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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

CD133 expression is a potential prognostic indicator in intrahepatic cholangiocarcinoma

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Abstract
Background and aim CD133 is one of the most important cancer-initiating (stem) cell markers and was confirmed to be expressed in solid cancers such as colon cancer. However, no one has investigated the role of CD133 in intrahepatic cholangiocarcinoma (IHCC). The aim of this study was to clarify the clinical role of CD133 expression in IHCC.
Patients and methods Twenty-nine patients with IHCC who underwent hepatic resection at our institution were enrolled in this study. Expression of CD133 was examined using anti-CD133 antibody. Staining was observed in the cytoplasm of cancer cells and CD133-positive cells distributed in the whole tumor. The patients were divided into two groups: the CD133-positive group ($n = 14$) and CD133-negative group ($n = 15$), in which no staining of CD133 was observed. Clinicopathological factors including hypoxia-inducible factor-1α expression were compared between the two groups. The prognostic factors were investigated by multivariate analysis using Cox's proportional hazard model.
Results The 5-year survival rate in the CD133-positive group (8.0%) was worse than that in the CD133-negative group (57.0%). In the CD133-positive group, the incidence of intrahepatic metastasis and positive expression of hypoxia-inducible factor-1α tended to be higher than that in the CD133 negative group. The multivariate analysis revealed CD133 expression was an independent prognostic indicator in IHCC.
Conclusions CD133 expression tended to be related to higher incidences of intrahepatic metastasis and positive expression of hypoxia-inducible factor-1α; furthermore, it was independently related to worse prognosis. Therefore, the CD133 expression is a potential prognostic indicator in IHCC.

Keywords Hypoxia-inducible factor-1α · Liver cancer · Intrahepatic metastasis · Multivariate analysis

Abbreviations

IHCC	Intrahepatic cholangiocarcinoma
HIF-1α	Hypoxia-inducible factor-1α
PBS	Phosphate-buffered saline

Introduction

Intrahepatic cholangiocarcinoma (IHCC) is well known to be one of the most malignant solid tumors in digestive organs [1–5]. We have already reported that lymph node metastases are seldom limited to the regional lymph nodes; most tumor recurrence occurs in the liver. Furthermore, lymph node dissection does not appear to improve patient survival [6]. Therefore, it is extremely important to elucidate the prognostic factors and establish treatment strategy to overcome the poor prognosis of IHCC.

Recently, cancer stem cells (CSC) have been reported to play important roles in various kinds of cancer. CD133 is reported originally to be one of the surface markers of hematopoietic stem cells and progenitor cells, and is known

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to be an important cancer-initiating (stem) cell marker, which has been confirmed to be expressed in solid cancers such as colon cancer and glioma [7–10]. Especially a CD133-positive subpopulation of colon cancer cells was recently demonstrated to be highly enriched in tumor-initiating CSCs that have the ability to self-renew and to recapitulate the bulk tumor population [7, 8].

Regarding the CD133-positive tumor cells in solid tumors, the following impressive reports have been published: (1) CD133-positive cells sorted from glioblastoma had the capacity of self-renewal and tumorigenesis [11]; (2) CD133/CXCR4 expressing cells are CSCs and correlated with metastasis in prostate cancer [12]; (3) CD133 expression is increased and is an important prognostic indicator in hepatocellular carcinoma, pancreas cancer and colon cancer [13–15]. Most recently, another impressive study has been reported in which hypoxia-inducible factor-1α (HIF-1α) regulates CD133 expression in cancer cells [16, 17]. However, to the best of our knowledge, there is one report about immunohistochemical CD133 expression in IHCC [18]. In this report, no clinical role of CD133 expression in IHCC as well as correlation between CD133 expression and HIF-1α expression was investigated. On the other hand, there are a few reports including ours regarding the CSCs (or progenitor cells) and cholangiolocellular carcinoma in the liver [19, 20].

The aim of this study was, therefore, to clarify both the clinical role of CD133 expression and correlation between CD133 expression and HIF-1α expression in IHCC.

Patients and methods

Among 37 patients who underwent hepatic resection for IHCC at Tokushima University Hospital between 1994 and 2007, 29 patients with available surgical specimens for immunohistochemistry, who didn't die in-hospital because of problems related to surgery itself such as postoperative liver failure, were included in this study. The mean follow-up was 29.2 months, and the regular follow-up included monthly measurement of carcinoembryonic antigen and carbohydrate 19-9 levels, and computed tomography and ultrasonography every 3 months.

Macroscopic and microscopic types, staging and curability were defined according to the Classification of Primary Liver Cancer by the Liver Cancer Study Group of Japan [21]. With respect to macroscopic typing, IHCC was classified into the following types: mass-forming type (characterized by the presence of a round-shaped mass in the liver parenchyma with a distinct border), periductal infiltrating type (characterized by tumor infiltration along the bile duct and occasionally involving the surrounding blood vessels or hepatic parenchyma) or intraductal growth type (characterized by papillary or granular growth, or both, within the bile duct lumen, occasionally involving superficial extension). Regarding the stage, the *T* factor was determined by tumor number (single or not), size (no more than 2 cm or not) and vascular infiltration (present of absent). Stage was finally determined by *T*, *N* and *M* factors. Curability was defined as follows: Curability A, no residual tumor; Curability B, no residual tumor, but not evaluated as "Curability A;" Curability C, definite residual tumors.

This study was authorized in advance by the Institutional Review Board of the University of Tokushima Graduate School (approval ID number: 266), and all patients provided written informed consent.

Immunohistochemistry

CD133

Surgical specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4-μm-thick sections. Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Slides were heated at 120°C in an autoclave in 10 mM sodium citrate (pH 6.0) for 10 min and cooled to room temperature. Sections were incubated in 3.0% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase action. Slides were incubated in 5% bovine serum albumin in phosphate-buffered saline (PBS) for 60 min at room temperature. Sections were incubated overnight at 4°C with mouse monoclonal anti-CD133 antibody (Abcam Inc, UK, mAbcam27699; diluted 1:100 in PBS), which has been reported previously [22]. Reactions were developed using the avidin-biotin immunoperoxidase technique. After washing, sections were overlaid with 2nd antibody (Dako Corporation, Dako ChemMate envision kit/HRP) for 60 min at 37°C. The peroxidase reaction was developed with 3,3′-diaminobenzidine as chromogen. The sections were counterstained with hematoxylin, dehydrated with ethanol, treated with xylene and enclosed in synthetic resin. Regarding the assessment of staining, normal bile duct epithelium was entirely negative in the non-cancer part. The tumor was defined as having positive staining when any cells stained in the cytoplasm were seen in the tumor (Fig. 1), according to previous studies [14, 18, 23]. Even in the tumor with CD133-positive cells, the frequency of CD133-positive cells in the tumor was 0.5–2.0%.

HIF-1α

The detailed method was described elsewhere [24]. Briefly, 4-μm-thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks. Sections were then

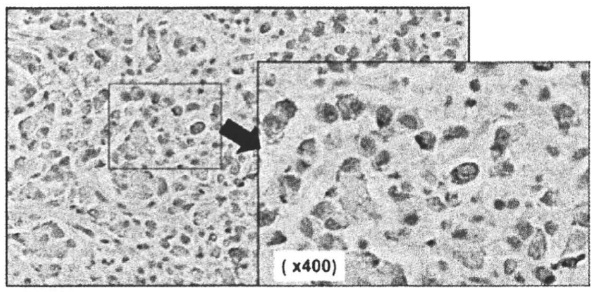

Fig. 1 CD133 expression in IHCC. The expression of CD133 was recognized in cytoplasm of cancer cells

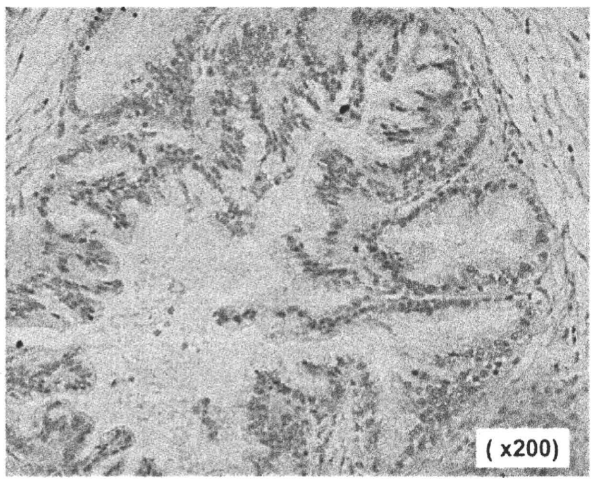

Fig. 2 Hypoxia-inducible factor-1α expression in IHCC. The expression of hypoxia-inducible factor-1α was recognized in the nucleus of cancer cells

irradiated in a domestic microwave oven for 20 min. After microwave irradiation, the slides were allowed to cool at room temperature. The sections required a primary mouse monoclonal antibody against HIF-1α (H1alpha67) NB100-105 (Novus Biological). The mouse monoclonal antibody HIF-1α diluted at 1:500 was used. The area counted in each section was randomly selected from a representative tumor field. For each section, eight areas were assessed; the counts were expressed as the mean percentage of positive tumor cells out of the total number of cells and high power fields. Regarding the assessment of staining, the tumor was evaluated as "positive staining" if there was nuclear staining in more than 10% of the tumor cells (Fig. 2). Concomitant cytoplasm staining was not counted because HIF-1α protein in the nucleus determined the functional activity of the HIF-1α complex [25, 26].

Immunofluorescent staining

In order to confirm both CD133 and HIF-1α expression in cancer cells of IHCC, immunofluorescent staining was performed. Sections of the IHCC specimens were used. For two-color immunofluorescent staining, primary antibody for CD133 was detected with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) (1:100 dilution), and the other primary antibody for HIF-1α was detected with Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen) (1:100 dilution) for 60 min. Finally, the slides were washed in 0.1% Triton X-100 in PBS. Slides were then viewed and photographed under a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

The clinicopathological variables including survival of patients who had positive CD133 expression ($n = 14$, 48.3%) were compared with those without CD133 expression ($n = 15$, 51.7%). Table 1 shows a comparison of background variables between the two groups. There is no difference in variables except for frequency of histological intrahepatic metastasis. The frequency of intrahepatic metastasis in the CD133-positive group (43%) tended to be higher than that in the CD133-negative group (13%).

Statistics

For comparison of continuous variables, the Mann-Whitney U test was used, and the chi-squared test was applied for categorical data. Patient survival was calculated by the product limit method of Kaplan and Meier, and differences in survival between the groups were compared using the log rank test. Prognostic factors were examined using univariate and multivariate analyses (Cox proportional hazards regression model). The continuous variables were generally classified into two groups, according to the median value of each variable. All statistical analysis was performed using statistical software (JMP 8.0.1., SAS Campus Drive, Cary, NC). Statistical significance was defined as a P value less than 0.05.

Results

Table 2 shows significant prognostic factors using univariate analysis. Among clinicopathological variables, curability (C: present residual tumor), staging III or IV, macroscopic tumor type (mass-forming and infiltration type), tumor size (≥5 cm), present lymph node metastasis, intrahepatic metastasis (present) and positive CD133 expression were significantly worse prognostic factors. Figure 3 shows the comparison of survival curves according to status of CD133 expression. The 5-year survival rate in the CD133-positive group (8%) was significantly worse than that in the CD133-negative group (57%).

Table 3 shows the relationship between recurrent patterns and CD133 expression. Incidence of recurrence in the liver in the CD133-positive group tended to be higher than

Table 1 Comparison of background variables between CD133-positive and -negative groups

Variables	CD133 expression		
	Positive (n = 14)	Negative (n = 15)	P value
Host variables			
Age: median (range)	75.1 (43–84)	64.1 (47–82)	0.15
Gender: male/female	10/4	10/5	0.54
Virus: negative: HBV: HCV: HB + HCV	9:3:0:2	10:2:1:2	0.75
Operative variables			
Major vs. minor	10 vs. 4	9 vs. 6	0.52
Lymph node dissection	7/14 (50%)	7/15 (47%)	0.86
Operative time (min)	504 (204–822)	446 (260–880)	0.462
Blood loss (g)	1160 (110–2850)	620 (120–3130)	0.462
Curability: A/B/C	1/9/4	6/6/3	0.59
Pathological variables			
Diameter: <5/≥5 cm	8/6	8/7	0.92
Macro: T/T + I	5/9	8/7	0.34
Differentiation: differentiated vs. undifferentiated	10/4	8/7	0.32
Lymph node metastasis: +/−	5/9	6/9	0.81
Vascular invasion: +/−	2/12	3/12	0.69
Perineural invasion: +/−	6/3	5/5	0.46
Intrahepatic metastasis: +/−	6/8	2/13	0.08
Stage: I, II/III, IV	2/12	6/9	0.12

Major means resection of 2 or more of segments and minor means resection of 1 or less segment. Curability was defined as follows: Curability A, no residual tumor; Curability B, no residual tumor, but not evaluated as "Curability A;" Curability C, definite residual tumors

Table 2 Significant prognostic factors: univariate analysis

Variables	3-year survival rate	P value
Curability: A, B vs. C	44.3 vs. 0%	0.0004
Staging: I, II vs. III, IV	87.5 vs. 11.3%	0.0019
Macroscopic type: T vs. T + I	58.6 vs. 14.3%	0.0280
Tumor size: <5 vs. ≥5 cm	40.6 vs. 19.4%	0.0288
Lymph nodes metastasis: − vs. +	53.1 vs. 0%	0.0016
Intrahepatic metastasis: − vs. +	45.6 vs. 0%	0.0461
CD133 expression: − vs. +	58.7 vs. 8.0%	0.0057

T: mass-forming type, T + I: mass-forming and infiltration type. Curability was defined as follows: Curability A, no residual tumor; Curability B, no residual tumor, but not evaluated as "Curability A;" Curability C, definite residual tumors

that in the CD133-negative group, although the difference was not statistically significant. No difference was observed between the two groups in other recurrent patterns such as lymph nodes and peritoneum.

Table 4 shows the result of the multivariate analysis of prognostic factors. Among the significant prognostic factors by the univariate analysis, only positive CD133 expression was an independent poor prognostic factor (relative risk 3.19).

Figure 4 shows coexpression of CD133 and HIF-1α in the serial section of IHCC. Expression of CD133 in cytoplasma (green) and HIF-1α in nuclei (red) was recognized. Thus, cancer cells were confirmed to express both CD133 and HIF-1alpha.

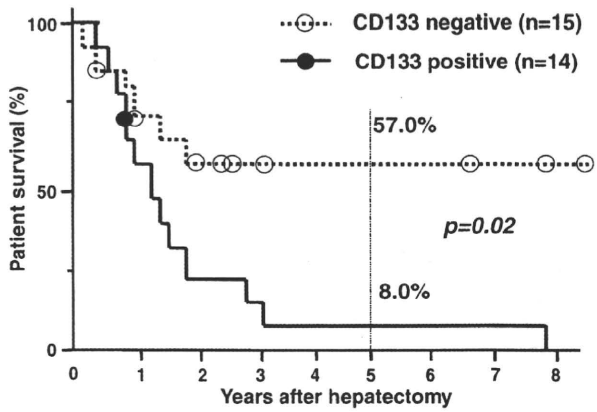

Fig. 3 The cumulative patient survival rate between CD133-positive and -negative groups. The survival rate in the CD133-positive group was significantly worse than that in the CD133-negative group

Regarding the correlation between CD133 expression and HIF-1α expression, a positive rate of HIF-1α expression in the CD133 positive group (80%) tended to be higher than that in the CD133 negative group (44%); however, the value was not statistically significant (Fig. 5).

Discussion

This is the first report to clearly demonstrate the important clinical role of CD133 expression in IHCC. Our important

Table 3 Correlation between CD133 expression and recurrent site in patients with Curability A and B resection ($n = 23$)

Sites of recurrence	CD133 pos ($n = 11$)	CD133 neg ($n = 12$)	P value
Liver ($n = 13$)	8 (72.7%)	5 (41.7%)	0.133
Lymph nodes ($n = 5$)	2 (18.2%)	3 (25.0%)	0.692
Peritoneum ($n = 1$)	1 (9.1%)	0 (0%)	0.286
Remote organ ($n = 2$)	1 (9.1%)	1 (8.3%)	0.949
Local ($n = 2$)	1 (9.1%)	1 (8.3%)	0.949

Curability was defined as follows: Curability A, no residual tumor; Curability B, no residual tumor, but not evaluated as "Curability A;" Curability C, definite residual tumors

Table 4 Prognostic factors: multivariate analysis

Variables	Relative risk	P value
CD133 expression: positive	3.19	0.038
Curability: C	2.70	0.146
Tumor size: ≥5 cm	2.34	0.194
Lymph node metastasis: positive	2.66	0.207
Staging: III, IV	3.86	0.270
Intrahepatic metastasis: present	1.34	0.668

Curability was defined as follows: Curability A, no residual tumor; Curability B, no residual tumor, but not evaluated as "Curability A;" Curability C, definite residual tumors

LNs lymph nodes

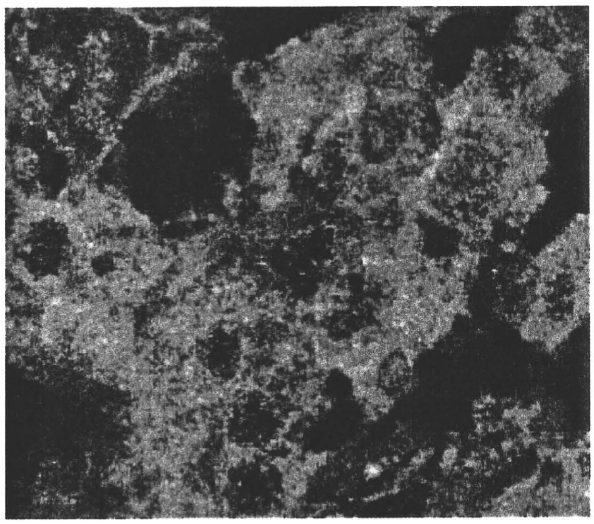

Fig. 4 Two-color immunofluorescent staining for CD133 and HIF-1α in cancer cells of IHCC. Expression of CD133 in cytoplasma (*green*) and HIF-1α in nuclei (*red*) was recognized. Thus, cancer cells were confirmed to express both CD133 and HIF-1α

findings are as follows: (1) CD133 expression was related to histological intrahepatic metastasis and HIF-1 expression; (2) CD133 expression was closely related to poor prognosis and was an independent prognostic indicator in IHCC. Regarding the correlation between clinicopathological

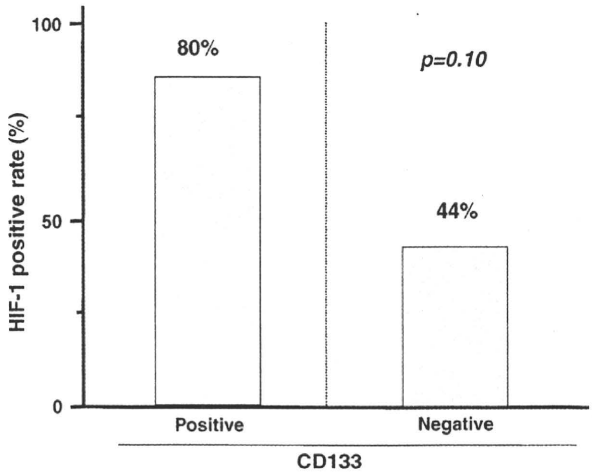

Fig. 5 Correlation between CD133 expression and HIF-1α expression. The expression of CD133 tended to correlate with HIF-1α expression

factors and CD133 expression, in pancreas cancer, CD133 expression was reported to be significantly associated with histological type (moderately or poorly differentiated type), lymphatic invasion and lymph node metastasis and expression of vascular endothelial factor C [14]. Hermann et al. [23] reported that a subpopulation of migrating CD133(+) CXCR4(+) CSCs is essential for tumor metastasis and that strategies aimed at modulating the SDF-1/CXCR4 axis may have important clinical applications to inhibit metastasis of CSCs. In our study, no significant correlation was observed between CD133 expression and tumor differentiation or lymph node metastasis. On the other hand, frequency of histological intrahepatic metastasis in CD133-positive expression was higher. The fact may be related to the relatively higher recurrence rate in the liver in the CD133-positive group.

Regarding the prognostic value of CD133, in pancreas cancer, CD133 expression was closely related to poor prognosis and was an independent prognostic indicator [14]. In colon cancer, Horst et al. [15] reported that CD133-positive cancer cells can be detected by immunohistochemistry; moreover, CD133 expression was an

independent prognostic marker that correlated with low survival. Maeda et al. [14] reported the 5-year survival rate of CD133-positive patients with pancreas head cancer was significantly lower than that of CD133-negative patients, and CD133 expression was an independent prognostic factor by multivariate analysis. Using immunohistochemical analysis of 63 HCC tissue specimens, Song et al. [27] reported that positive CD133 expression was found in 26 specimens (41.3%) and correlated with increased tumor grade, advanced disease stage and elevated serum alpha-fetoprotein levels. Furthermore, patients with positive CD133 expression had shorter overall survival and higher recurrence rates, and multivariate analysis revealed that positive CD133 expression was an independent prognostic factor for survival and tumor recurrence in patients with HCC. Those reports support our result that CD133 expression was an independent prognostic indicator in IHCC.

Regarding the immunohistochemical staining pattern of CD133, a previous study [17] reported multifocal minimal to mild cytoplasmic staining in IHCC. A relatively high percentage of tumor sample (67% in IHCC) was observed, similar to that in pancreas cancer (55–68%); however, in qualitative evaluation of the cancer cells within each tumor core, staining showed generally low expression intensity. In our study, no CD133 expression was observed in the normal biliary epithelium, while some cancer cells were distinctly stained by CD133. We detected positive cytoplasmic CD133 expression in 14 out of 29 cases (48.3%). In tumor with positive CD133 expression, the frequency of CD133-positive cells in each tumor was 0.5–2.0%. The positive cells were observed more in the peripheral region instead of central area in which necrosis was often noted.

On the other hand, in colorectal cancers, the immunohistochemical staining pattern was characterized by membranous (apical) as well as luminal staining of the gland-like tumor structures, although the nature of luminal staining is not known [15]. Such organ-specific patterns of CD133 expression still have to be clarified in further studies.

Regarding the relationship between CD133 and HIF-1, Matsumoto et al. [16] reported the inhibition of mTOR signaling up-regulated CD133 expression at both the mRNA and protein levels in a CD133-overexpressing cancer cell line. Hypoxic conditions up-regulated HIF-1α, a downstream molecule in the mTOR signaling pathway expression, and inversely down-regulated CD133 expression at both the mRNA and protein levels. Moreover, a strong inverse correlation between CD133 and HIF-1α was observed in gastric cancer samples. They concluded that HIF-1 alpha down-regulates CD133 expression and suggest that mTOR signaling is involved in the expression of CD133 in cancer cells. Soeda et al. [17] also reported that hypoxia (1% oxygen) promotes the self-renewal capacity of CD133-positive human glioma-derived CSCs. The enhanced self-renewal activity of the CD133-positive CSCs in hypoxia was preceded by upregulation of HIF-1α. Knockdown of HIF-1α abrogated the hypoxia-mediated CD133-positive CSC expansion. Finally, they concluded that response to hypoxia by CSCs involves the activation of HIF-1α to enhance the self-renewal activity of CD133-positive cells and to inhibit the induction of CSC differentiation. Therefore, we examined the correlation between HIF-1α expression and CD133 expression in IHCC. With certainty, some cancer cells of IHCC were proved to express both CD133 and HIF-1α (Fig. 4). Although HIF-1α expression tended to correlate with CD133 expression, Fig. 5 does not support the above-mentioned direct relationship between CD133 and HIF-1α in cancer cells. This result might suggest HIF-1α plays a crucial role in expansion of CSCs in IHCC. Therefore, to confirm the true role of HIF-1α in CSCs of IHCC, further investigation is necessary.

In conclusion, expression of CD133, a cancer-initiating cell marker, tended to be related to higher incidences of intrahepatic metastasis and positive expression HIF-1α, as well as independently worse prognosis. Therefore, the CD133 expression is suggested to be a potential prognostic indicator in IHCC, although study of a large number of patients is required before a definite conclusion can be made about this matter.

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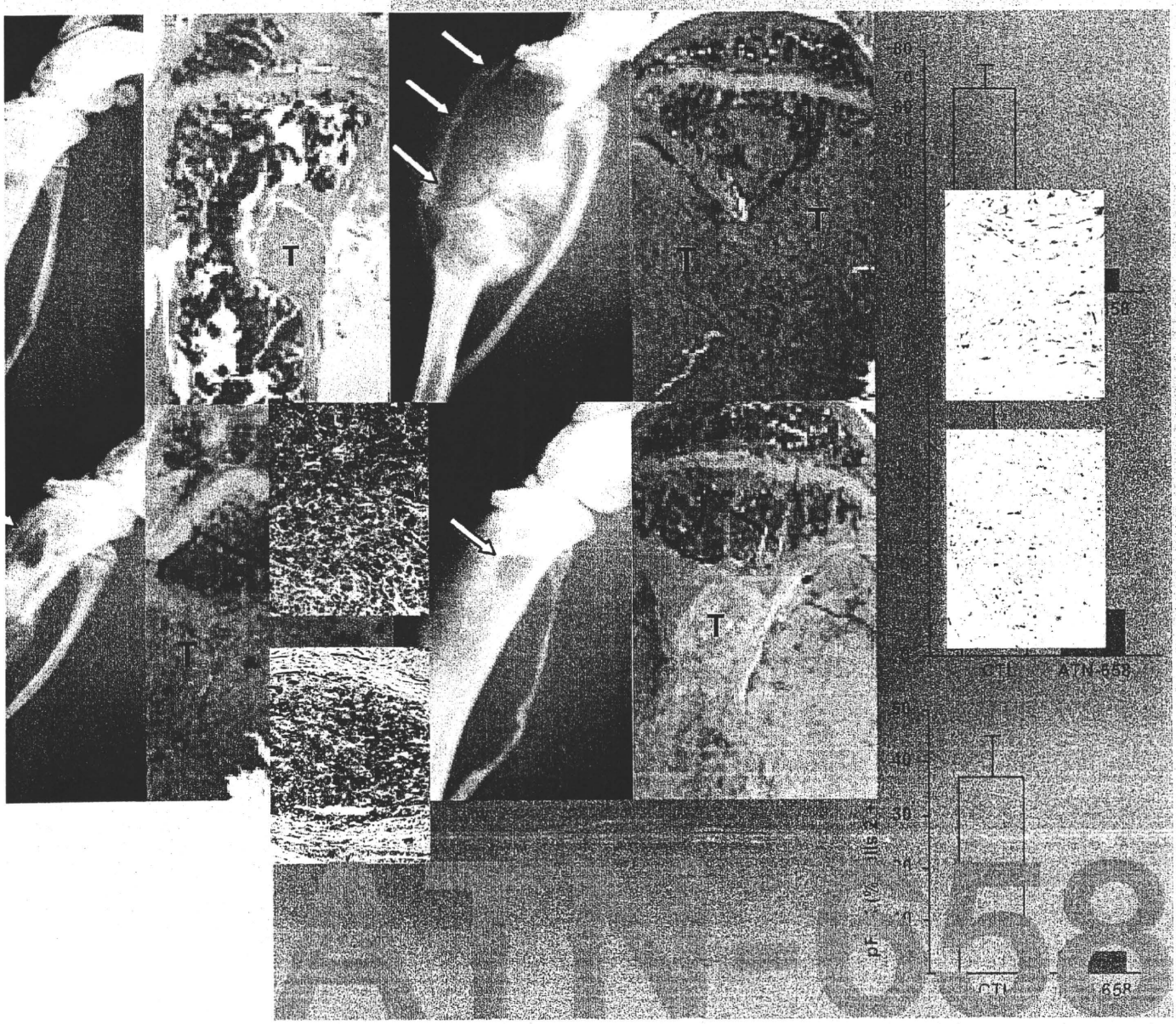

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Gene Expression Levels as Predictive Markers of Outcome in Pancreatic Cancer after Gemcitabine-Based Adjuvant Chemotherapy[1,2]

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Abstract

BACKGROUND AND AIMS: The standard palliative chemotherapy for pancreatic ductal adenocarcinoma (PDAC) is gemcitabine-based chemotherapy; however, PDAC still presents a major therapeutic challenge. The aims of this study were to investigate the expression pattern of genes involved in gemcitabine sensitivity in resected PDAC tissues and to determine correlations of gene expression with treatment outcome. *MATERIALS AND METHODS:* We obtained formalin-fixed paraffin-embedded (FFPE) tissue samples from 70 patients with PDAC. Of the 70 patients, 40 received gemcitabine-based adjuvant chemotherapy (AC). We measured *hENT1*, *dCK*, *CDA*, *RRM1*, and *RRM2* messenger RNA (mRNA) levels by quantitative real-time reverse transcription–polymerase chain reaction and determined the combined score (GEM score), based on the expression levels of *hENT1*, *dCK*, *RRM1*, and *RRM2*, to investigate the association with survival time. By determining the expression levels of these genes in endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) cytologic specimens, we investigated the feasibility of individualized chemotherapy. *RESULTS:* High *dCK* ($P = .0067$), low *RRM2* ($P = .003$), and high GEM score ($P = .0003$) groups had a significantly longer disease-free survival in the gemcitabine-treated group. A low GEM score (<2) was an independent predictive marker for poor outcome to gemcitabine-based AC as shown by multivariate analysis ($P = .0081$). Altered expression levels of these genes were distinguishable in microdissected neoplastic cells from EUS-FNA cytologic specimens. *CONCLUSIONS:* Quantitative analyses of *hENT1*, *dCK*, *RRM1*, and *RRM2* mRNA levels using FFPE tissue samples and microdissected neoplastic cells from EUS-FNA cytologic specimens may be useful in predicting the gemcitabine sensitivity of patients with PDAC.

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Abbreviations: 5′-NT, 5′-nucleotidase; AC, adjuvant chemotherapy; CDA, cytidine deaminase; dCK, deoxycytidine kinase; DFS, disease-free survival; EUS-FNA, endoscopic ultrasound-guided fine needle aspiration; FFPE, formalin-fixed paraffin-embedded; hENT1, human equilibrative nucleoside transporter 1; IC_{50}, 50% inhibitory concentration; NSCLC, non–small cell lung cancer; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, quantitative real-time reverse transcription–polymerase chain reaction; RR, ribonucleotide reductase; UICC, Union Internationale Contre le Cancer and the American Joint Committee on Cancer; WCP, whole cell pellet

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[2]This article refers to supplementary materials, which are designated by Tables W1 to W4 and Figure W1 and are available online at www.neoplasia.com.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal and aggressive human malignancies, being the fourth leading cause of tumor-related deaths in the industrialized world [1,2]. Most patients with PDAC have poor outcomes because of the aggressive biology of the tumor and the difficulties of early diagnosis because of a lack of early disease-specific signs and symptoms. Only 10% to 20% of patients with PDAC are candidates for curative resection [3], and even if the curative resection is performed, the postoperative 5-year survival rate is only 15% to 25% because of a high recurrence rate [4,5]. Although two recent randomized clinical phase 3 trials of adjuvant chemotherapy (AC) for PDAC showed significant increases in overall survival (OS) and disease-free survival (DFS) [6,7], there remain a substantial subset of cases in which AC efficacy is limited and insufficient. Recent studies have revealed that altered gene expression can at least partly explain responses and toxicity of cytotoxic agents [8]. To improve the prognosis of patients with PDAC, a helpful strategy would be to select subjects who are likely to respond to treatment based on gene expression profiles of the individual's own cancer tissues.

Gemcitabine (difluorodeoxycytidine; dFdC) is a deoxycytidine analog that has broad antitumor activity in various solid tumors, including pancreatic cancer [7] and non–small cell lung cancer (NSCLC) [9]. The drug is a prodrug that requires cellular uptake and intracellular phosphorylation to produce active diphosphate (dFdCDP) and triphosphate (dFdCTP). These phosphorylated forms function by inhibiting ribonucleotide reductase (RR) and DNA synthesis [10]. Gemcitabine is transported into cells predominantly by human equilibrative nucleoside transporter 1 (hENT1) [11]. A deficiency in hENT1 activity conferred high-level resistance to the toxicity of gemcitabine [12], and patients with PDAC that have detectable hENT1 or high *hENT1* gene expression have significantly prolonged survival after gemcitabine chemotherapy [13,14]. After cellular entry, gemcitabine must be phosphorylated by deoxycytidine kinase (dCK), which is a rate-limiting step. We previously demonstrated that down-regulation of *dCK* specifically enhanced acquired resistance to gemcitabine in pancreatic cancer cells [15], whereas transfection of wild-type *dCK* restored sensitivity to the drug [16]. Conversely, active metabolites of gemcitabine are reduced by 5′-nucleotidase (5′-NT), and gemcitabine itself is inactivated by cytidine deaminase (CDA). High levels of these catabolic enzymes are associated with resistance to the drug [17,18]. dFdCTP inhibits DNA synthesis by being incorporated into the DNA strand, but in addition, dFdCDP potently inhibits RR, resulting in a decrease of competing deoxyribonucleotide pools necessary for DNA synthesis [19]. RR is a dimeric enzyme composed of regulatory subunit M1 and catalytic subunit M2. Recurrent PDAC patients with high levels of *RRM1* expression had poor survival rates after gemcitabine treatment [20], and NSCLC patients with low levels of *RRM1* expression significantly benefited from gemcitabine/cisplatin neoadjuvant chemotherapy [21]. Moreover, *RRM2* gene silencing by RNA interference is an effective therapeutic adjunct to gemcitabine treatment [22]. These data suggest that the genes encoding proteins involved in the transport and metabolism of gemcitabine and in the metabolism of targets can be potential candidates to predict sensitivity to gemcitabine.

To develop individualized chemotherapy, the characterization of genes associated with tumor sensitivity or resistance to antitumor agents using cancer tissues from individuals plays a critical role in the selection of preferable treatments. In the current study, we investigated the correlation between the expression of genes involved in cellular uptake and metabolism of gemcitabine and the treatment outcome of patients with PDAC who underwent gemcitabine-based AC or no AC. Furthermore, to investigate the feasibility of individualized chemotherapy for patients with PDAC, even when the tumor is unresectable, we quantified the expression of genes in cytologic specimens obtained from endoscopic ultrasound-guided fine needle aspiration (EUS-FNA).

Materials and Methods

Cell Lines and Establishment of Gemcitabine-Resistant Cells

We used two pancreatic cancer cell lines, SUIT-2 (generously provided by Dr H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan) and Capan-1 (American Type Culture Collection, Manassas, VA). Gemcitabine-resistant Capan-1-GR and SUIT-2-GR cells were generated by exposing to gradually increasing concentrations of gemcitabine as described previously [15]. Cells were maintained as described previously [23].

Propidium Iodide Assay

To calculate the 50% inhibitory concentration (IC_{50}) of each cell line when exposed to gemcitabine, cells were seeded in 24-well plates (Becton Dickinson Labware, Bedford, MA) at a density of 2×10^4 per well, using cell numbers previously counted using a particle distribution analyzer CDA 500 (Sysmex, Kobe, Japan). Several different concentrations of gemcitabine (Wako, Osaka, Japan) were added 24 hours after seeding. Cell populations were evaluated by measuring the fluorescence intensity of propidium iodide after a further incubation for 72 hours, as described previously [24].

Patients and Pancreatic Tissues

Our study subjects consisted of 70 patients including 40 patients who received gemcitabine-based AC (GEM group) and 30 patients who received no AC (non-AC group) after pancreatic resection for PDAC at the Department of Surgery and Oncology, Kyushu University Hospital (Fukuoka, Japan) from 1992 to 2007. Although there were 48 patients who received gemcitabine-based AC, eight patients were excluded because they did not receive adequate AC. The GEM group patients ($n = 40$) received gemcitabine-based AC, consisting of two or more cycles of 1000 mg/m^2 per day of gemcitabine on days 1, 8, and 15 every 28 days, or three or more cycles of 1000 mg/m^2 per day of gemcitabine on days 1 and 8 every 21 days. The patients were 42 men and 28 women with a median age of 65 years (range, 36-86 years). We recommended that patients have follow-up visits every 3 months for 2 years, then visits every 6 months for 3 years, and then annual visits. DFS was defined as the time from the date of pancreatic resection to the date of local or distant recurrence. The date of recurrence was defined as the date of the first subjective symptom heralding relapse, or the date of documentation of recurrent disease, independent of site, as assessed by diagnostic imaging techniques (whichever occurred first). Data for patients without recurrence were censored at the time of the last follow-up visit. OS was measured from the date of pancreatic resection to the date of death. Fifty-seven patients died during follow-up, and the other patients were censored at the time of the last follow-up visit. Data were analyzed in December 2009, and follow-up data from all cases were available. The median observation time for DFS was 8.0 months (range, 0.5-114 months) and that for OS was 15.7 months (range, 0.5-114 months). The clinicopathologic characteristics of the tumors collected from a total of 70 patients are noted in Table W1.

All resected specimens were fixed in formalin and embedded in paraffin for pathologic diagnosis. All tissues adjacent to the specimens were evaluated histologically according to the criteria of the World Health Organization. Two pathologists were in agreement with regard to the pathologic features of all cases and both confirmed the diagnoses. The stage of tumors was assessed according to UICC (Union Internationale Contre le Cancer and the American Joint Committee on Cancer) guidelines [25]. The study was approved by the ethics committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Cytologic Specimens

Cytologic specimens were obtained at the time of cytologic examination and diagnosis from the pathologic laboratory of Kyushu University Hospital (Fukuoka, Japan). In brief, cytologic specimens were divided into whole cell pellets (WCPs) and into three or more smears as soon as possible after retrieval. Smears were processed in three different ways as described previously [26]. Two smears were mounted on standard glass slides for Hemacolor staining (Merck KGaA, Darmstadt, Germany) and Papanicolaou staining and were then used for rapid cytologic diagnosis and strict cytologic diagnosis, respectively. These two smears were examined histologically by cytopathologists, and diagnosis was confirmed according to Papanicolaou classification. The third smear of each specimen was mounted on membrane slides (P.A.L.M. Microlaser Technologies, Bernried, Germany) for laser capture microdissection. These smears were stained in 1% Toluidine blue staining solution or by Hemacolor staining. Fifteen cytologic specimens were obtained from patients at the Kyushu University Hospital (Fukuoka, Japan) who underwent endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) cytology and whose lesions were cytopathologically diagnosed as PDAC.

Isolation of RNA

Total RNA was extracted from cultured cells using a High-Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Total RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue samples using the RNeasy FFPE kit (Qiagen, Tokyo, Japan) with modification of the manufacturer's instructions after macrodissection based on a review of representative hematoxylin and eosin–stained slides as described previously [27]. Total RNA was extracted from cells isolated by microdissection according to the standard acid guanidinium thiocyanate-phenol-chloroform (AGPC) protocol [28], with or without glycogen (Funakoshi, Tokyo, Japan).

Quantitative Real-time Reverse Transcription–Polymerase Chain Reaction

Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) was performed using a Chromo4 Real-time PCR Detection System (BIO-LAD Laboratories, Hercules, CA) and a LightCycler 480 II Real-time PCR System (Roche Diagnostics) for 40 cycles for 15 seconds at 95°C and 1 minute at 55°C with a Quanti-Tect SYBR Green Reverse Transcription–PCR kit (Qiagen) according to the manufacturer's instructions [29]. We designed specific primers (Table 1) and performed BLASTN searches to confirm the primer specificities. The level of each mRNA was calculated from a standard curve constructed with total RNA from Capan-1, a human pancreatic cancer cell line. The level of each mRNA was normalized to that of *β-actin*. The PCR product sizes of each primer pair are small, which allowed accurate and sensitive qRT-PCR despite the fragmented RNA extracted from FFPE tissue specimens [30,31].

Statistical Analyses

Statistical analyses and graph presentations were made using JMP 7.01 software (SAS Institute, Cary, NC). Values are expressed as the mean ± SD. Comparisons between two groups were performed by Student's t test. Messenger RNA (mRNA) were split into high- and low-level groups using recursive descent partition analysis of all patients (n = 70) or the GEM group (n = 40), as described by Hoffmann et al. [32]. Categorical variables were compared using the χ^2 test (Fisher exact probability test). Survival curves were constructed using the Kaplan-Meier product-limit method and were compared by the log-rank test. To evaluate independent predictive or prognostic factors associated with survival time, multivariate Cox proportional hazards regression analysis was used. Statistical significance was defined as $P < .05$.

Results

Altered Expression of Genes Encoding Proteins Associated with Gemcitabine Sensitivity in Gemcitabine-Resistant Pancreatic Cancer Cell Lines

Gemcitabine-resistant SUIT-2 (SUIT-2-GR) and Capan-1 (Capan-1-GR) cells were generated by exposure to gradually increasing concentrations of gemcitabine. The IC_{50} values for gemcitabine of the gemcitabine-resistant cells were significantly higher than those of parental cells, respectively (Table 2). We also quantified the expression levels of genes involved in gemcitabine uptake and metabolism (Figure 1, *A-E*). SUIT-2-GR cells expressed significantly lower levels of *hENT1* and *dCK* and significantly higher levels of *RRM1* and *CDA* compared with parental cells. Capan-1-GR cells expressed significantly lower levels of *dCK* and significantly higher levels of *CDA* compared with parental cells, although the expression levels of *CDA* were lower than those of SUIT-2 cells. Therefore, alterations of *hENT1*, *dCK*, *RRM1*, and *CDA* expression were associated with the development of gemcitabine resistance in SUIT-2 cells, whereas only two genes, *dCK* and *CDA*, were associated in Capan-1 cells. These data suggest that there are different patterns of gene expression that can develop gemcitabine resistance, and evaluation of several genes is needed to predict gemcitabine sensitivity.

Univariate and Multivariate Analyses of Each mRNA Level Associated with Gemcitabine Sensitivity and Survival Time

To investigate predictive markers of sensitivity to gemcitabine-based AC in PDAC patients, we quantified *hENT1*, *dCK*, *RRM1*, *RRM2*,

Table 1. Primer Sequences and Product Sizes.

Primer	Forward	Reverse	Product Size (bp)
	Sequence 5′-3′	Sequence 5′-3′	
hENT1	gcaaaggagaggagccaagag	gggctgagagttggagactg	65
dCK	gctgcagggaagtcaacattt	ttcaggaaccacttcccaatc	69
RRM1	actaagcaccctgactatgctatcc	cttcctcacatcactgaacacttt	88
RRM2	ggctcaagaaacgaggactg	tcaggcaagcaaaatcacag	93
CDA	tcaaagggtgcaacatagaaaatg	cggtccgttcagcacagat	61
β-actin	tgagcgcggctacagctt	tccttaatgtcacgcacgattt	60

Table 2. IC_{50} of Each Cell Line.

Cell Line	IC_{50} (nM)		*P*
	Parental Cells	Gemcitabine-Resistant Cells	
SUIT-2	2.76 ± 0.19	8576.14 ± 156.41	<.01
Capan-1	62.62 ± 5.33	23,520.71 ± 680.72	<.01

and *CDA* mRNA levels in 70 FFPE tissue samples of resected PDAC using qRT-PCR. The relationship between gemcitabine-based AC and clinicopathologic characteristics or each mRNA level is summarized in Tables W2 and W3. The GEM and non-AC groups were composed of 40 and 30 cases, respectively. There was no significant correlation between gemcitabine-based AC and the clinicopathologic factors or any of the mRNA levels in PDAC patients. Also, there were no significant differences in the mRNA levels of any of the genes between the two groups (Figure W1, *A-E*).

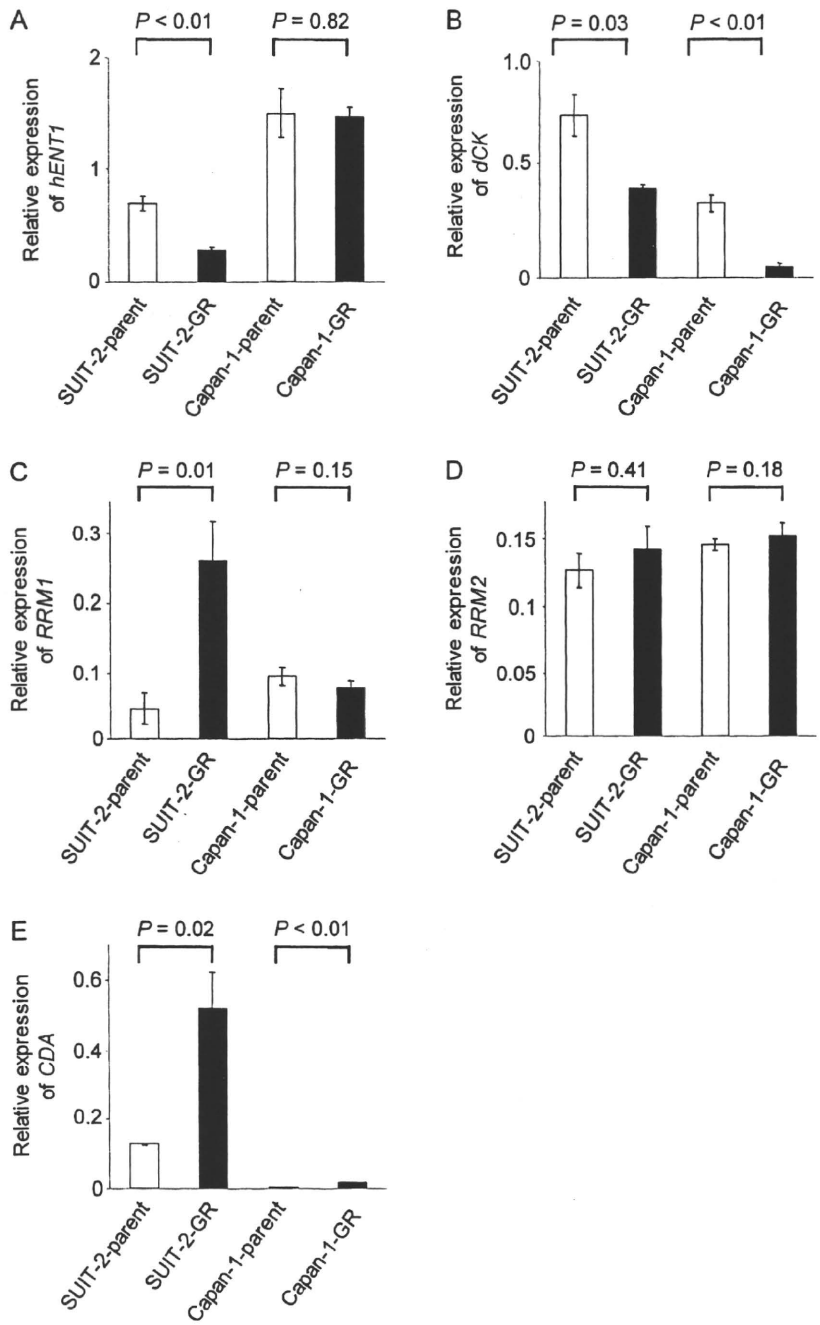

Figure 1. Quantitative analyses of mRNA associated with cellular uptake and metabolism of gemcitabine in gemcitabine-resistant cells. Quantitative analyses of *hENT1* (A), *dCK* (B), *RRM1* (C), *RRM2* (D), and *CDA* (E) mRNA in gemcitabine-resistant cell lines (SUIT-2-GR and Capan-1-GR) and parental cells. SUIT-2-GR cells expressed significantly lower levels of *hENT1* and *dCK* and significantly higher levels of *RRM1* and *CDA* compared with parental cells. Capan-1-GR cells expressed significantly lower levels of *dCK* and significantly higher levels of *CDA* compared with parental cells, although expression levels of *CDA* were lower than those of SUIT-2 cells.

Initially, we investigated independent markers that indicated poor prognosis in 70 PDAC patients. We obtained two groups for each gene showing high or low expression, respectively, after normalization to *β-actin* expression, using cutoff values calculated by recursive descent partition analyses [32] of all patients (N = 70; Table W3). In univariate analyses for OS, the conventional prognostic markers, gemcitabine-based AC (Figure 2; P = .0017), pN status (P = .049), histologic grade (P = .0043), residual tumor (P = .0009), and positive vessel invasion (P = .044) reached significance (Table 3), whereas low *dCK* (P = .019) and high *RRM2* (P = .015) levels normalized to *β-actin* were associated with a shorter OS (Table 4). Multivariate analysis for OS based on the Cox proportional hazard model was performed on all parameters that were found to be significant on univariate analyses (Table 5). Although OS was significantly dependent on history of gemcitabine-based AC (P = .0001), histologic grade (P < .0001), and R factor (P = .0003), the effects of low *dCK* and high *RRM2* levels did not reach statistical significance. Also, multivariate analysis for DFS (Table W4) showed that DFS was significantly dependent on the histologic grade (P = .0033) and R factor (P < .0001).

Next, to determine which parameters are predictive for gemcitabine sensitivity, we evaluated the correlation between each parameter, including gene expression, and DFS in the GEM and non-AC groups. Univariate survival analyses of the GEM group showed that pN status (P = .0052), UICC stage (P = .0066), residual tumor (P = .0002), and positive vessel invasion (P = .018) reached statistical significance for DFS, whereas low *dCK* (P = .0067) and high *RRM2* (P = .003) levels normalized to *β-actin* were associated with a shorter DFS (Table 6). Low *hENT1* (P = .11) and high *RRM1* (P = .069) groups tended to associate with a shorter DFS, although these markers did not reach statistical significance (Table 6; Figure 3, *A*, *C*, *E*, and *G*). In contrast, there was no significant correlation between these gene expression levels and DFS in the non-AC group (Figure 3, *B*, *D*, *F*, and *H*). Multivariate analysis of the GEM group (Table 7) showed that DFS was significantly dependent on the R factor (P = .0055) and *RRM2* level (P = .0055), whereas the effect of low *dCK* levels did not reach statistical significance.

Similarly, we also evaluated the correlation between each parameter and OS in the GEM and non-AC groups. Univariate survival analyses of the GEM group showed that the conventional prognostic markers pN status (P = .014) and residual tumor (P = .0012) reached statistical significance for OS (Table 6). Low *hENT1* (P = .011) and *dCK* (P = .0095) and high *RRM1* (P = .041) and *RRM2* (P = .030) levels, normalized to *β-actin*, were associated with a shorter OS (Table 6; Figure 4, *A*, *C*, *E*, and *G*). In contrast, there was no significant correlation between these gene expression levels and OS in the non-AC group (Figure 4, *B*, *D*, *F*, and *H*). Multivariate analysis of the GEM group (Table 8) showed that OS was significantly dependent on pN status (P = .029) and R factor (P = .0027), whereas altered gene expression did not reach statistical significance for any gene.

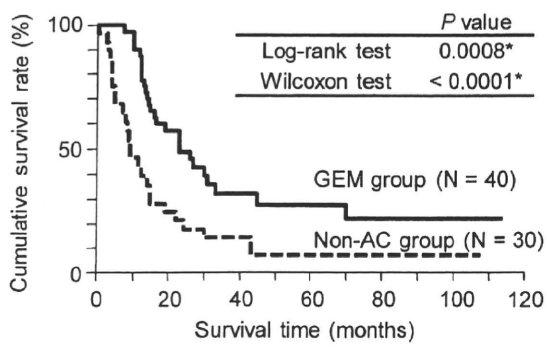

Figure 2. Correlation between gemcitabine-based AC and survival time. The patients who received gemcitabine-based AC (GEM group) showed a significantly prolonged OS time compared with the non-AC group (P = .0017). *P < .05.

Table 3. Univariate Survival Analysis of Conventional Prognostic Factors (N = 70).

Characteristics	No. Cases	Median DFS (Months)	P	Median OS (Months)	P	5-Year Survival Rate (%)
Age (years)			.95		.89	
≥65	36	8		15		18.7
<65	34	8		14.5		24.3
Sex			.94		.69	
Male	42	8		15		18.9
Female	28	7		16.5		20.8
Gemcitabine-based AC			.078		.0017*	
Yes	40	10		23		27.8
No	30	7		9		8.3
Radiotherapy			.81		.54	
Yes	19	8		22		25.3
No	43	8		14.7		9.4
pT category			.11		.063	
pT1/pT2	4	4		10		0
pT3/pT4	66	8		16.5		21.8
pN category			.0064*		.049*	
pN0	20	28		33		28.9
pN1	50	7		13.7		17.4
UICC stage			.022*		.29	
I	2	12.6		24.2		0
II	64	8		16.5		22.6
III/IV	4	2		12		0
Histologic grade			.0036*		.0043*	
G1/G2	41	14		26		27.8
G3	29	4		12		13.6
Residual tumor category			<.0001*		.0009*	
R0	39	19		24.2		33.2
R1	31	4		12		6.5
Vessel invasion			.0083*		.044*	
Positive	46	6		13.3		15.0
Negative	24	14		24.2		31.8
Neural invasion			.68		.56	
Positive	57	8		15		20.2
Negative	13	14		19		22.4

*P < .05.

Table 4. Univariate Survival Analysis of mRNA Expression Levels (N = 70).

Characteristics	No. Cases	Median DFS (Months)	P	Median OS (Months)	P	5-Year Survival Rate (%)
hENT1 (cutoff value: 0.5)			.13		.45	
High	27	12		23		31.8
Low	43	8		14.5		11.6
dCK (cutoff value: 1.25)			.028*		.019*	
High	19	20		30		34.5
Low	51	7		13.7		14.6
CDA (cutoff value: 0.034)			.26		.54	
High	32	7		14.7		21.2
Low	38	8		22		20.6
RRM1 (cutoff value: 0.032)			.15		.094	
High	19	4		12		23.7
Low	51	10		19		20.5
RRM2 (cutoff value: 0.017)			.047*		.015*	
High	50	7		13		17.1
Low	20	19		23		30.3

Cutoff values were determined with recursive descent partition analyses of all patients (N = 70).
*P < .05.

Table 5. Multivariate OS Analysis (Cox Regression Model) of Conventional Prognostic Factors and mRNA Expression Levels (N = 70).

Characteristics	Relative Risk	95% Confidence Interval	P
No AC	3.418	1.832-6.389	.0001*
pN status (pN1)	2.093	0.974-4.892	.059
Histologic grade (G3)	4.322	2.169-8.698	<.0001*
Residual tumor (pR)	3.328	1.746-6.398	.0003*
Positive vessel invasion	1.179	0.601-2.458	.64
Low *dCK* (<1.25)	0.811	0.411-1.682	.560
High *RRM2* (>0.017)	1.106	0.570-2.295	.770

Cutoff values were determined with recursive descent partition analyses of all patients (N = 70).
*P < .05.

Table 6. Univariate Survival Analysis of Conventional Prognostic Factors and of mRNA Expression Levels in the GEM Group (n = 40).

Characteristics	No. Cases	Median DFS (Months)	P	Median OS (Months)	P	5-Year Survival Rate (%)
Age (years)			.52		.56	
≥65	17	8		27		24.5
<65	23	10		23		32.7
Sex			.36		.33	
Male	26	14		27		29.1
Female	14	6		16.5		21.4
Radiotherapy			.20		.085	
Yes	9	19		27		41.7
No	27	8		19		12.9
pT category			.25		.084	
pT1/pT2	1	4		10		0.0
pT3/pT4	39	10		26		28.5
pN category			.0052*		.014*	
pN0	9	23		45		47.4
pN1	31	8		19		21.5
UICC stage			.0066*		.13	
I	0	—		—		—
II	37	12		23		30.3
III/IV	3	3		12		0
Histologic grade			.056		.075	
G1/G2	23	19		31		35.3
G3	17	8		16.5		24.2
Residual tumor category			.0002*		.0012*	
R0	26	20		45		46.6
R1	14	5		13.7		0.0
Vessel invasion			.018*		.054	
Positive	26	8		19		21.1
Negative	14	25		45		40.2
Neural invasion			.46		.89	
Positive	33	10		26		27.3
Negative	7	19		23		28.6
hENT1 (cutoff value: 0.5)			.11		.011*	
High	14	25		45		49.0
Low	26	8		16.5		11.5
dCK (cutoff value: 1.25)			.0067*		.0095*	
High	13	25		70		50.5
Low	27	8		16.5		13.7
CDA (cutoff value: 0.013)			.48		.79	
High	26	8		27		28.2
Low	14	12		23		28.6
RRM1 (cutoff value: 0.017)			.069		.041*	
High	12	8		19		47.6
Low	28	33		31		19.8
RRM2 (cutoff value: 0.027)			.003*		.03*	
High	27	8		16.3		18.8
Low	13	19		3.2		49.4

Cutoff value for each mRNA level was determined for the GEM group (n = 40).
*P < .05.

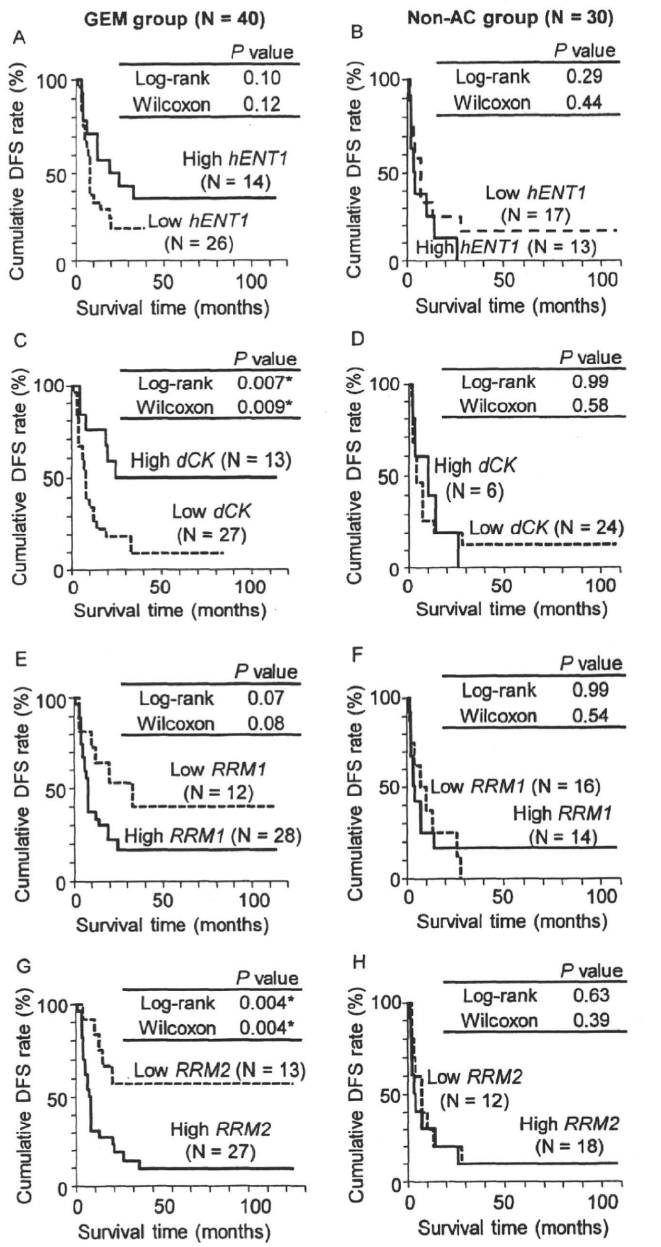

Figure 3. Correlation between the expression of each mRNA and DFS. Low *dCK* (P = .0067) and high *RRM2* (P = .003) levels, normalized to *β-actin*, were associated with a shorter DFS in the GEM group (A, C, E, G). In contrast, there was no significant correlation between these gene expression levels and DFS in the non-AC group (B, D, F, H). *P < .05.

Table 7. Multivariate DFS Analysis (Cox Regression Model) of Conventional Prognostic Factors and mRNA Expression Levels in the GEM Group (n = 40).

Characteristics	Relative Risk	95% Confidence Interval	P
pN status (pN1)	1.678	0.553-7.432	.39
UICC Stage III/IV	7.105	0.915-44.23	.059
Residual tumor (pR1)	3.683	1.474-9.536	.0055*
Positive vessel invasion	1.446	0.610-3.821	.41
Low *dCK* (<1.25)	2.381	0.891-7.069	.084
High *RRM2* (>0.027)	3.780	1.450-11.81	.0055*

Each cutoff value of mRNA expression level was determined with the GEM group (n = 40).
*P < .05.

Furthermore, we created a combined score, which was calculated from each gene expression score determined with recursive descent partition analysis of the GEM group, and evaluated the correlation between survival time and this combined score. Each gene expression was scored as follows: low *hENT1*, low *dCK*, high *RRM1*, and high *RRM2*, 1; high *hENT1*, high *dCK*, low *RRM1*, and low *RRM2*, 2. A combined score was created: GEM score = *hENT1* score × *dCK* score × *RRM1* score × *RRM2* score. As a result, high GEM score was correlated well with prolonged DFS (Figure 5*A*) and OS (Figure 5*B*) in GEM group patients. In univariate analyses of the GEM group, a low GEM score (<2) was associated with both of a shorter DFS ($P = .0003$) and a shorter OS ($P < .0001$). In multivariate analyses of the GEM group, DFS (Table 9) was significantly dependent on UICC stage III/IV ($P = .048$), *R* factor ($P = .030$), and low GEM score (<2, $P = .0081$), and OS (Table 10) was significantly dependent on pN status ($P = .0044$), *R* factor ($P = .011$), and low GEM score (<2, $P = .0002$). A low GEM score was an independent predictive and prognostic factor for poor survival in PDAC patients receiving gemcitabine-based AC, with a relative risk of 3.515 and 5.677, respectively.

Table 8. Multivariate OS Analysis (Cox Regression Model) of Conventional Prognostic Factors and mRNA Expression Levels in the GEM Group ($n = 40$).

Characteristics	Relative Risk	95% Confidence Interval	*P*
pN status (pN1)	4.411	1.146-23.90	.029*
Residual tumor (pR1)	3.574	1.561-8.470	.0027*
Low *hENT1* (<0.5)	2.980	0.964-10.86	.20
Low *dCK* (<1.25)	2.080	0.694-7.551	.058
High *RRM1* (>0.017)	2.803	0.998-9.113	.051
High *RRM2* (>0.027)	2.357	0.935-6.863	.070

Cutoff value for each mRNA level was determined for the GEM group ($n = 40$).
*$P < .05$.

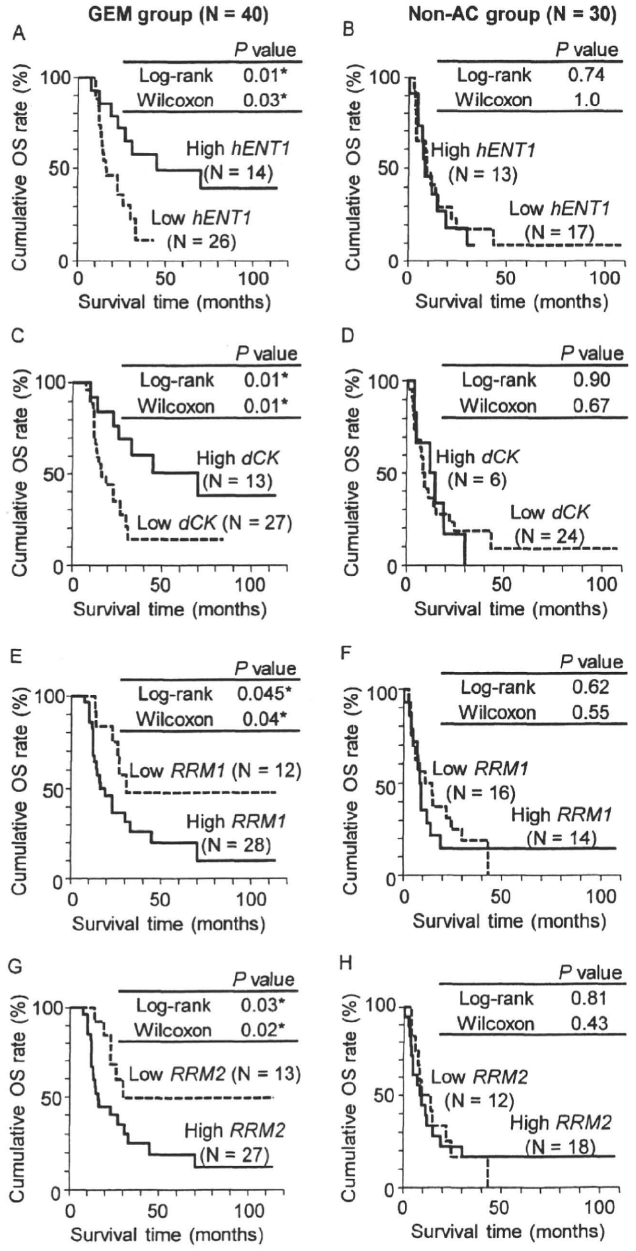

Figure 4. Correlation between the expression of each mRNA and OS. Low *hENT1* ($P = .011$), low *dCK* ($P = .0095$), high *RRM1* ($P = .041$), and high *RRM2* ($P = .030$) levels, normalized to *β-actin*, were associated with a shorter OS in the GEM group (A, C, E, G). In contrast, there was no significant correlation between these gene expression levels and OS in the non-AC group (B, D, F, H). *$P < .05$.

Quantitative Analyses of hENT1, dCK, and RRM2 Expression in Cells Microdissected from Cytologic Specimens

To apply the prediction of gemcitabine sensitivity, based on gene expression levels, to a clinical setting, we quantified *hENT1*, *dCK*,

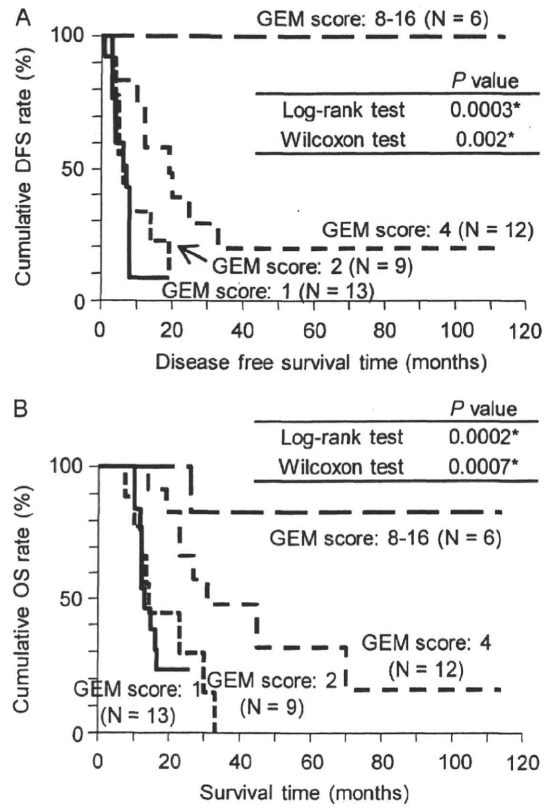

Figure 5. Correlation between GEM score and survival time. DFS time (A) and OS time (B) after resection of PDAC categorized by combined GEM score (*hENT1* score × *dCK* score × *RRM1* score × *RRM2* score) in GEM group patients. High GEM scores were well correlated with prolonged DFS time (A) and OS time (B). *$P < .05$.

Table 9. Multivariate DFS Analysis (Cox Regression Model) of Conventional Prognostic Factors and GEM Score in the GEM Group (n = 40).

Characteristics	Relative Risk	95% Confidence Interval	P
pN status (pN1)	3.111	0.968-14.10	.057
UICC Stage III/IV	7.935	1.026-49.18	.048*
Residual tumor (pR1)	2.668	1.099-6.480	.030*
Positive vessel invasion	1.202	0.488-3.310	.70
GEM score < 2	3.515	1.376-9.963	.0081*

Each cutoff value of mRNA expression level was determined with GEM group (n = 40).
*P < .05.

RRM1, and *RRM2* mRNA levels in cytologic specimens obtained from 15 patients with PDAC who underwent EUS-FNA cytologic examination in our institute. Although a few samples contained abundant neoplastic cells, most samples contained a large amount of blood and inflammatory cells and contained scarce clusters of neoplastic cells (Figure 6, *A* and *B*). Therefore, we quantified mRNA levels from WCPs and from microdissected neoplastic cells (laser capture microdissection) prepared from these samples and then compared expression levels between the two preparations. We were unable to detect clear differences in mRNA levels among the WCP samples; however, we could distinguish higher and lower expression levels of each gene among microdissected neoplastic cell samples (Figure 6, *C-F*). These data suggest that quantification of individual gene expression levels in microdissected neoplastic cells is a potent tool to predict gemcitabine sensitivity even when specimens contain abundant contaminating cells.

Discussion

PDAC remains a major therapeutic challenge. Recent randomized clinical trials showed a significant clinical benefit of gemcitabine-based chemotherapy in patients with both resected and unresectable PDAC [7,33]. Therefore, gemcitabine-based chemotherapy remains the standard palliative chemotherapy for PDAC. However, there remains a substantial subset of cases in which gemcitabine-based chemotherapy is insufficient, suggesting the importance of introducing individualized chemotherapy into the clinical setting. Individualized chemotherapy, based on the expression of genes involved in cellular uptake and metabolism of gemcitabine, will be a potent strategy.

We and other investigators have demonstrated that several altered gene expression profiles, including those of *hENT1*, *dCK*, *RRM1*, and *RRM2* correlated with the sensitivity to gemcitabine in cancer cell lines [12,15,20,22]. However, analysis of gene expression in two gemcitabine-resistant pancreatic cancer cell lines revealed that there were differences in gene expression between these cancer cell lines. These data suggest that there are different patterns of gene expression that can develop gemcitabine resistance, and combined evaluation of several genes may be required to predict gemcitabine sensitivity.

Table 10. Multivariate OS Analysis (Cox Regression Model) of Conventional Prognostic Factors and GEM Score in the GEM Group (n = 40).

Characteristics	Relative Risk	95% Confidence Interval	P
pN status (pN1)	4.907	1.560-22.48	.0044*
Residual tumor (pR1)	2.874	1.275-6.651	.011*
GEM score < 2	5.677	2.237-16.17	.0002*

Cutoff value for each mRNA level was determined for the GEM group (n = 40).
*P < .05.

In the current study, univariate analyses showed that low *hENT1*, low *dCK*, high *RRM1*, and high *RRM2* correlated well with poor outcome in patients treated with gemcitabine-based AC, although these altered expression levels did not reach statistical significance in multivariate analysis. Recent clinical studies, including two prospective clinical trials, revealed that PDAC patients with high hENT1 immunoreactivity or high *hENT1* expression gained significant benefit from gemcitabine-based AC [13,34,35], and these data are consistent with our results. However, although Akita et al. [36] and Zheng et al. [37] revealed that high RRM1 and high excision repair cross-complementation group 1–expressing patients with PDAC or NSCLC had prolonged survival regardless of AC, Nakahira et al. [20] and Akita et al. [36] also demonstrated that only patients with low RRM1 derive significant benefit from gemcitabine on disease recurrence. Therefore, RRM1 expression may contribute to gemcitabine resistance in PDAC. Moreover, although Sebastiani et al. [38] demonstrated that PDAC patients with high dCK expression had prolonged survival regardless of AC and concluded that genetic alterations of *dCK* are not a common mechanism of resistance to gemcitabine, previous *in vitro* studies [15,16,22] support our results showing that high *dCK* and low *RRM2* expressions are correlated with prolonged survival time in PDAC patients who received gemcitabine-based AC. Therefore, to introduce individualized AC into the clinical setting, based on gene expression profiles, the expression levels of several genes will need to be determined, and combined evaluation of these results may be needed. For this reason, we evaluated a simplified score, the GEM score, and found that a low GEM score was predictive for reduced DFS and prognostic for reduced survival in resected PDAC treated with gemcitabine-based AC. However, to evaluate the usefulness of this score, further studies, incorporating larger patient numbers, are required.

In contrast, we found that there was no evident correlation between *CDA* expression levels and survival time. Recently, a single-nucleotide polymorphism in the *CDA* gene, which was analyzed using the peripheral blood of cancer patients, was reported to influence the pharmacokinetics and toxicity of gemcitabine [39]. Bengala et al. [40] also demonstrated that high *CDA* expression and CDA activity levels in peripheral blood mononuclear cells were correlated with shorter survival in gemcitabine-treated patients with advanced pancreatic carcinoma. These data suggest that simple quantification of *CDA* mRNA in PDAC tissues is not helpful in predicting sensitivity of gemcitabine treatment.

Only 10% to 20% of patients with PDAC are candidates for curative resection [3]; therefore, the remaining 80% to 90% of patients with unresectable advanced PDAC need cytopathologic assessment of EUS-FNA specimens, or pancreatic juice specimens, to predict their sensitivity to chemotherapeutic agents for individualized chemotherapy. The present analysis of mRNA is quantitative (even considering the small amount of specimen available, including cytologic specimens). In addition, the present results revealed that quantification of mRNA in neoplastic cells microdissected from cytologic samples was more useful to distinguish between samples with higher and lower gene expression levels compared with the analysis of WCP samples. The reliability of tests based on tissue or cell extracts is often crucially dependent on the relative abundance of the target cell population, and sampling errors or a large number of "contaminating cells" can lead to false-negative results [26]. *hENT1* and *dCK* mRNA were reported to be expressed in human T lymphocytes and monocytes [41,42], and RRM1 and RRM2 are essential for DNA synthesis in somatic cells. For these reasons, quantification of gene expression

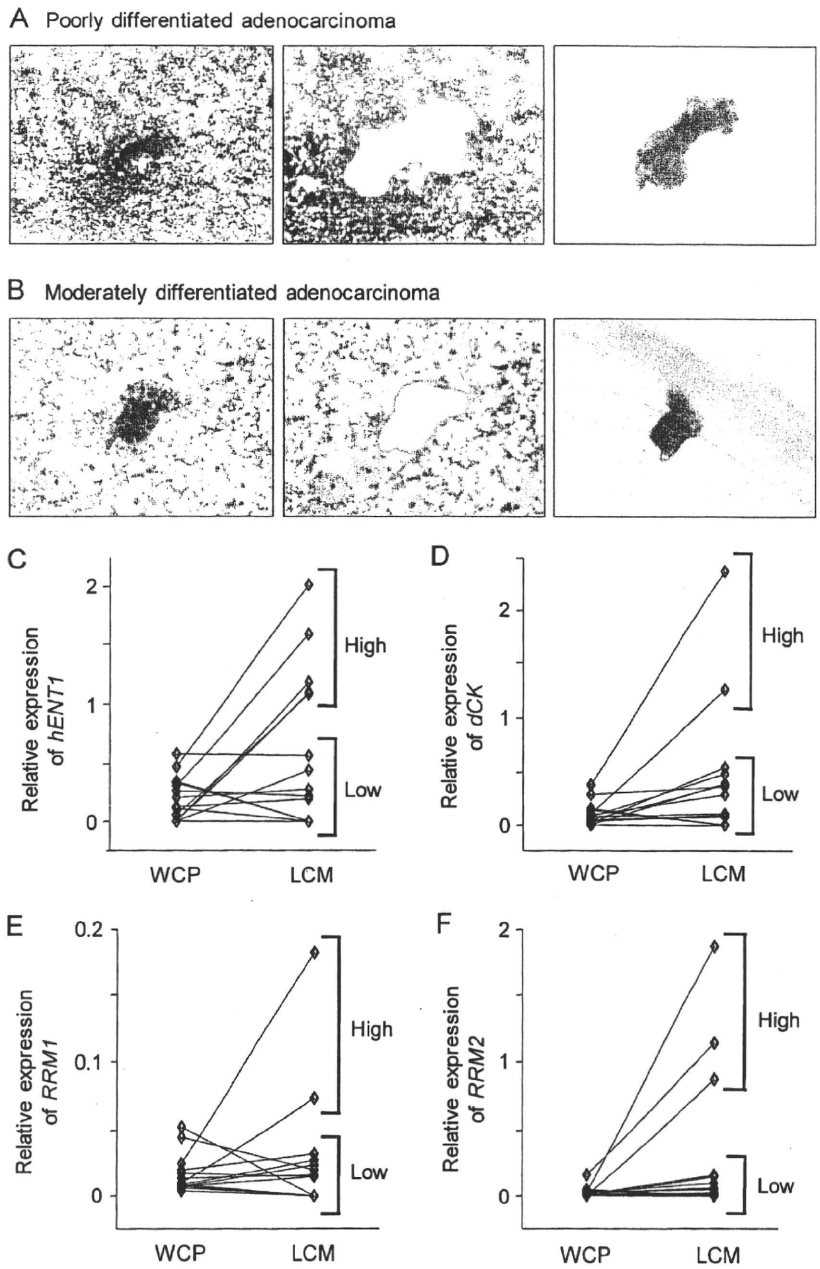

Figure 6. Quantitative analyses of mRNA associated with gemcitabine sensitivity in EUS-FNA cytologic specimens. Representative micrographs of cytologic specimens obtained from patients with PDAC who underwent EUS-FNA cytologic examination (A, B). Most samples consisted of a large amount of blood and inflammatory cells and contained scarce clusters of neoplastic cells. Quantitative analyses of *hENT1* (C), *dCK* (D), *RRM1* (E), and *RRM2* (F) mRNA in EUS-FNA cytologic specimens (n = 15). Although we could not detect clear changes of expression levels in WCP samples, we could distinguish samples having higher and lower expression levels of each gene in microdissected neoplastic cells (C-F).

in cells microdissected from EUS-FNA cytologic specimens is likely to be useful for predicting gemcitabine sensitivity in patients with PDAC, although further investigations are needed before this approach can be introduced into the clinical setting.

In conclusion, we demonstrated that quantitative analysis of *hENT1*, *dCK*, *RRM1*, and *RRM2* mRNA using FFPE tissue samples and evaluation of a combined GEM score were useful in predicting the sensitivity to gemcitabine-based AC in patients with PDAC. In addition, quantitative analysis of these genes in neoplastic cells microdissected from EUS-FNA specimens was useful in determining the treatment for patients with PDAC even when the tumor is unresectable. Quantitative analyses of genes related to cellular uptake and metabolism of cytotoxic agents can be a potent tool to perform individualized chemotherapy.

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