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Enhancement of gemcitabine sensitivity in pancreatic cancer by co-regulation of dCK and p8 expression

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Abstract. The purpose of this study was to improve the gemcitabine sensitivity in pancreatic cancer by adenovirus-mediated co-regulation of dCK and p8 expression. Firstly, we analyzed the sensitivity of three human pancreatic tumor cell lines (Capan-2, Panc-1 and BxPc-3) to gemcitabine using MTT assays, and found Panc-1 to be relatively resistant to gemcitabine. Further, we investigated the expression of dCK and p8 in different pancreatic cancer cell lines using real-time PCR and Western blot analysis, and found that the expression levels of these two genes were related to the gemcitabine sensitivity of pancreatic cancer cells. We constructed recombinant adenovirus vectors, Ad-dCK and Ad-p8-siRNA, that overexpressed dCK and knocked down p8, respectively. Using MTT assays, we observed that combined infection using Ad-dCK and Ad-p8-siRNA *in vitro* led to a significant decrease in the gemcitabine IC_{50} with an increase in apoptosis and caspase-3 activity in Panc-1 cells, which are relatively resistant to gemcitabine. Furthermore, in established subcutaneous pancreatic cancer models in nude mice, the tumor inhibition was markedly enhanced accompanied by elevation of the apoptosis index after intratumoral injection of Ad-dCK and Ad-p8-siRNA on the basis of intraperitoneal gemcitabine chemotherapy. Taken together, the present findings suggest that, dCK and p8 may be the important factors in the regulation of gemcitabine sensitivity in pancreatic cancer cells. Moreover, co-regulation of the two factors achieved better effects than regulation of either one alone.

Introduction

Pancreatic cancer is one of the most aggressive and lethal malignant diseases in humans. The lack of early detection and highly aggressive regional invasion render curative surgery rather difficult (1). 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% (2). Chemotherapy plays an important role in the systemic treatment of pancreatic cancer. Unfortunately, conventional chemotherapy has limited effect on the overall survival of patients with pancreatic cancer, because this neoplasm is resistant to almost all chemotherapeutic agents (3). Gemcitabine (2',2'-difluorodeoxycytidine) is the standard first-line anticancer agent for pancreatic cancer at the present time. However, although it produces a significant clinical benefit in patients, the response rate and prognosis remain dismal, with an objective response in <20% of clinical cases (4) and a 5-year survival rate of 1-4% (2). Therefore, any strategies that can enhance the sensitivity of pancreatic cancer to gemcitabine may improve the prognosis of this fatal disease.

As a pro-drug, gemcitabine must first be phosphorylated intracellularly before it can exert its antitumor activity. The first step in the phosphorylation is the rate-limiting step, and therefore essential for the activation of gemcitabine. For this reason, dCK plays a key role in the metabolism of gemcitabine (5). Several authors have described relationships between dCK activity and sensitivity to gemcitabine in tumor cells, including pancreatic cancer cells (6-8). Some studies also found that down-regulation of dCK enhanced acquired resistance to gemcitabine in pancreatic cancer (9) and that the pretreatment levels of dCK protein are highly correlated with overall survival following gemcitabine treatment (10). Overexpression of dCK in tumor cells deficient in this enzyme restored the sensitivity to gemcitabine (5). These findings suggest the feasibility of selecting dCK as a promising target for improving gemcitabine efficacy in pancreatic cancer. However, transfer of dCK alone does not result in complete restoration of gemcitabine sensitivity (11,12). Therefore, to obtain better improvement of the therapeutic efficacy of gemcitabine, we aimed to identify another target for combination with dCK.

During the process of gemcitabine-mediated injury to tumor cells, intracellular anti-injury factors work to protect the cells from adverse stimuli. Among these factors, the human p8 (NUPR1, COM-1) gene was recently shown to be involved in gemcitabine resistance. p8 is highly expressed in pancreatic cancer, and the extent of its expression is correlated with sensitivity to gemcitabine (13,14). Overexpression of p8 is inversely correlated with apoptosis in pancreatic cancer (15). Knockdown of p8 expression in gemcitabine-

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resistant cells improves their response to gemcitabine and increases their caspase-3 activity (14). We hypothesized that knockdown of p8 would contribute to the chemosensitization of pancreatic cancer to gemcitabine. Therefore, p8 was selected as the other candidate for our gemcitabine chemosensitization treatment.

The aim of our study was to render gemcitabine more efficient in pancreatic cancer by adenovirus-mediated co-regulation of the expressions of dCK and p8. Recombinant adenoviruses, Ad-dCK and Ad-p8-siRNA, were constructed and confirmed to be efficient in the corresponding gene regulation. The efficacies of the combined treatment for the relatively gemcitabine-resistant cell line Panc-1 and subcutaneous xenograft pancreatic cancer models in nude mice were evaluated. Overall, we observed enhancement of gemcitabine sensitivity in pancreatic cancer through this combined adenovirus-mediated gene therapy with increases in apoptosis both *in vitro* and *in vivo*. Moreover, co-regulation of the two genes achieved better effects than regulating either one of the genes alone.

Materials and methods

Cell lines. The human pancreatic cancer cell lines Panc-1, BxPc-3 and Capan-2 were obtained from the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS&PUMC) (Beijing, China). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a humidified incubator at 37˚C under 5% CO_2.

Dose-response curves for gemcitabine. Cells in 96-well plates were treated with gemcitabine (Eli Lilly, Suresnes, France) at final concentrations of 0.001-1000 μM for 72 h. The MTT reagent (Sigma, St. Louis, MO) was then added and the cells were incubated for 4 h before the absorbances were measured at 450 nm. Cell viability was calculated as the amount of MTT conversion relative to control cells without gemcitabine treatment.

Real-time polymerase chain reaction (PCR). Total RNA was extracted at specified time-points. The mRNA levels of dCK and p8 in the pancreatic cancer cells were quantified by real-time PCR as previously described (16). The primers used were as follows: dCK (forward primer: 5'-ACGATCTGTGTATAGTGACAGGT-3', reverse primer: 5'-ATGATTCCATCCAATTCAAGGCT-3'); p8 (forward primer: 5'-GCGGGCACGAGAGGAAAC-3', reverse primer: 5'-CTCAGTCAGCGGGAATAAGTC-3'); ß-actin (forward primer: 5'-GGCGGCACCACCATGTACCCT-3', reverse primer: 5'-AGGGGCCGGACTCGTCATACT-3'). The amounts of the PCR products were normalized by the ß-actin levels and expressed as percentages of the control values.

Western blot analysis. Cells were harvested and lysed at specified time-points. dCK protein was resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane while p8 protein was detected by Tricine-SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane as previously described (17,18). The mouse monoclonal antibodies used were all purchased from Abcam (Cambridge, UK). ß-actin was evaluated as an internal control.

Vector constructs and adenovirus production. The dCK-encoding recombinant adenovirus vector (Ad-dCK) and p8-silencing recombinant adenovirus vector (Ad-p8-siRNA) were constructed using an AdMax Adenovirus Creation Kit (Microbix Biosystems Inc., Toronto, Canada) according to the manufacturer's protocols. Briefly, for Ad-dCK construction, a cDNA corresponding to human dCK was recovered by RT-PCR from normal human lymph nodes using the following primers: 5'-GTAGAACGCAGATCGAATTCATGGCCACCCCGCCCAAG-3' and 5'-CCCTTGCTCACCATGAATTCCAAAGTACTCAAAAACTCTTTGAC-3'. The PCR fragments were then ligated into an *Eco*RI-linearized shuttle plasmid, pDC315-EGFP (Shanghai GeneChem Co., Ltd., Shanghai, China), using an In-Fusion™ PCR Cloning Kit (Clontech, Palo Alto, CA). The pDC315-EGFP plasmid with no inserted fragments was used as a negative control (NC-Ad-dCK). For Ad-p8-siRNA construction, the following target site was chosen: 5'-GAGGAAACTGGTGACCAAGCT-3'. Sense and antisense oligonucleotides based on this target site were synthesized by Shanghai GeneChem Co., Ltd. The oligonucleotides were annealed and ligated into an *Age*I/*Eco*RI-linearized pDC316-siRNA shuttle plasmid (Shanghai GeneChem Co., Ltd.), which contained hU6 and CMV promoters. A negative control (NC-Ad-p8-siRNA) for Ad-p8-siRNA was designed with a randomized sequence (5'-TTCTCCGAACGTGTCACGT-3') of the same composition as the target sequence that showed no homology with known genes. After identification and amplification, the shuttle plasmids and Ad genomic plasmids pBHG lox (δ) E1 and E3 Cre were cotransfected, respectively, into HEK293 cells to create adenoviruses. Recombinant viruses were propagated in HEK293 cells and purified using an Adeno-X™ Virus Purification Kit (Clontech). The infectious titers of the tertiary viral lysates were determined by end-point dilution assays according to the manufacturer's protocol (Clontech) and ranged from $1.0x10^{10}$ to $5.0x10^{10}$ plaque-forming units (pfu)/ml.

Infection using adenoviruses. The Panc-1 cell line was cultured as described above. After being seeded into 6-well plates and incubated overnight, the cells were infected with the corresponding adenoviruses at a MOI of 30 for 2 h. The virus-containing medium was then replaced with fresh medium. At 48 and 72 h after infection, total RNA and total protein were extracted, respectively, and subjected to real-time PCR and Western blot analyses as described above.

Animals. Athymic BALB/c-nu/nu female nude mice at 5 weeks of age were purchased from the Institute of Laboratory Animal Sciences, CAMS&PUMC (Beijing, China), housed in appropriate animal care facilities during the experimental period and handled according to the institutional guidelines for animal experimentation.

Subcutaneous pancreatic tumor xenograft model. Mice were subcutaneously inoculated at their left scapular area with Panc-1 cells ($1x10^7$ cells in 100 μl). When the tumor volumes had reached ~100 mm^3, the mice were randomly divided into 8 groups (n=8) and received an intratumoral injection of

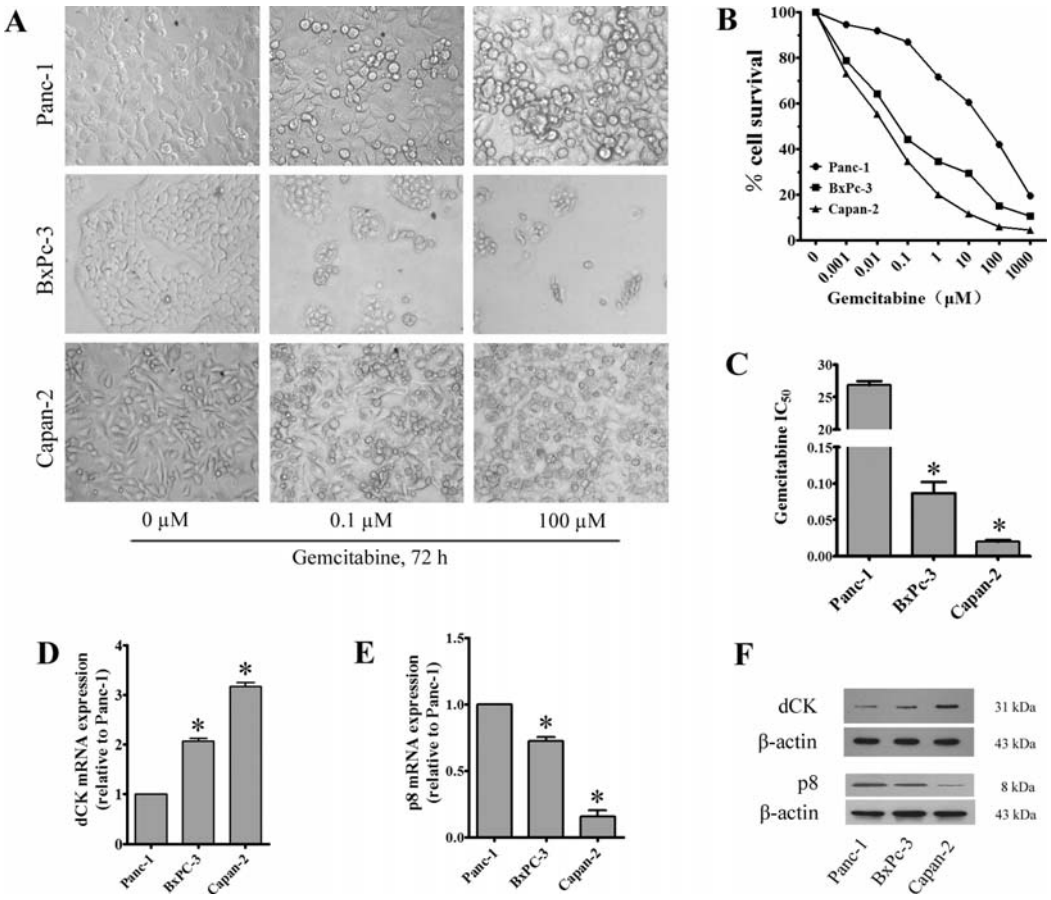

Figure 1. Sensitivity to gemcitabine differs in pancreatic cancer cell lines, and is related to the inherent expressions of dCK and p8. (A-C) Panc-1, BxPc-3 and Capan-2 cells were treated with increasing concentrations of gemcitabine (0.001-1000 μM) for 72 h. Representative microscopic images are shown [(A) original magnification, x200]. The cell viability ratios (B) and IC_{50} values (C) were measured by MTT assays. *P<0.01 vs. Panc-1 cells. (D-F) Expressions of dCK and p8 in the three pancreatic cancer cell lines evaluated by real-time PCR (D, E) and Western blot (F) analyses. *P<0.01 vs. Panc-1 cells. All the data represent the means ± SEM of four experiments performed in triplicate.

$1x10^9$ pfu of the corresponding recombinant adenoviruses once per week. Gemcitabine (150 mg/kg) was injected intraperitoneally in the therapeutic groups twice per week. The tumor dimensions were measured every 4 days using a linear caliper. The tumor volume was calculated using the following equation: volume = 0.523 x width^2 x length (19). After three adenovirus injections, real-time PCR and Western blot analyses were performed on tumor biopsies obtained from two mice per group. After 41 days of drug therapy, all mice were sacrificed by cervical dislocation. The subcutaneous tumors were excised, fixed with 4% formalin, embedded in paraffin, sectioned (4 μm) and evaluated by immunohistochemical staining.

Immunohistochemistry. After being routinely dewaxed and rehydrated, the sections were subjected to antigen retrieval using a microwave method as previously reported (20). The expressions of dCK and p8 in the tumors were detected using a PV-9002 Kit (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The primary antibodies against dCK and p8 were the same as those used in the Western blot assays, and were both diluted by 1:200.

Detection of apoptosis. *In vitro*, the early apoptosis rates and caspase-3 activities in treated and untreated Panc-1 cells were detected using an Annexin V-FITC Apoptosis Detection Kit (Biosea, Beijing, China) and a Caspase-3 Colorimetric Assay Kit (Keygen, Nanjing, China), respectively, according to the corresponding manufacturer's instructions. Briefly, after being infected with the adenoviruses at a MOI of 30 for 2 h, Panc-1 cells were incubated with gemcitabine (IC_{50} dose) for 72 h. After cell collection, the apoptosis rates and caspase-3 activities were analyzed by flow cytometry and an enzyme-labeled meter, respectively, as previously described (21,22). *In vivo*, the apoptosis rates were analyzed by TUNEL staining. Following the preparation of 4-μm tumor sections, apoptosis was detected using an ApopTag Kit (Chemicon, Temecula, CA) according to the manufacturer's protocol. The apoptosis index was calculated as previously described (23).

Statistical analysis. The data represent the means ± SEM. Differences between groups were examined for statistical significance by analysis of variance and/or the Student-Newman-Keuls test. Values of P<0.05 were considered to indicate statistical significance.

Results

Sensitivity to gemcitabine differs among pancreatic cancer cell lines, and is related to the inherent expressions of dCK

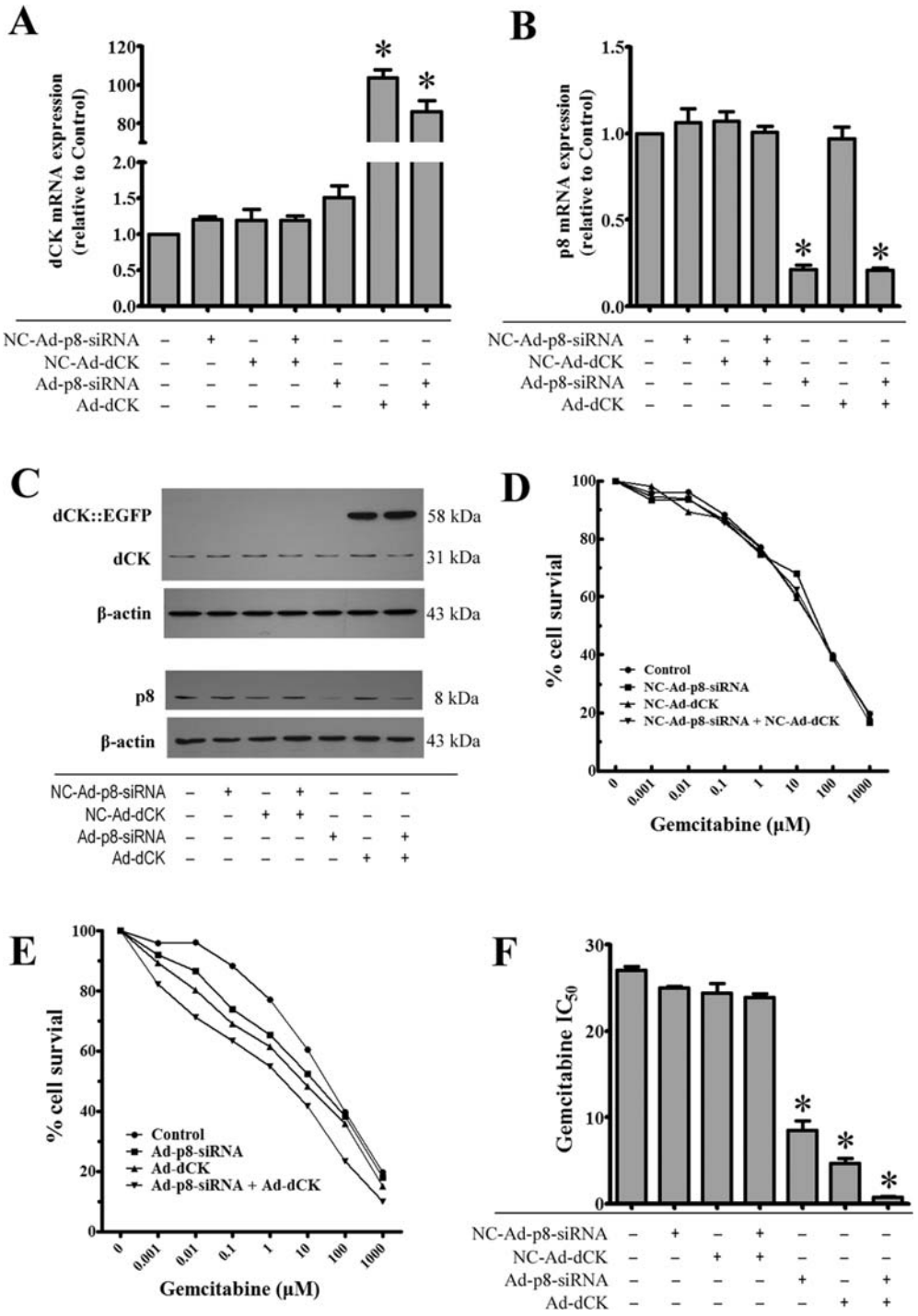

Figure 2. Overexpression of dCK and/or down-regulation of p8 improves the efficacy of gemcitabine in Panc-1 cells *in vitro*. (A-C) Gene regulation by recombinant adenovirus infections. Panc-1 cells were infected with the different recombinant adenoviruses for 2 h. At 48 and 72 h after infection, total RNA and total protein were extracted, respectively. The expressions of dCK and p8 were evaluated by real-time PCR (A, B) and Western blot (C) analyses. *P<0.01 vs. the virus-untreated group. (D-F) At 2 h after adenovirus infection, Panc-1 cells were treated with gemcitabine (0.001-1000 μM) for 72 h. The dose-response curves (D, E) and IC_{50} values (F) were measured by MTT assays. *P<0.01 vs. the virus-untreated group. All the data represent the means ± SEM of four experiments performed in triplicate.

and p8. First, we analyzed the sensitivities of three human pancreatic tumor cell lines (Capan-2, Panc-1 and BxPc-3) to gemcitabine using MTT assays (Fig. 1A and B), and found Panc-1 relatively resistant to gemcitabine. As shown in Fig. 1C, Panc-1 cells showed the highest resistance to gemcitabine with an IC_{50} of 27 μM, while BxPc-3 and Capan-2 cells exhibited intermediate (IC_{50}=0.09 μM) and low (IC_{50}=0.02 μM) resistance, respectively.

Real-time PCR (Fig. 1D and 1E) and Western blot analyses (Fig. 1F) were performed to measure the expressions of dCK and p8 in the three human pancreatic cancer lines, and identify the associations between the resistance of pancreatic cancer cells to gemcitabine and the expressions of the two genes. The basal expressions of dCK and p8 in the cell lines were related to the extent of resistance to gemcitabine. In other words, compared with the gemcitabine-sensitive cell

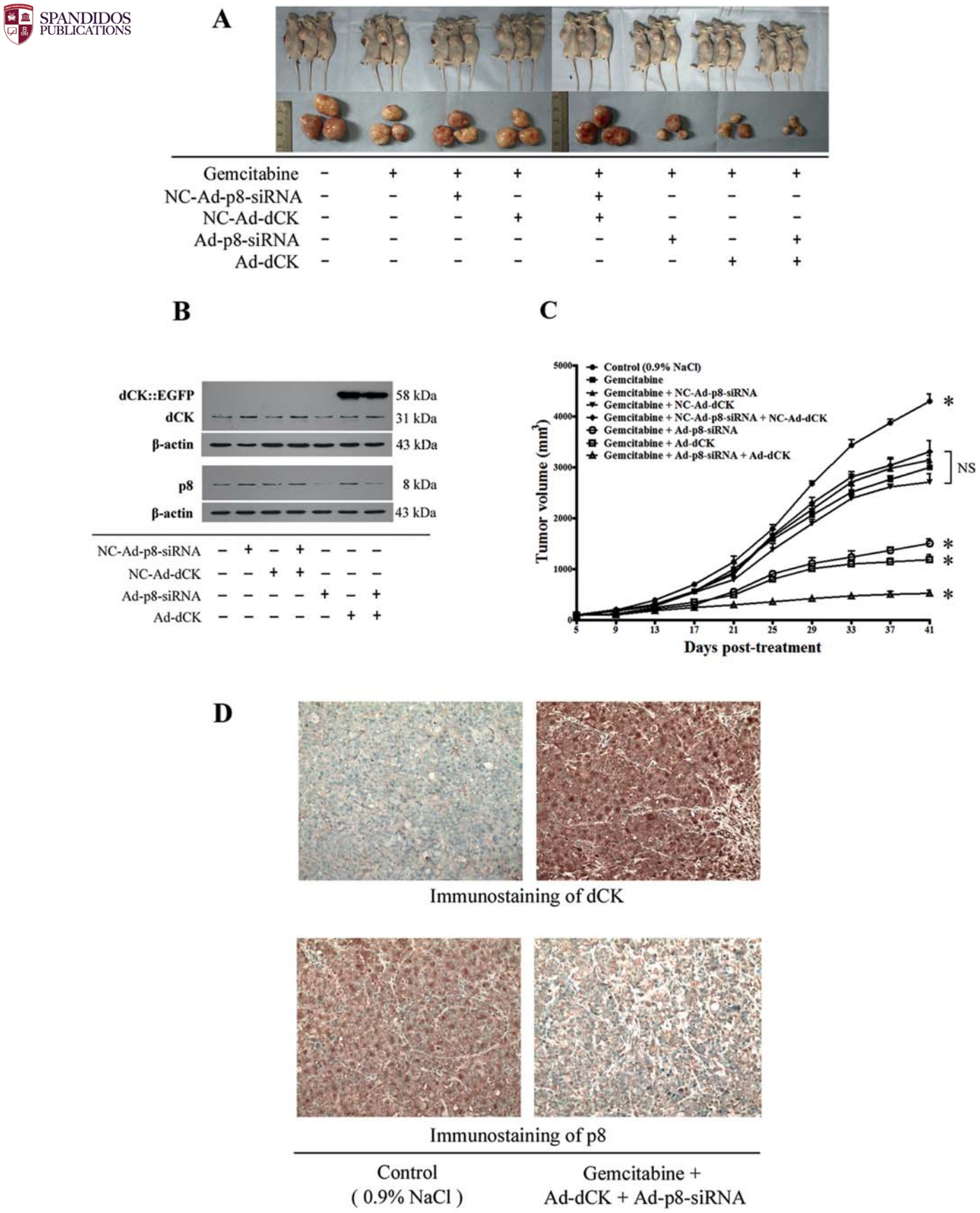

Figure 3. Adenovirus-mediated dCK overexpression and/or p8 down-regulation improves the therapeutic efficacy of gemcitabine *in vivo*. Subcutaneous pancreatic tumor xenograft models were established in 5-week-old female nude mice. When the tumor sizes reached ~100 mm^3, the mice received an intratumoral injection of $1x10^9$ pfu of the corresponding recombinant adenoviruses once per week. Gemcitabine (150 mg/kg) was injected intraperitoneally in the therapeutic groups twice per week. (A) Macroscopic samples of subcutaneous pancreatic tumors after 40 days of treatment. (B) Subcutaneous tumor growth. The tumor volumes were measured every 4 days from the beginning of treatment. NS, not significant. $^*P<0.01$ vs. the gemcitabine alone-treated group. (C) Western blot analyses were performed on tumor biopsies obtained from two mice per group after three adenovirus injections. (D) The expressions of dCK and p8 in the subcutaneous tumors after treatment were measured by immunohistochemistry. Original magnification, x200.

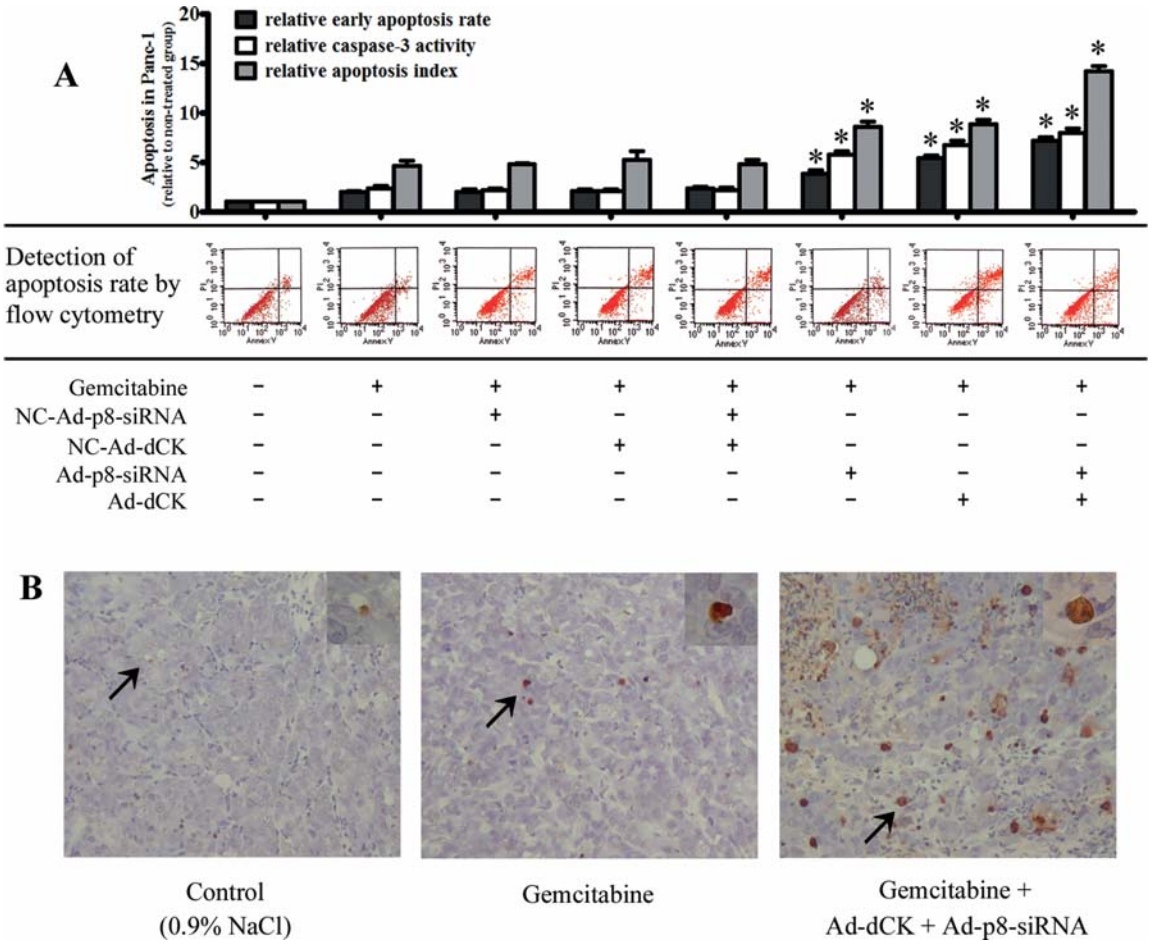

Figure 4. Detection of apoptosis. *In vitro*, after infection with the corresponding adenoviruses for 2 h, Panc-1 cells were incubated with gemcitabine (IC_{50} dose) for 72 h. After cell collection, the early apoptosis rates and caspase-3 activities were detected. *In vivo*, following preparation of 4-μm tumor sections, the apoptosis rates were detected. (A) The relative early apoptosis rates and caspase-3 activities *in vitro* as well as the relative apoptosis indexes *in vivo* are shown. All the data represent the means ± SEM of four experiments performed in triplicate. *P<0.01 vs. the gemcitabine alone-treated group. (B) TUNEL staining in sections of subcutaneous pancreatic tumors. The apoptosis index was calculated by dividing the number of TUNEL-positive cells (arrowhead) by the total number of nuclei counted in each field. Original magnification, x200.

lines Capan-2 and BxPc-3, dCK was expressed at a low level and p8 was expressed at a high level in the relatively gemcitabine-resistant cell line Panc-1.

Construction and efficiencies of the recombinant adenovirus vectors. Using the AdMax Adenovirus Creation Kit, we successfully constructed Ad-dCK and Ad-p8-siRNA, which expressed the fusion protein dCK::EGFP and knocked down p8 expression, respectively. In addition, we constructed the corresponding negative control adenoviruses (NC-Ad-dCK and NC-Ad-p8-siRNA, respectively). The constructed vectors all contained the reporter gene EGFP. The viral titers of the constructed recombinant adenoviruses were all higher than $1x10^{10}$ pfu/ml. The infection efficiencies of the viruses at a MOI of 30 for 2 h were all >80%.

Next, we tested the efficiencies of the recombinant adenoviruses after infection into Panc-1 cells (Fig. 2A-C). Infection of Panc-1 cells with Ad-dCK alone for 2 h resulted in an ~100-fold increase in the expression of dCK (P<0.01). Likewise, the expression of p8 was reduced to 22.3% of the level in untreated cells (P<0.01) after infection of Ad-p8-siRNA alone. Similar efficacies of gene regulation were observed when the two kinds of recombinant adenoviruses were co-infected into Panc-1 cells. Infection with the negative control adenovirus vectors influenced the expressions of the two genes only slightly.

Ad-dCK and/or Ad-p8-siRNA reduce the resistance of Panc-1 cells to gemcitabine in vitro. After infection with the different adenoviruses at a MOI of 30 for 2 h, Panc-1 cells were treated with gemcitabine (0.001-1000 μM) for 72 h. The percentages of viable cells were measured using MTT assays. The dose-response curves for gemcitabine are shown in Fig. 2D and E. As shown in Fig. 2F, Ad-p8-siRNA as well as Ad-dCK reduced the resistance of Panc-1 cells to gemcitabine, with reductions in the gemcitabine IC_{50} of ~68.4 and 82.6% (both P<0.01 vs. virus-untreated cells), respectively. As expected, co-infection of Ad-dCK and Ad-p8-siRNA caused the maximal reduction in the gemcitabine resistance, with a reduction in the gemcitabine IC_{50} of ~97.1% (P<0.01 vs. virus-untreated cells).

Combined treatment with Ad-dCK and Ad-p8-siRNA increases the gemcitabine efficacy in pancreatic cancer in vivo. In Panc-1 subcutaneous pancreatic tumor xenograft models in nude mice, we regulated the expressions of the two target

intratumoral injection of the recombinant adeno-The expressions of the two genes were analyzed in tumor biopsies by Western blot analyses and immunohistochemistry. As shown in Fig. 3B and D, Ad-dCK injection caused dCK overexpression in the tumor tissues. At the same time, Ad-p8-siRNA injection down-regulated p8 expression. On the basis of these gene regulations, we administered the gemcitabine chemotherapy intraperitoneally to the mice. As shown in Fig. 3A and C, after ~40 days of chemotherapy, the tumor inhibitions (vs. the control mice) in the mice treated with gemcitabine plus Ad-dCK (72.4% reduction in tumor volume, $P<0.01$) or Ad-p8-siRNA (64.9% reduction in tumor volume, $P<0.01$) were higher than those in the mice treated with gemcitabine alone (30.3% reduction in tumor volume, $P<0.01$). As expected, the most conspicuous tumor inhibition was observed in the mice treated with gemcitabine plus Ad-dCK and Ad-p8-siRNA (87.8% reduction in tumor volume, $P<0.01$ vs. the control mice). These findings were in general agreement with the *in vitro* findings.

The enhancements of gemcitabine sensitivity by adenovirus treatments are accompanied by increases in apoptosis and caspase-3 activation. In vitro, the apoptosis rates in Panc-1 cells with the corresponding treatments were analyzed by Annexin V/PI staining and flow cytometry. The results revealed that the early apoptosis rate was markedly elevated in the groups treated with gemcitabine combined with Ad-dCK and/or Ad-p8-siRNA, compared with the group treated with gemcitabine alone (Fig. 4A). Furthermore, increases in caspase-3 activation were observed (Fig. 4A), and were in line with the elevations in the apoptosis rates. Using TUNEL staining, apoptosis was detected in the subcutaneous tumors. As shown in Fig. 4B, more TUNEL-positive nuclei were observed in the treatment groups than in the control groups. In other words, the apoptosis indexes were elevated in the treatment groups (Fig. 4A).

Discussion

Gemcitabine has a pivotal role in the treatment of locally advanced and metastatic pancreatic cancer (24). Despite its relative success, the response rate is still low. Resistance to gemcitabine is a major cause of unsatisfactory improvement during pancreatic cancer treatment (9). A variety of factors have been shown to be correlated with gemcitabine resistance, including molecules associated with gemcitabine transport, metabolism and cell apoptosis (5). In the metabolism of gemcitabine, it is well known that gemcitabine phosphorylation by dCK is the rate-limiting step of gemcitabine pro-drug activation. Data from most studies have shown that deficiency of dCK is involved in gemcitabine resistance (6-8). However, some authors have different opinions, and found that the dCK levels are not directly related to gemcitabine sensitivity in pancreatic cancer cells (25). Consequently, at the beginning of our study, we evaluated the levels of dCK expression in three different pancreatic cancer lines. By real-time PCR and Western blot analyses, we demonstrated that the level of dCK expression is definitely related to gemcitabine sensitivity in pancreatic cancer cell lines, in relatively resistant Panc-1 cells, dCK is expressed at a relatively low level. In the following experiments, we also demonstrated that the overexpression of dCK reduced almost six times gemcitabine IC_{50} in Panc-1 cells. These findings confirmed clearly the crucial role of dCK in gemcitabine chemotherapy.

Since chemotherapeutic agents induce apoptotic tumor cell death and diminished apoptosis plays an important role in the resistance of tumor cells to anticancer agents (26,27), we focused on p8, which has been identified as a new target of gemcitabine in pancreatic cancer cells (14). The p8 gene is activated in the pancreas during the acute phase of pancreatitis, regeneration and pancreatic cancer development (13). In our study, different expression levels of p8 were observed in the three pancreatic cancer lines, and were negatively related to gemcitabine sensitivity. Some reports have described that p8 expression is induced by various proapoptotic stimuli and suggested that p8 may have an antiapoptotic function (28,29). Therefore, we chose p8 as another target to combine with dCK in our chemosensitization of pancreatic cancer to gemcitabine. Our data found that, the combination of Ad-p8-siRNA with Ad-dCK markedly strengthened the efficacy of gemcitabine in Panc-1 cells, as the gemcitabine IC_{50} was reduced approximately 97% vs. virus-untreated cells. Moreover, our data showed that both the early apoptosis rates and caspase-3 activities were increased in the combined treatment groups.

To further examine the findings of our *in vitro* experiments, we established Panc-1 subcutaneous xenograft pancreatic cancer models in nude mice. Compared with the gemcitabine alone-treated mice, the mice treated with gemcitabine plus Ad-dCK or Ad-p8-siRNA all showed significant reductions in tumor growth. As expected, the mice that received gemcitabine treatment combined with Ad-dCK and Ad-p8-siRNA showed the greatest inhibition of tumor growth, with a mean decrease of 87.8% in tumor growth compared with the gemcitabine alone-treated mice. Similar to the *in vitro* findings, the apoptosis index was markedly elevated in the mice treated with gemcitabine combined with Ad-dCK and Ad-p8-siRNA.

Taken together, we have demonstrated that the expressions of dCK and p8 in human pancreatic cancer cell lines are related to the cellular sensitivity to gemcitabine. Adenovirus-mediated overexpression of dCK and knockdown of p8 enhanced the gemcitabine sensitivity in pancreatic cancer cells with increases in apoptosis both *in vivo* and *in vitro*. Our encouraging data suggest that, dCK and p8 may be the important factors in the regulation of gemcitabine sensitivity in pancreatic cancer cells. Moreover, co-regulation of the two factors achieved better effects than regulating either one of them alone. Further investigations are warranted.

Acknowledgements

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References

1. Yeo TP, Hruban RH, Leach SD, Wilentz RE, Sohn TA, Kern SE, Iacobuzio-Donahue CA, Maitra A, Goggins M, Canto MI, Abrams RA, Laheru D, Jaffee EM, Hidalgo M and Yeo CJ: Pancreatic cancer. Curr Probl Cancer 26: 176-275, 2002.

2. Li D, Xie K, Wolff R and Abbruzzese JL: Pancreatic cancer. Lancet 363: 1049-1057, 2004.
3. Habiro A, Tanno S, Koizumi K, Izawa T, Nakano Y, Osanai M, Mizukami Y, Okumura T and Kohgo Y: Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells. Biochem Biophys Res Commun 316: 71-77, 2004.
4. Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML and Schilsky R: An investigational new drug treatment program for patients with gemcitabine: results for over 3000 patients with pancreatic carcinoma. Cancer 85: 1261-1268, 1999.
5. Bergman AM, Pinedo HM and Peters GJ: Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). Drug Resist Updat 5: 19-33, 2002.
6. Beausejour CM, Gagnon J, Primeau M and Momparler RL: Cytotoxic activity of 2',2'-difluorodeoxycytidine, 5-aza-2'-deoxycytidine and cytosine arabinoside in cells transduced with deoxycytidine kinase gene. Biochem Biophys Res Commun 293: 1478-1484, 2002.
7. Galmarini CM, Clarke ML, Jordheim L, Santos CL, Cros E, Mackey JR and Dumontet C: Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene. BMC Pharmacol 4: 8, 2004.
8. Bergman AM, Giaccone G, van Moorsel CJ, Mauritz R, Noordhuis P, Pinedo HM and Peters GJ: Cross-resistance in the 2',2'-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000 to standard and investigational drugs. Eur J Cancer 36: 1974-1983, 2000.
9. Ohhashi S, Ohuchida K, Mizumoto K, Fujita H, Egami T, Yu J, Toma H, Sadatomi S, Nagai E and Tanaka M: Down-regulation of deoxycytidine kinase enhances acquired resistance to gemcitabine in pancreatic cancer. Anticancer Res 28: 2205-2212, 2008.
10. Sebastiani V, Ricci F, Rubio-Viqueira B, Kulesza P, Yeo CJ, Hidalgo M, Klein A, Laheru D and Iacobuzio-Donahue CA: Immunohistochemical and genetic evaluation of deoxycytidine kinase in pancreatic cancer: relationship to molecular mechanisms of gemcitabine resistance and survival. Clin Cancer Res 12: 2492-2497, 2006.
11. Manome Y, Wen PY, Dong Y, Tanaka T, Mitchell BS, Kufe DW and Fine HA: Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside in vitro and in vivo. Nat Med 2: 567-573, 1996.
12. Van der Wilt CL, Kroep JR, Bergman AM, Loves WJ, Alvarez E, Talianidis I, Eriksson S, van Groeningen CJ, Pinedo HM and Peters GJ: The role of deoxycytidine kinase in gemcitabine cytotoxicity. Adv Exp Med Biol 486: 287-290, 2000.
13. Su SB, Motoo Y, Iovanna JL, Xie MJ, Mouri H, Ohtsubo K, Yamaguchi Y, Watanabe H, Okai T, Matsubara F and Sawabu N: Expression of p8 in human pancreatic cancer. Clin Cancer Res 7: 309-313, 2001.
14. Giroux V, Malicet C, Barthet M, Gironella M, Archange C, Dagorn JC, Vasseur S and Iovanna JL: p8 is a new target of gemcitabine in pancreatic cancer cells. Clin Cancer Res 12: 235-241, 2006.
15. Su SB, Motoo Y, Iovanna JL, Berthezene P, Xie MJ, Mouri H, Ohtsubo K, Matsubara F and Sawabu N: Overexpression of p8 is inversely correlated with apoptosis in pancreatic cancer. Clin Cancer Res 7: 1320-1324, 2001.
16. Kazuno H, Sakamoto K, Fujioka A, Fukushima M, Matsuda A and Sasaki T: Possible antitumor activity of 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine (ECyd, TAS-106) against an established gemcitabine (dFdCyd)-resistant human pancreatic cancer cell line. Cancer Sci 96: 295-302, 2005.
17. Veuger MJ, Heemskerk MH, Honders MW, Willemze R and Barge RM: Functional role of alternatively spliced deoxycytidine kinase in sensitivity to cytarabine of acute myeloid leukemic cells. Blood 99: 1373-1380, 2002.
18. Schagger H: Tricine-SDS-PAGE. Nat Protoc 1: 16-22, 2006.
19. Jiang WG, Martin TA, Lewis-Russell JM, Douglas-Jones A, Ye L and Mansel RE: Eplin-alpha expression in human breast cancer, the impact on cellular migration and clinical outcome. Mol Cancer 7: 71, 2008.
20. Jiang WG, Watkins G, Lane J, Cunnick GH, Douglas-Jones A, Mokbel K and Mansel RE: Prognostic value of rho GTPases and rho guanine nucleotide dissociation inhibitors in human breast cancers. Clin Cancer Res 9: 6432-6440, 2003.
21. Tang XY, Zhu YQ, Tao WH, Wei B and Lin XL: Synergistic effect of triptolide combined with 5-fluorouracil on colon carcinoma. Postgrad Med J 83: 338-343, 2007.
22. Wu C, Shan Y, Liu X, Song W, Wang J, Zou M, Wang M and Xu D: GalNAc-T14 may be involved in regulating the apoptotic action of IGFBP-3. J Biosci 34: 389-395, 2009.
23. Liau SS and Whang E: HMGA1 is a molecular determinant of chemoresistance to gemcitabine in pancreatic adenocarcinoma. Clin Cancer Res 14: 1470-1477, 2008.
24. Giovannetti E, Del TM, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del CM, Iannopollo M, Bevilacqua G, Mosca F and Danesi R: Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. Cancer Res 66: 3928-3935, 2006.
25. Nakano Y, Tanno S, Koizumi K, Nishikawa T, Nakamura K, Minoguchi M, Izawa T, Mizukami Y, Okumura T and Kohgo Y: Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells. Br J Cancer 96: 457-463, 2007.
26. Shrikhande SV, Kleeff J, Kayed H, Keleg S, Reiser C, Giese T, Buchler MW, Esposito I and Friess H: Silencing of X-linked inhibitor of apoptosis (XIAP) decreases gemcitabine resistance of pancreatic cancer cells. Anticancer Res 26: 3265-3273, 2006.
27. Denlinger CE, Rundall BK, Keller MD and Jones DR: Proteasome inhibition sensitizes non-small cell lung cancer to gemcitabine-induced apoptosis. Ann Thorac Surg 78: 1207-1214, 2004.
28. Mallo GV, Fiedler F, Calvo EL, Ortiz EM, Vasseur S, Keim V, Morisset J and Iovanna JL: Cloning and expression of the rat p8 cDNA, a new gene activated in pancreas during the acute phase of pancreatitis, pancreatic development, and regeneration, and which promotes cellular growth. J Biol Chem 272: 32360-32369, 1997.
29. Vasseur S, Vidal MG, Fiedler F, Bodeker H, Canepa E, Moreno S and Iovanna JL: Cloning and expression of the human p8, a nuclear protein with mitogenic activity. Eur J Biochem 259: 670-675, 1999.