The determinants of sensitivity and acquired resistance to gemcitabine differ in non–small cell lung cancer: a role of *ABCC5* in gemcitabine sensitivity

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Abstract

We examined the expression levels of the *multidrug resistance protein 5* (*ABCC5*) gene in non–small cell lung cancer (NSCLC) cell lines to clarify the relationship with the sensitivity to gemcitabine. The expression levels of *ABCC5* were inversely correlated with gemcitabine sensitivity significantly ($r = 0.628$; $P < 0.01$) in 17 NSCLC cells, whereas the expression of *ABCC5* in the gemcitabine-resistant NSCLC cell line H23/GEM-R was the same as that in parental NCI-H23 cells. Treatment with the ABCC5 inhibitor zaprinast altered the sensitivity to gemcitabine in ABCC5-expressing NSCLC cells. In addition, decreasing the expression of *ABCC5* by small interfering RNA altered the cytotoxicity to gemcitabine. These results indicate that modulation of ABCC5 activity could be used to increase the gemcitabine sensitivity in NSCLC. Previously, we found a decreased expression of deoxycytidine kinase in H23/GEM-R cells, and further investigation in this study showed an increased expression of ribonucleotide reductase subunit 1 in H23/GEM-R cells. We therefore also examined the effect of modifying the expression of both genes on gemcitabine resistance. We found that using small interfering RNA to decrease the expression of *ribonucleotide reductase subunit 1* resulted in a decreased resistance to gemcitabine in H23/GEM-R cells. Furthermore, pretreatment with pemetrexed resulted in an increased *deoxycytidine kinase* expression concomitant with the alteration of the resistance to gemcitabine in H23/GEM-R cells. The determinants for sensitivity and the acquired resistance in gemcitabine are quite different; nonetheless, modification of these factors may increase the efficacy of gemcitabine in the treatment of NSCLC. [Mol Cancer Ther 2006;5(7):1800–6]

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine) is a deoxycytidine analogue that has shown the efficacy as a treatment of many solid tumors (1). However, gemcitabine must first be transported into the cell via a nucleoside transporter, of which there are multiple forms. Once inside a cell, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form. This first stage of phosphorylation is the rate-limiting step for further phosphorylation to the active triphosphate form and thus is essential for the activation of gemcitabine (2). The subsequent incorporation of gemcitabine triphosphate into DNA, causing the addition of a single deoxynucleotide and chain termination, is most likely the major mechanism underlying the cytotoxicity of gemcitabine (3). The active diphosphate metabolite of gemcitabine also inhibits DNA synthesis indirectly through the inhibition of ribonucleotide reductase (RR). RR has two subunits (RRM1 and RRM2) and converts ribonucleoside 5′-diphosphates to the deoxyribonucleotide 5′-diphosphates that are essential for DNA synthesis. Gemcitabine blocks the *de novo* DNA synthesis pathway and decreases the intracellular concentrations of normal deoxynucleotide triphosphate pools (2).

The mechanism of sensitivity and/or resistance to gemcitabine is likely to be multifactorial, involving decreased intracellular accumulation and alteration of metabolism (4). The most active gemcitabine uptake has been found via the human equilibrative nucleoside transporter 1 (hENT1), with the hENT1 expression levels being related to the sensitivity and/or resistance to gemcitabine (5, 6). Acquired resistance to gemcitabine was shown to be associated with a dCK deficiency (6, 7). Overexpression of RR has also been shown to be a mechanism of resistance to gemcitabine (8, 9). Nonetheless, the mechanisms of sensitivity and resistance to the gemcitabine have not been clearly elucidated.

Several members of the ATP-binding cassette (ABC) transporter superfamily, such as multidrug resistance protein 1 (ABCC1), confer drug resistance to drug-sensitive cells by effluxing anticancer or antiviral agents or their metabolites from cells when expressed at high levels (10).

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Recently, ABCC5, which lacks a transmembrane domain that is present in another family member, ABCC1 (10), was shown to mediate the ATP-dependent transport of several anticancer agents and antiviral nucleosides (11) and confer resistance to gemcitabine (12). Because gemcitabine is also one of key agents in non–small cell lung cancer (NSCLC) chemotherapy (13), it is important to clarify the determinants of sensitivity and/or resistance to gemcitabine in NSCLC. Therefore, we examined the gene expression levels of ABCC5 in relation to sensitivity and/or resistance to gemcitabine in NSCLC cells. The gene expression of gemcitabine-resistant NSCLC cells and their parental cells was also compared, with the aim of elucidating the determinants of acquired resistance to this agent.

Materials and Methods

Cell Lines and Chemicals

The following human NSCLC cell lines were used in this study: 13 adenocarcinomas (A549, NCI-H23, PC-9, PC-14, VMRC-LCD, VMRC-LCF, RERF-LC-MT, RERF-LC-OK, RERF-LC-MS, NCU-LC-201, ACC-LC-94, ACC-LC-176, and SK-LC-10), 2 squamous cell carcinomas (PC10 and QG56), and 2 large cell carcinomas (NCI-H460 and SK-LC-6). Cells from the gemcitabine-resistant human lung adenocarcinoma cell lines H23/GEM1, H23/GEM10, and H23/GEM-R (IC_{50} = 47.55 nmol/L) were established as described previously (6). Cells were cultured in RPMI 1640 (or, for A549, in DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% (v/w) penicillin/streptomycin in a humidified chamber (37°C, 5% CO_2). Gemcitabine and pemetrexed were provided by Eli Lilly Pharmaceuticals (Indianapolis, IN). The ABCC5 inhibitor zaprinast was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Total RNA Extraction and Reverse Transcription-PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was done in a volume of 20 μL by SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen) according to the manufacturer's instructions. The sequences of the ABCC4 and ABCC5 primers and the PCR conditions were as described previously (14, 15). Amplified products were separated by 2% agarose gel electrophoresis, and bands were visualized by staining with ethidium bromide. We also did real-time PCR with the LightCycler FastStart DNA SYBR Green kit (Roche Diagnostics, Indianapolis, IN). We used melting curve analysis to control for specificity of the amplification products. The number of transcripts was calculated from a standard curve obtained by plotting the known input of six different concentrations versus the PCR cycle number at which the detected fluorescence intensity reached a fixed value. The sequences of dCK and RRM1 primers were as follows: dCK, 5′-tgcagggaagtcaacatt-3′ (forward) and 5′-tcccac-cattttctgag-3′ (reverse) and RRM1, 5′-catccacattgctgagcc-taac-3′ (forward) and 5′-cccagtcttcaaaccctgctt-3′ (reverse).

The PCR consisted of 45 cycles of 94°C for 15 seconds and 60°C for 1 minute. For each sample, the data were normalized to that for the housekeeping gene *glyceralde-hyde-3-phosphate dehydrogenase* (*GAPDH*).

Concentration of Gemcitabine for IC_{50}

Cells were cultured at 5,000 per well in 96-well tissue culture plates. To assess cell viability, stepwise 10-fold dilutions of the anticancer drug were added 2 hours after plating and the cultures were incubated at 37°C for 96 hours. At the end of the culture period, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) were added, the cells were incubated for a further 4 hours, and the absorbance was measured at 490 nm using an ELISA plate reader. Mean values were calculated from three independent experiments done in quadruplicate. Chemosensitivity is expressed here as the drug concentration for IC_{50} determined from the concentration-effect relationship using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

Inhibition of ABCC5 Activity by Zaprinast

ABCC5-expressed PC-9 and NCU-LC-201 cells (1×10^5) were plated in six-well tissue culture plates. As an ABCC5 inhibitor, we used zaprinast as referred previously (16). After 24 hours, the cells were exposed to the ABCC5 inhibitor zaprinast for 30 minutes and we added gemcitabine in the presence of zaprinast. After an additional 48 hours, we counted the viable cells with trypan blue staining to evaluate the cytotoxicity of gemcitabine.

Transfection and Small Interfering RNA Experiments

Approximately 2×10^5 cells were plated in six-well plates in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. After 24 hours, cells were transfected with small interfering RNA (siRNA) oligonucleotide or negative control siRNA using Oligofectamine (Invitrogen) to result in a final RNA concentration of 50 nmol/L in serum-free Opti-MEM (Invitrogen) according to the manufacturer's instructions. At 24 hours after transfection, we changed the medium to RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. After an additional 24 hours, total RNA was extracted, or the cells were treated with gemcitabine for 48 hours and then we counted viable cells with trypan blue staining to evaluate the cytotoxicity of gemcitabine. The siRNA oligonucleotides for ABCC5 and RRM1 (predesigned siRNA, ID number 117361 and 110817, respectively) were purchased from Ambion (Austin, TX). The negative control siRNA (Silencer Negative Control #1 siRNA), which does not induce nonspecific effects on gene expression, was also purchased from Ambion.

Effect of Pemetrexed on Gemcitabine Cytotoxicity

H23/GEM-R (1×10^5) cells were plated in six-well plates in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Cells were treated with 100 nmol/L or 1 μmol/L pemetrexed for 2 hours followed by 50 or 100 nmol/L gemcitabine for 48 hours, and then we counted viable cells with trypan blue staining to evaluate the cytotoxicity of gemcitabine.

Statistical Analysis

Spearman's test was used to analyze the correlation between the expression of *ABCC5* gene and the IC_{50} values for gemcitabine. The differences in the cell viability between samples were evaluated with Student's unpaired *t* test. The level of significance was set at 5% using a two-sided analysis.

Results

Relationship between Cytotoxicity of Gemcitabine and Expression of *ABCC5* Gene

Quantitative real-time RT-PCR was used to determine the expression levels of the *ABCC5* gene in 17 NSCLC cells. The *ABCC5* gene was expressed in all cell lines, and the IC_{50} values to gemcitabine of all cell lines were compared with their relative *ABCC5* expressions. IC_{50} values for gemcitabine in each cell line were as follows: A549, 11,360 nmol/L; NCI-H23, 0.1 nmol/L; PC-9, 545 nmol/L; PC-14, 20 nmol/L; VMRC-LCD, 15,169 nmol/L; VMRC-LCF, 13 nmol/L; RERF-LC-MT, 310 nmol/L; RERF-LC-OK, 996 nmol/L; RERF-LC-MS, 50 nmol/L; NCU-LC-201, 0.5 nmol/L; ACC-LC-94, 4 nmol/L; ACC-LC-176, 19 nmol/L; SK-LC-10, 1,068 nmol/L; PC10, 12,955 nmol/L; QG56, 1,976 nmol/L; NCI-H460, 5,388 nmol/L; and SK-LC-6, 36 nmol/L. We found a clear relation between the *ABCC5* gene expression and gemcitabine sensitivity in the NSCLC cells tested ($r = 0.628$; $P < 0.01$; Fig. 1A) but the expression of *ABCC4* were not (data not shown). In contrast, the expression of *ABCC5* gene in gemcitabine-resistant NSCLC cell line H23/GEM-R was the same as that in parental cells (Fig. 1B).

Inhibition of Gemcitabine Cytotoxicity

To elucidate whether ABCC5 actually affects gemcitabine sensitivity, we added the ABCC5 inhibitor zaprinast at a noncytotoxic concentration (1 or 10 μmol/L) to gemcitabine for two NSCLC cell lines: NCU-LC-201 cells (IC_{50} of gemcitabine, 0.5 nmol/L) and PC-9 cells (IC_{50} of gemcitabine, 545 nmol/L). We used RT-PCR to confirm that both cell types expressed the *ABCC5* gene (data not shown). As shown in Fig. 2, the sensitivity of NCU-LC-201 and PC-9 cells to gemcitabine was inhibited by 30 minutes of pretreatment with zaprinast in a dose-dependent manner. However, the expression of the *ABCC5* gene was not modulated significantly after treatment with zaprinast at any of the concentrations tested (data not shown).

To confirm the alteration of gemcitabine cytotoxicity by ABCC5, PC-9 cells were transfected with siRNAs directed against ABCC5, and control siRNA–transfected or untransfected cells were used as controls because zaprinast may also act as an ABCC4 inhibitor. Cells were harvested at 48 hours after transfection, and extracts were prepared and analyzed by quantitative real-time RT-PCR. The expression levels of the *ABCC5* gene were decreased at 48 hours after siRNA transfection (Fig. 3A) but the expression levels of *ABCC4* were not (data not shown). Forty-eight hours after transfection with ABCC5 siRNA or negative control siRNA or with no transfection, PC-9 cells were treated with
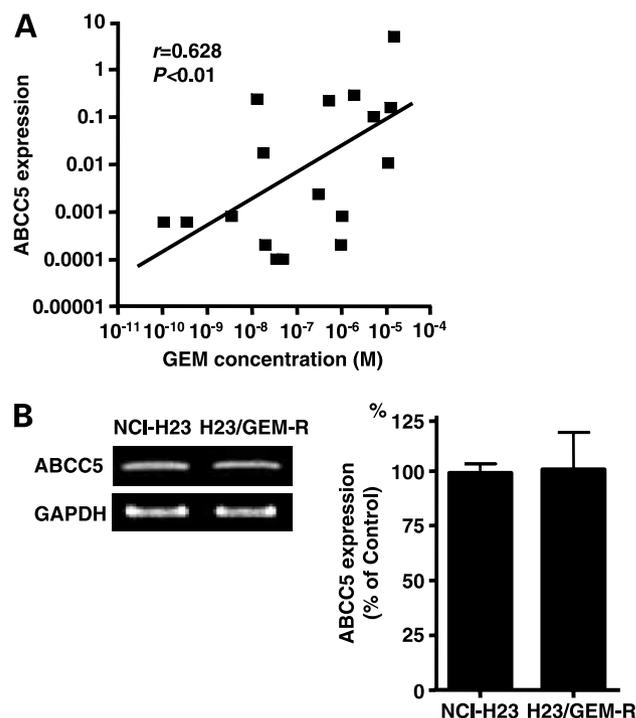

Figure 1. Gemcitabine cytotoxicity and expression level of *ABCC5* in NSCLC cells. **A,** there was a clear relation between the *ABCC5* expression and gemcitabine sensitivity in NSCLC cell lines ($n = 17$; $r = 0.628$; $P < 0.01$). *Points,* mean of three independent sensitivity tests done in quadruplicate. Expression levels are relative to expression by glyceraldehyde-3-phosphate dehydrogenase. Statistical significance of the correlations was determined by Spearman's correlation test. **B,** comparison of gene expression levels between NCI-H23 cells and the gemcitabine (*GEM*)-resistant cell line H23/GEM-R established as described previously (6). Expression levels of *ABCC5* in H23/GEM-R cells were the same as that in NCI-H23 cells by RT-PCR and real-time PCR. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

500 nmol/L gemcitabine for 48 hours. The cytotoxicity to gemcitabine was significantly higher for cells exposed to gemcitabine in the presence of ABCC5 siRNA than for cells transfected with negative control siRNA and for untransfected cells (Fig. 3B). Because negative control siRNA does not affect gene expression, these results indicate that decreasing the expression of ABCC5 by siRNA altered the cytotoxicity to gemcitabine.

RRM1 Suppression and Enhanced Cytotoxicity to Gemcitabine in H23/GEM-R Cells by RRM1 siRNA

Davidson et al. (9) showed that increased expression of RRM1 was related to gemcitabine resistance in NSCLC cell lines; hence, we investigated RRM1 expression in H23/GEM-R cells. We isolated gemcitabine-resistant subline H23/GEM1 cells produced by the exposure of NCI-H23 cells to 1 nmol/L gemcitabine for 6 weeks, then isolated H23/GEM10 cells by exposing the produced H23/GEM1 cells to 10 nmol/L gemcitabine, and then we finally cloned H23/GEM-R cells as described previously (6). Quantitative real-time RT-PCR analysis showed a direct relationship between the concentration of gemcitabine selected for the

NCI-H23 sublines and the gene expression level of *RRM1*, with the expression level of *RRM1* being ∼2.5-fold higher in gemcitabine-resistant cells than in parental cells (Fig. 4A). We also examined the gene expression levels of *RRM1* in 17 NSCLC cells but found no relationship between RRM1 expression levels and gemcitabine sensitivity (data not shown).

H23/GEM-R cells were then transfected with siRNAs directed against RRM1, and control siRNA–transfected or untransfected cells were used as controls. Cells were harvested at 48 hours after transfection, and extracts were prepared and analyzed by quantitative real-time RT-PCR. The expression levels of *RRM1* gene were decreased at 48 hours after siRNA transfection (Fig. 4B). We then investigated whether RRM1 siRNA would enhance the cytotoxicity to gemcitabine in H23/GEM-R cells. Forty-eight hours after transfection with RRM1 siRNA or negative control siRNA or with no transfection, H23/GEM-R cells were treated with 50 or 100 nmol/L gemcitabine for 48 hours. The cytotoxicity to gemcitabine was significantly higher for cells exposed to gemcitabine in
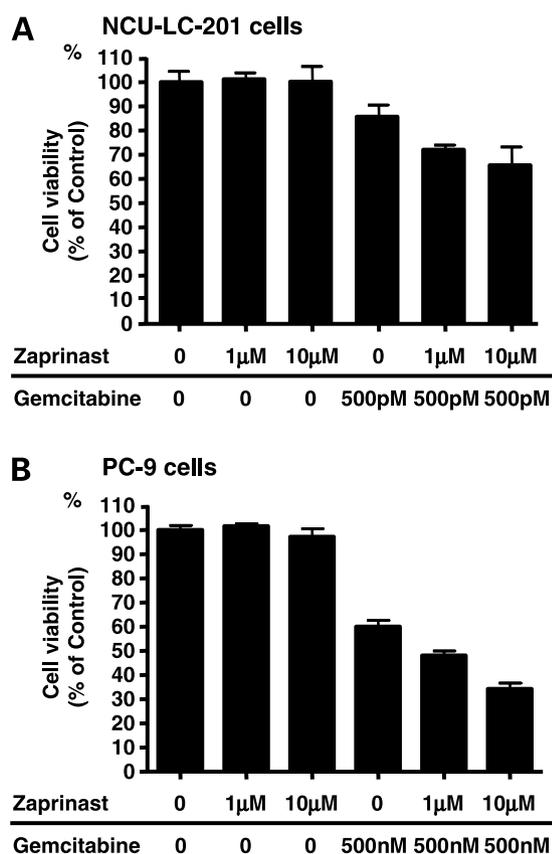
Figure 3. Modification of gemcitabine cytotoxicity by ABCC5 siRNA. **A,** gene expression levels of *ABCC5* in PC-9 cells transfected with siRNAs directed against ABCC5 and in control siRNA–transfected or untransfected PC-9 cells. Cytotoxicity to 500 nmol/L gemcitabine in PC-9 cells transfected with siRNA directed against ABCC5 and in control siRNA–transfected or untransfected PC-9 cells. **B,** cell viability following gemcitabine treatment for 48 h in PC-9 cells transfected with ABCC5 differed significantly from that in control siRNA–transfected or untransfected PC-9 cells. *, $P < 0.05$.

Figure 2. Modification of gemcitabine cytotoxicity by the ABCC5 inhibitor zaprinast. NCU-LC-201 (IC$_{50}$ of gemcitabine, 0.5 nmol/L) and PC-9 (IC$_{50}$ of gemcitabine, 545 nmol/L) cells were incubated in 1 or 10 μmol/L zaprinast for 30 min, and we added gemcitabine in the presence of zaprinast. After an additional 48 h, we counted the viable cells with trypan blue staining to evaluate the cytotoxicity of gemcitabine.
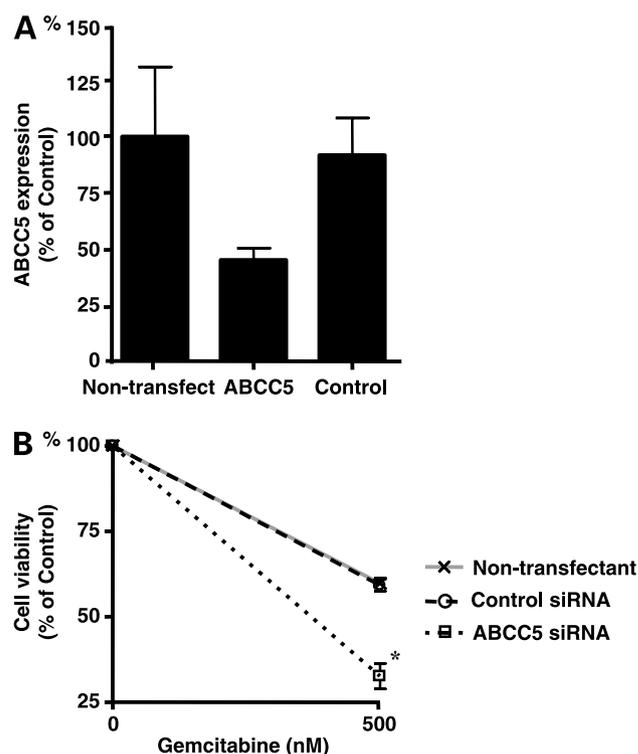
the presence of RRM1 siRNA than for cells transfected with control siRNA and for untransfected cells (Fig. 4C). Because negative control siRNA does not affect gene expression, these results indicate that decreasing the expression of RRM1 by siRNA altered the cytotoxicity to gemcitabine.

Modification of dCK Expression by Pemetrexed and Gemcitabine Cytotoxicity

Previously, we found that the expression of dCK was significantly lower in H23/GEM-R cells than in parental cells (6). Recent studies have found that pretreatment with pemetrexed enhanced the dCK expression, which resulted in a synergic effect on gemcitabine cytotoxicity in cancer cells (17, 18). We therefore examined the gene expression levels of *dCK* in NCI-H23 and H23/GEM-R cells after treatment with 100 nmol/L or 1 μmol/L pemetrexed for 2 hours and found that the expression of the *dCK* gene was decreased in H23/GEM-R cells and that pemetrexed treatment enhanced the *dCK* expression levels in a dose-dependent manner (Fig. 5A). In contrast, pemetrexed treatment did not affect ABCC5 expression (data not shown). Pemetrexed treatment at either 100 nmol/L or 1 μmol/L for 48 hours had no effect on

the viability of H23/GEM-R cells (Fig. 5B). We found that pretreatment with pemetrexed for 2 hours altered the cytotoxicity of H23/GEM-R cells to gemcitabine for 48 hours (Fig. 5C).

Discussion

In this study, we found that the expression levels of the *ABCC5* gene were inversely correlated with gemcitabine sensitivity significantly in NSCLC cells. In contrast, the expression of the *ABCC5* gene was the same in gemcitabine-resistant NSCLC cells and parental cells.
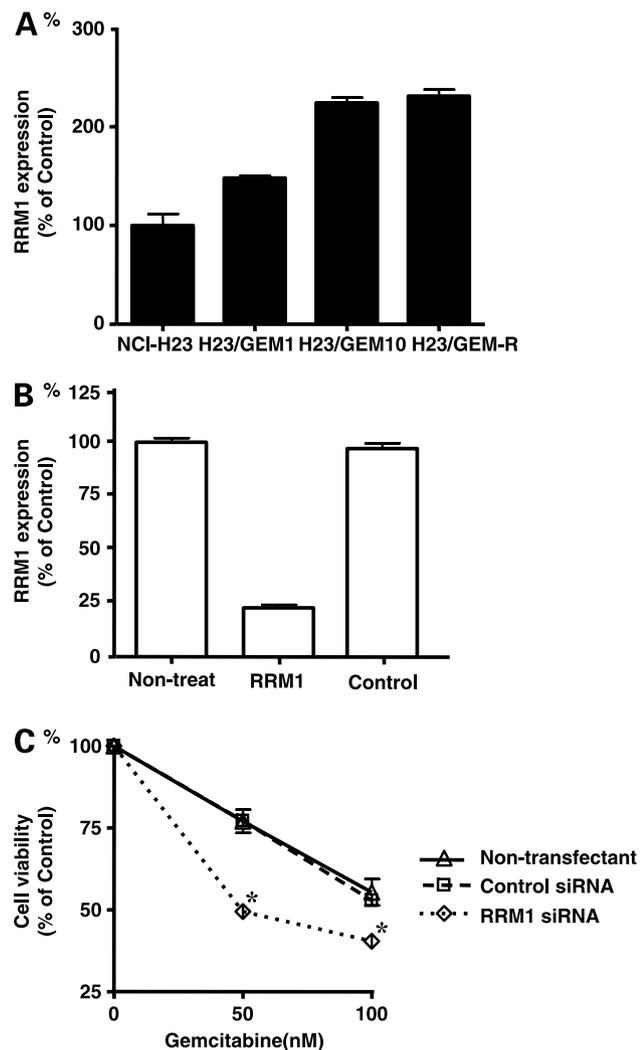

Figure 4. RRM1 modification and enhanced cytotoxicity to gemcitabine in H23/GEM-R cells. **A,** gene expression levels of *RRM1* in NCI-H23, their sublines continuously exposed to gemcitabine (H23/GEM1 and H23/GEM10), and gemcitabine-resistant clone H23/GEM-R. **B,** gene expression levels of *RRM1* in H23/GEM-R cells transfected with siRNAs directed against RRM1 and in control siRNA – transfected or untransfected H23/GEM-R cells. Cytotoxicity to 50 or 100 nmol/L gemcitabine in H23/GEM-R cells transfected with siRNAs directed against RRM1 and in control siRNA – transfected or untransfected H23/GEM-R cells. **C,** cell viability following gemcitabine treatment for 48 h differed significantly between H23/GEM-R cells transfected with RRM1 siRNA and control siRNA – transfected or untransfected H23/GEM-R cells. *, $P < 0.05$.
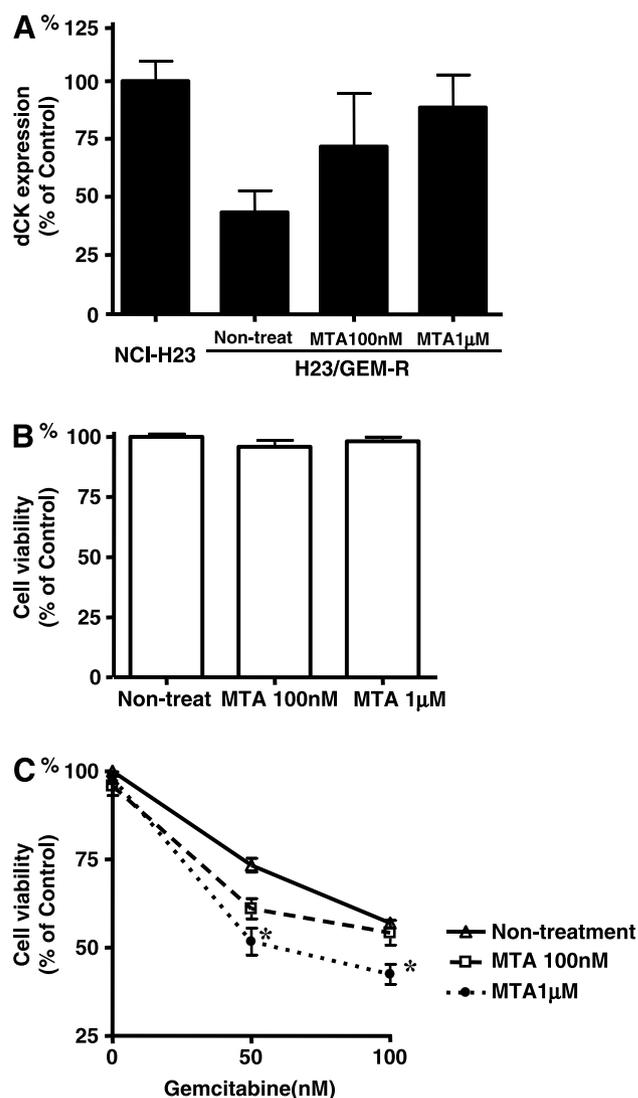

Figure 5. Modification of *dCK* expression by pemetrexed and gemcitabine cytotoxicity in H23/GEM-R cells. **A,** *dCK* gene expression in NCI-H23 and H23/GEM-R cells and dose-dependent modification of *dCK* expression in H23/GEM-R cells by 2 h of treatment with pemetrexed. **B,** pemetrexed treatment at either 100 nmol/L or 1 μmol/L for 48 h had no effect on the viability of H23/GEM-R cells. Pretreatment with pemetrexed at 100 nmol/L or 1 μmol/L for 2 h altered the cytotoxicity of H23/GEM-R cells to gemcitabine. **C,** cell viability following gemcitabine treatment for 48 h differed significantly between H23/GEM-R cells pretreated with pemetrexed at 1 μmol/L and H23/GEM-R cells without pretreatment with pemetrexed. *, $P < 0.01$.

Multidrug resistance in cancer cells is often attributed to ABCC transporters via the cotransport of anticancer drugs with glutathione or glutathione-drug or glucuronide-drug conjugates (10, 14, 15). ABCC5 lacks the NH_2-terminal hydrophobic extension that is present in ABCC1, with the structure varying among different transport proteins. When transfected into drug-sensitive cells, ABCC5 confers resistance to several anticancer and antiviral nucleosides by active efflux of the monophosphorylated metabolites of these drugs (11, 19). Reid et al. (19) reported that ABCC5

transfectants of HEK 293 cells were cross-resistant to 6-thioguanine, 6-mercaptopurine, and 9-(2-phosphonylme-thoxynyl)-adenine but not to several other agents, including gemcitabine and 5-fluorouracil. In contrast, recent studies have shown that ABCC5 confers resistance to gemcitabine or 5-fluorouracil by efflux of the monophos-phorylated metabolites of these anticancer nucleosides (12, 20). In the present study, we found that the ABCC5 inhibitor zaprinast mediated sensitivity to gemcitabine. In addition, a decreased expression of *ABCC5* by siRNA altered the cytotoxicity to gemcitabine. The clear relationship found between the expression of *ABCC5* and sensitivity to gemcitabine suggests that ABCC5 is associated with the efflux of gemcitabine and that ABCC5 expression is a determinant to the gemcitabine sensitivity in NSCLC.

In contrast, the expression of *ABCC5* was the same in gemcitabine-resistant and parental cells. Consistent with the ABCC5 results, we previously found that the expression levels of *hENT1* were significantly correlated with gemcitabine sensitivity in NSCLC cells, whereas the expression of *hENT1* in H23/GEM-R cells did not change compared with that in parental NCI-H23 cells (6). We did not find the relationship between the expression of *hENT1* and *ABCC5* (data not shown), suggesting that these two transporters do not regulated coordinately. Nucleoside transporters mediate the cellular uptake of physiologic nucleosides for nucleic acid synthesis in the salvage pathways in many cell types. These transporters also play an important role in the *in vivo* disposition and intracellular targeting of many nucleoside analogues used in anticancer therapies. Mammalian cells contain two major nucleoside transporter gene families: (*a*) equilibrative nucleoside transporters and (*b*) concentrative nucleoside transporters (21). hENT1 is the most active transporter for gemcitabine uptake, and hENT1 expression levels are correlated with gemcitabine cytotoxicity (5, 6). Taking these previous observations with the present result that ABCC5 may be associated with efflux of gemcitabine, we postulate that an increased intracellular concentration of gemcitabine is one of the most important factors influencing the sensitivity to gemcitabine in NSCLC.

To elucidate the determinants of acquired resistance to gemcitabine, we compared the gene expression of *RRM1* between H23/GEM-R and NCI-H23 cells and found that it was higher in the former. Previous studies have shown that overexpression of RR, especially RRM1, is also a mechanism of resistance to gemcitabine (8, 9). To confirm the previous results, in this study we examined the relationship between the expression of *RRM1* and the cytotoxicity to gemcitabine. Interestingly, *RRM1* expression increased gradually in the process of establishing gemcitabine-resistant cells. Furthermore, suppression of *RRM1* expression by siRNA restored gemcitabine cytotoxicity. Our results indicate that the induction of RRM1 expression by exposure to gemcitabine resulted in the acquisition of resistance to gemcitabine. Rosell et al. (22) have recently shown that *RRM1* gene expression is a predictive marker of

survival in gemcitabine- and cisplatin-treated patients. These results strongly suggest that RRM1 expression is an important determinant of the responses to gemcitabine-containing chemotherapy.

We previously used real-time PCR to show decreased expression of *dCK* in gemcitabine-resistant cells (6), and this was confirmed in the present study. dCK mediates the rate-limiting step of the first phosphorylation of gemcitabine for further phosphorylation to the active triphosphate form; hence, down-regulation of dCK by a negative feedback pathway is thought to be one of the mechanisms of resistance to nucleotide analogues (2). It is thought that decreased gemcitabine metabolism to its active form by dCK inactivation is one of the mechanisms underlying the development of acquired resistance.

Because recent studies have shown the enhancement of dCK expression levels by treatment with pemetrexed (17, 18), we used this agent in the present study. Similar to the previous studies, we found that treatment with pemetrexed enhanced *dCK* expression concomitant with altering the cytotoxicity to gemcitabine. These results indicate that modification of dCK expression recovers the cytotoxicity to gemcitabine. In addition, the combination of gemcitabine and pemetrexed exhibits order-dependent synergistic cytotoxic activity concomitant with enhancing *dCK* expression, thus suggesting the efficacy of gene expression modulation by chemotherapy combinations in NSCLC (17, 18). Indeed, the administration of pemetrexed followed by gemcitabine met the protocol-defined efficacy criteria and was less toxic than administering gemcitabine followed by pemetrexed (23). These results indicate a new treatment option for NSCLC chemotherapy, although further evaluation in randomized trials remains necessary.

Increased RR expands the size of the deoxynucleotide triphosphate pools that competitively inhibit the incorporation of gemcitabine triphosphated into DNA (2). These expanded deoxynucleotide triphosphate pools further down-regulate the activity of dCK via a negative feedback pathway. A recent study showed that down-regulation of dCK increased the resistance of human leukemia cells to cladribine and clofarabine and increased the activity of RR in cells resistant to fludarabine (24, 25). These results strongly suggest that decreased phosphorylation of gemcitabine (induced by decreased dCK expression) and increased deoxynucleotide triphosphate pools (induced by increased RR expression) together result in the development of acquired resistance to gemcitabine. Therefore, we postulate that the alteration of intracellular gemcitabine activity has a crucial role in the acquisition of resistance to gemcitabine.

We found that the mechanisms of sensitivity and resistance to gemcitabine are multifactorial, with the determinants differing greatly. However, modification of the determinants may increase the efficacy of gemcitabine treatment in NSCLC. Future clinical studies should investigate whether these factors could be used as predictive markers of the sensitivity and/or resistance to gemcitabine in NSCLC.

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