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Clinical implications of ribonucleotide reductase subunit M1 in patients with pancreatic cancer who undergo curative resection followed by adjuvant chemotherapy with gemcitabine

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Abstract. To the best of our knowledge, the clinical implications of using ribonucleoside reductase subunit M1 (RRM1) in patients who undergo curative resection and adjuvant chemotherapy have not been established. In the present study, the clinical data from 101 consecutive patients who underwent macroscopically curative resection, and who received adjuvant gemcitabine chemotherapy for pancreatic cancer at the Kanagawa Cancer Centre (Yokohama, Kanagawa, Japan) between April 2005 and December 2014 were retrospectively analyzed. The association between the RRM1 status and survival and clinicopathological features were assessed. Of the 101 patients, 41 patients expressed high levels of RRM1 expression (40.6%). Although a significant difference was observed in lymphatic invasion, there was no difference between the two groups with regard to any other clinicopathological parameters. The median follow-up period was 67.3 months. There was a significant difference between the recurrence-free survival (RFS) rates at 5 years after surgery, which were 12.9 and 0% in the high RRM1 and low RRM1 groups, respectively (P=0.042). Furthermore, there was a significant difference in the 5-year overall survival (OS) rates following surgery, which were 5.1 and 21.5% in the high RRM1 and low RRM1 groups, respectively (P=0.015). The results of the present study indicated that out of the factors assessed, RRM1 was the most important prognostic factor for OS and RFS in patients with pancreatic cancer who underwent curative resection followed by adjuvant chemotherapy with gemcitabine. Adjuvant chemotherapy with gemcitabine alone may be insufficient for the treatment of pancreatic cancer, particularly in patients with relevant risk factors.

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Key words: pancreatic cancer, ribonucleoside reductase subunit M1, adjuvant chemotherapy, gemcitabine

Introduction

Pancreatic cancer, which has a 5-year patient survival rate of <5%, is a major cause of cancer-associated mortality worldwide (1,2). Complete resection is an essential part of treatment for patients with pancreatic cancer. However, only 10-20% of patients are candidates for curative resection. Furthermore, due to the high rate of recurrence, the postoperative 5-year survival rate is 15-25% when curative resection is performed (3-5). Several studies have conducted randomized controlled studies on adjuvant chemotherapy following pancreatic cancer resection (6-8). The European Study Group for Pancreatic Cancer 1 and 3 trials, and the Charite Onkologic 001 trial demonstrated that the administration of gemcitabine or fluorouracil plus folinic acid significantly improves overall survival following surgical resection in patients with pancreatic cancer in comparison to surgery alone (6-8). Based on these results, adjuvant chemotherapy with gemcitabine is now considered to be the standard treatment and is routinely recommended following curative resection for pancreatic cancer. However, adjuvant chemotherapy with gemcitabine is unable to completely prevent the development of recurrence. The selection of patients who would benefit most from gemcitabine treatment may be an important step towards improving the clinical outcomes associated with pancreatic cancer.

Ribonucleotide reductase subunit M1 (RRM1) is a multimeric enzyme that converts ribonucleotides to deoxyribonucleosides, both of which are required for DNA polymerization and repair (9,10). It has previously been reported that the overexpression of the RRM1 gene is associated with gemcitabine resistance. Patients with advanced pancreatic carcinoma who exhibited high levels of RRM1 expression were demonstrated to have poor survival rates following gemcitabine treatment, while patients with non-small cell lung cancer who had low levels of RRM1

expression were revealed to benefit significantly from gemcitabine/cisplatin neoadjuvant chemotherapy (11,12). However, few published studies have evaluated the prognostic value of RRM1 expression in patients with pancreatic cancer who undergo resection followed by adjuvant chemotherapy with gemcitabine, and no definite conclusions have been made regarding the prognostic value of RRM1 in such patients (13,14). Using cancer tissue samples from individuals, the characterization of the genes that are associated with tumor sensitivity or resistance and antitumor agents serves an essential role in the development and provision of individualized adjuvant chemotherapy treatments.

In the present study, RRM1 expression was investigated in consecutive patients who underwent curative resection followed by adjuvant chemotherapy with gemcitabine. In addition, the association between RRM1 expression and the clinicopathological parameters and survival rates of patients were evaluated.

Patients and methods

Patients. Consecutive patients were selected from the medical records of those who underwent pancreatic surgery at the Kanagawa Cancer Centre (Yokohama, Kanagawa, Japan) between April 2005 and December 2014. The following inclusion criteria were applied: i) A pathologically common type of pancreatic adenocarcinoma according to the definitions of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) 6th edition (15); ii) the patient had initially undergone curative resection, with the resected specimen available from the archive; and iii) the patient had received adjuvant chemotherapy with gemcitabine. The resected specimens were histopathologically examined and were staged according to the UICC TNM 6th edition (15). Patients with other pancreatic and periampullary neoplasms, including intraductal papillary mucinous neoplasm, cystadenocarcinoma and endocrine tumors, and patients who had undergone R2 resection were excluded from the present study. The present study was approved by the Institutional Review Board Committee of the Kanagawa Cancer Center.

Surgical procedure. All pancreatic surgeries were performed in accordance with standardized procedures that have been previously described (16-19). Briefly, for distal pancreatectomy cases, lymph node dissection was performed in the region of the celiac trunk, and the superior mesenteric artery and vein, in addition to behind the pancreas along the left side of the renal vein and the left adrenal gland. In each case, intraperitoneal drains were placed close to the pancreatic anastomosis and stump. For pancreaticoduodenectomy cases, pylorus-preserving pancreaticoduodenectomy was performed as the standard procedure. Lymph node dissection along the hepatoduodenal ligament, common hepatic artery, vena cava, superior mesenteric vein and the right side of the superior mesenteric artery was performed as part of the standard procedure. Multiple intraperitoneal drains were placed, with the first being posterior to the hepaticojejunostomy and the second on the anterior surface of the pancreaticojejunostomy or the closed remnant of the pancreas.

Adjuvant chemotherapy. Gemcitabine treatment was initiated within 8 weeks of surgery. The patients received a weekly dose via intravenously of 1,000 mg/m^2 for 3 weeks, followed by 1 week of rest. Gemcitabine treatment was continued for 6 months.

Follow-up. Patients were followed up at outpatient clinics. Hematological tests and physical examinations were performed at least every 2 weeks during adjuvant chemotherapy treatment, and at least every 3 months for 5 years following the end of the adjuvant chemotherapy course. The carcinoembryonic antigen and cancer antigen 19-9 tumor marker levels were measured at least every 3 months for 5 years. Patients underwent a computed tomography examination every 3 months during the first 3 years after surgery, and then every 6 months until 5 years after surgery. Peritoneal recurrence was defined as positive when imaging results revealed at least one of the following findings: Massive ascites, ascites confirmed by cytology, enhanced abdominal nodules, abnormal intestinal wall thickness, increased fat density of the intestinal mesentery, diffuse hydronephrosis or an intraabdominal mass. Imaging results were assessed by a radiologist and two staff physicians at Kanagawa Cancer Center (Kanagawa, Japan). When liver metastasis was suspected based on the imaging results, gadolinium-ethoxybenzyl-diethylentriamine-penta-acetate-enhanced magnetic resonance imaging or contrast-enhanced ultrasonography was performed to confirm the diagnosis.

Immunohistochemical analysis of RRM1 expression. Hematoxylin and eosin-stained 5-μm slides containing specimens from each pancreatic adenocarcinoma sample were reviewed, and a representative tumor region and the corresponding formalin-fixed paraffin-embedded tissue block was selected for use in a tissue microarray. RRM1 expression was evaluated using human mouse monoclonal antibody directed against RRM1 (dilution, 1:100; #60073-2; Proteintech Group, Inc., Chicago, IL, USA) and the horseradish peroxidase secondary antibody was Histofine® Simple Stain MAX-PO (#424151; Nichirei Biosciences, Inc., Tsukiji, Japan). The immunohistochemical staining procedure was performed as described previously (14,20). Images were captured using light microscopy. The intensity of the RRM1 staining was scored as follows: Grade 0, unstained; grade 1, slightly stained; grade 2, weakly stained in comparison to plasma and stroma cells; and grade 3, stained as strongly as plasma and stroma cells. For the evaluation of intratumoral RRM1 expression, if grade 2 or 3 staining was observed in >50% of the neoplasm, the sample was considered to have high RRM1 expression, whereas if grade 0 or 1 staining was observed in >50% of tumor cells, the sample was considered to have low RRM1 expression (Fig. 1). The cut-off value used was determined on the basis of previous study results (14,20). The immunohistochemical evaluation of RRM1 expression was confirmed independently by two observers and a consensus was reached by joint review.

Statistical analysis. The significance of the correlations between RRM1 expression and clinicopathological parameters was determined using Fisher's exact or χ^2 tests. Overall survival (OS) rate was defined as the period between surgery and

Table I. Association between the clinicopathological characteristics of patients with pancreatic cancer and high (n=41) or low (n=60) ribonucleotide reductase M1.

Clinicopathological characteristic	Low RRM1 group, n (%)	High RRM1 group, n (%)	P-value
Gender			0.725
Male	33 (55.0)	24 (58.5)	
Female	27 (45.0)	17 (41.5)	
Age, years			0.955
<65	26 (43.3)	18 (43.9)	
≥65	34 (56.7)	23 (56.1)	
R status			0.404
R0	52 (86.7)	33 (80.5)	
R1	8 (13.3)	8 (19.5)	
Tumor location			0.233
Head	46 (76.7)	27 (65.9)	
Body/tail	14 (23.3)	14 (34.1)	
Pathological differentiation			0.154
Well	52 (86.7)	31 (75.6)	
Moderate/poor	8 (13.3)	10 (24.4)	
UICC pT factor			0.142
T1/T2	6 (10.0)	1 (2.4)	
T3	54 (90.0)	40 (97.6)	
Lymph node metastasis			0.259
N0	16 (26.7)	7 (17.1)	
N1	44 (73.3)	34 (82.9)	
Lymphatic invasion			0.021
No	33 (55.0)	13 (31.7)	
Yes	27 (45.0)	28 (68.3)	
Vascular invasion			0.551
No	24 (40.0)	14 (34.1)	
Yes	36 (60.0)	27 (65.9)	

RRM1, ribonucleotide reductase subunit M1; UICC, International Union Against Cancer; pT, pathological T factor, tumor factor.

mortality. Recurrence-free survival (RFS) was defined as the period between surgery and recurrence or mortality. The data of the patients who had not experienced an event were censored at the date of the final observation. The OS and RFS rates were evaluated using univariate and multivariate analyses. OS and RFS curves were calculated using the Kaplan-Meier estimator method and compared using the log-rank test. The univariate and multivariate survival analyses were performed using Cox's proportional hazards model. P<0.05 was considered to indicate a statistically significant difference. The survival data were obtained from hospital records or from the city registry system. All statistical analyses were performed using SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA).

Results

Patients. A total of 201 patients underwent surgical resection between April 2005 and December 2014. Of these patients, 101 were eligible for inclusion in the present study. The patients were aged between 40 and 78 years (median, 66 years), with 57 men and 44 women. In total, 28 patients underwent distal pancreatomy, 70 underwent pancreaticoduodenectomy and 3 underwent total pancreatic resection. The median follow-up period was 67.3 months (range, 22.2-122.7 months).

Association between clinicopathological factors and RRM1 expression. High RRM1 expression was observed in 41 (40.6%) patients (Table I). The clinicopathological factors were compared between patients with high and low RRM1 expression. In total, 9 clinicopathological factors were evaluated. The incidence of lymphatic invasion was significantly higher in the patients with high RRM1 expression compared with that in the low RRM1 expression group (P=0.021; Table I).

Survival analysis. The OS rates at 3 and 5 years post-surgery in the patients with high RRM1 expression were 10.5 and

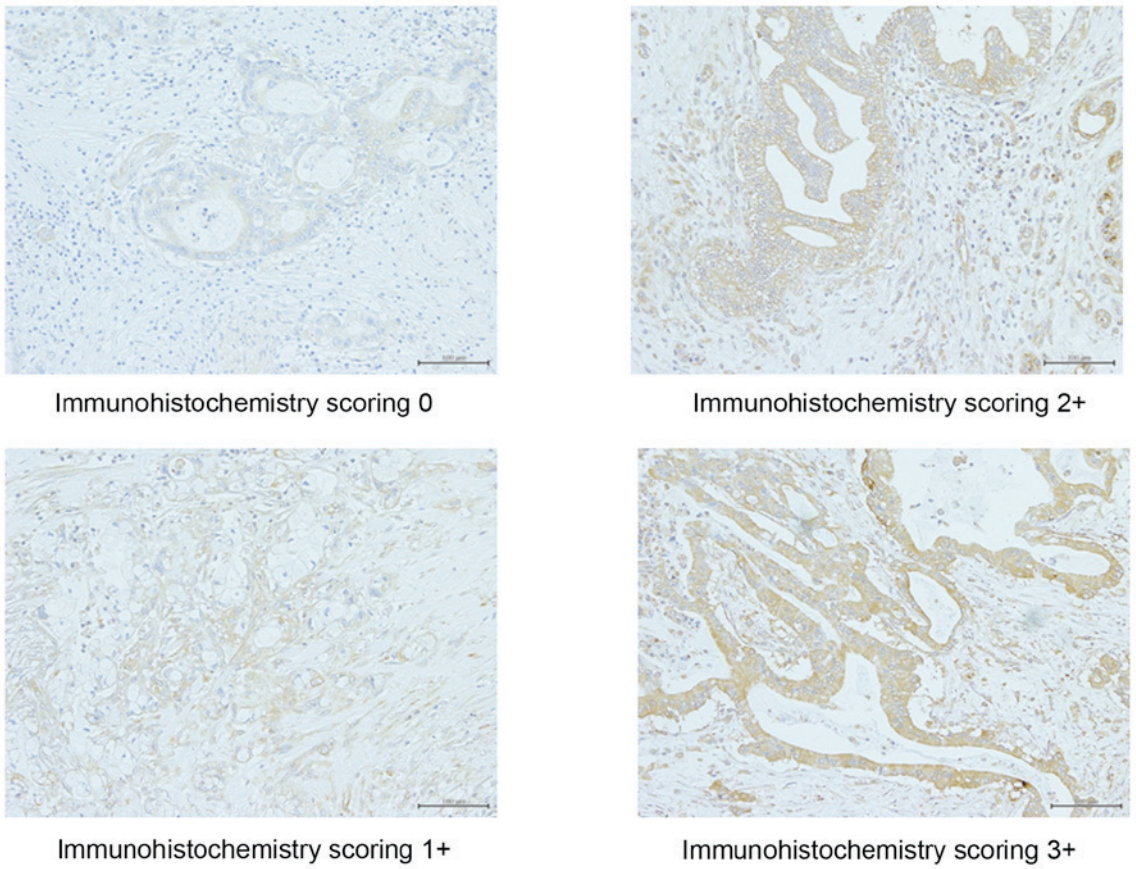

Figure 1. Representative results of the immunohistochemical staining of ribonucleotide reductase M1 in tissue samples from patients with pancreatic cancer (scale bar, 100 μm).

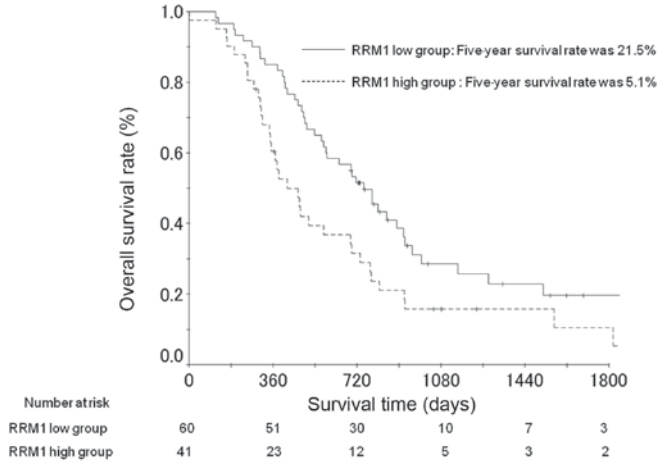

Figure 2. A comparison of the overall survival in the high and low RRM1 groups of patients with pancreatic cancer. The data below the graph represents the overall survival rate patients during the indicated time. RRMI, ribonucleotide reductase M1.

5.1%, respectively; and 25.7 and 21.5% in the patients with low RRM1 expression (Fig. 2). The difference between OS rates for patients with high and low RRM1 expression was identified to be significant following multivariate analysis (P=0.015; Table II). Multivariate analysis also demonstrated that tumor location and lymphatic invasion were significant risk factors for OS (Table II).

The RFS rates at 3 and 5 years post-surgery in the patients with high RRM1 expression were 7.8 and 0%, respectively (Fig. 3). For patients with low RRM1 expression, the RFS rates were 20.7 and 12.9%, respectively (Fig. 3). The difference between RFS rates for patients with low and high expression was significant (P=0.042; Table III). Multivariate analysis also demonstrated that tumor location, lymphatic invasion and resection status were significant risk factors for RFS (Table III).

Discussion

The present study evaluated the RRM1 status in patients with pancreatic adenocarcinoma who underwent curative resection followed by adjuvant chemotherapy with gemcitabine, and found that 40% of these patients exhibited high RRM1 expression. Furthermore, the OS and RFS rates of the patients differed significantly based on their RRM1 status. These results suggest that gemcitabine alone was insufficient as an adjuvant therapy, particularly in the patients with high RRM1 expression. Thus, these patients should be a target group for future clinical trials using novel treatments for pancreatic cancer.

Numerous studies have examined the presence and effect of RRM1 protein overexpression or gene amplification in patients with pancreatic adenocarcinoma. These studies reported that RRM1 is highly expressed in 20.4-87.3% of patients (13,14,20-22). However, the measurement of RRM1

Table II. Univariate and multivariate analyses of risk factors for the overall survival of patients with pancreatic cancer.

		Univariate analysis			Multivariate analysis		
Factor	n	OR	95% CI	P-value	OR	95% CI	P-value
Gender				0.561			0.900
Female	44	1.000			1.000		
Male	57	1.143	0.728-1.795		1.033	0.620-1.724	
Age, years				0.740			0.626
<65	44	1.000			1.000		
≥65	57	1.081	0.683-1.709		1.123	0.703-1.794	
R status				0.041			0.197
R0	85	1.000			1.000		
R1	16	1.850	1.026-3.336		1.555	0.795-3.041	
Tumor location				0.024			0.013
Body/tail	28	1.000			1.000		
Head	73	1.840	1.085-3.120		1.980	1.153-3.400	
Pathological differentiation				0.892			0.932
Well	83	1.000			1.000		
Moderate/poor	18	1.042	0.572-1.898		1.029	0.533-1.988	
UICC pT factor				0.035			0.273
T1/T2	7	1.000			1.000		
T3	94	4.545	1.113-18.559		2.284	0.522-9.997	
Lymph node metastasis				0.038			0.704
N0	23	1.000			1.000		
N1	78	1.802	1.034-3.140		1.131	0.599-2.136	
Lymphatic invasion				0.001			0.009
No	46	1.000			1.000		
Yes	55	2.192	1.374-3.498		1.898	1.174-3.066	
Vascular invasion				0.032			0.283
No	38	1.000			1.000		
Yes	63	1.678	1.044-2.695		1.358	0.776-2.377	
RRM1 status				0.009			0.015
Low	60	1.000			1.000		
High	41	1.814	1.160-2.837		1.777	1.116-2.830	

RRM1, ribonucleotide reductase subunit M1; UICC, International Union Against Cancer; pT, pathological tumor; CI, confidence interval; OR, odds ratio.

expression was not standardized and the background of the patients with pancreatic cancer was heterogeneous, as it included patients with stage I-IV tumors. Nakagawa *et al* (14) evaluated the incidence of RRM1 in resectable pancreatic cancer cases using immunohistochemical methods in 109 Japanese patients with pancreatic carcinoma who were treated with adjuvant gemcitabine-based chemotherapy following operative resection. It was demonstrated that RRM1 expression was observed in 44 (40.4%) patients. In addition, Xie *et al* (22) measured RRM1 expression using reverse transcriptase-quantitative polymerase chain reaction analysis in 122 patients with resectable pancreatic adenocarcinoma. It was revealed that high RRM1 expression was observed in 44 (36.1%) patients. These results were similar to the results of the present study. Thus, the incidence of high RRM1 expression is ~40% in patients with resectable pancreatic cancer.

Regarding the association between RRM1 expression and clinicopathological factors, Akita *et al* (23) reported that in an analysis of 64 patients with resected pancreatic carcinoma, there were no significant differences in clinicopathological factors, including UICC pT factor, and lymph node status, between patients with high and low RRM1 expression. Nakagawa *et al* (14) reported similar results. In the current study, a significant difference was only observed in lymphatic

Table III. Univariate and multivariate analyses of risk factors for the recurrence-free survival of patients with pancreatic cancer.

		Univariate analysis			Multivariate analysis		
Factor	n	OR	95% CI	P-value	OR	95% CI	P-value
Gender				0.874			0.380
Female	44	1.000			1.000		
Male	57	1.035	0.674-1.590		1.239	0.768-1.997	
Age, years				0.293			0.272
<65	44	1.000			1.000		
≥65	57	1.264	0.817-1.954		1.294	0.817-2.048	
R status				0.001			0.007
R0	85	1.000			1.000		
R1	16	2.668	1.469-4.845		2.322	1.261-4.276	
Tumor location				0.016			0.014
Body/tail	28	1.000			1.000		
Head	73	1.816	1.118-2.949		1.850	1.132-3.025	
Pathological differentiation				0.775			0.747
Well	83	1.000			1.000		
Moderate/poor	18	1.083	0.627-1.869		1.099	0.620-1.946	
UICC pT factor				0.148			0.730
T1/T2	7	1.000			1.000		
T3	94	1.778	0.814-3.883		1.167	0.484-2.812	
Lymph node metastasis				0.074			0.715
N0	23	1.000			1.000		
N1	78	1.597	0.956-2.669		1.122	0.606-2.075	
Lymphatic invasion				0.001			0.031
No	46	1.000			1.000		
Yes	55	2.238	1.438-3.482		1.704	1.049-2.767	
Vascular invasion				0.204			0.818
No	38	1.000			1.000		
Yes	63	1.330	0.856-2.065		1.066	0.618-1.838	
RRM1 status				0.008			0.042
Low	60	1.000			1.000		
High	41	1.784	1.164-2.735		1.610	1.017-2.549	

RRM1, ribonucleotide reductase subunit M1; UICC, International Union Against Cancer; pT, pathological tumor; CI, confidence interval; OR, odds ratio.

invasion. However, there was no difference between the two groups in any of the other clinicopathological parameters of the patients with high and low RRM1 expression, including UICC pT factor and lymph node status. Thus, RRM1 expression appears to be independent from the other clinicopathological factors.

In the present study, the OS and RFS rates differed significantly based on the patients' RRM1 status. It is hypothesized that RRM1 is an essential enzyme that encodes the regulatory subunit of ribonucleotide reductase and catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides for use in *de novo* DNA synthesis (24,25). There is a good rationale for this as gemcitabine is converted into gemcitabine diphosphate, an active metabolite capable of inhibiting ribonucleoside reductase, and RRM1 has been demonstrated to be a determinant of gemcitabine resistance in pancreatic cancer cells under *in vitro* conditions (11). Nakagawa *et al* (14) evaluated 109 patients with resected pancreatic cancer who underwent adjuvant chemotherapy with gemcitabine and were divided into 2 groups based on their RRM1 levels. A significant association was identified between disease-free survival and RRM1 expression (P=0.009). Furthermore, the patients with high RRM1 levels experienced poorer overall survival following gemcitabine treatment

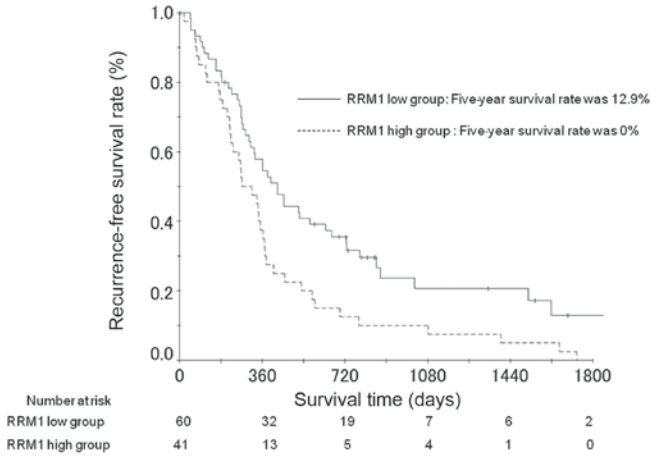

Figure 3. A comparison of the recurrence free survival in the high and low RRM1 groups of patients with pancreatic cancer. The data below the graph represents the recurrence-free survival rate of patients during the indicated time. RRMI, ribonucleotide reductase M1.

compared with those with low RRM1 levels (P=0.019). In addition, Akita *et al* (23) reported that patients with low RRM1 expression experienced significantly improved OS rate compared with patients with high RRM1 expression in an analysis of 68 patients with pancreatic carcinoma who underwent resection and received gemcitabine chemotherapy. A similar result was observed in a study of patients with advanced pancreatic cancer (11). Nakahira *et al* (11) evaluated 18 patients with recurrent pancreatic cancer who were treated with gemcitabine and who were divided into 2 groups based on RRM1 levels. A significant association was observed between gemcitabine response and RRM1 expression (P=0.018). Additionally, patients with high RRM1 levels exhibited poorer survival times following gemcitabine treatment compared with those patients with low RRM1 levels (P=0.016). The median survival time following gemcitabine treatment was 6.0 months in the patients with high RRM1 levels, while it was 14.6 months in the patients with low RRM1 levels. However, Giovannetti *et al* (26) demonstrated that there was no correlation between RRM1 expression and the clinical outcome of patients with pancreatic cancer. These controversial findings are probably associated with a range of factors, including the interaction with other genes, environmental effects on gene expression and differences in the detection methods, sample sizes and study design.

Particular attention is required when interpreting the results of the current study as there are several associated potential limitations. Firstly, the present study was a retrospective analysis and was performed at a single institution. Thus, the possibility that these findings were observed by chance cannot be excluded. Secondly, there was a selection bias in the patients in this series. Surgeons often avoid performing pancreatomy in certain patients, as the procedure is associated with high rates of morbidity (40-60%) and mortality (1-1.5%) (27-31). Thus, the fact that certain patients in this study received pancreatectomy could be considered a potential bias. In addition, the hospital is a specialized cancer center. Finally, the evaluation of RRM1 expression was not standardized. The appropriate RRM1 cutoff value remains unclear. Considering these limitations, the results must be confirmed in another cohort or in a prospective multicenter-study.

In conclusion, the OS and RFS rates of patients with pancreatic cancer who underwent curative resection followed by adjuvant chemotherapy with gemcitabine differed significantly based on their RRM1 expression. These results suggest that gemcitabine was insufficient, particularly for the patients with high RRM1 expression. Thus, these patients should be a target group for future clinical trials using novel treatments for pancreatic cancer.

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