Immunohistochemical and Genetic Evaluation of Deoxycytidine Kinase in Pancreatic Cancer: Relationship to Molecular Mechanisms of Gemcitabine Resistance and Survival

Valeria Sebastiani,[1] Francesca Ricci,[1] Belen Rubio-Viquiera,[2] Piotr Kulesza,[3] Charles J. Yeo,[4] Manuel Hidalgo,[2] Alison Klein,[2] Daniel Laheru,[2] and Christine A. Iacobuzio-Donahue[2,3]

Abstract Gemcitabine is considered the standard first-line therapy for patients with advanced pancreatic cancer. More recent strategies have focused on improving the efficacy of gemcitabine by either improving the method of delivery or by combining gemcitabine with other non-cross-resistant chemotherapy agents or with small-molecule drugs. However, the clinical benefits, response rates, and duration of responses have been modest. Deoxycytidine kinase (dCK) is the rate-limiting enzyme involved in the metabolism of gemcitabine. The expression of dCK has been postulated to be correlative of gemcitabine resistance. We determined the relationship of dCK immunohistochemical protein expression and/or genetic status of dCK in a panel of human pancreatic cancer tissues and pancreatic cancer cell lines and determined the relationship of these variables to the clinical outcome of patients treated with gemcitabine. We report that dCK protein expression is expressed in the majority of pancreatic cancers analyzed (40 of 44 cases, 91%) and showed a range of labeling intensities ranging from 1+ (labeling weaker in intensity than normal lymphocytes present in same section) to 3+ (labeling greater in intensity than normal lymphocytes present in same section). When labeling intensity was compared with survival, low dCK expression (1+ labeling) was correlated with both overall survival ($P < 0.009$) and progression-free survival following gemcitabine treatment ($P < 0.04$). Low dCK labeling intensity was also significantly correlated with patient age (70.3 ± 8.1 versus 59.8 ± 7.4 years; $P < 0.0006$), suggesting that age-related methylation of the *dCK* gene may account in part for the observed differences. Sequencing of the entire dCK coding sequence in 17 cell lines and 9 patients' cancer tissues with disease progression while on gemcitabine did not identify any mutations, suggesting that genetic alterations of dCK are not a common mechanism of resistance to gemcitabine for this tumor type. Moreover, dCK labeling showed similar patterns and intensities of labeling among matched pretreatment and post-treatment tissues. In summary, pretreatment levels of dCK protein are most correlated with overall survival following gemcitabine treatment and are stable even after resistance to gemcitabine is clinically documented.

The anticancer agent gemcitabine (2′,2′-difluorodeoxycytidine, Gemzar, Eli-Lilly, Indianapolis, IN) is a cell cycle–dependent deoxycytidine analogue of the antimetabolite class (1). On transport into the cell, gemcitabine is phosphorylated to its mononucleotide moiety by deoxycytidine kinase (dCK), a rate-limiting enzyme in the salvage of deoxyribonucleosides that provides deoxynucleotide triphosphates for replicative and repair DNA synthesis. These gemcitabine mononucleotides, subsequently activated by nucleotide kinase to gemcitabine diphosphate and gemcitabine triphosphate, are then incorporated into DNA leading to masked chain termination (2, 3). Active metabolites of gemcitabine can also inhibit ribonucleotide reductase, dCMP deaminase, and CTP synthetase, thus enhancing gemcitabine activation. Gemcitabine is inactivated by deamination catalyzed by deoxycytidine deaminase (4).

Gemcitabine has significant therapeutic potential against a variety of hematopoetic and solid tumors, including carcinomas of the pancreas (5). Among patients with advanced pancreatic cancer, gemcitabine is the standard of care first-line therapy (6, 7). Clinical trials to determine the utility of adding a second cytotoxic drug or radiotherapy to this treatment regimen have shown modest improvements in disease-free survival but has not resulted in statistically significant improvements in survival with reported survival rates reaching 20% to 40% at 1 year (8, 9). Thus, despite its clinical benefit for some patients,

Authors' Affiliations: [1]Department of Pathology, University "La Sapienza," Rome, Italy; Departments of [2]Oncology and [3]Pathology, Johns Hopkins Hospital, Baltimore, Maryland; and [4]Department of Surgery, Jefferson Medical College, Philadelphia, Pennsylvania

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Note: D. Laheru and C.A. Iacobuzio-Donahue are both senior authors.

Requests for reprints: Christine A. Iacobuzio-Donahue, Department of Gastrointestinal/Liver Pathology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins Hospital, 720 Rutland Street, Ross 632, Baltimore, MD 21205. Phone: 410-955-3511; Fax: 410-614-0671; E-mail: ciacobu@jhmi.edu.

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the recognition of those patients whose tumors will not respond to gemcitabine therapy remains a hurdle in successful adjuvant management.

A variety of factors have been described as correlates of gemcitabine resistance, including mutations of the *dCK* gene (1, 10–13), low levels of dCK enzyme activity (7, 14), and increased levels of the active metabolite gemcitabine triphosphate. In addition, studies in lung cancer have shown an association between expression levels of RRM1 and resistance to the agent (15). However, comprehensive evaluations of these factors and their relationship to dCK protein expression, gemcitabine resistance, and disease progression of pancreatic cancer are lacking. Toward this end, we determined the relationship of *DCK* genetic status to immunohistochemical protein expression of dCK in a panel of human pancreatic cancer tissues or pancreatic cancer cell lines and determined the relationship of these variables to the clinical outcome of patients treated with gemcitabine.

Materials and Methods

Patient specimens. Paraffin-embedded tissues of 44 primary or metastatic infiltrating ductal adenocarcinomas of the pancreas were obtained from the Surgical Pathology and Cytopathology archives of the Johns Hopkins Hospital or from the tissues obtained from the Gastrointestinal Cancer Rapid Medical Donation Program (16). For each case, one block was chosen that was representative of the infiltrating primary or metastatic carcinoma and adjacent normal tissue. Clinicopathologic and treatment data were obtained for each case from the patient's medical records. The project was approved by the institutional review board.

Cell culture. Human pancreatic cancer cell lines AsPC1, BxPC3, Capan1, Capan2, CFPAC, Hs766t, Hs578, MiaPaca2, and Panc1 cell lines were obtained from the American Type Culture Collection (Manassas, VA) or were generously provided by Dr. Elizabeth Jaffee (Department of Oncology, Johns Hopkins Hospital; ref. 17). All cell lines were grown at 37°C in DMEM containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2.

PCR amplification and sequencing of DCK. Microdissection of pancreatic carcinoma tissues was done to enrich for neoplastic cells as described previously (16). Genomic DNA was extracted from cell lines and tissues using a DNeasy kit (Qiagen, Valencia, CA) and the last 100 bases of the 5′-untranslated region and complete coding sequence of exons 1 to 7 of the human *dCK* gene were amplified using intronic primers flanking each exon. All primer sequences are shown in Table 1. PCR-amplified products were purified using QIAquick columns (Qiagen) and automated sequencing was done using nested primers and an ABI Prism model 3700 (Applied Biosystems, Foster City, CA). Sequence analysis employed Sequencher version 4.0.5 software (Gene Codes, Ann Arbor, MI).

Immunohistochemistry. Unstained 4-μm sections of each pancreatic cancer were deparaffinized by routine techniques before placing in 200 mL Target Retrieval Solution (pH 6.0; Envision Plus Detection kit, Dako, Carpinteria, CA) for 20 minutes at 100°C. After cooling for 20 minutes, the slides were quenched with 3% H_2O_2 for 5 minutes followed by incubation at room temperature with a 1:200 dilution of polyclonal rabbit anti-dCK antibody for 1 hour (antibody generously provided by Dr. Iannis Talianidis, Crete, Greece). This antibody was generated against amino acids 246 to 260 corresponding to the COOH-terminal portion of the human dCK protein (18). Labeling was detected with the Dako Envision system following the manufacturer's protocol. A negative control was used in each run in which the antibody was replaced by an equal volume of PBS. Immunohistochemical labeling of each tissue section was scored on an intensity scale of 0 to 3, with 0 corresponding to no positive labeling neoplastic epithelial cells, 1 corresponding to weak positive labeling of neoplastic epithelium (labeling weaker in intensity to lymphocytes present in the same section), 2 corresponding to positive labeling of epithelial cells that was equal in intensity to lymphocytes present in the same section, and 3 corresponding to intense positive labeling that was greater in intensity to lymphocytes present in the same section. The percentage of neoplastic epithelial cells with positive labeling was also scored from 1% to 100%. Scoring was done by two of the authors (V.S. and C.A.I-D.) with agreement in all cases. At the time of scoring, information regarding treatment history and survival were unknown.

Statistics. All summary values are expressed as mean ± SD unless otherwise indicated. For correlations of growth inhibition by gemcitabine, a Pearson correlation coefficient was used. For correlations of dCK immunolabeling to clinical and pathologic variables, a Student's *t* test was used for parametric measurements, whereas for frequency distributions a χ^2 test was used with modification by the Fisher's exact test to account for frequency values less than 5. For determinations of survival following gemcitabine treatment in relation to dCK immunohistochemical labeling, event time distributions

Table 1. dCK primer sequences

Exon	Sequence	Product size (bp)
5′-Untranslated region/1	Forward 5′-CAGGTCAGGATCTGGCTTAG-3′	208
	Reverse 5′-GAAGGGAACATCGGTAAGGA-3′	
2	Forward 5′-ATTGGGCAGGGAGCCTTTTC-3′	646
	Reverse 5′-CTATTTGGGAGGATATATGC-3′	
3	Forward 5′-GGAAAGAGTATGAGAAAGGGAAC-3′	387
	Reverse 5′-CTTGTACGTGGCCATTTGAA-3′	
4	Forward 5′-CTGCTTTCCACGGCACTATG-3′	618
	Reverse 5′-GAATCCCAGACTGGGCAAGT-3′	
5	Forward 5′-GCCACCAAGTGGCTGAAAAG-3′	485
	Reverse 5′-GCAATGAGATCCTATTTAC-3′	
6	Forward 5′-TTGGGCTCCATATGGTAAATC-3′	508
	Reverse 5′-GAATCTCAGAATCTGACAGAT-3′	
7	Forward 5′-CAATGGCATTGTGGTAGTTAC-3′	418
	Reverse 5′-GAAATGTAGAGGTAGATGGTT-3′	

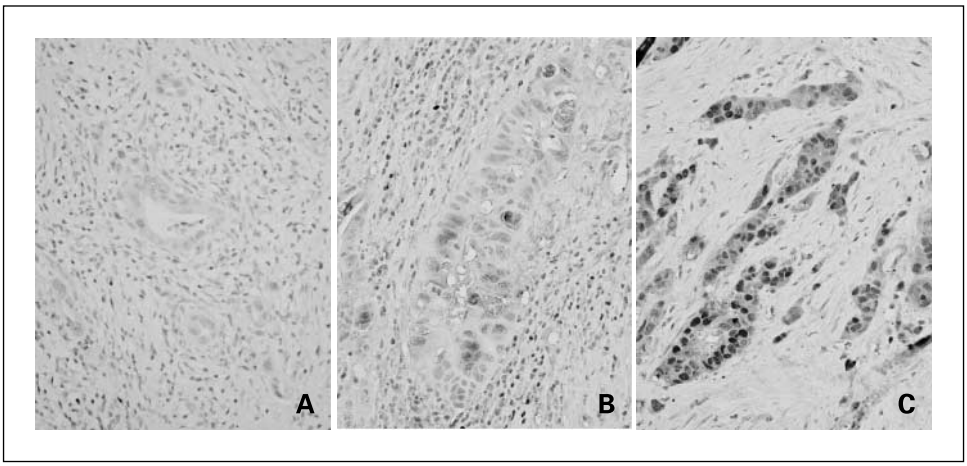

Fig. 1. Immunohistochemical labeling patterns of dCK protein in resected pancreatic cancer tissues. Examples of 1+ staining (*A*), 2+ staining (*B*), and 3+ staining (*C*). In all cases, note the presence of positive labeling lymphocytes within the desmoplastic stroma that serve as a useful internal control for evaluating the intensity of dCK immunolabeling.

were estimated using the method of Kaplan and Meier and compared using the log-rank statistic (19). *P*s ≤ 0.05 were considered statistically significant.

Results

Immunohistochemical labeling of DCK in resected pancreatic cancer tissues. We determined the immunohistochemical protein expression of dCK in 44 resected primary or metastatic infiltrating pancreatic cancers that were gemcitabine naive and correlated these findings to the clinical outcome data available for each patient.

Among the 44 resected pancreatic cancer tissues, positive labeling for dCK was noted in 40 of 44 (91%) cases. Among these 40 positive cases, 9 of 40 were scored as 1+ intensity (weak positive), 20 of 40 were scored as 2+ intensity (moderate positive), and 11 of 40 were scored as 3+ intensity (strong/intense positive). For all positive cases, the percentage of positive labeling cells was ≥75%. In 36 of these 40 (90%) positive cases, labeling was predominantly nuclear with or without cytoplasmic labeling, and in the remaining 4 of 40 (10%) positive cases, labeling was present within the cytoplasm only. Positive labeling was also observed within normal lymphocytes, acinar cells, and islets, although the most consistent labeling for dCK was noted within lymphocytes. As lymphocytes are a component of desmoplastic stroma within pancreatic cancers and in some sections normal pancreas was not present, lymphocytes thus served as useful internal controls when evaluating labeling patterns of the neoplastic cells (Fig. 1).

For 14 of the infiltrating carcinomas, matched normal ductal epithelium was present within the same section. Positive immunolabeling for dCK protein was observed in the normal pancreatic ductal epithelium in all 14 cases analyzed. In all cases, positive labeling was predominantly nuclear in location with varying amounts of cytoplasmic labeling. A comparison of the labeling intensity among the matched normal ductal epithelium present for 14 infiltrating cancers indicated that for 11 cases the identical staining intensity could be seen in both the normal duct epithelium and the matched infiltrating carcinoma. In the remaining 3 cases, the infiltrating carcinoma was stronger in intensity than the matched normal duct epithelium.

Correlation of DCK immunolabeling with survival. For 32 of the 44 patients, a complete treatment history, including overall survival, was available that allowed an evaluation of the relationship of dCK immunolabeling patterns to outcome following gemcitabine treatment. Nine of these patients showed low dCK immunolabeling in their pancreatic cancer tissues (staining scored as negative to 1+ intensity) and 23 patients showed high dCK immunolabeling (2+ to 3+ intensity). The relationship of dCK immunostaining to clinicopathologic features of these 32 patients are shown in Table 2. Patients with low dCK immunostaining were significantly older than patients with high dCK immunostaining ($P < 0.0006$), and more male patients were included in the high dCK immunostaining group ($P < 0.04$). No correlation was found for patterns of dCK immunolabeling and tumor differentiation, margin status, or clinical stage of disease; neither were differences in the proportion of patients receiving single-agent gemcitabine versus a combined adjuvant regimen. A significant difference in overall survival was found between low and high dCK immunogroups ($P < 0.009$). When corrected for progression-free survival following gemcitabine treatment, a significant difference was still present among low and high dCK groups ($P < 0.04$; Fig. 2).

Genetic status and immunohistochemical labeling of DCK in pancreatic cancers from patients with acquired gemcitabine resistance. To determine if loss of dCK expression is selected for within infiltrating pancreatic carcinomas during gemcitabine treatment, we determined the genetic status and immunohistochemical labeling patterns of dCK among 9 patients for which gemcitabine-treated pancreatic cancer tissues were available and who showed disease progression while on gemcitabine. Positive immunolabeling was noted for all 9 (100%) cases examined, with 8 of 9 cases showing strong positive dCK labeling (2+ to 3+ intensity) and 1 of 9 cases showing weak positive dCK labeling (1+ intensity). For 6 of these 9 cases, the matched pretreatment (gemcitabine-naive) pancreatic cancer tissues were also available for comparison, and in all 6 cases, the intensity and percentage of dCK immunolabeling was strongly positive (2-3+). A comparison of the pretreatment and post-treatment dCK labeling for these 6 patients indicated that in only one case a significant decrease in dCK immunolabeling could be shown in the post-treatment tissue (1+ intensity) compared with the pretreatment tissue (3+ intensity; Fig. 3).

Genetics of DCK in pancreatic cancer tissues and cell lines. Studies of hematopoetic neoplasms have suggested that genetic alterations of dCK may account for the decreased response to gemcitabine observed for some patients (1, 10–13). Thus, we sequenced the last 100 bases of the 5′-untranslated region and entire coding region of dCK in all 9 pancreatic cancer tissues from patients with disease progression while undergoing gemcitabine treatment as well as in 17 pancreatic cancer cell lines with a wide range of sensitivities to gemcitabine *in vitro* (data not shown). No mutations were found in any of the tissues or cell lines analyzed, indicating that genetic alterations of dCK may not be a frequent mechanism of gemcitabine resistance.

Discussion

In 2004 in the United States, >31,000 patients were diagnosed with pancreatic cancer and most all of these patients will succumb to their disease within 1 year (20). Treatment with gemcitabine, either alone or in combination with other agents, is the standard of care in this disease (6, 7). However, as some patients show limited or no response to gemcitabine treatment, a better understanding of the mechanisms of gemcitabine resistance is required, including a determination of these factors in the pretreatment period so that patients can be better stratified into appropriate treatment regimens.

Although several small studies have shown that the pretreatment levels of dCK enzyme activity or protein may be important determinants of gemcitabine resistance (7, 14, 21), the *in vivo* protein expression of dCK has not been explored in detail. Our data indicate that the immunohistochemical labeling patterns of dCK enzyme may also provide valuable information regarding gemcitabine efficacy as recently shown for hematologic or pediatric malignancies (22). dCK protein was found to be expressed in the majority of normal and neoplastic tissues analyzed, consistent with the ubiquitous role for this enzyme in cellular DNA synthesis (4). However, the intensity of dCK protein labeling was widely variable among these same tissues, with low levels of immunohistochemical labeling for dCK significantly correlated with both overall survival ($P < 0.009$) and progression-free survival on gemcitabine ($P < 0.04$). A correlation of dCK protein levels and enzyme activity has been noted previously (14) and was shown to relate to observed gemcitabine sensitivities in xenograft models. Thus, our findings indicate that a similar correlation may be found in patient samples among pretreatment levels of dCK immunolabeling and observed clinical outcome. However, as some patients and cancer cell lines showed no obvious sensitivity to gemcitabine despite strong positive dCK immunolabeling, we cannot rule out that for some patients other factors may play a role in gemcitabine sensitivity as well (23).

One unexpected finding in this study was the highly significant relationship of dCK immunolabeling intensity and patient age, in that patients with low levels of dCK labeling were at least 10 years older than those with strong positive labeling ($P < 0.0006$), a finding that may in part relate to the observed decrease in survival in these patients. Age-related methylation in nonneoplastic and neoplastic pancreaticoduodenal tissues has been well described (24). Thus, our finding of low-level dCK protein immunolabeling in older patients may indicate epigenetic silencing of this gene. Limited data addressing the DNA methylation status of dCK indicated that

Table 2. Clinicopathologic features of patients treated with gemcitabine

	Low dCK	High dCK	*P*
Age (y)	70.3 ± 8.1	59.8 ± 7.4	<0.0006
Gender (M/F)	3/6	17/6	<0.04
Race (white/black)	8/1	20/3	NS
Tumor differentiation*			
Well/moderate ($n = 12$)	4	8	NS
Poor ($n = 10$)	2	8	
Positive margins*			
Yes ($n = 3$)	1	2	NS
No ($n = 18$)	4	14	
Clinical stage			
I	0	2	NS
II	3	1	
III	5	14	
IV	1	6	
CA19-9 decrease ≥ 25%			
Yes ($n = 7$)	3	4	NS
No ($n = 15$)	2	13	
Overall survival (mo)	14.6 ± 7.3	21.7 ± 13.8	<0.009
Overall survival following gemcitabine initiation (mo)	10.0 ± 5.3	12.0 ± 9.3	<0.04
Gemcitabine single agent			
Yes ($n = 11$)	3	8	NS
No ($n = 21$)	6	15	

*This information was only available for patients who underwent surgical resection.

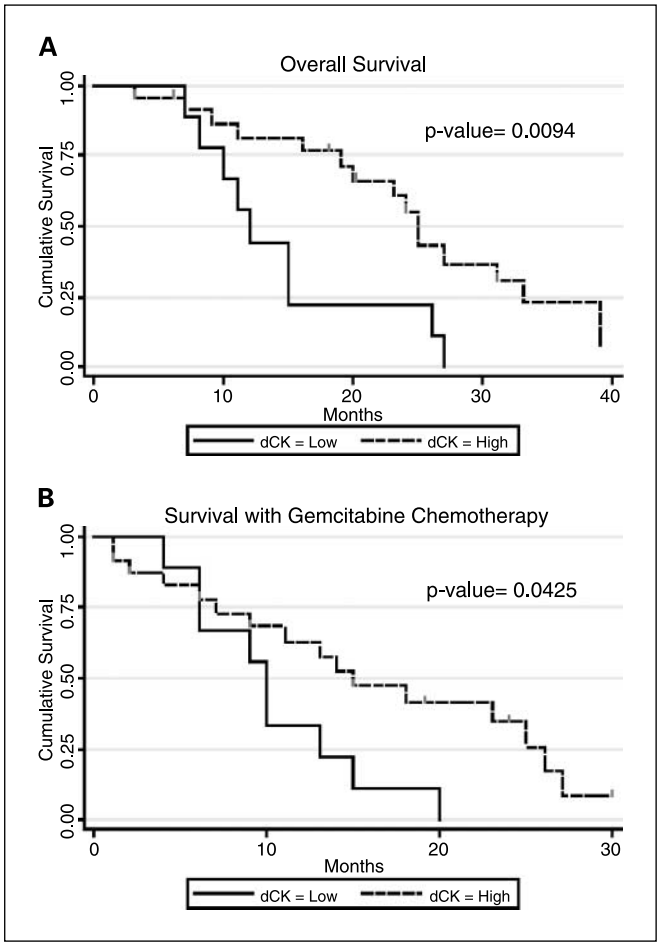

Fig. 2. Kaplan-Meier survival curves in relation to dCK immunolabeling status. Low levels of dCK immunolabeling show a significant relationship toward shorter overall survival (*A*) and survival following gemcitabine (*B*).

epigenetic silencing was not a common feature of acute lymphoblastic leukemia cell lines that were resistant to gemcitabine (25), whereas incubation of the dCK-deficient HL-60 cell line with 5-azacytidine increased dCK activity 12-fold (26). As neither of these studies was done with regard to clinical features of patients or on neoplastic tissues obtained from patients with gemcitabine resistance, dCK methylation status may best be interpreted in regard to patient age among other features.

Genetic mutations or coding polymorphisms of *DCK* are not a common mechanism of innate resistance to gemcitabine in pancreatic cancers. This is not entirely unexpected, as it is unlikely a mutation in this DNA salvage pathway would offer a clonal advantage during tumorigenesis. In fact, in most other reports, resistance was acquired *in vitro* (1, 11, 12, 27). However, we also show that *DCK* mutation is also not a common mechanism of acquired resistance in pancreatic cancer as has been reported for acute myeloid leukemia (13) and consistent with our findings of similar dCK immunolabeling patterns in matched pretreatment and post-treatment gemcitabine-treated tissues. In only one case of matched pretreated primary carcinoma and post-treatment metastasis did we find a marked reduction in dCK immunolabeling, suggesting a down-regulation of dCK expression with pancreatic cancer progression. Variations in expression of nucleoside transporters have been reported as critical for gemcitabine influx and maintenance of toxicity (2, 28, 29). Thus, in the absence of genetic mutations of *DCK* to account for gemcitabine resistance, alterations in expression of the nucleoside transporters *CNT1* or *ENT1* may affect gemcitabine sensitivity with disease progression to provide one possibility (30).

We propose that routine immmunohistochemical labeling for dCK in pancreatic cancer tissue specimens may have value in the clinical setting. Other approaches to pretreatment determinations of potential gemcitabine resistance have included measurements of dCK mRNA or enzyme activity (7, 14, 21). Although these measurements may be robust, they rely on laboratory infrastructure that may not be available in some institutions. Moreover, many require high-quality material that is collected in a sterile fashion and that contains adequate neoplastic cellularity. Immunohistochemical analysis can overcome the problem of low cellularity as it is done *in situ*, it preserves tissue morphology (thus allowing a direct correlation between gene expression and morphology), it is less labor intensive and can be done in any histology laboratory, and it can be applied to clinical materials already available for the patient. Thus, determinations of dCK immunolabeling before initiation of adjuvant gemcitabine therapy may improve overall survival by identifying those patients less likely to respond to this treatment.

In summary, we have shown that genetic alterations of dCK are not a common mechanism of resistance to gemcitabine in pancreatic cancer cell lines or pancreatic cancer tissues. Rather, pretreatment levels of dCK protein are most correlated with

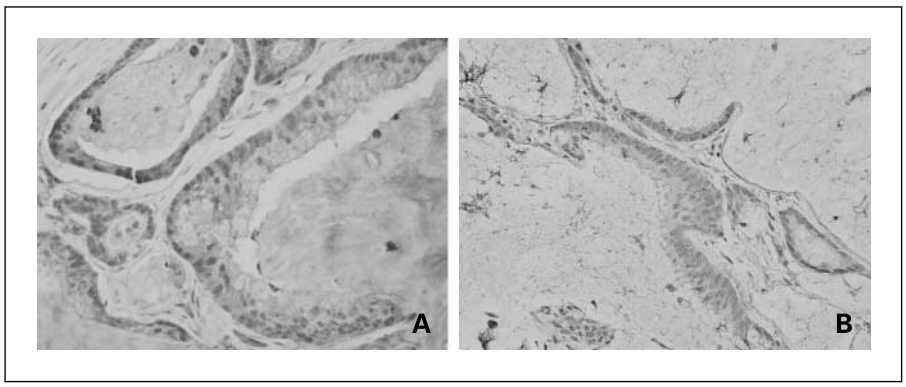

Fig. 3. dCK immunolabeling patterns in an untreated primary infiltrating pancreatic cancer (*A*) and a matched lung metastasis that developed two years later following gemcitabine therapy (*B*). Strong positive (3+) immunolabeling of the nucleus and cytoplasm is seen within the neoplastic cells of the untreated primary tumor. In contrast, a significant reduction in dCK immunolabeling (1+) was found in the metastatic disease from this patient (original magnification, ×200).

overall survival following gemcitabine treatment and are stable even after resistance to gemcitabine is clinically documented. Future studies in a larger cohort of patients are planned to determine the utility of pretreatment dCK immunolabeling on stratification of patients into adjuvant regimens that include gemcitabine either alone or in combination with other agents as well as among age- and gender-matched controls.

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Correction: Article on Deoxycytidine Kinase in Pancreatic Cancer

In the article on deoxycytidine kinase in pancreatic cancer in the April 15, 2006 issue of *Clinical Cancer Research*, the name of an author, Belen Rubio-Viqueira, was spelled incorrectly.

Sebastiani V, Ricci F, Rubio-Viqueira, et al. Immunohistochemical and genetic evaluation of deoxycytidine kinase in pancreatic cancer: relationship to molecular mechanisms of gemcitabine resistance and survival. Clin Cancer Res 2006;2:2492–7.

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