Combined Analyses of hENT1, TS, and DPD Predict Outcomes of Borderline-resectable Pancreatic Cancer

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Abstract. *Background: Predicting chemosensitivity to neoadjuvant chemoradiotherapy (NACRT) in pancreatic cancer is desired. The present study aimed to examine the relationship between intratumoral expression of human equilibrative nucleoside transporter 1 (hENT1), thymidylate synthase (TS), and dihydropyrimidine dehydrogenase (DPD) and the outcomes of NACRT with gemcitabine (GEM) combined with S-1 in patients with borderline-resectable pancreatic cancer (BRPC). Materials and Methods: Forty-seven patients who underwent NACRT with GEM plus S-1, following curative surgery, were recruited in our Institution between 2009 and 2012. Immunohistochemical expressions of hENT1, TS, and DPD in fine-needle aspiration (FNA) biopsies and resected specimens were examined. The correlation between these enzyme expressions and long-term outcome was analyzed. Results: In 21 FNA specimens, no relationship between clinical responses to NACRT and long-term survival was found. However, in 47 resected specimens, patients were classified according to the number of favorable hENT1, TS, and DPD expression factors (hENT1 positive/TS negative/DPD negative). The presence of three favorable factors was strongly associated with improved partial response rates to NACRT ($p=0.002$). Patients with 2 or more favorable factors showed a significantly longer overall survival than the other patients ($p=0.002$). Conclusion: Combined expression analyses of hENT1, TS, and DPD may predict long-term outcomes in patients with BRPC after NACRT.*

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Pancreatic cancer is a highly lethal disease (1). Complete surgical resection of tumors remains the only treatment with curative potential. However, up to 20% of patients are eligible for surgical resection at diagnosis (2). Furthermore, surgical resection cannot guarantee a cure, although the 5-year survival rate improves to approximately 10% following resection (3). Several randomized controlled studies of adjuvant chemotherapy after pancreatic resection have demonstrated survival benefits (4-6). According to these results, adjuvant chemotherapy is currently considered to be a standard treatment and is routinely recommended.

Evans *et al.* (7) first reported the use of neoadjuvant chemoradiotherapy (NACRT) for pancreatic cancer to improve the R0 resection and patient survival rates. In 2008, Katz *et al.* (8) proposed the concept of borderline-resectable pancreatic cancer (BRPC), which is at a high risk for a margin-positive resection with surgery alone. In practice, a Japanese multicenter study found that the R0 resection rate for patients with BRPC was 65.9% and that the 3- and 5-year survival rates in resected patients were 22.8% and 12.5%, respectively (9). In this study, only 9.4% of patients underwent preoperative adjuvant treatment. To increase the R0 resection and overall survival (OS) rates, NACRT has been introduced for BRPC. Moreover, in 2009, our department adopted neoadjuvant gemcitabine (GEM) plus S-1 combination chemoradiotherapy.

Chemotherapy with GEM (2',2'-difluorideoxycytidine), a deoxycytidine analog that inhibits DNA replication and repair, is one of the standard treatment for pancreatic cancer. The human equilibrative nucleoside transporter 1 (hENT1) protein plays an important role for transporting GEM to intracellular space (10) and can also predict chemosensitivity for GEM-based treatment. Less hENT1 expression in cancer cells induces lower intracellular concentration of GEM, resulting in drug resistance (11). Our previous reports revealed that human pancreatic cancer cells with hENT1-expression are shown to be sensitive to GEM (12). Furthermore, a randomized study demonstrated that hENT1

protein expression was associated with prolonged OS and disease-free survival (DFS) rates in patients who received postoperative GEM (13). Therefore, hENT1 expression may be a predictive factor of the survival rate in patients with pancreatic cancer undergoing GEM chemotherapy.

S-1, an oral fluoropyrimidine derivative comprising of tegafur and the two modulators 5-chloro-2,4-dihydroxypyridine and potassium oxonate, has been used to treat patients with pancreatic cancer since the early 2000s in Japan. The GEST study, a multicenter, prospective, randomized study for unresectable pancreatic cancer, demonstrated that S-1 and GEM had similar efficacies in the disease (14). Furthermore, GEM plus S-1 did not demonstrate superiority with respect to OS. However, the response rate for GEM plus S-1 was 29.3%, which is higher than that for GEM or S-1 alone. Hence, assessing pancreatic tumor cell sensitivity to 5-fluorouracil (5-FU) is important and is usually performed by measuring the expression levels of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) in cancer cells (15, 16). Studies have evaluated the correlation between TS and DPD expression levels in tumors and clinical outcomes for patients with gastric cancer (17, 18).

To evaluate the prognostic value of hENT1, TS, and DPD expression for long-term outcomes of patients with BRPC undergoing NACRT with GEM plus S-1, we assessed the protein expression levels of these enzymes in tumor samples using fine-needle aspiration (FNA) and resected specimens and identified the correlations with clinical parameters and disease outcomes.

Patients and Methods

Patients. Fifty-seven patients with pancreatic cancer visited the Department of Gastroenterological Surgery, Yokohama City University Hospital between January 2009 and December 2012 and were enrolled in our study. All patients gave their informed consent, and the study was conducted according to the Declaration of Helsinki. The Ethics Committee of the hospital approved the present study (No. B090312028). Diagnoses of pancreatic cancer were confirmed by cytology in 27 patients and by histology of specimens using endoscopic ultrasonography-guided FNA (EUS-FNA) biopsies for 30 patients.

In this study, the indication for NACRT was as follows: patients with BRPC, defined by the National Comprehensive Cancer Network Guidelines Version 1.2013 (19), and with Eastern Cooperative Oncology Group performance status 0-1. On completing chemoradiotherapy, restaging CT and positron emission tomography were performed. If distant metastasis or local progression were not present, surgery was performed.

Treatment planning and assessments of clinical efficacy. NACRT comprised of combined chemotherapy of GEM and S-1 followed by radiotherapy. GEM plus S-1 comprised of the intravenous administration of 1,000 mg/m² GEM on days 8 and 15 and daily oral administration of 60 mg/m² S-1 on days 1-14. After two

courses of the chemotherapy, radiotherapy (30 Gy) was combined with 60 mg/m² S-1. Patients received restaging interventions at 4-6 weeks after completing chemoradiotherapy. If there was no disease progression, the patients underwent surgery. Clinical responses to NACRT were assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (20).

Resection procedures and postoperative chemotherapy. Ten patients were excluded from this study: six had liver metastases and four had peritoneal dissemination after chemoradiotherapy. Thus, 47 (82%) patients underwent surgical resection after NACRT. After surgery, postoperative chemotherapy was planned, comprising of a regimen of intravenously administered 1,000 mg/m² GEM on days 8 and 15 plus orally-administered 60 mg/m² S-1 on days 1-14 for 6 months. After surgical resection, all the patients were followed up monthly, and serum carcinoembryonic antigen and CA19-9 levels were determined every 2 or 3 months. MDCT was performed every 3 months for 2 years and every 6 months thereafter. Patients with elevated tumor markers were immediately examined by MDCT. When recurrence occurred, the recurrent site was recorded.

Initial histopathological assessment. Sections were routinely histologically examined. Residual tumors were considered as R1 if histological infiltration of carcinoma was present at the pancreatic cut end margin, at the bile duct cut end margin, or in the dissected peripancreatic tissue margin. Tumor stage, lymph node metastasis, and final stage were classified according to the 7th edition of the International Union Against Cancer (UICC) tumor, node, and metastasis system (TNM) system (21). Histological responses to NACRT were determined following Evans classification (3); grade 1, slight tumor cell destruction (<10 %); grade 2a, 10%-50% non-viable tumor cells; grade 2b, 50-90% non-viable tumor cells; grade 3, ≤10% viable tumor cells; and grade 4, no viable tumor cells.

Immunohistochemical staining. Tissue sections of 4 μm were deparaffinized, and endogenous peroxidase activity of specimens was blocked by immersing the slides in a solution of absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature. To retrieve antigens, slides were autoclaved at 105°C for 10 min in the Dako Target Retrieval Solution High pH x1 for hENT1 or at 121°C for 10 min in 1 M methylenediamine tetraacetic acid buffer for DPD and TS. After autoclaving, sections were put at room temperature for cooling. After blocking endogenous peroxidases, tissue slices were incubated with 1:100 dilutions of anti-hENT1 rabbit polyclonal antibody (Abnova, Taipei, Taiwan), 1:50 dilutions for anti-DPD mouse monoclonal antibody (IBL, Gunma, Japan), or 1:200 dilutions for anti-TS mouse monoclonal antibody (IBL, Gunma, Japan) at 4°C overnight. Labeled antigens were visualized using a Histo Fine kit (Nichirei, Tokyo, Japan), followed by reaction with DAB (Wako, Osaka, Japan). Finally, sections were counterstained with hematoxylin and observed under a microscope. Negative control was prepared in the absence of primary antibodies.

Immunohistochemical evaluation. Expression levels of hENT1, TS, and DPD were evaluated in 21 FNA specimens and 47 resected specimens by immunohistochemistry. Staining grades were evaluated by two observers (Y.Y. and R.M.) under the direction of

Figure 1. Immunohistochemical analysis of hENT1, TS, and DPD expression in pancreatic cancer. (a) Positive hENT1 expression. (b) Negative hENT1 expression stained adjacent islet cells (arrow) were used as a positive internal control. (c) Positive TS expression. (d) Positive DPD expression. Scale bar, 50 μ m.

a pathologist. Clinical information was blinded at the time of evaluation. Islets of Langerhans cells were selected as the internal positive control for hENT1 and DPD expression. The intensity of hENT1 and DPD staining was scored as follows: grade 0, no staining; grade 1, faintly stained; grade 2, weakly stained compared with islet cells; and grade 3, stained as strongly as islet cells. TS expression level was quantified using a visual grading system on the basis of the extent of staining. The intensity of TS staining was scored from grades 0 to 3 as previously reported (grade 0, no staining; grade 1, light yellow; grade 2, yellow or deep yellow; and grade 3, brown or tan) (22). Samples were considered hENT1 positive if grade 2 or 3 staining was observed in >50% of carcinoma cells. Samples were considered TS- or DPD-positive if grade 2 or 3 staining with 30% or more of carcinoma cells. These cutoff values were decided according to previous reports (13, 23). Samples that did not fulfill these criteria were determined as negative for the expression of tumor markers (Figure 1).

Statistical analyses. Statistical analyses were performed using the SPSS version 20 software (SPSS Inc., Chicago, IL, USA). Observation period and patients' age were presented as median and

range. Comparisons of categorical variables were tested by χ^2 or Fisher's exact tests. Significant differences within groups were identified using paired *t*-tests and across groups using the Mann-Whitney *U*-test. DFS and OS curves were estimated using Kaplan-Meier analyses, and differences between patient groups were evaluated using the log-rank test. Multivariate Cox proportional hazard analyses were performed to determine the influences of variables on outcomes using a stepwise variable selection process. Differences were considered statistically significant at $p < 0.05$.

Results

Patient population and clinical outcomes. Patient characteristics are summarized in Table I. Thirty-one (66%) patients were males and 16 (34%) were females, with a median age of 65 (37-81) years. Pancreatic tumors were confined to the head and body-tail of the pancreas in 36 (77%) and 11 (33%) patients, respectively. Forty-six patients completed the two planned courses of chemotherapy. Because of an allergic reaction, one patient underwent NACRT without oral

Table I. Characteristics of the 47 patients.

Characteristics	N=47
Age	
<65	23
≥65	24
Gender	
Male	31
Female	16
Tumor location	
Head	36
Body-Tail	11
UICC T classification	
T3	31
T4	16
Operative procedure	
Pancreaticoduodenectomy	36
Distal pancreatectomy	11
Total pancreatectomy	0
Major vessel resection	
Yes	37
PV/SMV	29
SMA	2
CHA	2
CA	8
No	10
Surgical margin	
R0	43
R1	4
Pathological differentiation	
Well	7
Others	40
Lymph node metastasis	
Negative	24
Positive	23

administration of S-1. Twenty-one patients provided sufficient volumes of specimen for FNA analyses. The average number of FNA specimens from each patient was four (range=2-6). Resection procedures included pancreaticoduodenectomy in 36 (77%) patients and distal pancreatectomy in 11 (33%). Combined resection of major vessels was performed in 37 patients: PV/SMV in 29, SMA in two, common hepatic artery in two, and celiac artery in eight. The surgical margin was assessed as negative for malignancy (R0) in 43 (91%) patients and microscopically positive (R1) in four (9%). Tumors were identified as well-differentiated adenocarcinomas in seven (15%) patients, moderately differentiated in 29 (62%), poorly differentiated in 10 (21%), and adenosquamous carcinoma in one (2%). According to the TNM classification, 31 (66%) and 16 (34%) patients had T3 and T4 tumors, respectively, and 23 (49%) patients had lymph node metastases. According to the RECIST criteria, NACRT produced partial response (PR) in 11 (23%) patients, but stable disease in 36 (77%). Pathological assessments using Evans criteria classified histological

responses as grade 1 in two (4%) patients, grade 2a in 22 (47%), grade 2b in 18 (38%), and grade 3 in five (11%). Twenty-seven (57%) patients underwent postoperative chemotherapy with GEM plus S-1. Treatment was completed in 6 months for 14 (52%) patients. The other 13 patients changed chemotherapy because of disease recurrence (n=7, 26%) and side effects (n=6, 22%). Four (9%) patients received GEM alone, four (9%) received S-1 alone, and 12 (26%) did not received postoperative chemotherapy as per the patients' decision.

Median follow-up was 20 (6.5-51.9) months after the initial treatment. Median duration of DFS and OS after initial treatment were 15.4 and 24.8 months, respectively, and 1- and 3-year DFS and OS rates of the entire cohort were 62.2% and 32.2% and 91.1% and 34.0%, respectively.

Clinicopathological factors and hENT1, TS, and DPD expression. Among 21 FNA specimens, seven (33%) were hENT1 positive, five (24%) were TS positive, and 13 (62%) were DPD positive. Among eight clinicopathological factors (age, sex, tumor location, UICC classification, major vessel resection, surgical margin, pathological differentiation, and lymph node metastasis), no significant differences in characteristics were observed between the positive and negative groups (Table II). Relationships between hENT1, TS, and DPD expression levels in FNA and in resected specimens are presented in Table III. No correlations between expression levels in FNA and resected specimens were found.

Among 47 resected tumors, 26 (55%) were hENT1 positive, whereas 26 (55%) were TS positive and 32 (68%) were DPD positive. However, no significant differences were observed in eight clinicopathological factors between these marker expression groups (Table IV).

Patients were classified into one of the following four groups according to the number of favorable hENT1, TS, and DPD expression factors: three favorable factors (hENT1 positive/TS negative/DPD negative; n=6), any two favorable factors (hENT1 positive/TS negative/DPD positive, hENT1 positive/TS positive/DPD negative, or hENT1 negative/TS negative/DPD negative; n=13), any one favorable factor (hENT1 positive/TS positive/DPD positive, hENT1 negative/TS negative/DPD positive, or hENT1 negative/TS positive/DPD negative; n=21), or no favorable factors (hENT1 negative/TS positive/DPD positive; n=7). Forty (85%) patients revealed the presence of more than one favorable factor, 19 (40%) the presence of more than two, and six (13%) the presence and absence of three favorable factors. The tumor location of patients with three favorable factors was significantly more frequent in the body-tail of the pancreas (Table V).

Relationships between tumor response to NACRT and hENT1, TS, and DPD expression and their combination. Relationships between hENT1, TS, and DPD expression and tumor responses

Table II. Comparison of clinicopathological factors based on intratumoral hENT1, TS, and DPD expression in FNA specimens (n=21).

	hENT1		p-Value	TS		p-Value	DPD		p-Value
	Positive (n=7)	Negative (n=14)		Positive (n=5)	Negative (n=16)		Positive (n=13)	Negative (n=8)	
Age			0.438			0.185			0.392
<65	4	6		1	9		7	3	
≥65	3	8		4	7		6	5	
Gender			0.557			0.656			0.310
Male	4	9		2	6		6	2	
Female	3	5		3	10		7	6	
Tumor location			0.681			0.258			0.160
Head	4	8		4	8		9	3	
Body-Tail	3	6		1	8		4	5	
UICC T classification			0.676			0.080			0.528
T3	3	6		4	5		6	3	
T4	4	8		1	11		7	5	
Major vessel resection			0.274			0.579			0.316
Yes	7	11		4	14		12	6	
No	0	3		1	2		1	2	
Surgical margin			0.667			0.381			0.381
R0	7	13		5	15		13	7	
R1	0	1		0	1		0	1	
Pathological differentiation			0.433			0.571			0.133
Well	0	2		0	2		0	2	
Others	7	12		5	14		13	6	
Lymph node metastasis			0.056			0.262			0.664
Negative	2	11		2	11		8	5	
Positive	5	3		3	5		5	3	

to NACRT were evaluated. Clinical efficacy, including radiographic and histological responses, did not significantly differ between patients with marker-positive and -negative FNA specimens. In contrast, resected specimens that were DPD negative tended to demonstrate treatment effects in radiographic assessments ($p=0.073$). Evans' histopathological criteria did not significantly differ between patients grouped according to marker expression (Table VI). The PR rates of NACRT were strongly associated with the presence of all three favorable factors ($p=0.002$). Furthermore, any ≥ 2 favorable factors (n=19, 40%) tended to demonstrate PR ($p=0.074$).

Relationship between survival and hENT1, TS, and DPD expression. No relationships were found between expressions of marker proteins in FNA specimens and survival. However, patients with three favorable factors in resected specimens had significantly longer OS ($p=0.005$) than patients in the other groups (Figure 2). Patients with more than two favorable factors tended to have longer DFS ($p=0.056$) and OS ($p=0.002$) rates than patients in the other groups (Figure 2).

Univariate analyses of DFS and OS are presented in Table VII. Patients with tumor in the body–tail had a significantly longer OS rate ($p=0.028$). One- and 3-year OS rates were

Table III. Correlation between the expression in FNA specimens and resected specimens.

	N	Resected specimens		p-Value
		Positive (%)	Negative(%)	
FNA-hENT				0.572
Positive	7	5 (71)	2 (29)	
Negative	14	9 (64)	5 (36)	
FNA-TS				0.258
Positive	5	4 (80)	1 (20)	
Negative	16	8 (50)	8 (50)	
FNA-DPD				0.590
Positive	13	9 (69)	4 (31)	
Negative	8	6 (75)	2 (25)	

significantly higher in patients with TS-negative resected specimens (90% and 65%, respectively) than in those with TS-positive resected specimens (92% and 10%, respectively, $p=0.013$). The DPD-negative group achieved significantly longer OS rate both at 1-year and 3-year (85% and 59%, respectively) than the DPD-positive group (94% and 22%,

Table IV. Comparison of clinicopathological factors based on intratumoral hENT1, TS, and DPD expression in resected specimens (n=47).

	hENT1		p-Value	TS		p-Value	DPD		p-Value
	Positive (n=26)	Negative (n=21)		Positive (n=26)	Negative (n=21)		Positive (n=32)	Negative (n=15)	
Age			0.454			0.454			0.299
<65	14	9		14	9		14	9	
≥65	12	12		12	12		18	6	
Gender			0.477			0.252			0.555
Male	16	15		19	12		22	9	
Female	10	6		7	9		10	6	
Tumor location			0.390			0.610			0.229
Head	19	17		20	16		26	10	
Body – Tail	7	4		6	5		6	5	
UICC T classification			0.477			0.927			0.944
T3	16	15		17	14		21	10	
T4	10	6		9	7		11	5	
Major vessel resection			0.512			0.488			0.397
Yes	20	17		21	16		26	11	
No	6	4		5	5		6	4	
Surgical margin			0.610			0.227			0.619
R0	24	19		25	18		29	14	
R1	2	2		1	3		3	1	
Pathological differentiation			0.377			0.307			0.394
Well	3	4		5	2		4	3	
Others	23	17		21	19		28	12	
Lymph node metastasis			0.312			0.454			0.401
Negative	15	9		12	12		15	9	
Positive	11	12		14	9		17	6	

respectively, $p=0.035$). However, no relationship was found between hENT1 expression and survival.

Multivariate Cox proportional models were fitted using prognostic factors which were significant in the univariate analysis. TS and DPD expressions were omitted from this analysis because “more than two favorable factors” included these factors. “Three favorable factors” was not added to this analysis because the number of patients was small. The two prognostic factors tumor location and more than two favorable factors were included in multivariate analyses of OS. These analyses revealed that the presence of more than two favorable factors was an independent factor influencing long-term survival rate (hazard ratio, 3.40; 95% confidence interval, 1.36-8.68; $p=0.010$; Table VI).

Discussion

This study evaluated the influence of hENT1, TS, and DPD expression on NACRT with GEM and S-1 based regimen for patients with BRPC. A relationship between more than two favorable factors of three enzymes and survival benefit was observed. However, the 3-year OS rate of patients with an absence of more than two favorable factors was only 10%.

Recently, the OS rates in patients with pancreatic cancer undergoing surgical resections following neoadjuvant therapy have increased. Since the clinical introduction of GEM (24) in the late 1990s, numerous randomized studies have suggested that GEM supersedes 5-FU which was the standard chemotherapeutic agent for pancreatic cancer. Although various studies indicated that efficacy of GEM was superior to 5-FU, 5-FU has been clearly demonstrated as an effective drug in patients with pancreatic cancer (5). Trials of adjuvant treatments, including the RTOG 9704 study (25), which was a large prospective randomized trial, and the CONKO-001 study (26), provided strong supporting evidence for the efficacy of adjuvant therapy. In particular, Evans *et al.* (27) reported an actual 5-year OS rate of 36% in patients with resectable pancreatic head cancer who underwent surgical resection following preoperative GEM-based chemoradiotherapy. Moreover, Motoi *et al.* (28) reported a median OS of 35 months in patients who received resection following neoadjuvant therapy with GEM plus S-1. Thus, GEM and fluoropyrimidine are pivotal agents in current adjuvant and neoadjuvant therapies. A Japanese multicenter study (9) found that the 3- and 5-year OS rates in patients with borderline-resected pancreatic cancer were 22.8 and 12.5 %, respectively. The present study shows a 3-year OS rate of 34% and a median

Table V. Comparison of clinicopathological factors based on intratumoral combined expression of hENT1-positive, T-negative, and DPD-negative in resected specimens. (n=47)

	hENT1/TS/DPD		<i>p</i> -Value	hENT1/TS/DPD		<i>p</i> -Value	hENT1/TS/DPD		<i>p</i> -Value
	≥1 matched (n=40)	The Other (n=7)		≥2 matched (n=19)	The other (n=28)		3 matched (n=6)	The Other (n=41)	
Age			0.475			0.312			0.312
<65	19	4		11	12		4	19	
≥65	21	3		8	16		2	22	
Gender			0.229			0.739			0.092
Male	25	6		12	19		2	29	
Female	15	1		7	9		4	12	
Tumor location			0.197			0.229			0.021
Head	32	4		13	23		2	34	
Body - Tail	8	3		6	5		4	7	
UICC T classification			0.477			0.769			0.092
T3	27	4		13	18		2	29	
T4	13	3		6	10		4	12	
Major vessel resection			0.164			0.634			0.378
Yes	30	7		15	22		4	33	
No	10	0		4	6		2	8	
Surgical margin			0.512			0.536			0.568
R0	36	7		17	26		6	37	
R1	4	0		2	2		0	4	
Pathological differentiation			0.276			0.400			0.643
Well	5	2		2	5		1	6	
Others	35	5		17	23		5	35	
Lymph node metastasis			0.475			0.172			0.104
Negative	21	3		12	12		5	19	
Positive	19	4		7	16		1	22	

Table VI. Correlation between clinical efficacy of NACRT and expression of biomarkers.

	hENT1		<i>p</i> -Value	TS		<i>p</i> -Value	DPD		<i>p</i> -Value	hENT1/ TS/DPD		<i>p</i> -Value	hENT1/ TS/DPD		<i>p</i> -Value	hENT1/ TS/DPD		<i>p</i> -Value
	Pos (n=26)	Neg (n=21)		Pos (n=26)	Neg (n=21)		Pos (n=32)	Neg (n=15)		Any ≥1 matched (n=40)	The other (n=7)		Any ≥2 matched (n=19)	The other (n=28)		3 matched (n=6)	The other (n=41)	
Radiographic response			0.164			0.136			0.073			0.473			0.074			0.002
PR	8	3		4	7		5	6		10	1		7	4		5	6	
SD	18	18		22	14		27	9		30	6		12	24		1	35	
Evans' criteria			0.552			0.448			0.540			0.226			0.676			0.413
Grade I, IIa	13	11		14	10		16	8		19	5		9	15		4	20	
Grade IIb, III	13	10		12	11		16	7		21	2		10	13		2	21	

Pos: positive, Neg: negative.

OS of 25 months in patients with BRPC who underwent resection after NACRT at our institute.

A study (29) suggested that hENT1 is a potential biomarker for long-term outcome of GEM-based chemotherapy for pancreatic cancer. The hENT1 expression affected therapeutic

outcome of GEM-based NACRT (30). Other clinical studies (16, 31) of pancreatic cancer have also revealed that low DPD expression predicts efficacy and prognosis of fluoropyrimidine-treated patients. In addition, TS expression was reported to be a poor prognostic factor for pancreatic cancer patients treated

Table VII. Univariate and multivariate survival analysis of prognostic factors.

	N	DFS 3 year rate (%)	p-Value	OS 3 year rate (%)	p-Value	HR	95%CI	p-Value
Age			0.641		0.648			
<65	23	34		31				
≥65	24	30		38				
Gender			0.734		0.930			
Male	31	33		30				
Female	16	28		45				
Tumor location			0.747		0.028	2.539	0.861-7.489	0.091
Head	36	35		25				
Body - Tail	11	32		59				
Major vessel resection			0.843		0.709			
Yes	37	33		36				
No	10	30		27				
Surgical margin			0.832		0.438			
R0	43	33		36				
R1	4	25		25				
Lymph node metastasis			0.243		0.134			
Negative	24	40		53				
Positive	23	24		17				
Pathological differentiation			0.283		0.630			
Well	7	0		0				
Others	40	38		38				
Evan's criteria			0.396		0.190			
Grade IIIa	24	38		42				
Grade IIb III	23	28		25				
Adjuvant Chemotherapy			0.301		0.250			
Yes	35	33		34				
No	12	32		30				
hENT1			0.565		0.224			
Positive	26	32		37				
Negative	21	30		30				
TS			0.090		0.013			
Positive	26	15		10				
Negative	21	48		65				
DPD			0.524		0.035			
Positive	32	30		22				
Negative	15	38		59				
hENT1/TS/DPD								
Positive/Negative/Negative								
Any ≥1 matched	40	36	0.209	37	0.339			
the other	7	14		17				
Any ≥2 matched	19	52	0.056	70	0.002	3.403	1.335-8.675	0.010
the other	28	17		10				
3 matched	6	44	0.421	100	0.005			
the other	41	31		23				

with 5-FU-based adjuvant therapy (16). Thus, hENT1, TS, and DPD are candidate biomarkers for GEM concomitant with fluoropyrimidine treatments. In our study, combination analysis of these biomarkers appeared useful for patient selection.

Unexpectedly, expression profiles in FNA specimens were not predictive of outcomes in the patients. Specimens from endoscopic ultrasound-guided FNA may have been too small to

reflect the characteristics of the whole tumor. However, the positive predictive values of FNA specimens were relatively high at 71, 80, and 69%, respectively. Yamada *et al.* reported that pre-treatment hENT1 expressions in FNA specimens were concordant with those in the resected specimen after GEM-based chemoradiotherapy (32). Further studies are required to confirm the importance of enzyme expression in FNA specimens.

Figure 2. Correlation between combined expression analysis and both of DFS and OS. Patients with more than two favorable factors tended to have longer DFS (c) $p=0.056$ and had significantly longer OS (d) $p=0.002$ than patients in other groups. Patients with three favorable factors had significantly longer OS (f) ($p=0.005$) than patients in other groups.

S-1 treatments induced an increase of hENT1 mRNA levels and GEM uptake in pancreatic cancer xenografts (33). Treatment with GEM increased hENT1 expression and reduced 5-FU sensitivity in human pancreatic cancer cell lines (34). Thus, in the present study, NACRT using GEM plus S-1 may also have caused changes in the expression of these factors in FNA specimen ie pretreatment tumor cells.

Recent studies indicate a relationship between hENT1 expression and NACRT outcomes in patients with pancreatic cancer. Murata *et al.* (30) reported positive correlation between hENT1 expression and improved outcome. In contrast, another report described that hENT1 expression did not predict long-term outcomes in patients with pancreatic cancer who underwent NACRT with GEM (35). This discrepancy may reflect the use of GEM as an adjuvant chemotherapy in the former study and the contrasting use of continuous liver perfusions of 5-FU as adjuvant chemotherapy in the latter study. In the present study, adjuvant chemotherapy comprised GEM plus S-1 for 57%, GEM alone for 9%, and S-1 alone for 9% of patients, and 25% of patients did not undergo any adjuvant chemotherapy. This heterogeneity may have influenced the relationship between hENT1, TS, and DPD expression and long-term survival. TS and DPD were more reliable prognostic factors than hENT1 expression. According to this finding, it is widely accepted that 5-FU metabolism-associated enzymes affect the efficacy of 5-FU-based chemoradiotherapy (36, 37). In view of the present results, patients who lack favorable factors may consider adjuvant chemotherapy using drugs other than GEM or S-1, such as modified FOLFIRINOX or GEM and nab-paclitaxel.

Previous reports described independent associations of positive lymph node metastasis and surgical margin status with longer OS in patients with pancreatic cancer who were treated with NACRT (27, 30). In the present study, the presence of more than two favorable factors (more than two from hENT1 positive, TS negative, and DPD negative) was selected as an independent favorable prognostic factor of OS for patients with pancreatic cancer who underwent NACRT with GEM plus S-1.

This study had limitations, such as its retrospective nature and a small number of participants. Thus, further prospective validation with a larger patient cohort is required to confirm the results of the present study.

In summary, combined analysis of hENT1, TS and DPD in resected specimens may predict therapeutic efficacy of NACRT with GEM plus S-1 in patients with BRPC. Further studies are required to determine the efficacy of these biomarkers in FNA specimens. Adjuvant chemotherapy with drugs other than GEM or S-1 should be considered for patients in whom any three factors are absent.

Conflicts of Interest

The Authors report no potential conflict of interest regarding this study.

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