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Human Equilibrative Nucleoside Transporter 1 Levels Predict Response to Gemcitabine in Patients With Pancreatic Cancer

JAMES J. FARRELL,* HANY ELSALEH,‡ MIGUEL GARCIA,§ RAYMOND LAI,‖ ALI AMMAR,* WILLIAM F. REGINE,¶ ROSS ABRAMS,# A. BOWEN BENSON,** JOHN MACDONALD,‡‡ CAROL E. CASS,‖ ADAM P. DICKER,§§ and JOHN R. MACKEY‖

*Division of Digestive Diseases, University of California Los Angeles School of Medicine, Los Angeles, California; ‡The Australian National University, Canberra, ACT, Australia; §Radiation Therapy Oncology Group, Philadelphia, Pennsylvania; ‖Cross Cancer Institute, University of Alberta, Edmonton, Canada; ¶University of Maryland Medical Systems, Baltimore, Maryland; #Rush University Medical Center, Chicago Illinois; **Northwestern University, Chicago, Illinois; ‡‡St. Vincent's Comprehensive Cancer Center, New York, New York; and §§Thomas Jefferson University, Philadelphia, Pennsylvania

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Background & Aims: **The human equilibrative nucleoside transporter (hENT1) protein transports gemcitabine into cells. Small retrospective studies in pancreatic cancer suggest that levels of hENT1 protein or messenger RNA may have prognostic value. We studied the predictive value of hENT1 levels in a cohort of pancreatic adenocarcinoma patients from the large prospective randomized adjuvant treatment trial RTOG9704.** ***Methods:*** **In RTOG9704, 538 patients were assigned randomly, after surgical resection, to groups that were given either gemcitabine or 5-fluorouracil (5-FU). Immunohistochemistry for hENT1 was performed on a tissue microarray of 229 resected pancreatic tumors from RTOG9704 and scored as having no staining, low staining, or high staining. Associations between hENT1 protein and treatment outcome were analyzed by unconditional logistic regression analysis using the chi-square test and the Cox proportional hazards model.** ***Results:*** **HENT1 expression was associated with overall and disease-free survival in a univariate (hazard ratio [HR], 0.51; 95% confidence interval [CI], 0.29–0.91; $P = .02$; and HR, 0.57; 95% CI, 0.32–1.00; $P = .05$) and multivariate model in the group given gemcitabine (HR, 0.40; 95% CI, 0.22–0.75; $P = .004$; and HR, 0.39; 95% CI, 0.21–0.73; $P = .003$). hENT1 expression was not associated with survival in the group given 5-FU.** ***Conclusions:*** **In this prospective randomized trial, hENT1 protein expression was associated with increased overall survival and disease-free survival in pancreatic cancer patients who received gemcitabine, but not in those who received 5-FU. These findings are supported by preclinical data; the gemcitabine transporter hENT1 is therefore a molecular and mechanistically relevant predictive marker of benefit from gemcitabine in patients with resected pancreatic cancer.**

Pancreatic cancer remains one of the most lethal human cancers. This is caused, in part, by resistance to most chemotherapeutic drugs. The nucleoside pyrimidine analogue gemcitabine is the most effective single agent in the palliation of advanced pancreatic cancer, where it has been shown to improve clinical symptoms and modestly extend survival.[1] Gemcitabine, one of the most commonly used chemotherapeutic agents, also is approved for use in non–small-cell lung cancer, breast cancer, and ovarian cancer.

It is likely that genetic variability of key enzymes in gemcitabine transport and metabolism may impact on treatment response and toxicity of gemcitabine agents.[2] Gemcitabine and physiologic nucleosides are hydrophilic, and diffusion through the plasma membrane lipid layer is slow. Efficient cellular uptake therefore requires the presence of specialized integral membrane nucleoside transporter proteins.[3,4] Two general processes of nucleoside transport have been identified: the equilibrative bidirectional facilitators and the concentrative sodium/nucleoside symporters.[2] However, the major routes for transporting gemcitabine are human equilibrative nucleoside transporter (hENT1) (also known as SLC29A1) and, to a lesser extent, hCNT1 and hCNT3.[2,4–7]

As a prodrug, gemcitabine must be phosphorylated to its active diphosphate and triphosphate metabolites. Deoxycytidine kinase is the rate-limiting enzyme in the biotransformation of nucleoside analogues by phosphorylation to its mononucleotide. Cytidine deaminase, 5′ nucleotidase, and uridine monophosphate-cytidine monophosphate kinase also are key enzymes in this pathway.

Gemcitabine has many anticancer mechanisms of action. For example, gemcitabine triphosphate incorpo-

Abbreviations used in this paper: CI, confidence interval; 5-FU, 5-fluorouracil; hENT1, human equilibrative nucleoside transporter; HR, hazard ratio; SNP, single nucleotide polymorphism; TMA, tissue microarray.

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rates into DNA with a subsequent addition of a natural nucleotide, thereby making the strand less vulnerable to DNA repair by base-pair excision.[8,9] The de novo DNA synthesis pathway is blocked through inhibition of ribonucleotide reductase (RRM1 and RRM2 subunits) by gemcitabine diphosphate.[10] At high cellular concentration, gemcitabine triphosphate inhibits deoxycytidine monophosphate deaminase and cytidine triphosphate synthetase, thereby lowering the opposing deoxycytidine triphosphate pool.[11,12] Finally, gemcitabine also has self-potentiating mechanisms that achieve higher intracellular concentrations and increase cytotoxicity.

There are limited data about hENT1 in pancreatic cancer cell lines. Studies involving pancreatic cancer cell lines (NP9, NP18, NP 29, NP31) have indicated that hENT1 is the major gemcitabine transporter in which it is overexpressed.[6] It is speculated that populations of cells with lower hENT1 abundance may be relatively gemcitabine resistant owing to reduced intracellular accumulation.[2,7] In one study, pharmacologic inhibition of hENT1 in cells has been reported to render them gemcitabine resistant.[2] Preclinical studies, including studies involving pancreatic cancer cells lines, have suggested a positive correlation between hENT1 gene expression and chemosensitivity.[13–16] In a small retrospective surgical series, patients with hENT1-positive pancreatic cancer tumor tissue by immunohistochemistry had significantly longer survival after gemcitabine chemotherapy than patients with pancreatic tumors without detectable hENT1.[17] Furthermore, hENT1 messenger RNA (mRNA) expression in pancreatic cancer resection specimens has been associated with longer overall survival, disease-free survival, and time to disease.[18] Although these retrospective studies have shown the possibility of hENT1 as a prognostic marker, they did not show its predictive role because they lacked an appropriate control group who did not receive gemcitabine.

The RTOG 9704 study was a phase III randomized postoperative adjuvant study in resectable pancreatic cancer patients comparing 5-fluorouracil with gemcitabine before and after chemoradiation.[19] All patients received 5-FU as a radiation sensitizer during radiation. After closure of the study we used pretreatment, resected, formalin-fixed tumor tissue from patients enrolled in this study to analyze hENT1 expression and correlate it with patient demographics and treatment outcome. We further studied hENT1 single nucleotide polymorphisms (SNPs) to seek relationships with the variable hENT1 expression in pancreatic adenocarcinoma.

Methods

Patient Selection and Consent

Patients entering RTOG 9704 gave consent for use of formalin-fixed tissue for future planned translational research as part of the formal informed consent process. The RTOG tissue bank received tumor blocks from a total of 229 of the 538 patients who had undergone surgical resection and were entered in the RTOG 9704 prospective adjuvant treatment trial. Tissue microarrays (TMAs) were constructed from these blocks. Clinicopathologic factors were obtained as part of the patients' enrollment in the study. Treatment schedules and follow-up clinical information including outcome (overall survival, diseases-free survival) and toxicity were recorded as end points in the study. Permission to perform this study was obtained by the study sites' and investigators' institutional review boards.

TMA Construction

A TMA was constructed using tissue core samples from the patients enrolled in the RTOG 9704 study. Each patient's tumor was represented by 3 cores (each core is 6 mm in size) from different regions within the tumor block and placed on 3 separate arrays to exclude effects of heterogeneous antigen expression. H&E staining was performed on each of the 3 TMAs to confirm tumor presence.

Immunohistochemistry

Anti-hENT1 monoclonal antibody was developed and characterized as described previously.[17,20,21] Goat antimouse antibodies and horseradish-peroxidase–labeled dextran polymer (DAKO EnVision+) were purchased from DAKO Corporation (Carpinteria, CA). All other reagents were of analytic grade and available commercially.

Three separate formalin-fixed, paraffin-embedded pancreatic TMA sections (4- to 6-um thick, each containing the 229 patients' pancreatic tumor cores) were deparaffinized with 3 immersions in xylene baths (10 minutes each) followed by serial washes in graded alcohol from 100% to 50%. After rinsing in water, slides were placed in 250 mL of high pH 1× DAKO target antigen retrieval solution and microwaved in TT-mega Milestone (ESBE Scientific, Markham, Ontario, Canada) under controlled temperatures and high pressure for 10 minutes at 100°C. After cooling in water for 6 minutes, the slides were rinsed with water, peroxidase was blocked in 3% H_2O_2 solution with methanol for 10 minutes, and then washed in running water for 10 minutes. Phosphate-buffered saline (PBS) (pH 7.2) was used for rinsing before incubation with appropriate dilutions of anti-hENT1 monoclonal antibodies. Slides with anti-hENT1 were incubated in a humidified chamber overnight at 4°C. The sections then were rinsed with PBS, immersed in buffer for 5 minutes, incubated with goat anti-mouse dextran conjugate (DAKO Envision+) for 30 minutes, followed by soaking in PBS. DAKO diaminobenzidine liquid chromagen (DAKO Corp., Carpenteria, CA) was placed on the samples for 5 minutes and rinsed, after which the slides were soaked in 1% $CuSO_4$ for another 5 minutes. Subse-

quently, the sections were rinsed, counterstained with hematoxylin, dehydrated through graded alcohol and xylene, and finally coverslipped. Negative controls were provided by omitting the primary antibodies.

Two readers experienced with hENT1 expression and hENT1 staining patterns assessed and scored the hENT1 immunostaining intensities. All hENT1 staining was scored, including membranous and cytoplasmic. All hENT1 immunostaining was performed twice and both sets of scores were consistent. Both readers were blinded to the clinical characteristics and outcomes data. Scoring for hENT1 was based on relative intensities of staining of the pancreatic tumor with reference to the normally strong hENT1 staining of lymphocytes, using a previously established system.[17] These internal references then were used as internal positive controls between slides and samples as well as for the staining procedure. Pancreatic tumor tissue then was evaluated by comparison with the internal controls. A score of *high hENT1 staining* was given for strong reactivity in greater than 50% of neoplastic cells. A score of *no hENT1 staining* was given if there was no staining in greater than 50% of cells. A score of *low hENT1 staining* was given to all cases in between. Because each patient's tumor was represented on each of the 3 TMAs, the maximum score for all 3 of the TMAs was used as the final hENT1 score for that patient (No hENT1, Low hENT1, or High hENT1).

Statistical Analyses

The hENT1 immunohistochemistry and SNP genotype scores were submitted to the RTOG Statistical Core for analysis without knowledge of patient demographics, treatment arm randomization, or outcome. A statistical comparison to assess whether missing hENT1 data were associated with baseline characteristics was performed using the chi-square test.

hENT1 expression was dichotomized as No hENT1 vs combined Low and High hENT1. hENT1 also was broken down into 2 dummy variables with a value of No hENT1 as the reference level: No hENT1 vs Low hENT1, and No hENT1 vs High hENT1. The following pretreatment characteristics were dichotomized: pathologic t-stage (stage T1 and T2 vs stage T3 and T4), American Joint Committee on Cancer stage (stage I and II vs stage III and IV), and primary tumor location (head vs everything else). Race was categorized as white, African American, or other. The following toxicities also were dichotomized: worst overall (grades 1, 2 vs grade 3 or higher), worst hematologic (grades 1, 2 vs grade 3 or higher), and worst nonhematologic (grades 1, 2 vs grade 3 or higher). The failure event for overall survival was defined as death from any cause. Survival time was measured from the date of randomization to the date of death or last follow-up evaluation. The failure event for disease-free survival was defined as disease relapse (local or regional), distant disease (including abdominal ascites, peritoneal seeding, and other abdominal sites), second primary or death from any cause. Disease-free time was measured from the date of randomization to the date of first disease-free failure event occurrence.

Association between hENT1 protein expression, either dichotomized (No hENT1 vs combined Low and High hENT1) or ungrouped (No hENT1 vs Low hENT1 vs High hENT1) with tumor demographic details, toxicity, and treatment outcome (overall survival and disease-free survival) were sought by unconditional logistic regression analysis using the chi-square test and the Cox proportional hazards model. Both treatment arms of the study were analyzed. Univariate and multivariate analysis for correlation between overall survival and disease-free survival and patient clinical features were calculated according to the Kaplan–Meier method and compared by the log-rank test based on the pattern of hENT1 immunostaining. The following variables were included in the multivariate analyses: nodal involvement (no vs yes), tumor diameter (<3 vs ≥ 3 cm), Karnofsky Performance Scale (100, 90 vs 60, 70, 80), surgical tumor stage (I, II vs III, IV), and surgical margin status (negative vs positive and negative vs unknown). Because there were 3 possible responses for surgical margin status (negative, positive, or unknown), this variable was broken into 2 dummy variables with a value of negative as the reference level. Results were expressed as a hazard ratio (HR) (HR < 1 denoting survival benefit) and were considered significant at a P value of .05 or less. By using statistical power calculations for survival models, our analysis was designed such that, with the available tissue (100 samples in each arm) and 20%–25% of patient samples having no hENT1 expression (control arm), it would provide an 80% power to detect a 40% decrease in HR for all patients with a P value of .05.

Results

Patient Population

The study opened July 20, 1998, and closed on July 26, 2002, with a total of 538 patients, and the final results have been published.[19] Eighty-seven percent and 86% of patients assigned to the 5-FU group completed chemotherapy and radiotherapy, respectively, as planned. Ninety percent and 88% of patients assigned to the gemcitabine group completed chemotherapy and radiotherapy, respectively, as planned. Of the 268 patients entered in the gemcitabine arm, 91 were eligible and had analyzable hENT1. Of the 177 cases excluded, 47 were ineligible (including 12 patients with analyzable tissue hENT1 expression) and 130 had no analyzable tissue hENT1 (Figure 1). Possible associations between baseline characteristics and the determination of hENT1 levels were investigated to determine if missing data may have influenced the analysis. Tumor location of head (head vs everything else) was the only baseline characteristic to

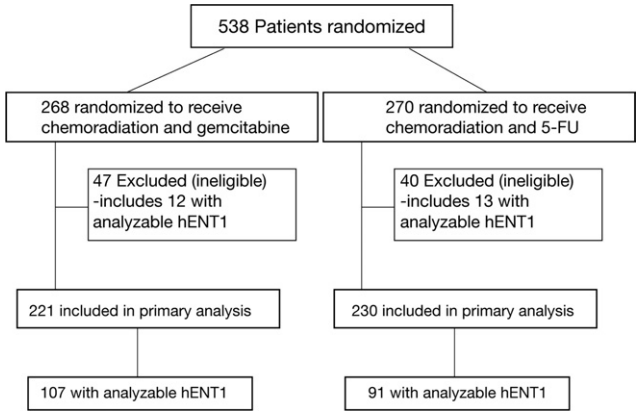

Figure 1. Flow of patients through study.

have a positive statistical association between missing and determined hENT1 in the gemcitabine treatment arm ($P = .02$).

Of the 270 patients entered in the 5-FU arm, 107 were eligible and had analyzable hENT1. Of the 163 cases excluded, 39 were ineligible (including 13 patients with analyzable tissue hENT1 expression), 1 patient withdrew, and 123 had no analyzable tissue hENT1 (Figure 1). Possible associations between baseline characteristics and the determination of hENT1 levels were investigated to determine if missing data may have influenced the analysis. No positive statistical associations existed for this grouping between missing and determined hENT1 in the 5-FU treatment arm.

hENT1 Immunohistochemistry

Of the 229 patient tumor samples evaluated, 6 patient samples were not analyzable for hENT1 immunohistochemistry because of inadequate tissue on the TMA. Of the remaining 223 patients, 25 were excluded from the analysis because they were excluded from the main trial analysis because of ineligibility rules, leaving 198 with analyzable hENT1 immunostaining. The localization of the hENT1 immunostaining was predominantly membrane, although occasional cytoplasmic staining was seen. For patients randomized to the gemcitabine arm with analyzable hENT1 (n = 91), No hENT1 staining was seen in 18 patients, Low hENT1 staining was seen in 39 patients, and High hENT1 staining was seen in 34 patients (Table 1). For those randomized to the 5-FU arm

Table 1. Characteristics of Patients Entered Into Gemcitabine and 5-FU Treatment Arm Based on hENT1 Immunohistochemistry Staining Score (No Staining, Low Staining, and High Staining)

	Gemcitabine				5-FU			
hENT IHC score	No stain	Low	High	*P* values[a]	No stain	Low	High	*P* values[a]
	18	39	34		26	41	40	
Age (*y*)								
Median	53	63	65	N/A	60	61	63	N/A
Gender				.27				
Male	11 (61%)	16 (41%)	19 (56%)		16 (62%)	23 (56%)	23 (58%)	.91
Female	7 (39%)	23 (59%)	15 (44%)		10 (38%)	18 (44%)	17 (43%)	
Primary location				.97				
Head	14 (78%)	30 (77%)	27 (79%)		23 (88%)	37 (90%)	31 (78%)	.23
T-stage				.99				.55
T1, T2	4 (22%)	9 (23%)	8 (24%)		9 (35%)	12 (29%)	9 (23%)	
T3, T4	14 (78%)	30 (77%)	26 (76%)		17 (65%)	29 (71%)	31 (78%)	
N-stage				.57				.54
N0	6 (33%)	16 (41%)	10 (29%)		11 (42%)	16 (39%)	12 (30%)	
N1	12 (67%)	23 (59%)	24 (71%)		15 (58%)	25 (61%)	28 (70%)	
Surgical margins				.51				.96
Complete resection/negative margins	4 (22%)	16 (41%)	15 (44%)		11 (42%)	18 (44%)	18 (45%)	
Complete resection/positive margins	7 (39%)	12 (31%)	12 (35%)		8 (31%)	15 (37%)	13 (33%)	
Complete resection/unknown margins	7 (39%)	11 (28%)	7 (21%)		7 (27%)	8 (20%)	9 (23%)	
Largest tumor dimension				.75				.43
<3 cm	6 (33%)	17 (44%)	13 (38%)		10 (38%)	18 (44%)	12 (30%)	
>3cm	12 (67%)	22 (56%)	21 (62%)		16 (62%)	23 (56%)	28 (70%)	
KPS (Categorized)				.07				.38
60, 70, 80	4 (22%)	20 (51%)	11 (32%)		8 (31%)	19 (46%)	14 (35%)	
90, 100	14 (78%)	19 (49%)	23 (68%)		18 (69%)	22 (54%)	26 (65%)	
Race				N/A[b]				N/A[b]
White	17 (94%)	36 (92%)	30 (88%)		25 (96%)	37 (90%)	32 (80%)	
African American	1 (6%)	1 (3%)	3 (9%)		0 (0%)	1 (2%)	5 (13%)	
Other	0 (0%)	2 (5%)	1 (3%)		1 (4%)	3 (7%)	3 (8%)	

KPS, Karnofsky Performance Scale.
[a]*P* value for chi-square test.
[b]Chi-square test not valid due to small cell counts.

with analyzable hENT1 (n = 107), 26 patients had No hENT1 staining, 41 patients had Low hENT1 staining, and 40 patients had High hENT1 staining (Table 1).

Analysis of Baseline Demographics

There were no positive statistical associations between baseline characteristics and hENT1 levels, both ungrouped (No hENT1, Low hENT1, and High hENT1) and grouped (No hENT1 and combined Low and High hENT1) in either the gemcitabine or 5-FU treatment arms (Table 1).

Analysis of Toxicity

Relationships between toxicities and hENT1 levels, both ungrouped (No hENT1, Low hENT1, and High hENT1) and grouped (No hENT1 and combined Low and High hENT1) were sought. A logistic regression analysis did not show a difference in incidence of grade 3 or higher toxicities (worst overall, worst hematologic, and worst nonhematologic) for the patients with combined Low and High hENT1) when compared with patients with No hENT1 for either the gemcitabine or 5-FU treatment arm (data not shown).

Analysis of Outcome

Univariate analysis. hENT1 protein levels were associated significantly with both overall and disease-free survival in the univariate models assessing patients in the gemcitabine treatment arm (Table 2). Among patients receiving gemcitabine, disease-free survival was prolonged in patients with combined Low and High hENT1 as compared with patients with No hENT1 (HR, 0.57; 95% confidence interval [CI], 0.32–1.00; $P = .05$). There was also an improvement in disease-free survival for patients with High hENT1 as compared with patients with No hENT1 for all patients (HR, 0.51; 95% CI, 0.27–0.97; $P = .04$). There was longer overall survival among gemcitabine-treated patients with combined Low and High hENT1 when compared with patients with No hENT1 (HR, 0.51; 95% CI, 0.29–0.91; $P = .02$). Similarly, overall survival was longer among patients with High hENT1 compared with patients with No hENT1 for all patients (HR, 0.42; 95% CI, 0.22–0.81; $P = .01$).

Conversely, hENT1 expression was not associated significantly with overall and disease-free survival in these univariate models for the dichotomized hENT1 level variable (No hENT1 vs combined Low and High hENT1) or for the ungrouped hENT1 level variable (No hENT1 vs Low hENT1 vs High hENT1) among those patients randomized to the 5-FU treatment arm.

Multivariate analysis. Multivariate analyses for dichotomized hENT1 (No hENT1 vs combined Low and High hENT1) and ungrouped hENT1 (No hENT1 vs Low hENT1 vs High hENT1) was performed for all patients. hENT1 expression was associated independently and significantly with overall and disease-free survival despite adjusting for baseline characteristics in these multivariate models for both groupings (Table 2). We observed prolonged disease-free survival for patients with combined Low and High hENT1 as compared with patients with No hENT1 for all patients (HR, 0.39; 95% CI, 0.21–0.73; $P = .003$) (Figure 2*A*). There was also an improvement in disease-free survival for patients with High hENT1 as compared with patients with No hENT1, and for patients with Low hENT1 as compared with patients with No hENT1 for all patients (adjusted HR, 0.36; $P = .003$; and adjusted HR, 0.43; $P = .01$) (Figure 2*C*). There was prolonged overall survival among patients with combined Low and High hENT1 as compared with patients with No hENT1 for all patients treated with gemcitabine (HR, 0.40; 95% CI, 0.22–0.75; $P = .03$) (Figure 3*A*). There was an independent improvement in overall survival for patients with Low hENT1 as compared with patients with No hENT1, and for patients with High hENT1 compared with patients with No hENT1 for all patients treated with gemcitabine (adjusted HR, 0.47; $P = .03$; and adjusted HR, 0.34; $P = .002$, respectively) (Figure 3*C*).

Table 2. HENT 1 Immunohistochemistry Score and Survival in Patients Treated With Gemcitabine or 5-FU Expressed as Hazard Ratio (H.R.) (95% CI)

	Gemcitabine arm		5-FU arm	
	Low/High hENT1 vs no hENT1	High hENT vs no hENT1	Low/High hENT1 vs no hENT1	High hENT vs no hENT1
Univariate analysis				
Overall survival	0.51 (0.29, 0.91) ($P = .02$)	0.42 (0.22, 0.81) ($P = .01$)	0.93 (0.58, 1.49) ($P = .75$)	0.78 (0.46, 1.34) ($P = .37$)
Disease free survival	0.57 (0.32, 1.001) ($P = .05$)	0.51 (0.27, 0.97) ($P = .04$)	0.88 (0.56, 1.40) ($P = .60$)	0.82 (0.49, 1.37) ($P = .44$)
Multivariate analysis				
Overall survival	0.40 (0.22, 0.75) ($P = .03$)	0.47 (0.24, 0.92) ($P = .04$)	0.78 (0.47, 1.27) ($P = .31$)	0.68 (0.40, 1.19) ($P = .18$)
Disease free survival	0.39 (0.21, 0.73) ($P = .003$)	0.36 (0.18, 0.71) ($P = .003$)	0.72 (0.45, 1.16) ($P = .18$)	0.71 (0.41, 1.21) ($P = .20$)

H.R., hazard ratio. H.R. < 1 denotes survival benefit.

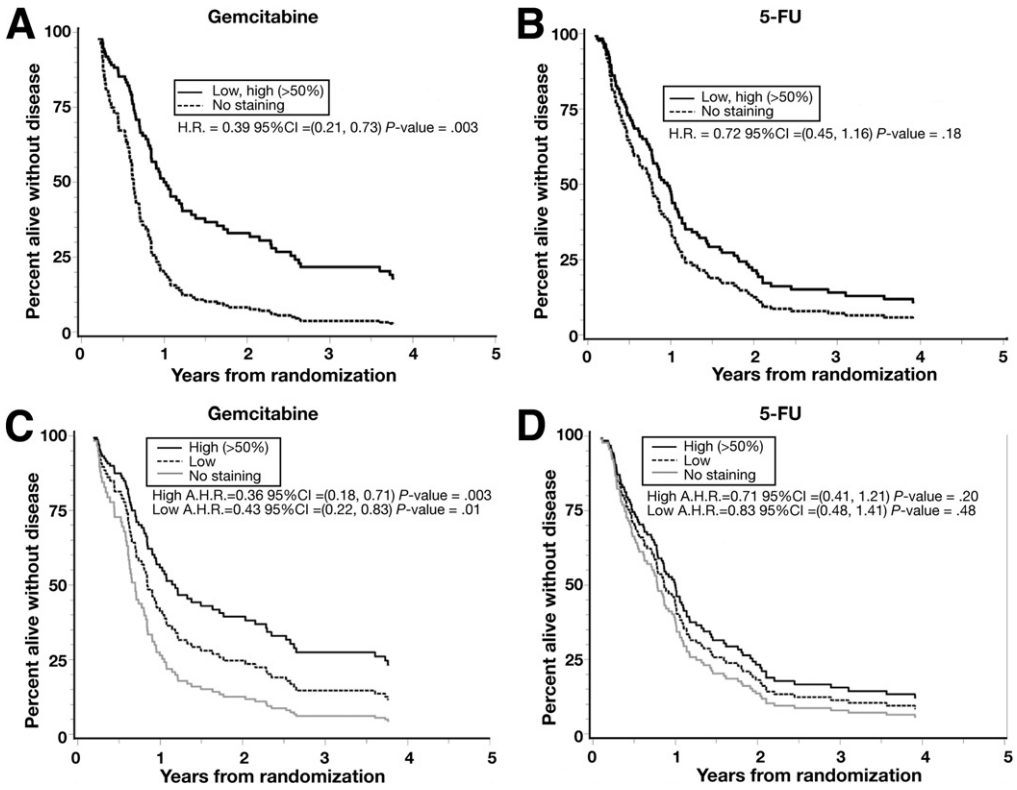

Figure 2. Disease-free survival for patients receiving either gemcitabine or 5-FU by multivariate analysis. (*A*) Disease-free survival in patients in the gemcitabine treatment arm comparing combined Low and High hENT1 (*solid line*) with No hENT1 (*dashed line*). From adjusted Cox proportional hazard model, adjusted for surgical margin. (*B*) Disease-free survival in patients in the 5-FU treatment arm comparing combined Low and High hENT1 (*solid line*) with No hENT1 (*dashed line*). From adjusted Cox proportional hazard model, adjusted for nodal involvement and Karnofsky Performance Scale. (*C*) Disease-free survival in patients in the gemcitabine treatment arm comparing No hENT1 (*thin line*) vs Low hENT1 (*dashed line*) vs High hENT1 (*thick line*). From adjusted Cox proportional hazard model, adjusted for surgical margin. No hENT1 staining is used as a reference value. (*D*) Disease-free survival in patients in the 5-FU treatment arm comparing No hENT1 (*thin line*) vs Low hENT1 (*dashed line*) vs High hENT1 (*thick line*). From adjusted Cox proportional hazard model, adjusted for nodal involvement and Karnofsky Performance Scale. No hENT1 staining was used as a reference value.

In the 5-FU treatment arm, hENT1 expression was not associated significantly with overall and disease-free survival after adjusting for baseline characteristics in these multivariate models for both groupings; dichotomized hENT1 (No hENT1 vs combined Low and High hENT1) (Figures 2*B* and 3*B*) and ungrouped hENT1 (No hENT1, Low hENT1, and High hENT1) (Figures 2*D* and 3*D*).

Discussion

This current study studied hENT1 in a phase III adjuvant therapy trial in early stage pancreas cancer. Patients were randomized to receive gemcitabine or 5-FU as part of their systemic therapy. Although slightly less than 50% of the patients entered into the trial had tissue available for hENT1 analysis, the missing data are not expected to bias our results because there were no significant imbalances between patient and tumor baseline characteristics (including tumor and nodal stage) in the analyzed and nonanalyzed patients.

Our data showed a strong relationship between hENT1 protein expression in pancreatic cancer and treatment outcome including overall survival and disease-free survival in patients with resected pancreatic cancer treated with adjuvant gemcitabine. This finding is statistically significant by univariate and multivariate analyses after adjustment for standard clinicopathologic prognostic factors. This correlation remained valid for high expressing hENT1 tumors in the univariate analysis, and both the low and high staining tumors (when compared with no staining) by multivariate analysis. The survival benefit of hENT1 protein expression was not seen in the 5-FU treatment arm, either by univariate or multivariate analyses. Although both arms received radiosensitizing amounts of 5-FU, the total amount of 5-FU given to patients who received gemcitabine is not thought to influence clinical outcomes compared with the nongemcitabine 5-FU arm. Furthermore, the inclusion of 5-FU in this arm would be more important if predictors of response to 5-FU were being evaluated. The current data do not support hENT1 as a prognostic marker with neither a statistical trend nor a statistically significant difference achieved in the patients treated with 5-FU alone. The

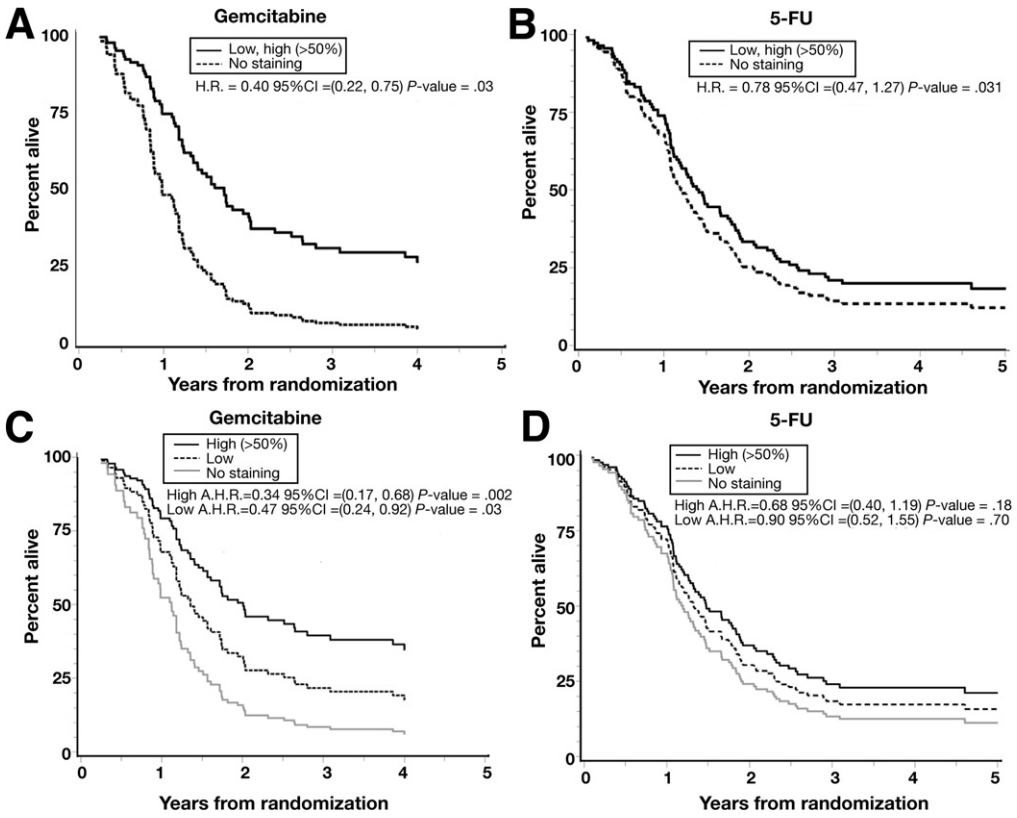

Figure 3. Overall survival for patients receiving either gemcitabine or 5-FU by multivariate analysis. (*A*) Overall survival in patients in the gemcitabine treatment arm comparing combined Low and High hENT1 (*solid line*) with No hENT1 (*dashed line*). From adjusted Cox proportional hazard model, adjusted for surgical margin. (*B*) Overall survival in patients in the 5-FU treatment arm comparing combined Low and High hENT1 (*solid line*) with No hENT1 (*dashed line*). From adjusted Cox proportional hazard model, adjusted for nodal involvement and Karnofsky Performance Scale. (*C*) Overall survival in patients in the gemcitabine treatment arm comparing No hENT1 (*thin line*) vs Low hENT1 (*dashed line*) vs High hENT1 (*thick line*). From adjusted Cox proportional hazard model, adjusted for surgical margin. No hENT1 staining was used as a reference value. (*D*) Overall survival in patients in the 5-FU treatment arm comparing No hENT1 (*thin line*) vs Low hENT1 (*dashed line*) vs High hENT1 (*thick line*). From adjusted Cox proportional hazard model, adjusted for nodal involvement and Karnofsky Performance Scale. No hENT1 staining was used as a reference value.

current data support the concept of hENT1 as a predictive marker.

Although kinetic studies of human cell lines with defined nucleoside transporter processes have shown that gemcitabine intracellular uptake can be mediated by hENT1, hENT2, hCNT1, and hCNT3, the hENT1 protein mediates the majority of gemcitabine transport in preclinical models.[2,4–6] The relationship between hENT1 RNA expression and gemcitabine sensitivity in pancreatic cancer cells remains unclear. One recent study did not show a correlation between hENT1 mRNA levels and median inhibitory values of gemcitabine in 8 pancreatic cancer cell lines studied ex vivo (PC143, PK1, KLM1, PK9, PK8, MIAPaCa2, KPIN, BxPC3), and hENT1 gene expression was not reduced after the development of acquired resistance to long-term continuous gemcitabine exposure.[14] Under these conditions, hENT1 mRNA expression alone does not appear to mediate inherent and acquired resistance to gemcitabine. The increase in hENT1 expression in PK1, PCI, and KLM1 pancreatic cancer cells lines after exposure to gemcitabine may reflect a compensatory adaptation to higher chemoresistance to gemcitabine.[14] Furthermore, this particular study suggested that acquired and inherent chemoresistance to gemcitabine of pancreatic cancer cells is determined by the balance of deoxycytidine kinase, RRM1, RRM2, and hENT1 gene expression, but not that of any individual gene.[14] It is likely that posttranslational modification may account for some of the inconsistencies seen between hENT1 gene expression and gemcitabine sensitivity in vitro.

When the hENT1 protein, the major mediator of gemcitabine cellular uptake, has been studied by immunohistochemistry in normal pancreas and pancreatic cancer tissue, hENT1 is readily detected in normal Langerhans cells, lymphocytes, and pancreatic adenocarcinoma cells, but not in normal glandular elements.[17] The hENT1 protein previously has been evaluated as a prognostic marker in gemcitabine-treated pancreatic cancer. Spratlin et al[17] studied 21 patients with pancreatic adenocarcinoma treated with gemcitabine (stage III, 1; stage IVA, 9; and stage IVB, 11 at time of initiation of gemcitabine). Nine patients had uniformly detectable hENT1 immunostaining and 12 patients possessed a proportion (10%–100%) of adenocarcinoma cells without detectable hENT1.

Patients lacking uniformly detectable hENT1 immunostaining had a median survival of 4 months (95% CI, 1.5–6.9 mo) from the time of initiation of gemcitabine, whereas patients with uniformly detectable hENT1 had a 3-fold longer median survival of 13 months (95% CI, 4.2–20.4 mo). Kaplan–Meier analysis of survival from gemcitabine initiation revealed a statistically significant separation in the survival curves ($P = .01$). Univariate analysis revealed no significant relationships between patient clinical characteristics (age, sex, performance status). A similar retrospective study evaluated hENT1 RNA expression in microdissected pancreatic tissue from 105 patients. Forty-seven of 62 resected patients received adjuvant treatment with gemcitabine and 36 of 43 patients received palliative treatment with gemcitabine. By using tertiles to define hENT1 gene expression, high hENT1 gene expression (>1.38) was associated significantly with increased time to disease progression in the palliative setting ($P = .02$) and disease-free survival in the adjuvant setting ($P < .01$).[18] Although both of these retrospective studies showed a prognostic role for hENT1 in pancreatic carcinoma, neither studied controls who did not receive gemcitabine. Consequently, the predictive value of hENT1 in gemcitabine-treated pancreatic carcinoma (ie, the ability to identify those patients most likely to benefit from gemcitabine) could not be assessed.

The regulation of hENT1 RNA expression and protein levels is poorly understood. hENT1 is expressed variably within normal tissues and malignant cells. Various factors that influence ENT1 expression have been identified, and include hypoxia and differentiation status.[22–24] Although the variability of hENT1 expression in pancreatic cancer is shown in this and other studies, the reason for the increased and variable hENT1 expression in pancreatic adenocarcinoma is not known.[17,18] One possibility includes hENT1 SNPs. Recently, in a study of 247 ethnically diverse individuals, several SNPs in hENT1 (SLC29A1), including 2 nonsynonymous ones: Ile216Thr (T216C) in exon 6 and Glu391Lys in exon 12, were identified in 1.2% and 0.4% of African Americans, Caucasians, and Mexican Americans. However, no functional alterations in hENT1 with these 2 nonsynonymous SNPs were found.[25] It is possible that other hENT1 SNPs may account for the variability in hENT1 expression.[26,27] Further, the modulation of hENT1 expression induced by thymidylate synthase inhibitors, as well as by the multitargeted antifolate pemetrexed, may represent a new way to explore effective modalities for pancreatic cancer treatment.[28,29] Although there is preclinical evidence suggesting the synergistic effect of gemcitabine and pemetrexed in pancreatic cancer cells that includes up-regulation of hENT1 as a mechanism of action, there are limited clinical data available for pancreatic cancer.[30,31] One trial comparing gemcitabine with gemcitabine and permetrexed for advanced pancreatic cancer did show a statistical difference in overall response rate but not for survival.[32]

Other key enzymes involved in gemcitabine metabolism, such as deoxycytidine kinase, cytidine deaminase, RRM1, and RRM2, also may have a key role in altering intracellular disposition of the drug and determining response to gemcitabine. However, there are limited clinical data available on the predictive value of these markers in pancreatic cancer. Single, small, retrospective, clinical studies have suggested the poor prognostic value of high levels of RRM1 or RRM2 gene expression and low deoxycytidine kinase protein expression in patients with pancreatic cancer treated with gemcitabine.[33–35] However, further study using larger prospectively collected data is required to determine the true predictive value of these markers as well as their influence on hENT1 expression.

In summary, the current study reports the predictive value of hENT1 immunohistochemistry for assessing benefit from gemcitabine adjuvant chemotherapy in patients with early stage pancreatic cancer. Although we have not been able to explain the variability of hENT1 expression, it is possible that nonsynonymous hENT1 SNP may account for some of this. Evaluation of hENT1 testing on pancreatic cancer tissue acquired with minimally invasive procedures (endoscopic ultrasound–guided fine-needle aspiration or computerized tomography–guided biopsy) warrants further study to determine the potential to individualize gemcitabine therapy in the majority of pancreatic cancer patients who present with locally advanced or metastatic disease. Finally, for other types of tumors for which gemcitabine is approved but there exist multiple other effective nongemcitabine regimens, our findings should prompt investigation to determine if oncologists can rationally choose between gemcitabine and nongemcitabine regimens based on hENT1 expression.

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Address requests for reprints to: James J. Farrell, MD, Division of Digestive Diseases, University of California Los Angeles School of Medicine, Los Angeles, California 90405. e-mail: jfarrell@mednet.ucla.edu; fax: (310) 794-9718.

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