Gemcitabine and Cytosine Arabinoside Cytotoxicity: Association with Lymphoblastoid Cell Expression

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Abstract

Two cytidine analogues, gemcitabine (dFdC) and 1-β-D-arabinofuranosylcytosine (AraC), show significant therapeutic effect in a variety of cancers. However, response to these drugs varies widely. Evidence from tumor biopsy samples shows that expression levels for genes involved in the cytidine transport, metabolism, and bioactivation pathway contribute to this variation in response. In the present study, we set out to test the hypothesis that variation in gene expression both within and outside of this "pathway" might influence sensitivity to gemcitabine and AraC. Specifically, Affymetrix U133 Plus 2.0 GeneChip and cytotoxicity assays were performed to obtain basal mRNA expression and IC_{50} values for both drugs in 197 ethnically defined Human Variation Panel lymphoblastoid cell lines. Genes with a high degree of association with IC_{50} values were involved mainly in cell death, cancer, cell cycle, and nucleic acid metabolism pathways. We validated selected significant genes by performing real-time quantitative reverse transcription-PCR and selected two representative candidates, NT5C3 (within the pathway) and FKBP5 (outside of the pathway), for functional validation. Those studies showed that down-regulation of NT5C3 and FKBP5 altered tumor cell sensitivity to both drugs. Our results suggest that cell-based model system studies, when combined with complementary functional characterization, may help to identify biomarkers for response to chemotherapy with these cytidine analogues. [Cancer Res 2008;68(17):7050–8]

Introduction

Studies of gene expression may make it possible to identify biomarkers that will help predict clinical response to antineoplastic drug therapy. Many of these drugs have narrow therapeutic indexes. Therefore, it is crucial to identify biomarkers that might help to maximize efficacy and minimize drug-related toxicity. Most previous studies have focused on the relationship of expression signatures in tumor tissue to therapeutic response (1–3). However, individual variation in expression patterns controlled by germline DNA can also play an important role in response. Genes encoding proteins involved in drug transport, metabolism, activation, deactivation, or drug targets and downstream signaling pathways could all potentially influence drug response phenotypes (4). To test the hypothesis that variation in basal gene expression might affect sensitivity or resistance to chemotherapy, we have used a Human Variation Panel lymphoblastoid cell line model system consisting of 197 cell lines for which we obtained gene expression data using Affymetrix U133 Plus 2.0 GeneChips and drug-related cytotoxicity phenotypes. Although their expression profiles are not identical with those of tumor cells, these cell lines provide an opportunity to query the effect on drug response of common variation across the genome, providing data that cannot be obtained with other model systems such as the NCI-60 cell lines. Therefore, these Human Variation Panel cell lines can serve as an initial screen to identify candidate genes for which variation in expression might contribute to variation in drug response phenotypes.

We used two cytidine analogues, gemcitabine (dFdC) and 1-β-D-arabinofuranosylcytosine (AraC), to determine whether individual variation in basal gene expression might influence drug sensitivity. These drugs have been used to treat many cancers, and they share similar chemical structures, metabolic pathways, and mechanisms of action (5–8). Both are prodrugs that must be transported into cells, activated by kinases to form active diphosphorylated and triphosphorylated metabolites, and inactivated by dephosphorylation. Seventeen genes are involved in this cytidine analogue "pathway" (Supplementary Table S1). The triphosphates, AraCTP and dFdCTP, can be incorporated into DNA, terminating DNA synthesis (8, 9). dFdCDP can also inhibit ribonucleotide reductases, enzymes that catalyze the conversion of ribonucleotides to deoxyribonucleotidase (10, 11).

Gemcitabine is used to treat solid tumors (6, 12), whereas AraC is a major component of the therapy of acute myelogenous leukemia (AML; refs. 6, 7, 13). Clinical response to both drugs varies widely (8, 14, 15). Most previous studies have focused on variation in the expression of genes within the known cytidine analogue metabolism and activation pathway (1, 16, 17). However, very little information is available with regard to genes outside of that pathway. Therefore, we have used these lymphoblastoid cell lines to explore the possible contribution of individual variation in basal gene expression to gemcitabine and AraC sensitivity.

Specifically, drug cytotoxicity and basal expression array data were obtained for 197 lymphoblastoid cell lines. Selected genes significantly associated with cytotoxicity were then validated functionally. A series of functional analyses were performed for two candidate genes, *NT5C3*, within the cytidine analogue pathway, and *FKBP5*, outside of that pathway. Specific siRNA "knockdown" confirmed the results of the association study, and the functional effects of both genes on response to gemcitabine and AraC was explored. Therefore, the use of these cell lines to identify pharmacogenomic candidate genes for cytidine analogue cytotoxicity resulted in novel hypotheses that can now be tested in clinical translational studies.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines. Lymphoblastoid cell lines from 60 Caucasian-American, 54 African-American, 60 Han Chinese-American, and 23 Centre d' Etude du Polymorphisme Humain (CEPH; Caucasian-American) unrelated subjects were purchased from the Coriell Institute. Human SU86 pancreatic cancer cells were a gift from Dr. Daniel D. Billadeau, Mayo Clinic, Rochester, MN. Human breast cancer MDA-MB-231 cells were obtained from the American Type Culture Collection.

Drugs and cell proliferation assays. AraC was purchased from Sigma-Aldrich, and gemcitabine was provided by Eli Lilly. Drugs were dissolved in DMSO and were frozen at −20°C. Assays were performed in triplicate at each drug concentration using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega Corporation) in 96-well plates (Corning) at a density of 5×10^4 cells per well. One hour after plating, 10 μL of gemcitabine (0.1 nmol/L–1 mmol/L) or AraC (1 nmol/L–10 mmol/L) was added to the wells, and plates were read in a Safire^2 microplate reader (Tecan AG) after 72-h incubations. Twelve randomly selected lymphoblastoid cell lines were used to repeat the cytotoxicity studies 3 mo later. Human tumor cell line cytotoxicity was determined in a similar fashion except the cells were incubated overnight before the addition of drug.

Expression array data. Total RNA was extracted using Qiagen RNeasy Mini kits (QIAGEN, Inc.). RNA quality was tested using an Agilent 2100 Bioanalyzer, followed by hybridization to Affymetrix U133 Plus 2.0 GeneChips. Only 26,653 probe sets that could be verified using the RefSeq RNA database were used in our analyses.

Real-time quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (QRT-PCR) was performed with the 1-step, Brilliant SYBR Green QRT-PCR kit (Stratagene) using primers purchased from Qiagen. All experiments were performed in triplicate, with β-actin as an internal control. Reverse transcribed Universal Human reference RNA (Stratagene) was used to generate a standard curve.

Transient transfection and RNA interference. Human breast cancer MDA-MB-231 and SU86 pancreatic cancer cell lines were used to perform siRNA studies. Cells were grown to 30% to 50% confluence in 6-well plates, and lipofectamine RNAMAX reagent (Invitrogen) was used to perform the transfections.

Western blot analysis. Western blot analysis was performed with lysates from cells transiently expressing siRNA 48 h after transfection with control, NT5C3, FKBP5, NT5C2, or FKBP1A siRNA. Specifically, 30 μg of protein was subjected to electrophoresis on 12% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes that were incubated overnight at 4°C with primary antibodies, followed by the secondary antibody. Bands were detected with enhanced chemiluminescence (Amersham). Antibodies were obtained from GenWay Biotech, Abcam, and Novus Biologicals.

Intracellular gemcitabine and AraC metabolites. High performance liquid chromatography (HPLC) was used to measure intracellular AraCDP, AraCTP, dFdCDP, and dFdCTP concentrations. Nucleotide extracts were prepared with a modification of the method of Van Haperen and colleagues (18) after treatment for 8 h with the average IC_{50} concentration for each drug. Cells (5×10^6) were then centrifuged and washed with ice-cold PBS, followed by resuspension in 135 μL PBS with 15 μL of 100 μmol/L AraCTP or dFdCTP as internal standards. Subsequently, 50 μL of 40% TCA was added, followed by vortexing and centrifugation. The supernatant was neutralized with 400 μL of trioctylamine/trichlorotrifluoroethane (1:4). After centrifugation, the aqueous phase was subjected to HPLC using a ZirChrom SAX HPLC column with photo-diode array detection and a gradient from 100% 10 mmol/L K_2HPO_4 and 40 mmol/L NaCl (pH 6.8), to 65% 100 mmol/L K_2HPO_4 and 400 mmol/L K_2HPO_4 (pH 6.8).

Caspase-3/7 activity assay. Caspase-3/7 activity was determined as a measure of apoptosis using the Caspase-Glo 3/7 Assay kit (Promega BioSciences). siRNA-transfected cells were then treated for 72 h with increasing concentrations of gemcitabine or AraC. One hundred microliters of Caspase-Glo 3/7 Reagent was added, and the cells were incubated at room temperature for 1 h, followed by the measurement of luminescence.

Statistical methods. Three different logistic functions were used to fit the cytotoxicity data. The logistic model with the lowest mean square error was used to determine IC_{50} values. Expression array data were normalized

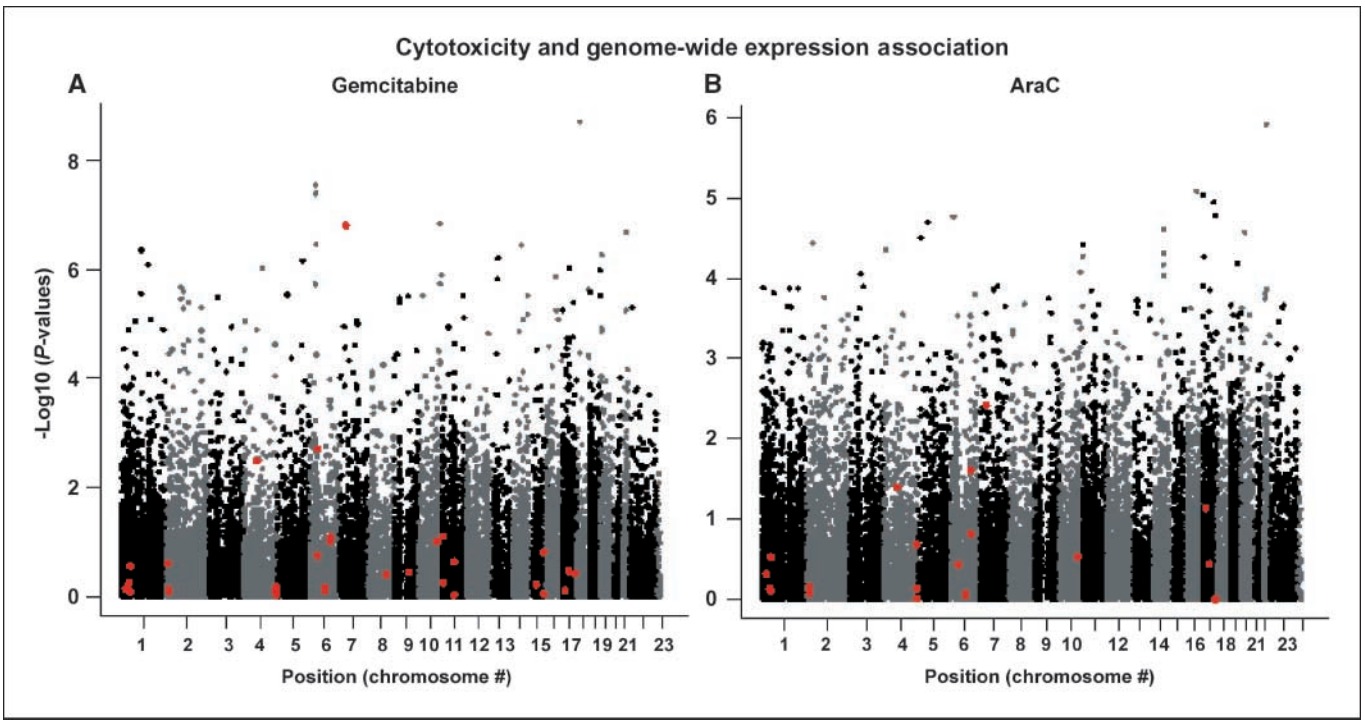

Figure 1. Association between expression array data and IC_{50} values for gemcitabine (*A*) and AraC (*B*). Each dot on the *y*-axis represents the $-\log_{10}$ (*P* value) for the probe set with the lowest *P* value for each gene. Probe sets are plotted on the *x*-axis with regard to the chromosomal location of their genes. *Red dots,* probe sets for genes listed in Supplementary Table S1 that encode proteins within the cytidine analogue metabolism and target pathway.

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Table 1. Significant genes with expression that was associated with gemcitabine or AraC cytotoxicity (IC_{50} values)

Status of probe sets	Gene name	Chromosome	Correlation between QRT-PCR and microarray (*P*)	Gemcitabine			AraC		
				P	R	Q	*P*	R	Q
Specific Probe Sets	*FKBP5* (gemcitabine)	6	0.045	4.12E-08	−0.38	2.04E-04	—	—	—
	FKBP5 (gemcitabine)	6	0.028	4.15E-08	−0.38	2.04E-04	—	—	—
	NT5C3 (gemcitabine)	7	0.007	1.60E-07	0.365	5.23E-04	—	—	—
	C14orf169	14	0.067	8.45E-06	−0.313	3.25E-03	4.84E-05	−0.285	6.96E-02
	ESR2	14	0.023	3.61E-07	0.355	7.87E-04	3.40E-04	0.253	9.59E-02
	GCAT	22	0.011	2.08E-07	−0.361	5.83E-04	1.39E-04	−0.268	8.92E-02
	INPP5F	10	0.001	1.89E-06	−0.334	1.69E-03	8.35E-05	−0.277	8.92E-02
	MYBBP1A	17	0.009	5.65E-06	−0.319	2.44E-03	9.24E-06	−0.31	5.79E-02
	MYBBP1A	17	0.311	3.72E-05	−0.291	7.30E-03	2.97E-04	−0.255	8.92E-02
	TLE4	9	0.003	3.11E-06	−0.327	1.85E-03	1.77E-04	−0.264	8.92E-02
	ZNF278	22	0.251	5.67E-06	−0.319	2.44E-03	1.20E-06	−0.338	2.48E-02
Nonspecific Probe Sets	*ARL2BP*	16	0.339	1.37E-06	−0.338	1.41E-03	8.20E-06	−0.312	5.79E-02
	CENPB	20	0.677	5.52E-07	−0.349	9.85E-04	4.37E-04	−0.248	1.01E-01
	MAP4K4	2	0.118	2.57E-06	−0.329	1.85E-03	1.74E-04	−0.264	8.92E-02
	MGMT	10	0.232	1.30E-06	0.338	1.41E-03	2.21E-04	0.26	8.92E-02
	TRRAP	7	0.955	8.85E-06	−0.312	3.26E-03	1.26E-04	−0.27	8.92E-02
	FKBP5 (gemcitabine)	6	0.085	3.47E-07	−0.35	7.8E-0.4	—	—	—
	TPMT (AraC)	6	0.387	—	—	—	1.72E-05	0.301	5.93E-02

NOTE: R values represent the correlation coefficients. The Q value represents the false discovery rate.

on a log 2 scale, using both GCRMA and Fastlo (19, 20). Normalized expression data were regressed on gender, race, and time since the Coriell Institute acquired the cell line. Residuals were then standardized by subtracting the mean residual for individual probe sets and dividing by the SD to derive a "standardized adjusted expression value." Analyses were based on adjusted standardized values for both expression array and log-transformed IC_{50} data. Pearson correlation coefficients were calculated, and a Wald test was used to test for a nonzero correlation. Multiple testing using 10,000 permutations was performed for selected probe sets. The contribution of pathway genes to expression was based on the coefficient of determination (R^2) using a multiple regression model. Differences in intracellular metabolites between randomly selected resistant and sensitive cell lines were determined with Student's *t* test. Correlations between expression array and QRT-PCR or intracellular metabolites were also determined. Agreement between cytotoxicity performed at different times was determined using an intraclass correlation coefficient. Ingenuity pathway analysis was performed by using Fischer's exact test to calculate *P* values to identify genes within given pathways.

Results

Gemcitabine and AraC cytotoxicity. Gemcitabine and AraC cytotoxicity studies were performed to determine the range of variation in IC_{50} values as an indication of individual cell line variation in drug sensitivity. Average unadjusted IC_{50} values for gemcitabine and AraC in these 197 cell lines were 25.3 ± 30.7 nmol/L and 8.4 ± 14.3 μmol/L (mean ± SD), respectively. IC_{50} differed among the groups of subjects studied ($P < 0.01$ for both drugs), with CEPH samples appearing to be more resistant to both drugs when compared with the three other groups. Gender did not seem to have a significant effect on IC_{50} values for either gemcitabine ($P = 0.39$) or AraC ($P = 0.88$). The time since the Coriell Institute acquired the cell lines had a slight effect on gemcitabine ($P = 0.037$) but not AraC IC_{50} values ($P = 0.18$). We also performed a replication study to exclude the possibility that this phenotype might vary over time. Specifically, 12 ethnically diverse cell lines were selected randomly and cytotoxicity assays were repeated 3 months after the initial experiments. There was good agreement between results at the two different times. The intraclass correlation coefficient was 0.83 [95% confidence interval (95% CI), 0.51–0.95] for gemcitabine and 0.71 (95% CI: 0.26–0.91) for AraC.

Association between expression and cytotoxicity. Correlations between basal gene expression and IC_{50} values for gemcitabine and AraC were determined to identify genes that might contribute to variation in cytotoxicity. The 26,653 RefSeq validated sequences among the 54,000 Affymetrix probe sets were used to perform these correlation studies. The *P* values for association for gemcitabine tended to be smaller than those for AraC (Fig. 1*A*). With the exception of *NT5C3*, genes encoding proteins in the "cytidine analogue pathway" (Supplementary Table S1), shown as red points in Fig. 1, did not display highly significant *P* values. NT5C3 had only one probe set, and it was significantly associated with gemcitabine IC_{50} values ($P = 1.6 \times 10^{-7}$; Fig. 1*A*). *NT5C3* encodes a member of the nucleotidase family that catalyzes the dephosphorylation of monophosphorylated drug metabolites, thus decreasing the concentration of active drug metabolites. NT5C3 expression showed a less significant association with AraC IC_{50} values, although among all genes within the "pathway" (Supplementary Table S1), it also had the smallest *P* value for AraC ($P = 0.004$). Because most previous studies have focused only on pathway genes, we also estimated the effect of variation in expression for all known pathway genes on variation in IC_{50} values. Approximately 27% of the variation in gemcitabine IC_{50} values and ~11% of the variation for AraC could be explained by variation in gene expression within this intensively studied metabolic pathway.

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Among the 26,653 probe sets tested, 55 had P values of $\leq 10^{-6}$ for gemcitabine (adjusted multiple testing P value = 0.0002) and 21 had P values of $\leq 10^{-5}$ (adjusted multiple testing P value = 0.047). Because gemcitabine and AraC function in a similar fashion as antineoplastic drugs, we overlapped significant genes for both drugs. To identify top candidate genes for each drug that might be further characterized functionally, we used P values as a way to rank genes with regard to their association with drug cytotoxicity rather than establishing a particular "cutoff" value because very few of the candidate genes could pass Bonferroni correction. Therefore, we arbitrarily used a P value cutoff of $<10^{-3}$ for AraC and $<10^{-4}$ for gemcitabine to obtain a similar number of genes for each drug, realizing that we might still miss some true candidate genes. Thirty-one probe sets were identified that were common to both gemcitabine and AraC. Among those 31 probe sets, 14 encoding 12 genes were replicated when we used a different method of expression array normalization, Fastlo (Table 1). In addition, three "nonoverlapping" genes with highly significant associations for either gemcitabine or AraC are also listed in Table 1.

To verify expression array data for these 15 genes (18 probe sets), 20 lymphoblastoid cell lines were randomly selected to perform real-time QRT-PCR, and the results were compared with the expression array data. However, before performing QRT-PCR, we determined the specificity of these 18 probe sets by aligning the sequences of individual probes with the sequences of their presumed gene targets. We only verified probe specificity for top candidate genes that were considered for further functional validation because it was practically difficult to verify specificity for all 26,653 probe sets. Seven probe sets (Table 1) lacked specificity, defined as at least 5 of 11 probes that were "nonspecific." When we performed real-time QRT-PCR and correlated RT-PCR with expression array for these nonspecific probe sets, they did not correlate significantly (Table 1). Among the remaining 11 probe sets for 9 genes, 9 showed significant or near significant correlations between QRT-PCR and expression array data ($P < 0.05$),

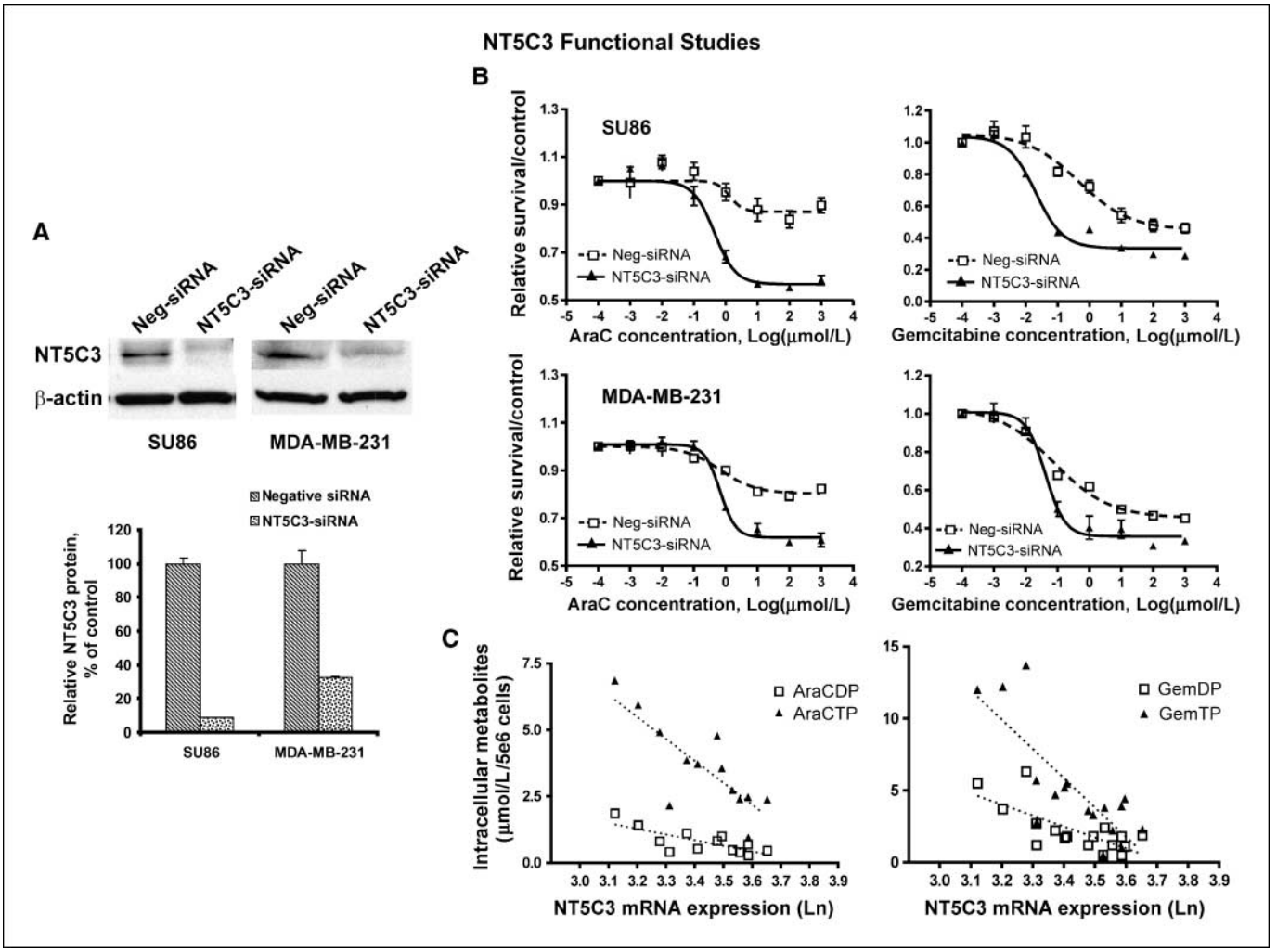

Figure 2. NT5C3 functional validation in two tumor cell lines. *A,* Western blot analyses showing significantly decreased levels of NT5C3 protein in SU86 pancreatic cancer and MDA-MB-231 breast cancer cells after treatment with specific siRNAs. *Insert,* average levels of protein as a percentage of control. *Columns,* mean values for three experiments; *bars,* SE. *B,* SU86 and MDA-MB-231 cytotoxicity. Both cell lines were sensitized to gemcitabine and AraC as determined by MTS assay after the down-regulation of NT5C3 gene expression. *Points,* mean values for three independent experiments; *bars,* SE. *C,* levels of intracellular phosphorylated gemcitabine and AraC metabolites were correlated with NT5C3 gene expression in 14 randomly selected sensitive and resistant lymphoblastoid cell lines. Rp and P values for metabolites, with expression adjusted for IC_{50}, were $R_{AraCDP} = -0.50$, $P_{AraCDP} = 0.084$; $R_{AraCTP} = -0.65$, $P_{AraCTP} = 0.016$; $R_{GemDP} = -0.49$, $P_{GemDP} = 0.045$; and $R_{GemTP} = -0.52$, $P_{GemTP} = 0.033$.

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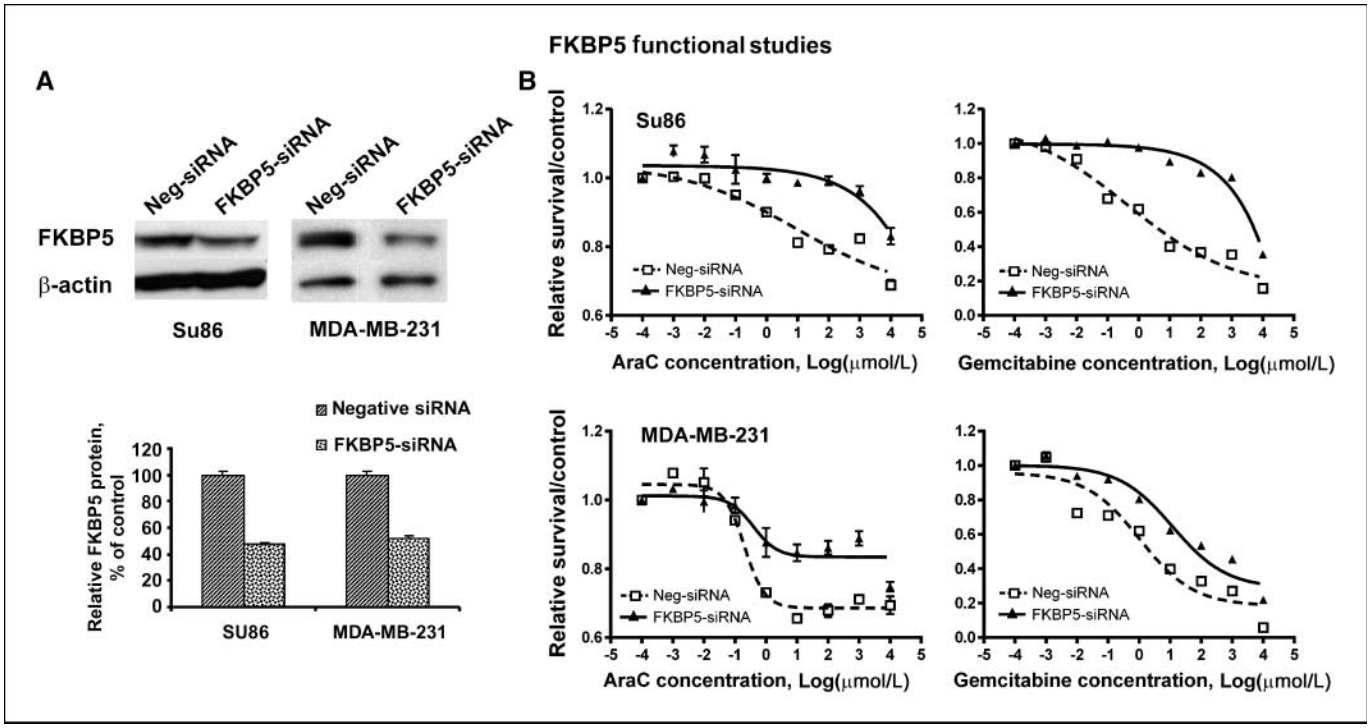

Figure 3. FKBP5 functional validation in two tumor cell lines. *A,* Western blot analyses showing significantly decreased levels of FKBP5 protein in SU86 pancreatic cancer and MDA-MB-231 breast cancer cells after treatment with FKBP5-specific siRNA. *Insert,* average levels of expressed protein as a percentage of control. *Columns,* mean for three experiments; *bars,* SE. *B,* SU86 and MDA-MB-231 cytotoxicity. Both cell lines became more resistant to gemcitabine and AraC as determined by MTS cytotoxicity assay after the down-regulation of FKBP5. *Points,* mean for three independent experiments; *bars,* SE.

whereas two (MYBBP1A and ZNF278) failed to show a significant correlation (Table 1).

NT5C3 and FKBP5 candidate gene validation. To confirm results obtained during the association study, we selected two candidate genes for functional validation with specific siRNA, followed by cytotoxicity studies. These genes were selected on the basis of the significance of the observed association and whether the gene was within or outside of the cytidine analogue metabolism pathway. The first was a pathway gene, *NT5C3*, a gene encoding an enzyme that dephosphorylates active cytidine analogue metabolites to form the inactive parent drug. Although *NT5C3* is a pathway gene, no previous reports had suggested that NT5C3 might play a role in sensitivity to cytidine analogues, although other members of the nucleotidase family, e.g., NT5C and NT5C1A, have been associated with clinical response (21, 22). Our association study had shown a positive correlation between the NT5C3 expression levels and gemcitabine IC_{50} values ($P = 1.6 \times 10^{-7}$; Bonferroni-corrected P value = 0.0004; $r = 0.365$), indicating—as anticipated—that high expression of NT5C3 was associated with gemcitabine resistance.

The second gene tested was from outside of the metabolic pathway, FKBP5, a gene encoding a 51 kDa immunophilin. FKBP5 is involved in steroid receptor maturation, and it is a binding partner for rapamycin (23–25). However, there had been no previous indication that FKBP5 might be involved in response to cytidine analogues. In contrast to NT5C3, expression levels for FKBP5 were negatively correlated with gemcitabine IC_{50} values ($r = -0.38$), indicating that increased FKBP5 transcription resulted in increased sensitivity to these drugs. Three Affymetrix probe sets targeted FKBP5, although we found that one probe set was not specific (Table 1). Both specific probe sets showed highly significant associations with gemcitabine IC_{50} values ($P = 4.12 \times 10^{-8}$ and 4.15×10^{-8}, respectively). After Bonferroni correction, the P values for these two FKBP5 probe sets remained significant ($P = 0.0001$). However, neither NT5C3 nor FKBP5 were significantly associated with AraC cytotoxicity ($P = 3.47 \times 10^{-3}$ for NT5C3 and $P = 1.26 \times 10^{-2}$ for FKBP5); although, as described subsequently, both proved to influence AraC response when tested functionally.

To confirm the possible functional significance of these two genes, we performed siRNA knockdown studies, followed by cytotoxicity assays, using two tumor cell lines to confirm the results of the association study and to extend our results beyond the lymphoblastoid cell lines to include cancer cell lines. Although neither gene displayed as significant an association with AraC as with gemcitabine cytotoxicity, functional studies were performed with both drugs. Although AraC is mainly used clinically to treat AML, previous studies have also used solid tumor cell lines for the analysis of both gemcitabine and AraC cytotoxicity (26). Two human cancer cell lines, the MDA-MB-231 breast cancer cell line and the pancreatic cancer SU86 cell line, were used to perform functional studies because gemcitabine is used to treat both types of cancers and because NT5C3 and FKBP5 showed their most significant associations with IC_{50} values for gemcitabine.

We first performed transient transfections with NT5C3 and FKBP5-specific siRNAs. Western blots verified that both genes were knocked down in both tumor cell lines (Figs. 2*A* and 3*A*). Gemcitabine and AraC cytotoxicity studies were then performed after transient transfection with siRNA. Down-regulation of NT5C3 with specific siRNA shifted the dose response curve to the left compared with control siRNA transfection, indicating increased sensitivity to gemcitabine in both cell lines (Fig. 2*B*), consistent

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with results obtained during the genome-wide expression association study. In contrast, FKBP5 had shown a negative correlation between level of expression and IC_{50} values. That relationship was confirmed by knockdown experiments performed with FKBP5-specific siRNA because down-regulation of FKBP5 in the both tumor cell lines desensitized the cells to both gemcitabine and AraC compared with nonspecific siRNA transfection (Fig. 3*B*). We also performed siRNA studies with two genes that were not significantly associated with IC_{50} values for either drug as "negative controls." One of those genes was *FKBP1A*, encoding FKBP12, a family member related to FKBP5, and the other was *NT5C2*, a gene encoding a family member of nucleotidases. Knockdown performed with specific siRNA for FKBP1A and NT5C2 did not significantly alter response to either gemcitabine or AraC (Supplementary Fig. S1).

Characterization of NT5C3 and FKBP5 cytotoxicity mechanisms. The 5′-nucleotidases catalyze the dephosphorylation of nucleoside monophosphates and, as a result, inactivate active phosphorylated drug metabolites (22). Both clinical and *in vitro* studies suggest that an increase in nucleotidase activity can reverse nucleoside analogue metabolic activation, resulting in drug resistance (22, 27). Furthermore, NT5C3 hydrolyzes pyrimidine monophosphates such as the active metabolites of gemcitabine and AraC (22, 28). As a result, the effect of NT5C3 on gemcitabine and AraC cytotoxicity could result from alterations in levels of active intracellular drug metabolites. Therefore, we randomly selected seven sensitive and seven resistant lymphoblastoid cell lines for gemcitabine and another seven sensitive and seven resistant lymphoblastoid cell lines for AraC to measure levels of active intracellular diphosphate and the triphosphate metabolites for both drugs. Sensitive and resistant cell lines were defined as cell lines with IC_{50} values of >0.85 SD from the mean of the IC_{50} distribution curve. Using this definition, we identified 32 resistant and 31 sensitive cell lines for gemcitabine and 35 resistant and 38 sensitive cell lines for AraC. The sensitive and resistant cell lines used to measure intracellular metabolites were selected randomly from these two groups. HPLC was used to measure levels of intracellular phosphorylated metabolites in these cells after 3 days of treatment with gemcitabine or AraC. Concentrations of active metabolites were higher in sensitive than in resistant cell lines ($P < 0.05$; Table 2). These results imply that genes within the cytidine analogue metabolic pathway, including the 5′-NTs, may contribute to the variation in cytotoxicity that we had observed. Equally important was the fact that intracellular metabolite concentrations were inversely related to levels of NT5C3 mRNA (Fig. 2*C*). This result was consistent with the conclusion that higher

Table 2. Intracellular metabolites determined by HPLC in lymphoblastoid cells after incubation with AraC (*A*) or gemcitabine (*B*)

A.

	AraC IC_{50} (μmol/L)		AraCDP		AraCTP	
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
1	7.2	0.24	0.28	1.42	0.95	5.94
2	13.8	0.65	0.70	1.87	2.47	6.86
3	20.0	0.96	0.49	0.81	2.73	4.92
4	20.4	1.06	0.41	1.00	2.39	3.56
5	26.0	1.99	1.34	0.42	2.64	2.16
6	51.3	2.73	0.46	1.11	2.39	3.87
7	76.6	2.77	0.83	0.54	4.78	3.72
Average ± SE	30.8 ± 8.7	1.49 ± 0.36	0.65 ± 0.13	1.02 ± 0.18	2.62 ± 0.40	4.43 ± 0.56
P	0.020*		0.133		0.032*	

B.

	Gemcitabine IC_{50} (nmol/L)		GemDP		GemTP	
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
1	32.2	1.24	0.5	3.7	0.5	12.2
2	34.3	3.88	0.5	5.5	1.0	12.0
3	40.7	6.43	1.1	6.3	4.4	13.7
4	58.6	7.32	1.2	2.7	3.6	5.7
5	80.7	12.0	1.9	2.2	2.3	4.7
6	103	12.2	1.2	1.2	2.2	2.8
7	273.1	13.4	2.4	1.8	3.8	5.5
Average ± SE	80.5 ± 26.8	7.61 ± 1.58	1.34 ± 0.24	3.14 ± 0.66	2.71 ± 0.51	7.49 ± 1.55
P	0.032*		0.030*		0.017*	

NOTE: Metabolites are μmol/L/5 × 10^6 cells.
*$P < 0.05$ between sensitive and resistant cell lines.

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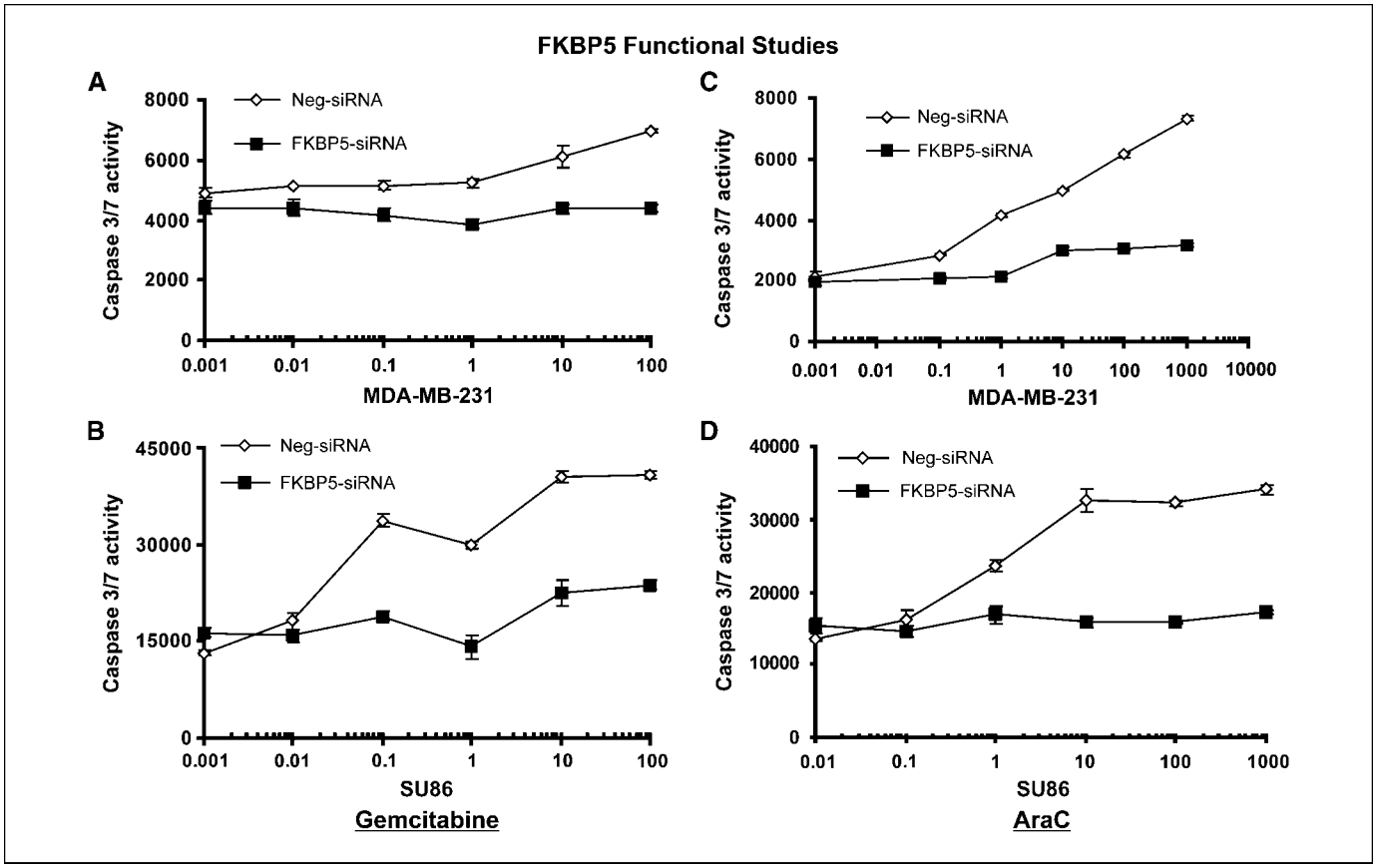

Figure 4. Caspase activity in SU86 and MDA-MB-231 cells after FKBP5 siRNA treatment. Caspase activity was measured using the Caspase-Glo 3/7 activity assay in *A* and *C* for MDA-MB-231 cells and in *B* and *D* for SU86 cells treated with FKBP5 siRNA in the presence of gemcitabine (*A* and *B*) or AraC (*C* and *D*). *Points,* mean for three independent experiments; *bars,* SE.

NT5C3 expression in these cells was associated with AraC and gemcitabine dephosphorylation, with decreased concentrations of active drug metabolites, resulting in drug resistance. These findings were also consistent with results of our NT5C3-siRNA functional studies (Fig. 2*B*).

We next attempted to test hypotheses with regard to mechanisms by which FKBP5 might influence sensitivity to gemcitabine and AraC. One possibility would involve the blockade of apoptosis signaling pathways as a result of FKBP5 knockdown. We also performed caspase-3/7 activity assays to determine whether alterations in FKBP5 expression might influence apoptotic signaling because previous studies had suggested that FKBP5 might be involved in apoptosis (29). Caspase-3/7 activity in FKBP5 siRNA–treated cells was significantly decreased after treatment with increasing concentrations of both gemcitabine and AraC when compared with cells treated with negative siRNA (Fig. 4), suggesting that activation of the apoptotic pathway was affected by the down-regulation of FKBP5 expression.

Gene network analysis. In an attempt to help define biological relationships among candidate genes identified during our study, we also applied Ingenuity Pathway Analysis (30). That analysis was focused on top ranked genes based on *P* values, using *P* values of $<10^{-5}$ for gemcitabine and $<10^{-4}$ for AraC that were significant with both GCRMA and Fastlo normalization methods. Eighty-four probe sets for gemcitabine and 75 for AraC were used to perform this analysis. A total of 9 and 20 networks were identified for gemcitabine and AraC, respectively. The "top" networks, with *P* values of <0.05 based on Fisher's exact text were associated with cell death, cancer, cell growth and proliferation, cell signaling, DNA replication, DNA recombination, DNA repair, and nucleic acid metabolism (Supplementary Table S2). On the basis of pathways with the highest number of significant candidate genes, cancer was identified as the major disease, and cell signaling and the cell cycle were the major molecular and cellular functions identified. These preliminary results are clearly consistent with the use of these drugs to treat cancer.

Discussion

Drug response predictors have evolved significantly in the postgenomic era. Rather than using single genes, transcripts, proteins, or metabolites to predict response, information from across the genome is now available to help predict drug response (4). Variation in response to chemotherapy results from a combination of factors that include gene sequence variation, ultimately resulting in differences in mRNA and protein expression, but also including differences in gender, ethnic group, or environmental factors. Variation in mRNA is one important factor that may contribute to variation in response to chemotherapy (31). To screen for possible pharmacogenomic candidate genes that might contribute to variation in drug response, we tested the relationship between variation in basal gene expression and sensitivity to gemcitabine and AraC using Human Variation Panel cell lines, a model system designed to study common human genetic variation.

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Gemcitabine and AraC share similar chemical structures and pathways of metabolism, but they are used in very different ways clinically (8, 32). Gemcitabine is mainly used to treat solid tumors, whereas AraC is first line chemotherapy for AML (7, 12, 13, 33, 34). Clinical response to these two cytidine analogues varies widely (8, 14, 15, 35). We set out to use the Human Variation Panel model system to identify candidate genes that might contribute to this variation. It should be emphasized once again that, although the tumor genome is critical for understanding disease pathophysiology and response to therapy, the germline genome is also critical, especially for response to drug therapy. In addition, variation in gene expression in lymphoblastoid cells is strongly influenced by inheritance (36). Therefore, these cell lines represent one model system suitable for the study of the contribution of "pharmacogenomic" variation in expression to individual variation in drug response.

Previous studies have shown that variation in the expression of genes within the cytidine analogue metabolism and bioactivation pathway is associated with variation in response to gemcitabine and AraC (37–39). However, little information is available with regard to genes outside of this pathway. Therefore, rather than focus only on the known pathway, we used 26,635 expression probe sets to perform association analysis with drug response, in this case, cytotoxicity. However, this type of approach will also produce false-positive results if functional validation experiments, such as those that we applied, are not performed. Therefore, we first identified candidate genes, both within and outside of the known pathway, which might be important for response to these two cytidine analogues, followed by functional validation.

As a first step, our strategy involved obtaining expression data for 197 cell lines, followed by gemcitabine and AraC cytotoxicity assays. By correlating expression and IC_{50} values for both drugs, we identified genes both within and outside of the currently known cytidine analogue metabolic pathway that were significantly associated with cytotoxicity (Table 1). One pathway gene, *NT5C3*, a gene not previously identified as a factor in response to gemcitabine or AraC, showed a significant association with cytotoxicity. However, the overall contribution of expression for all pathway genes to variation in IC_{50} values was only $\sim$27% for gemcitabine and 11% for AraC. These observations emphasize the potential advantage of genome-wide analyses rather than focusing entirely on known biological pathways. Although none of the genes listed in the Table 1 have been previously reported to be associated with gemcitabine or AraC response, our results complement and extend several previous reports of correlations of response to gemcitabine with gene expression profiles in cancer cell lines or tumor tissue samples obtained from patients (3, 40–43).

The lymphoblastoid cell lines used in our study are EBV transformed, so they are neither tumor cell lines nor tumor tissue. Therefore, one potential problem with the use of these cell lines is that EBV transformation could influence drug sensitivity and/or expression profiles, so we might miss some genes of importance, either because they are not expressed in these cell lines or, after transformation, are down-regulated. There is also evidence showing that gemcitabine and doxorubicin can induce the lytic form of EBV-transformed cells (44). However, the fact that we were able to functionally validate the two candidate genes that we studied supports the feasibility of our approach. Furthermore, lymphoblastoid cells have been used to identify genetic variation associated with cytotoxicity for other antineoplastic drugs, including daunorubicin, another drug that also potentially induces EBV lytic forms (45, 46). Finally, in any high-throughput association study, candidate genes identified require functional validation, as performed for our two candidate genes.

NT5C3 encodes a protein involved in cytidine metabolism, whereas *FKBP5* lies outside of that pathway. Their novelty, and the fact that one of these genes is within, whereas the other is outside of our current sphere of knowledge, highlights the potential of this model system for hypothesis generation. Although several 5′-NT isoforms have been reported to be associated with AraC and gemcitabine response (22, 47, 48), the role of the "pyrimidine-specific" nucleotidase NT5C3 in response to these drugs is unexplored (28, 49). FKBP5 is a 51-kDa immunophilin (23, 50) that, just as NT5C3, has never previously been reported to influence gemcitabine cytotoxicity. Our studies indicated that decreased expression of NT5C3 and FKBP5 after specific siRNA treatment altered response to both gemcitabine and AraC but through different mechanisms. The functional characterization of both NT5C3 and FKBP5 provided biological evidence in support of their involvement in variation in gemcitabine and AraC cytotoxicity.

Finally, although *P* values for AraC were not as significant as those for gemcitabine, functional studies of NT5C3 and FKBP5 showed similar effects for both drugs. That observation reminds us of the limitations of *P* values when determining true associations, as well as the fact that genome-wide association studies represent only one step in the identification of biomarkers to help predict clinically relevant variation in response to chemotherapy or new targets that might be used to enhance treatment outcomes. These results can now be applied in translational studies designed to test the hypothesis that variation in the expression of these two genes might be associated with clinically relevant variation in response to therapy with gemcitabine and/or AraC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Gemcitabine and Cytosine Arabinoside Cytotoxicity: Association with Lymphoblastoid Cell Expression

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