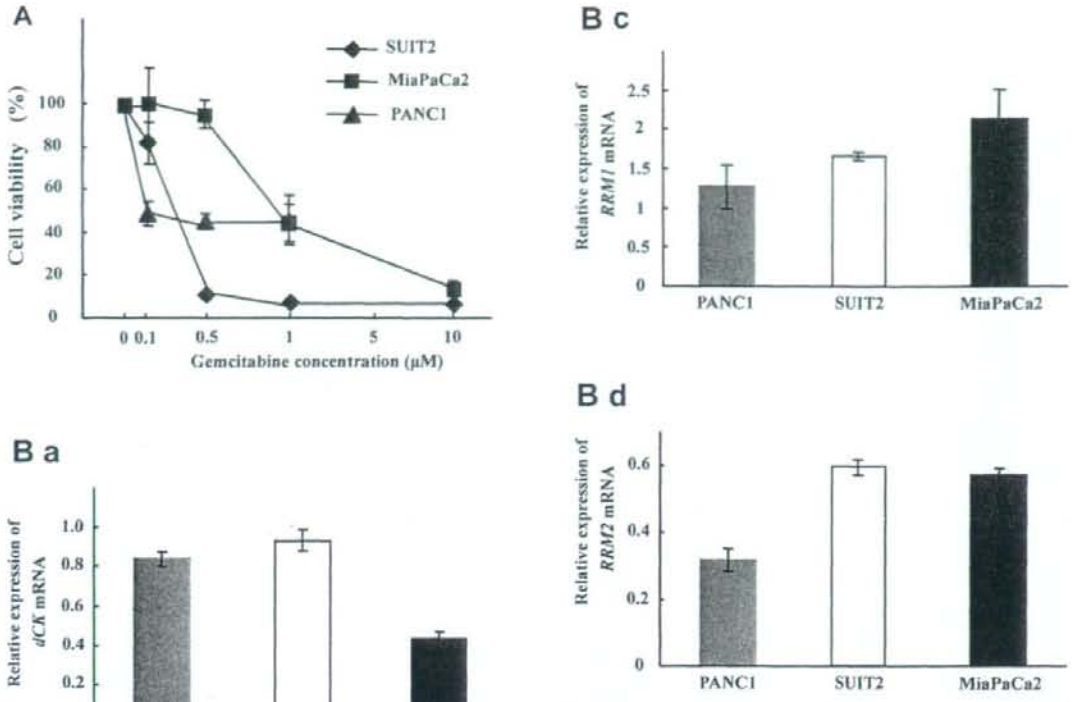

Figure 1. *Gemcitabine sensitivities and expressions of gemcitabine transporter and metabolism-related genes in pancreatic cancer cell lines. A, Gemcitabine sensitivities of pancreatic cancer cell lines. The IC_{50} of PANC1 cells was 98.3±21.9 nM, that of SUIT2 cells was 200.9±24.7 nM and that of MiaPaCa2 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 ($p<0.01$).

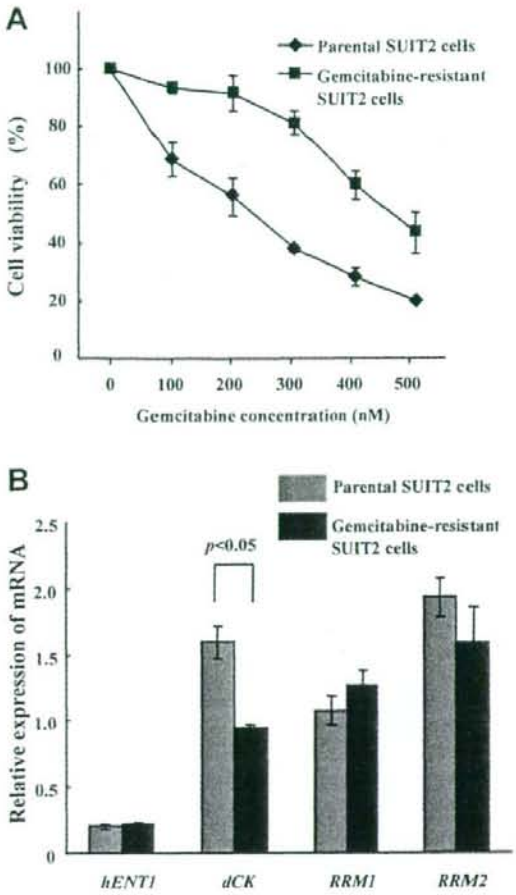

Figure 2. *Cell viabilities and expressions of gemcitabine transporter and metabolism-related genes in parental and gemcitabine-resistant SUIT2 cells. A, The changes in viability of parental and gemcitabine-resistant SUIT2 cells were evaluated by PI assays and are shown as gemcitabine dose-response curves. 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.

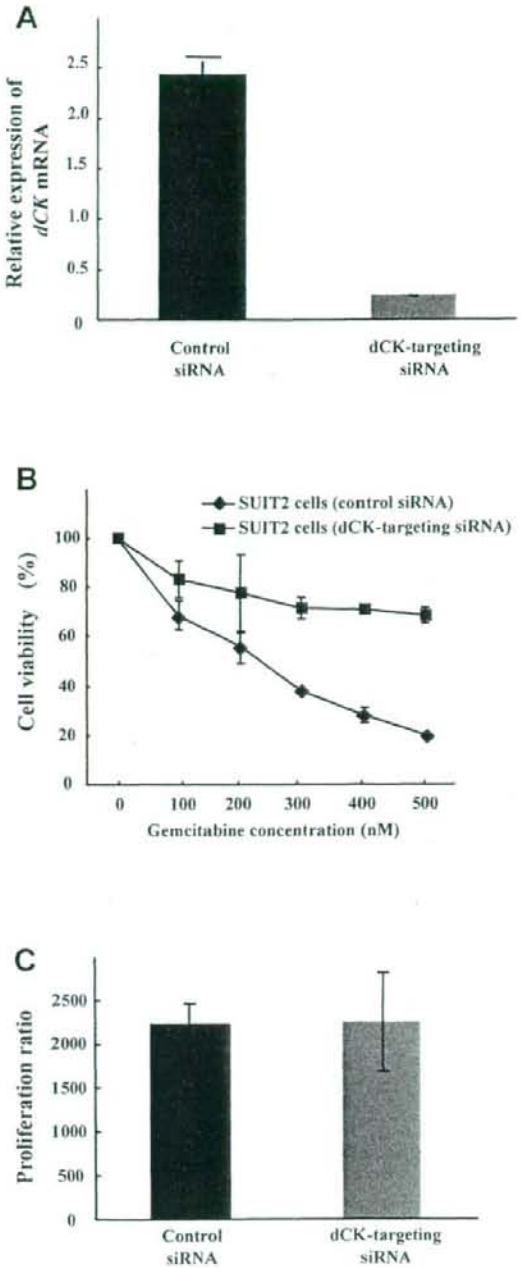

Figure 3. *Inhibition of dCK expression in SUIT2 cells by a dCK-targeting siRNA. A, Expression of dCK is suppressed to less than 10% by the dCK-targeting siRNA. B, The dCK-targeting siRNA significantly reduced the gemcitabine sensitivity of SUIT2 cells ($p<0.01$ for 300, 400 and 500 nM gemcitabine). 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

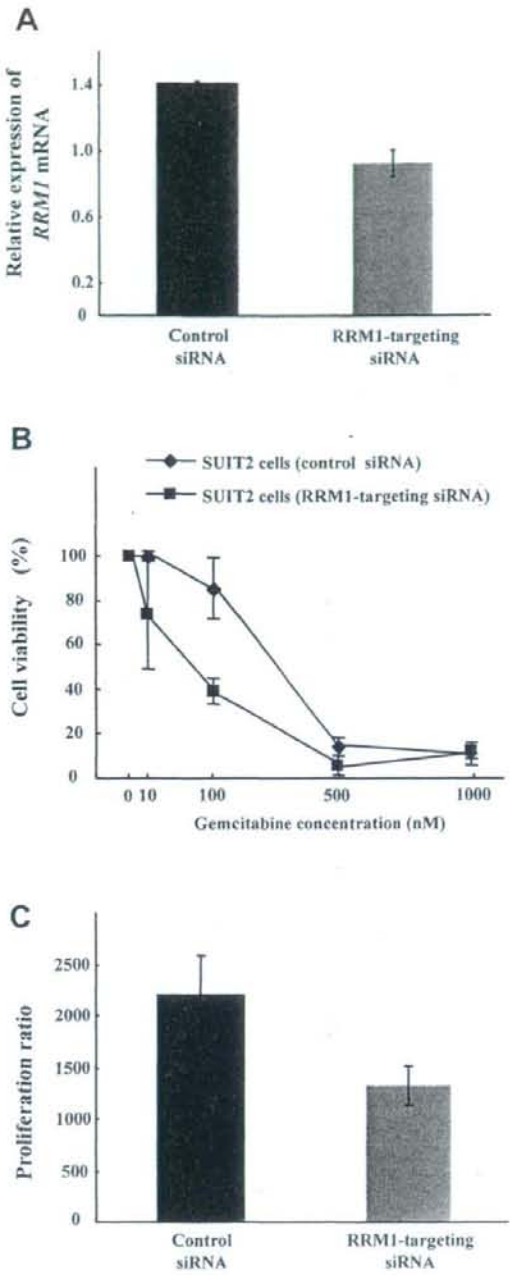

Figure 4. *Inhibition of RRM1 expression in SUIT2 cells by an RRM1-targeting siRNA. A, Expression of RRM1 was suppressed to 65% by the RRM1-targeting siRNA. B, The RRM1-targeting siRNA increase the gemcitabine sensitivity of SUIT2 cells ($p=0.01$ for 100 nM gemcitabine). C, The RRM1-targeting siRNA reduced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

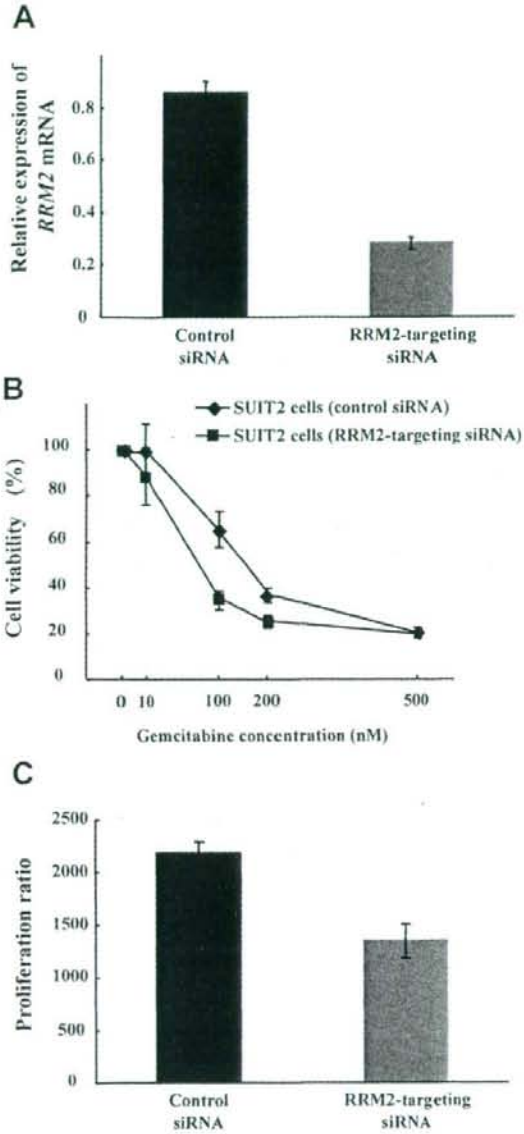

Figure 5. *Inhibition of RRM2 expression in SUIT2 cells by an RRM2-targeting siRNA. A, Expression of RRM2 was suppressed to 33% by the RRM2-targeting siRNA. B, The RRM2-targeting siRNA increase the gemcitabine sensitivity of SUIT2 cells ($p<0.01$ for 100 and 200 nM gemcitabine). C, The RRM2-targeting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

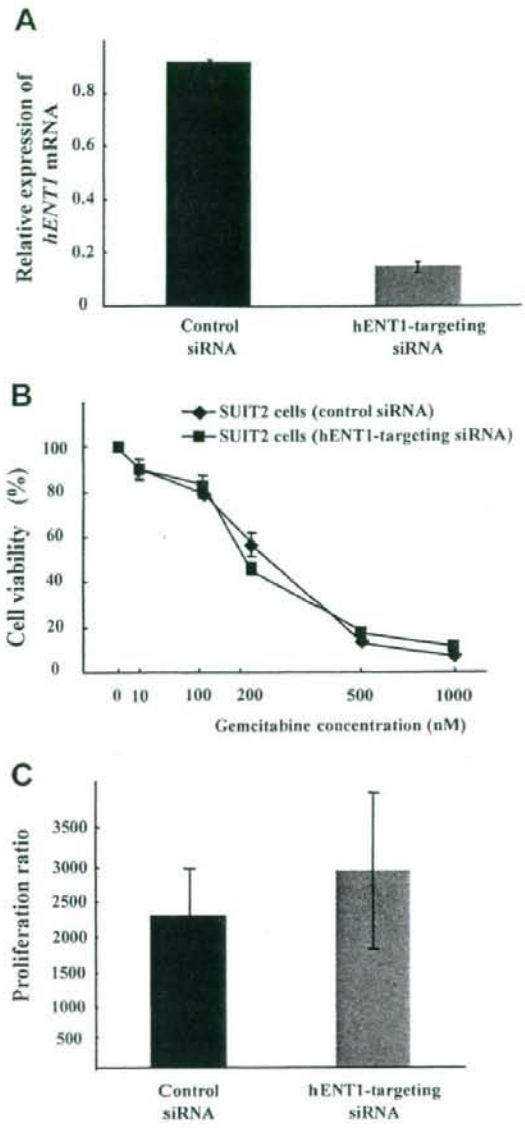

Figure 6. *Inhibition of hENT1 expression in SUIT2 cells by a hENT1-targeting siRNA. A, Expression of hENT1 was suppressed to 85% by the hENT1-targeting siRNA. B, The hENT1-targeting siRNA did not change the gemcitabine sensitivity of SUIT2 cells ($p>0.05$ for 10, 100 and 200 nM gemcitabine). C, The 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.

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