Combined analysis of intratumoral human equilibrative nucleoside transporter 1 (hENT1) and ribonucleotide reductase regulatory subunit M1 (RRM1) expression is a powerful predictor of survival in patients with pancreatic carcinoma treated with adjuvant gemcitabine-based chemotherapy after operative resection

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Background. *Although postoperative adjuvant chemotherapy for pancreatic carcinoma improves survival in some patients, its efficacy varies among individuals. The aim of this study was to determine the usefulness of intratumoral expression of human equilibrative nucleoside transporter 1 (hENT1) and ribonucleotide reductase regulatory subunit M1 (RRM1) as predictive markers of the efficacy of adjuvant gemcitabine-based chemotherapy for pancreatic carcinoma after operative resection.*
Methods. *The expression of intratumoral hENT1 and RRM1 was examined immunohistochemically in 109 patients with pancreatic carcinoma who received adjuvant gemcitabine-based chemotherapy after operative resection. Relationships between clinicopathologic factors, including hENT1 and RRM1 expression, and disease-free and overall survival (DFS and OS) were evaluated by univariate and multivariate analyses.*
Results. *The 5-year DFS and OS rates for the 109 patients were 26% and 31%, respectively. In univariate analysis, both hENT1 and RRM1 expression were significantly associated with DFS (hENT1,* P = *.004; RRM1,* P = *.011) and OS (hENT1,* P = *.001; RRM1,* P = *.040). In multivariate analysis, both were independent factors for DFS (hENT1,* P = *.001; RRM1,* P = *.009) and OS (hENT1,* P = *.001, RRM1,* P = *.019). Evaluation of the combination analysis of both was also identified as a powerful independent predictor of DFS (*P $<$ *.001) and OS (*P $<$ *.001).*
Conclusion. *Expression of hENT1 and RRM1 is predictive of the efficacy of adjuvant gemcitabine-based chemotherapy for pancreatic carcinoma after operative resection. In addition, their combined analysis has greater predictive value than either factor alone. (Surgery 2013;153:565-75.)*

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Pancreatic carcinoma is one of the most lethal human malignancies; it has an extremely poor prognosis.[1] Operative resection offers the only chance of cure or long-term survival for patients with this disease; however, the actuarial 5-year survival rate has been reported to be less than 20%, even after operative resection with curative intent.[2-6] Therefore,

surgery alone is not a sufficient treatment for pancreatic carcinoma, and effective adjuvant therapies have an impact on long-term survival.[7] In 1997, Burris et al[8] reported that gemcitabine (difluorodeoxycytidine; dFdC) yielded significant improvements in survival for unresectable pancreatic carcinoma, and recently, large-scale randomized controlled trials have demonstrated that adjuvant gemcitabine chemotherapy has a beneficial effect in patients with pancreatic carcinoma after operative resection.[9,10] Gemcitabine has been accepted as the current standard anticancer drug for patients with unresectable or resected pancreatic carcinoma.

Gemcitabine is a deoxycytidine analog that has broad antitumor activity in various solid neoplasms, including pancreatic cancer[8-10] and non–small cell lung cancer.[11] Gemcitabine is transported into cells predominantly by human equilibrative nucleoside transporter 1 (hENT1).[12] A deficiency in hENT1 activity conferred high-level resistance to the toxicity of gemcitabine,[13] and patients with pancreatic carcinoma who have detectable hENT1 or high hENT1 gene expression have significantly prolonged survival after gemcitabine chemotherapy.[14,15] As a prodrug, gemcitabine must be phosphorylated to its active diphosphate (dFdCDP) and triphosphate (dFdCTP) that, respectively, inhibit ribonucleotide reductase (RR) and DNA synthesis.[16]

Deoxycytidine kinase (dCK) is the rate-limiting enzyme in the biotransformation of nucleoside analogues, and an increase in dCK activity may improve the efficacy of gemcitabine.[17] In contrast, the active metabolites of gemcitabine are reduced by 5′-nucleotidase, and gemcitabine itself is inactivated by cytidine deaminase. dFdCTP inhibits DNA synthesis by being incorporated into the DNA strand, but in addition, dFdCDP potently inhibits RR, resulting in a decrease in competing deoxyribonucleotide pools necessary for DNA synthesis.[18] RR is a dimeric enzyme composed of a regulatory subunit M1 and a catalytic subunit M2. Recurrently, patients with pancreatic carcinoma who had high levels of RRM1 expression had poor survival rates after gemcitabine treatment,[19] and patients with non–small cell lung cancer who had low levels of RRM1 expression significantly benefited from gemcitabine/cisplatin neoadjuvant chemotherapy.[20]

Since 2002, postoperative adjuvant gemcitabine-based chemotherapy has been administered to patients with pancreatic carcinoma at our institution, and we have already reported that this approach improves long-term survival.[21-23] However, the efficacy of gemcitabine-based chemotherapy varies among individuals. Therefore, we have tried to identify predictive markers of the efficacy of adjuvant gemcitabine-based chemotherapy to help improve the survival of patients with resected pancreatic carcinoma. We hope that our findings will lead to an optimized adjuvant chemotherapy protocol. The aim of this study was to determine the usefulness of hENT1 and RRM1 expression as predictive markers of adjuvant gemcitabine-based chemotherapy for pancreatic carcinoma after operative resection.

METHODS

Study design. One hundred nine patients with pancreatic adenocarcinoma who received adjuvant gemcitabine-based chemotherapy after curative operative resection (R0 or R1 resection) at the Department of Surgery, Hiroshima University Hospital from January 2002 to May 2011 were enrolled in this study. A diagnosis of pancreatic adenocarcinoma was confirmed histologically in all cases. Other histologic variants, such as pancreatic carcinoma derived from mucinous cystic neoplasms and intraductal papillary-mucinous neoplasms, were excluded from this analysis. Patients with distant metastasis and peritoneal dissemination also were excluded from this analysis, even if they had undergone resection. However, patients with para-aortic lymph node metastasis, which was diagnosed by postoperative histologic examination and not by preoperative imaging examinations, were included. Formalin-fixed, paraffin-embedded tumor tissues from the resected specimens were collected from all patients, and immunohistochemical analysis of hENT1 and RRM1 expression was performed. Relationships between clinicopathologic factors, including immunohistochemical hENT1 and RRM1 expression and disease-free survival (DFS) and overall survival (OS), were evaluated with univariate and multivariate survival analyses. Written informed consent was obtained from all patients for operative treatment, adjuvant chemotherapy and pathologic examinations according to the institutional guidelines.

Operative procedures. Types of operative resections included pancreatoduodenectomy, pylorus-preserving pancreatoduodenectomy, distal pancreatectomy, and total pancreatectomy. Patients with carcinoma in the pancreatic head usually underwent pylorus-preserving pancreatoduodenectomy. Patients with carcinoma in the pancreatic body or tail underwent distal pancreatectomy with splenectomy. All patients underwent regional and para-aortic lymph node dissection. Partial resection of the portal vein was performed if the surgeon observed invasion of the portal vein by neoplasm at the time of the operation. Intraoperative pathologic assessment of proximal or distal pancreatic margins was performed with

frozen-tissue sections. If the pancreatic margin was positive for cancer cells, further resection of the pancreas was performed to the maximum extent possible. Total pancreatectomy was performed only in cases in which negative margins could only be achieved with total pancreatectomy, based on preoperative or intraoperative diagnosis.

Pathologic investigation. After the neoplasms were resected, all specimens were examined histologically, and each neoplasms was classified as well-differentiated, moderately differentiated, or poorly differentiated adenocarcinoma according to the predominant pathological grading of differentiation. Anterior serosal invasion, retropancreatic tissue invasion, splenic or portal vein invasion, splenic artery invasion, lymph node metastasis, and extrapancreatic nerve plexus invasion all were examined pathologically. Residual tumor (R factor) was considered R1 if infiltrating adenocarcinoma was present at the proximal or distal pancreatic transaction line or in dissected peripancreatic soft-tissue margins. The final stage of pancreatic carcinoma was examined pathologically according to the Tumor, Node, Metastasis (ie, TNM) classification system of malignant neoplasms published by the International Union Against Cancer (UICC), 7th edition.[24]

Postoperative adjuvant gemcitabine-based chemotherapy. The regimen of adjuvant chemotherapy with gemcitabine was reported previously.[21-23] Patients who received postoperative adjuvant gemcitabine-based chemotherapy had 2 options after operative resection: intravenous chemotherapy alone or intravenous and oral chemotherapy. Intravenous chemotherapy consisted of gemcitabine 700 mg/m^2 administered biweekly for 30 minutes by intravenous drip infusion. Patients who received intravenous and oral chemotherapy were given intravenous gemcitabine 700 mg/m^2 on day 1 and oral S1 50 mg/m^2 for 7 consecutive days; this cycle was repeated every 14 days. S1 is a novel oral fluoropyrimidine combination that includes tegafur (a prodrug of 5-fluorouracil), dihydropyrimidine dehydrogenase inhibitor (5-chloro-2,4-dihydroxypyrimidine), and orotate phosphoribosyltransferase inhibitor (potassium oxonate).[25] Neither external-beam radiation nor intraoperative irradiation was administered to any of the patients. Patients who had to switch to other chemotherapies before 10 cycles because of recurrent disease were included in this study. Patients who received gemcitabine-based chemotherapy because of recurrent disease after completion of adjuvant gemcitabine-based chemotherapy were also included.

Immunohistochemical analysis of hENT1 and RRM1 expression. Hematoxylin and eosin-stained slides containing specimens from each pancreatic carcinoma were reviewed, and a representative tumor region and the corresponding formalin-fixed, paraffin-embedded tissue block was selected for use in a tissue microarray. Immunohistochemistry was performed with the streptavidin-peroxidase technique and the Dako Envision+ system (Dako Cytomation GmbH, Hamburg, Germany).[15,26] To evaluate hENT1 expression, an affinity-purified polyclonal rabbit antibody against human hENT1 was purchased from Abnova Co., Taipei, Taiwan; RRM1 expression was evaluated with a polyclonal rabbit antibody against human RRM1 (ab81085) purchased from Abcam (Cambridge, UK). The immunohistochemical staining procedure was as follows: tissues were cut as 4-μm serial sections from tissue microarray paraffin blocks, deparaffinized in xylene, and rehydrated through a series of graded ethanol solutions. After antigen retrieval by autoclaving (100°C for 10 minutes in Dako Target Retrieval Solution High pH x1 for hENT1; 121°C, 10 min in 0.01 M citrate buffer for RRM1), sections were immersed in methanol containing 3% hydrogen peroxide for 15 minutes and incubated in protein blocking solution (Dako, Carpinteria, CA) for 10 minutes. Sections were incubated with appropriate dilutions of hENT1 antibody (1:100 dilution) overnight at 4°C and RRM1 antibody (1:150 dilution) for 60 minutes at room temperature.

After being washed 3 times in phosphate-buffered saline, samples were incubated in labeled streptavidin-biotin polymer (Envision Plus, Dako) at room temperature for 60 minutes as a secondary antibody. After being washed 3 times in phosphate-buffered saline, the slides were immersed for 10 min in 0.01% 3,3-diaminobenzidine solution in 50 mM Tris-HCl buffer with 10 mM hydrogen peroxide as a substrate. Sections were counterstained with Mayer's hematoxylin solutions, dehydrated through graded ethanol and xylene solutions, and finally mounted. Negative control consisted of sections incubated without the primary antibodies.

Staining intensity was evaluated by light microscopy and Image-Pro Plus version 4.0 software (Media Cybernetics, Silver Spring, MD). Pancreatic islet cells were used as an internal positive control for anti-hENT1 staining because hENT1 is strongly expressed in islet cells and lymphocytes.[15,26] Plasma and stromal cells were used as an internal positive control for anti-RRM1 staining because RRM1 is strongly expressed in plasma and stromal

cells.[27-31] Negative controls were obtained by replacement of the primary antibody with buffer. The intensity of hENT1 staining was scored as follows: grade 0, not stained; grade 1, faintly stained; grade 2, weakly stained compared with islet cells; and grade 3, stained as strongly as islet cells. The intensity of RRM1 staining was scored as follows: grade 0, not stained; grade 1, faintly stained; grade 2, weakly stained compared with plasma and stromal cells; and grade 3, stained as strongly as plasma and stromal cells. For evaluation of intratumoral hENT1and RRM1 expression, if grade 2 or 3 staining was observed in greater than 50% of the neoplasms, the sample was considered to have high hENT1 and RRM1 expression, and if grade 0 or 1 staining was observed in greater than 50% of tumor cells, the sample was considered to have low hENT1 and RRM1 expression (Fig 1). This cutoff value was determined on the basis of a previous report.[26,32] Immunohistochemical evaluation of hENT1 and RRM1 expression was confirmed independently by 2 observers (N.N. and Y.M.) in a blinded manner. In cases of disagreement, consensus was reached by joint review.

Survival. All patients were followed regularly in outpatient clinics by undergoing a blood test or computed tomography every 3 to 6 months. Diagnosis of recurrence was made on the basis of imaging findings. Information on outcomes beyond 5 years after surgery was collected by telephone or personal interview. For patients who died, survival time after surgery and the cause of death were recorded. For surviving patients, postoperative survival time and recurrence status were recorded. Clinical data were available from 109 patients who were followed-up until June 14, 2011, with follow-up periods ranging from 2 to 122 months (median, 39.7) after surgery.

Statistical analysis. The χ^2 test or Fisher exact test was used for univariate comparison between the 2 groups. Survival curves were constructed on the basis of the Kaplan-Meier method, and differences in survival curves were compared with a univariate log-rank (Mantel-Cox) test. Factors found to be significant by univariate analysis were subjected to multivariate analysis with a Cox proportional hazards model. Data were analyzed with SPSS for Windows (IBM SPSS Statistics 19.0). Statistical significance was set at $P < .05$.

RESULTS

Patient demographic and neoplasm characteristics. Of the 109 patients, 52 (48%) were males and 57 (52%) were females with a median age of 67 years (range, 41–83). Pancreatoduodenectomy, distal pancreatectomy, and total pancreatectomy were performed for 74 (68%), 30 (28%), and 5 patients (4%), respectively. The pancreatic neoplasm was confined to the head and to the body/tail of the pancreas in 72 patients (66%) and 37 patients (34%), respectively. Twenty-three patients (21%) had undergone R1 resection. Neoplasms were identified as well-differentiated adenocarcinoma in 52 patients (48%), moderately differentiated adenocarcinoma in 43 patients (39%), and poorly differentiated adenocarcinoma in 14 patients (13%).

According to the TNM classification, 7 (6%), 6 (6%), and 96 (88%) patients had T1, T2, and T3 neoplasms, respectively, and 70 patients (64%) had lymph node metastases. Finally, 5 (5%) patients had stage IA disease, whereas 3 (3%) had stage IB, 29 (26%) had stage IIA, 63 (58%) had stage IIB, and 9 (8%) had stage IV, respectively. All 9 patients with stage IV disease had para-aortic lymph node metastases detectable only on postoperative histologic examination but not on preoperative imaging examinations.

Delivery of adjuvant gemcitabine-based chemotherapy. Of the 109 patients, 96 (88%) received 10 or more cycles of adjuvant gemcitabine-based chemotherapy. The other 13 (12%) had to switch to other chemotherapy regimens before 10 cycles because of recurrent disease; however, they received at least 6 cycles of adjuvant gemcitabine-based chemotherapy (1 patient with 6 cycles, 4 patients with 7 cycles, 7 patients with 8 cycles, and 1 patient with 9 cycles). The median total dose of gemcitabine administered to the 109 patients was 16,033 mg (range, 6,000–40,000). No treatment-related deaths were reported in any of the patients.

Relationship between clinicopathologic factors and intratumoral hENT1 and RRM1 expression. High intratumoral expression of hENT1 and RRM1 was observed in 78 (72%) and 44 (40%) cases, respectively. Clinicopathologic factors were compared between patients with high hENT1 expression and those with low hENT1 expression as well as between patients with high RRM1 expression and those with low RRM1 expression. Among the 8 clinicopathologic factors evaluated, no significant differences were observed between these 2 pairs of patient groups (Table I). There were no significant correlations between intratumoral hENT1 expression and intratumoral RRM1 expression.

Relationship between patient survival and intratumoral hENT1 and RRM1 expression. DFS rates for all 109 patients were 59% at 1 year, 42% at 2 years, and 26% at 5 years, and OS rates were 81%

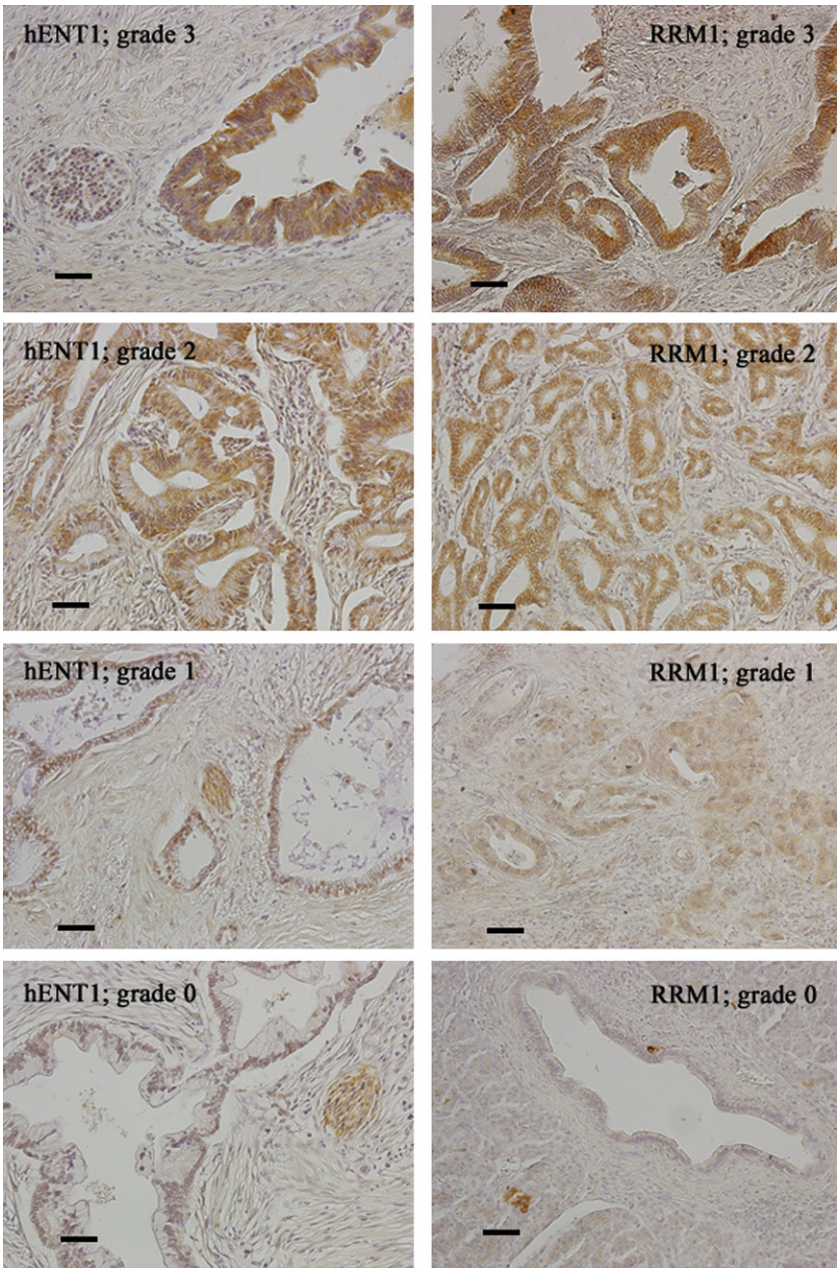

Fig 1. Immunohistochemical analysis of hENT1 and RRM1 expression in pancreatic carcinoma. The intensity of hENT1 staining was scored as follows: grade 0, not stained; grade 1, faintly stained; grade 2, weakly stained compared with islet cells; and grade 3, stained as strongly as islet cells. The intensity of RRM1 staining was scored as follows: grade 0, not stained; grade 1, faintly stained; grade 2, weakly stained compared with plasma and stromal cells; and grade 3, stained as strongly as plasma and stromal cells. (Bar = 20 μm).

at 1 year, 61% at 2 years, and 31% at 5 years. The median DFS and OS were 17.8 months and 34.9 months, respectively.

The results of univariate DFS and OS analyses are shown in Table II. UICC pT factor ($P = .033$), lymph node metastasis ($P < .001$), UICC final stage ($P = .025$), hENT1 expression ($P = .004$, Fig 2A), and RRM1 expression ($P = .011$, Fig 2B) were significantly associated with DFS. Furthermore, R factor ($P = .042$), UICC pT factor ($P = .019$), lymph node metastasis ($P = .001$), hENT1 expression ($P = .001$, Fig 2C), and RRM1 expression ($P = .040$, Fig 2D) were significantly associated with OS. We classified each of the 109 patients into 1 of 4 groups according to hENT1 and RRM1 expression: high hENT1/low RRM1 expression ($n = 42$), high

Table I. Comparison of clinicopathologic factors based on intratumoral hENT1 and RRM1 expression ($N = 109$)

Factors	*No. patients*		P *value*	*No. patients*		P *value*
	High hENT1 (n = *78*)	*Low hENT1* (n = *31*)		*High RRM1* (n = *44*)	*Low RRM1* (n = *65*)	
Age						
≤70	43	18	.781	24	37	.806
>70	35	13		20	28	
Sex						
Male	36	16	.607	22	30	.693
Female	42	15		22	35	
Location of neoplasm						
Head	51	21	.815	33	39	.105
Body/tail	27	10		11	26	
R factor						
R0	65	21	.072	32	54	.194
R1	13	10		12	11	
Pathologic differentiation						
Well	39	13	.447	24	28	.240
Moderate/poor	39	18		20	37	
UICC pT factor						
T1/T2	10	3	.648	3	10	.176
T3	68	28		41	55	
Lymph node metastasis						
Yes	46	24	.070	33	37	.053
No	32	7		11	28	
UICC final stage						
IA/IB	7	1	.299	2	6	.357
IIA/IIB/IV	71	30		42	59	

hENT1, Human equilibrative nucleoside transporter 1; *RRM1*, ribonucleotide reductase regulatory subunit M1; *UICC*, International Union Against Cancer.

hENT1/high RRM1 expression ($n = 36$), low hENT1/low RRM1 expression ($n = 23$), and low hENT1/high RRM1 expression ($n = 8$). This combined classification was significantly associated with both DFS ($P < .001$, Fig 3A) and OS ($P < .001$, Fig 3B) by univariate analysis. Patients with high hENT1/low RRM1 expression experienced significantly longer DFS ($P = .009$) and OS ($P = .041$) than those with high hENT1/high RRM1 expression, significantly longer DFS ($P = .007$) and OS ($P = .014$) than those with low hENT1/low RRM1 expression, and significantly longer DFS ($P < .001$) and OS ($P < .001$) than those with low hENT1/high RRM1 expression (Table II).

A multivariate proportional hazards regression model was fit using the prognostic factors identified as significant in the univariate analysis. The UICC final stage was not included in the multivariate analysis because the UICC stage itself depends upon pT factor and lymph node metastasis and seems to be confounded by them. First, we subjected the 4 (UICC pT factor, lymph node metastasis, hENT1 expression, and RRM1 expression) and 5 (R factor, UICC pT factor, lymph node metastasis, hENT1 expression, and RRM1 expression) significant prognostic factors to multivariate DFS and OS analyses, respectively. Multivariate DFS analysis identified lymph node metastasis ($P < .001$), hENT1 expression ($P = .001$), and RRM1 expression ($P = .009$) as independent factors. Multivariate OS analysis identified UICC pT factor ($P = .047$), lymph node metastasis ($P = .016$), hENT1 expression ($P = .001$), and RRM1 expression ($P = .019$) as independent factors (Table III, Model 1).

Next, we subjected the combined hENT1 and RRM1 classification to multivariate analysis. As a result, our multivariate DFS analysis identified lymph node metastasis ($P = .001$) and the combined hENT1 and RRM1 classification ($P < .001$) as independent factors. Multivariate OS analysis also identified lymph node metastasis ($P = .018$) and the combined hENT1 and RRM1 classification ($P < .001$) as independent factors (Table III, Model 2).

Table II. Univariate disease-free and overall survival analyses of prognostic factors for 109 patients who received adjuvant gemcitabine-based chemotherapy

		Disease-free survival		*Overall survival*	
Factors	*No. patients*	*5-year survival rate (%)*	P *value*	*5-year survival rate (%)*	P *value*
Age					
≤70	61	29	.804	26	.562
>70	48	23		35	
Sex					
Male	52	31	.868	20	.457
Female	57	24		38	
Location of neoplasm					
Head	72	20	.350	27	.527
Body/tail	37	37		38	
R factor					
R0	86	29	.128	37	.042
R1	23	16		11	
Pathologic differentiation					
Well	52	28	.191	21	.056
Moderate/poor	57	24		30	
UICC pT factor					
T1/T2	13	57	.033	59	.019
T3	96	20		25	
Lymph node metastasis					
Yes	70	13	<.001	17	.001
No	39	49		55	
UICC final stage					
IA/IB	8	69	.025	64	.054
IIA/IIB/IV	101	21		29	
hENT1 expression					
High	78	30	.004	38	.001
Low	31	17		13	
RRM1 expression					
High	44	16	.011	24	.040
Low	65	32		37	
high hENT1/low RRM1	42	38	<.001	47	<.001
high hENT1/high RRM1	36	18		30	
low hENT1/low RRM1	23	20		17	
low hENT1/high RRM1	8	0		0	

hENT1, Human equilibrative nucleoside transporter 1; *RRM1*, ribonucleotide reductase regulatory subunit M1; *UICC*, International Union Against Cancer.

DISCUSSION

Gemcitabine still plays an important role in adjuvant chemotherapy for patients with resected pancreatic carcinoma, as determined by the results of large randomized phase 3 trials, including the Charite Onkologie 001 (CONKO-001) study[9] and the European Study Group for Pancreatic Cancer 3 (ESPAC-3) study.[10] However, the efficacy of adjuvant gemcitabine chemotherapy is unsatisfactory, with a median survival time of 22–24 months in these studies,[9,10] because a substantial number of patients are resistant to gemcitabine. Therefore, in the current study, we focused on intratumoral hENT1 and RRM1 expression as predictive biomarkers of the efficacy of adjuvant gemcitabine chemotherapy and investigated the relationships between intratumoral hENT1 and RRM1 expression and DFS and OS in patients with resected pancreatic carcinoma who received adjuvant gemcitabine-based chemotherapy. We found that both hENT1 and RRM1 expression were independent predictive biomarkers of efficacy in patients with resected pancreatic carcinoma treated with adjuvant gemcitabine-based chemotherapy. Moreover, the combined hENT1 and RRM1 classification was a more powerful predictor of OS and DFS than either factor alone in this cohort.

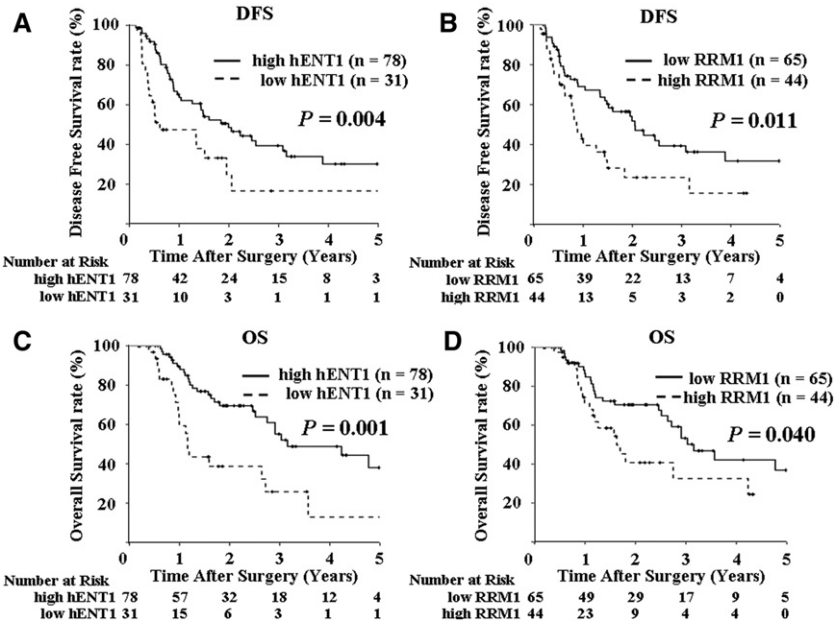

Fig 2. DFS and OS curves stratified by intratumoral hENT1 and RRM1 expression. (*A*) DFS curves stratified by hENT1 expression ($P = .004$). (*B*) DFS curves stratified by RRM1 expression ($P = .011$). (*C*) OS curves stratified by hENT1 expression ($P = .001$). (*D*) OS curves stratified by RRM1 expression ($P = .040$).

Reports regarding intratumoral hENT1 and RRM1 expression in resected specimens of pancreatic carcinoma are scarce. According to a few reports in which authors evaluated intratumoral hENT1 and RRM1 expression by immunochemical staining, the proportions of high hENT1 and RRM1 expression were observed in 59–78%[32-34] and 50%,[30] respectively. In the current study, 72% and 40% of patients with resected pancreatic carcinoma had high intratumoral hENT1 and RRM1 expression, respectively, which is similar to that found in the previous reports.

With regard to the relationships between hENT1 or RRM1 expression and clinicopathological factors, Farrell et al[26] reported that there were no positive statistical correlations between hENT1 expression levels and clinicopathologic factors in an analysis of 198 patients with resected pancreatic carcinoma. Another investigator also reported that there were no relationships between hENT1 expression levels and clinicopathological factors.[33] In addition, Akita et al[30] reported that there were no significant differences in clinicopathologic factors, including UICC pT factor and lymph node status, between patients with high RRM1 expression and those with low RRM1 expression in an analysis of 64 patients with resected pancreatic carcinoma. Similar to these reports, the current study demonstrated that no significant differences in clinicopathologic factors were found between patients with high hENT1 expression and those with low hENT1 expression as well as between patients with high RRM1 expression and those with low RRM1 expression. Both hENT1 and RRM1 expression seem to be independent from other clinicopathologic factors.

The prognostic impact of intratumoral hENT1 and RRM1 expression in patients with pancreatic carcinoma who received gemcitabine-based chemotherapy was demonstrated by several investigators.[14,15,26,30,32-34] Farrell et al[26] reported that hENT1 protein expression was independently associated with increased DFS and OS in patients with resected pancreatic carcinoma who received gemcitabine in the adjuvant setting, but not in those who received 5-fluorouracil. In an analysis of 55 patients with resected pancreatic carcinoma who received gemcitabine-based chemoradiotherapy, Murata et al[33] reported that the 1-and 3-year OS rates were significantly greater in the high hENT1 expression group than in the low hENT1 expression group. Other studies also demonstrated prolonged survival for patients with high hENT1 expression in patients with pancreatic carcinoma, who were treated with gemcitabine.[14,15,32,34] In addition, Akita et al[30] reported that patients with low RRM1 expression had significantly better OS than patients with high RRM1 expression in an analysis of 68 patients with resected pancreatic carcinoma who received gemcitabine chemotherapy.

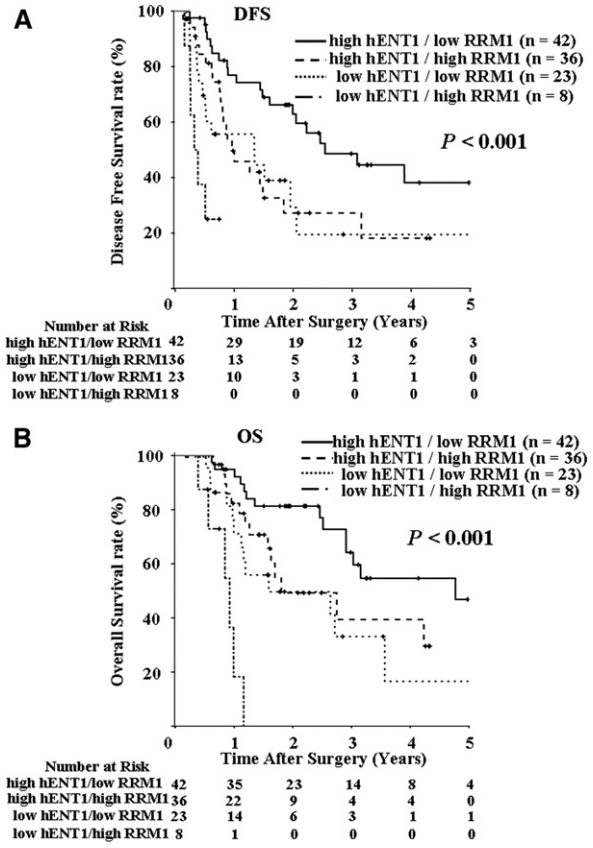

Fig 3. DFS and OS curves stratified by the combined analysis of intratumoral hENT1 and RRM1 expression. (*A*) DFS ($P < .001$). (*B*) OS ($P < .001$).

Similar to these reports, we demonstrated that high hENT1 or low RRM1significantly prolonged DFS and OS rates of patients with resected pancreatic carcinoma, who received adjuvant gemcitabine-based chemotherapy, and both hENT1 and RRM1 expression were independent predictors in this cohort. We believe that intratumoral hENT1 expression and intratumoral RRM1 expression are useful biomarkers for predicting the survival of patients with pancreatic carcinoma who are treated with gemcitabine after operative resection.

The mechanisms of gemcitabine resistance with hENT1 and RRM1 are different, as mentioned above.[12-18] Therefore, the intratumoral expression of hENT1 is thought to be theoretically independent from that of RRM1. In fact, there was no correlation between intratumoral hENT1 expression and intratumoral RRM1 expression, and both biomarkers were independent predictors of DFS and OS in patients with resected pancreatic carcinoma who received adjuvant gemcitabine-based chemotherapy in this study. On the basis of these findings, combined analysis of intratumoral hENT1 and RRM1 expression was expected to bring more useful information for predicting patient survival. In the current study, the combined hENT1 and RRM1 classification was a more powerful independent predictor of DFS and OS in patients with resected pancreatic carcinoma who received adjuvant gemcitabine-based chemotherapy. Surprisingly, no patient with low hENT1 expression and high RRM1 expression, which are both unfavorable factors, survived longer than 2 years after operative resection, despite administration of adjuvant gemcitabine-based chemotherapy. Recently, new anticancer regimens that did not include gemcitabine were reported to have a beneficial survival effect in patients with unresectable or resected pancreatic carcinoma.[10,35] These regimens may be recommended for patients with low hENT1 expression and high RRM1 expression in the adjuvant setting instead of adjuvant gemcitabine chemotherapy.

With regard to the combined biomarker analysis, Maréchal et al[36] recently evaluated the prognostic values of immunohistochemical assessment of hENT1, RRM1, and dCK in 222 patients with pancreatic carcinoma who were treated with adjuvant gemcitabine-based chemotherapy. They reported that hENT1 and dCK expression, but not RRM1 expression, was significantly associated with OS and the combined analysis of hENT1 and dCK expression might provide the most powerful predictive signal to inform decisions regarding treatment with gemcitabine. The reason for differences in results between their study and the current study is unknown. Further studies in a larger number of patients are needed to determine the prognostic significance of intratumoral hENT1 and RRM1expession.

The limitations of this study are its retrospective design and the relatively small number of patients evaluated. In addition, other biomarkers, including dCK, 5′-nucleotidase, and cytidine deaminase have been reported to be associated with resistance to gemcitabine in pancreatic cancer.[14,36-39] Further prospective validation with an adequate number of patients is needed to clarify the association between intratumoral hENT1 and RRM1 expression and survival of patients with pancreatic carcinoma who receive adjuvant gemcitabine-based chemotherapy.

In conclusion, our study demonstrated that both intratumoral hENT1 and RRM1 expression were useful as predictive markers of the efficacy of adjuvant gemcitabine-based chemotherapy for pancreatic carcinoma after operative resection, and the combined analysis of hENT1 and RRM1

Table III. Multivariate disease-free and overall survival analysis for 109 patients who received adjuvant gemcitabine-based chemotherapy

Factors	*Disease-free survival*			*Overall survival*		
	HR	*95% CI*	P *value*	*HR*	*95% CI*	P *value*
Model 1: Multivariate analysis including separate hENT1 and RRM1 expression factors						
R factor						
R0				1.0	0.57–2.14	.709
R1				1.13		
UICC pT factor						
T1/T2	1.0	0.81–5.62	.173	1.0	1.02–9.01	.047
T3	1.91			3.03		
Lymph node metastasis						
Yes	3.08	1.64–5.75	<.001	2.45	1.18–5.08	.016
No	1.0			1.0		
hENT1 expression						
High	1.0	1.52–4.83	.001	1.0	1.65–6.06	.001
Low	2.70			3.16		
RRM1 expression						
High	2.09	1.24–3.70	.009	2.20	1.14–4.24	.019
Low	1.0			1.0		
UICC pT factor						
T1/T2				1.0	0.99–8.77	.052
T3				2.94		
Lymph node metastasis						
Yes	3.04	1.62–0.71	.001	2.44	1.17–5.08	.018
No	1.0			1.0		
Combined hENT1 and RRM1 classification						
high hENT1/low RRM1	1.0		<.001	1.0		<.001
high hENT1/high RRM1	1.89	1.01–0.56		1.64	0.76–3.51	
low hENT1/low RRM1	2.40	1.21–4.76		2.35	1.09–5.03	
low hENT1/high RRM1	6.69	2.44–18.34		9.79	3.32–28.86	

CI, Confidence interval; *HR*, hazard ratio; *hENT1*, human equilibrative nucleoside transporter 1; *RRM1*, ribonucleotide reductase regulatory subunit M1; *UICC*, International Union Against Cancer.

expression was even more useful as a predictive marker. These results may enable optimal adjuvant chemotherapy for patients with pancreatic carcinoma after operative resection.

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