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Original Article

Ribonucleotide reductase M2 does not predict survival in patients with resectable pancreatic adenocarcinoma

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Abstract: Background: Ribonucleotide reductase M2 (RRM2) was associated with pancreatic tumor progression and resistance to gemcitabine. This study aimed to determine if RRM2 protein expression was prognostic in patients with resectable pancreatic adenocarcinoma and predictive of adjuvant gemcitabine benefit. Methods: 117 patients underwent tumor resection for pancreatic adenocarcinoma from 10/1999 to 12/2007. We constructed tissue microarrays from paraffin-embedded tumors and determined RRM2 protein expression using immunohistochemistry and grouped as negative or positive. We estimated overall survival (OS) and progression-free survival (PFS) using the Kaplan-Meier method and examined the prognostic and predictive value of RRM2 expression using Cox proportional hazards model. Results: RRM2 expression showed no prognostic value in the entire group regarding OS (median OS 30.9 months in RRM2-positive versus 13.7 months in RRM2-negative, $P = 0.26$) and PFS (median OS 20.6 months in RRM2-positive versus 11.8 months in RRM2-negative, $P = 0.46$). RRM2 expression did not predict adjuvant gemcitabine benefit in the subgroup of 44 patients who received gemcitabine therapy (median OS 31.2 versus 15.2 months, $P = 0.62$; median PFS 11.3 versus 14.0 months, $P = 0.35$). Cox proportional hazards regression showed no prognostic effect of RRM2 expression on OS and PFS in the subgroup of 44 patients. However, the number of positive lymph nodes and perineural invasion were prognostic factors for OS (HR 1.2, $P = 0.005$) and for PFS (HR 5.5, $P = 0.007$), respectively. Conclusion: RRM2 protein expression in pancreatic adenocarcinoma is neither prognostic nor predictive of adjuvant gemcitabine benefit in patients with resectable pancreatic adenocarcinoma.

Keywords: Ribonucleotide reductase M2, pancreatic cancer, resectable, gemcitabine

Introduction

Approximately 40,000 new cases of pancreatic ductal adenocarcinoma were diagnosed every year [1]. The median overall survival is only 18 months even for the 15-20% patients with resectable tumor at initial presentation [2, 3]. Although gemcitabine-based chemotherapy and chemoradiation therapy are used widely as an adjuvant treatment for resectable pancreatic adenocarcinoma [4–6], many patients show poor response. Once local recurrence or metastasis occurs, the options for these patients are often limited to palliative or supportive care [2, 3].

Efforts to address gemcitabine resistance mechanism of pancreatic adenocarcinoma have been focusing on the role of key players involved in the transport and metabolism of gemcitabine [7]. Among these proteins such as deoxycytidine kinase, human equilibrative nucleoside transporter 1 (hENT1), and ribonucleotide reductase M1 (RRM1), ribonucleotide reductase small subunit M2 (RRM2), the catalytic subunit of ribonucleotide reductase [8], is also associated with tumor progression and resistance to gemcitabine [9]. Preclinical studies in pancreatic cancer cell lines demonstrated the relationship between gemcitabine resistance and RRM2 overexpression [10]. The transfection of siRNA to RRM2 decreased pancreatic cancer cell invasiveness as well as gemcitabine resistance [11], suggesting that RRM2 is a potential therapeutic target in treating pancreatic adenocarcinoma.

From the results of these *in vitro* studies, RRM2 seemed to have features that are independent of RRM1, which has been demonstrated as a

predictive factor for adjuvant gemcitabine benefit to overall survival in pancreatic adenocarcinoma [12]. However, the clinical evidence presented so far were contradictory. Giovannetti and coworkers [13] studied 102 patients with pancreatic adenocarcinoma and 67 of them treated with adjuvant gemcitabine. The results showed neither prognostic nor predictive value of RRM2 mRNA level for survival. However, a recent study by Fujita and coworkers [14] on 40 patients treated with adjuvant gemcitabine showed that low mRNA expression of RRM2 was predictive of treatment benefit of gemcitabine in patients with resected pancreatic adenocarcinoma. Furthermore, RRM2 expression was also prognostic for survival in a univariate analysis of the entire cohort of 70 patients.

To elucidate the role of RRM2, we evaluated 117 patients with resectable pancreatic adenocarcinoma. We aimed to determine whether RRM2 protein expression level assessed by immunohistochemistry is prognostic in patients with resectable pancreatic adenocarcinoma or is predictive of adjuvant gemcitabine benefit.

Materials and methods

We retrospectively reviewed the medical records of 117 patients who underwent surgical resection for pancreatic adenocarcinoma at the Cleveland Clinic from October 1999 to December 2007. We included the patients with the diagnosis of pancreatic ductal adenocarcinoma and pathologic stage of T(1-3)N(0,1,x)M0. We excluded the patients with unresectable or metastatic disease, R2 resection, ampullary carcinoma, and indolent pancreatic tumors such as mucinous cystadenoma, mucinous cystadenocarcinoma, and islet cell tumor. This study was approved by the institutional review board at the Cleveland Clinic.

All 117 pancreatic adenocarcinoma tissue samples were either formalin-fixed (103 patients, 88.0%) or Hollande's-fixed (14 patients, 12.0%), and paraffin-embedded. We centrally reviewed the tumor-containing hematoxylin and eosin stained slides. Tissue microarrays of tumors (duplicate, 2 mm core from the area with the highest density of tumor cells) were constructed and used for the determination of RRM2 protein. Immunohistochemistry staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO LSAB+ and diaminobenzadine (DAB) as the chromogen. De-paraffinized sections at five-micron thickness were labeled with RRM2 antibody (Goat polyclonal antibody, SC-10846, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Microwave citric acid epitope retrieval was employed. Appropriate negative (no primary antibody) were stained in parallel with each set of tumors studied. RRM2 immunoreactivity was assessed for the percentage of nuclear immunoreactivity in tumor cells. Results were grouped into the following categories: no nuclear staining, negative; with nuclear staining, positive.

Statistical analysis was primarily descriptive in nature in this retrospective study. Thus, we did neither sample size calculation nor power estimation. Categorical data were summarized as frequency counts and percentages. Continuous measures were summarized as means, standard deviations (s.d.), medians, and ranges. We compared continuous data with student t-test and categorical data with Fisher's exact test. OS was measured from the time of tumor resection to death. PFS was measured from the time of tumor resection to disease progression or death. Time-to-event data was plotted using the Kaplan-Meier method with surviving patients censored at the date of last follow-up. The different groups of patients were evaluated using the log-rank tests. We used Cox proportional hazards model for multivariable analyses of potential prognostic indicators in a stepwise fashion. The criterion for entry variables was $P < 0.1$. The retention criterion was $P < 0.05$. Hazard ratio (HR), 95% confidence interval (CI) for the HR, and corresponding *P*-value were used to present Cox proportional hazards regression results. All the statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

Results

Prognostic value of RRM2 in the entire cohort of patients with resectable pancreatic adenocarcinoma

A total of 117 patients were diagnosed with pancreatic ductal adenocarcinoma and underwent tumor resection from October 1999 to December 2007. As shown in **Table 1**, 68 patients (58.1%) were male. The median age at the time of tumor resection was 65 years (range: 35-93). The surgical procedures included Whipple procedure in 96 patients

Table 1. Patient Demographics, Clinical Characteristics, and Histopathologic Features (n = 117)

Variables	Number of Patients (%)
Gender	
male/female	68 (58.1)/ 49 (41.9)
Age	
median [range]	65 [35-93]
Surgical Procedure	
Whipple	96 (82.1)
Distal pancreatectomy	19 (16.2)
Subtotal pancreatectomy	1 (0.9)
Uncinate excision	1 (0.9)
Tumor size, cm	
mean ± s.d.	3.4 ± 1.4
median [range]	3.2 [0.1-8]
Pathologic stage	
T1/T2/T3	11 (9.4)/ 50 (42.7)/ 56 (47.9)
N0/N1/Nx	47 (40.1)/ 69 (59.0)/ 1 (0.9)
M0	117 (100)
Overall stage	
IA/IB/IIA/IIB	5 (4.3)/ 17(14.7)/ 24 (20.7)/ 71 (60.7)
Differentiation	
well/moderate/poor	5 (4.3)/ 67 (57.3)/ 45 (38.5)
Number of positive lymph nodes	
mean ± s.d.	2.2 ± 3.3
median [range]	1 [0-21]
Lymphovascular invasion	
Yes/No	56 (47.9)/ 61 (52.1)
Perineural invasion	
Yes/No	75 (64.1)/ 42 (35.9)
Surgical margins	
negative/positive	81 (69.2)/ 36 (30.8)
Adjuvant therapy	
Chemoradiation	59 (50.4)
Chemotherapy only	16 (13.7)
None	24 (20.5)
Unknown	15 (12.8)
Adjuvant gemcitabine	
yes/no/unknown	44 (37.6)/ 55 (47.0)/ 18 (15.4)
Status at follow-up	
alive/dead	19 (16.2)/98 (83.8)

(82.1%), distal pancreatectomy in 19 patients (16.2%), subtotal pancreatectomy in 1 patient (0.9%), and uncinate excision in 1 patient (0.9%). 36 patients (30.8%) had a positive surgical margin. The mean tumor size was 3.4 cm (s.d. 1.4 cm). As to pathologic stage, the tumor of 11 patients (9.4%) was T1, of 50 patients (42.7%) was T2, of 56 patients (47.9%) was T3, of 47 patients (40.1%) was N0, and of 69 (59.0%) patients was N1. The tumors were well differentiated in 5 patients (4.3%), moderately differentiated in 67 patients (57.3%), and poorly differentiated in 45 patients (38.5%). The mean number of positive lymph nodes was 2.2 (s.d. 3.3). Lymphovascular invasion was present in the tumor of 56 patients (47.9%). Perineural invasion was present in the tumor of 75 patients (64.1%). 24 patients (20.5%) did not undergo any form of adjuvant treatment. 59 patients (50.4%) underwent adjuvant chemoradiation therapy. 16 patients (13.7%) had adjuvant chemotherapy only. The treatment history of 15 patients (12.8%) was unknown. 44 patients (37.6%) received adjuvant gemcitabine treatment or gemcitabine-based regimens. The median follow-up time was 13.4 months (range: 0.2-105.6). At the time of the last follow-up, 19 patients (16.2%) were still alive, whereas 98 (83.8%) patients were dead.

For OS analysis, 97 patients were grouped into RRM2 negative expression group, 20 patients into RRM2 positive expression group. For PFS analysis, 67 patients were grouped into RRM2 negative expression group, 13 patients into RRM2 positive expression group. These were carried on to univariate analysis of prognostic factors for survival. The variables included RRM2 protein expression, T stage, tumor size, number of positive lymph nodes, overall stage, lymphovascular invasion, perineural invasion, adjuvant therapy, gender, age, N stage, tumor differentiation, status of surgical margins, and adjuvant gemcitabine. As shown in **Table 2**, number of positive lymph nodes (HR 1.1 per 1 more, $P = 0.0005$), overall stage IIA/I (HR 2.4, $P = 0.01$), IIB/I (HR 2.4, $P = 0.004$), perineural invasion (HR 2.0, $P = 0.002$), N stage (HR 1.6, $P = 0.03$) were associated with decreased OS. Similarly, number of positive lymph nodes (HR 1.1 per one more, $P = 0.02$), tumor size (HR 1.2, per 1 cm increase, $P = 0.04$), perineural invasion (HR 2.9, $P = 0.0009$), and N stage (HR 2.5, $P = 0.002$) were associated with decreased PFS. RRM2 expression and adjuvant gemcitabine were not prognostic factors for either OS or PFS. As depicted in **Figure 1**, patients with negative RRM2 expression in tumors did not have significantly longer OS than patients with positive RRM2 expression in tumors (median OS

Table 2. Univariate Analysis for Overall Survival and Progression-Free Survival in the Entire Cohort

	Overall Survival (n =117)			Progression-Free Survival (n = 80)		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
RRM2 expression						
positive/negative	0.7	0.4-1.2	0.3	0.8	0.4-1.6	0.5
T stage						
T2/T1	1.7	0.8-3.8	0.2	2.6	0.6-10.9	0.2
T3/T1	2.3	1.05-5.1	0.04	2.9	0.7-12.2	0.2
Tumor size						
per 1 cm increase	1.1	0.99-1.3	0.06	1.2	1.01-1.5	0.04
Number of positive lymph nodes						
per 1 node increase	1.1	1.05-1.18	0.0005	1.1	1.02-1.2	0.02
Overall stage						
IIA/I	2.4	1.2-4.8	0.01	1.5	0.6-3.7	0.4
IIB/I	2.4	1.3-4.3	0.004	2.5	1.2-5.1	0.01
Lymphovascular invasion						
yes/no	1.4	0.9-2.0	0.1	1.7	1.0-3.0	0.05
Perineural invasion						
yes/no	2.0	1.3-3.0	0.002	2.9	1.5-5.3	0.0009
Adjuvant therapy						
yes/no	0.6	0.4-1.0	0.06	0.4	0.2-0.8	0.01
Gender						
female/male	1.1	0.8-1.7	0.5	1.1	0.7-2.0	0.6
Age						
per 10-y increase	1.0	0.8-1.2	0.7	0.8	0.6-1.02	0.07
N stage						
N1/N0, Nx	1.6	1.05-2.4	0.03	2.5	1.4-4.5	0.002
Differentiation						
poor/moderate	1.1	0.8-1.7	0.5	0.9	0.5-1.7	0.8
Surgical margin						
positive/negative	1.3	0.9-2.1	0.2	0.9	0.5-1.8	0.8
Adjuvant gemcitabine						
yes/no	1.3	0.8-2.0	0.2	1.0	0.6-1.7	0.9

13.7 months versus 30.9 months, $P = 0.26$). The same conclusion stayed true for PFS (median PFS 11.8 months versus 20.6 months, $P = 0.46$).

Multivariable analyses identified prognostic factors for survival based on the pre-defined entry and retention criteria (**Table 3**). The presence of perineural invasion was associated with both decreased OS (HR 1.9, $P = 0.009$) and decreased PFS (HR 2.9, $P = 0.0009$). In addition, number of positive lymph nodes (HR 1.1 per 1 more, $P = 0.0003$) was associated with decreased OS. The presence of adjuvant therapy was associated with increased OS (HR 0.6, $P = 0.02$) and increased PFS (HR 0.4, $P = 0.01$). RRM2 expression was not associated with either OS or PFS.

Prognostic value of RRM2 in the subgroup of resectable pancreatic adenocarcinoma patients treated with adjuvant gemcitabine

Similar analyses for a subpopulation of 44 patients who received adjuvant gemcitabine treatment were also performed. Univariate analysis revealed that number of positive lymph nodes (HR 1.2 per 1 more, $P = 0.005$) was associated with decreased OS. Only perineural invasion (HR 5.52, $P = 0.007$) was associated with decreased PFS. RRM2 expression did not predict the treatment benefit of adjuvant gemcitabine for either OS or PFS (**Table 4**). As depicted in **Figure 2**, patients with negative RRM2 expres-

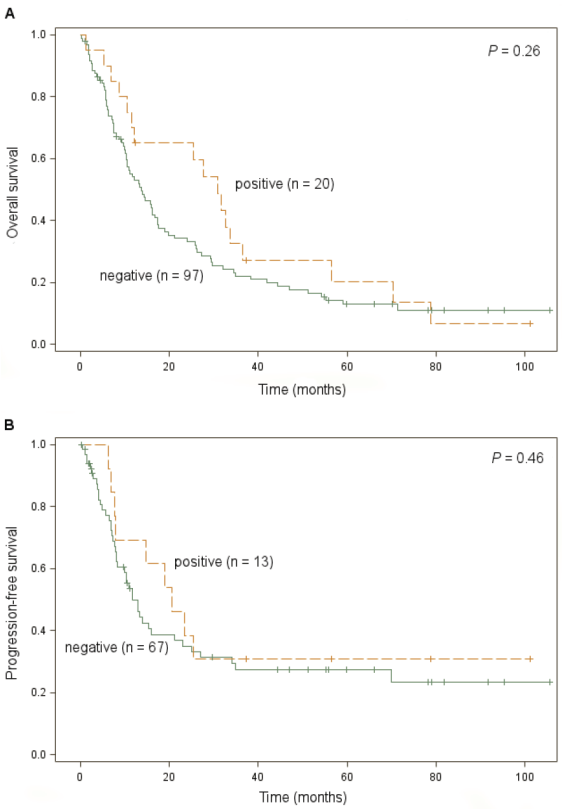

Figure 1. Overall survival and progression-free survival by RRM2 expression in the entire cohort (A) Overall survival by RRM2 expression (RRM2-negative: median OS 13.7 months; RRM2-positive: median OS 30.9 months). (B) Progression-free survival by RRM2 expression (RRM2-negative: median PFS 11.8 months; RRM2-positive: median PFS 20.6 months).

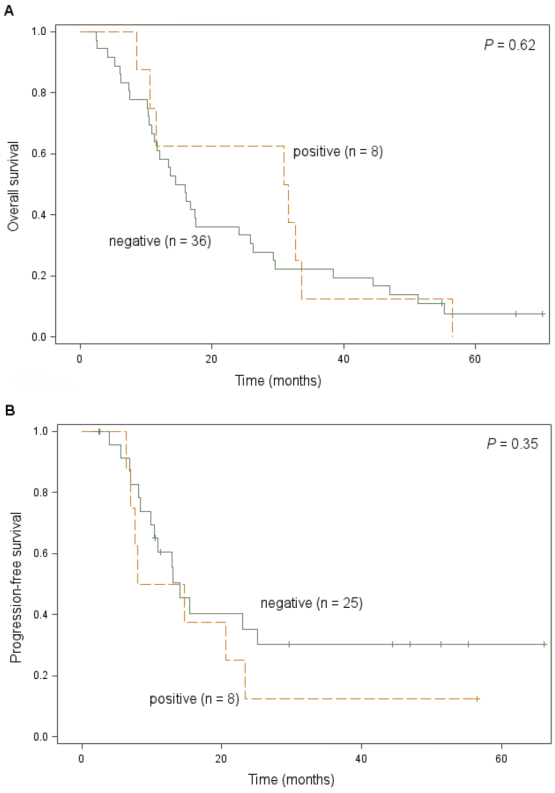

Figure 2. Overall survival and progression-free survival by RRM2 expression in adjuvant gemcitabine group (A) Overall survival by RRM2 expression (RRM2-negative: median OS 15.2 months; RRM2-positive: median OS 31.2 months). (B) Progression-free survival by RRM2 expression (RRM2-negative: median PFS 14 months; RRM2-positive: median PFS 11.3 months).

sion in tumors did not have significantly longer OS than patients with positive RRM2 expression (median OS 15.2 months versus 31.2 months, $P = 0.62$). The same conclusion stayed true for PFS (median PFS 14.0 months versus 11.3 months, $P = 0.35$).

The results from multivariable analyses shown in **Table 5** were consistent with the aforementioned findings. Number of positive lymph nodes and perineural invasion were predictors of shorter OS (HR 1.2 per 1 more, $P = 0.005$) and shorter PFS (HR 5.5, $P = 0.007$), respectively. RRM2 expression was not associated with either OS or PFS.

Discussion

In this retrospective study, we reviewed the medical records of patients from a single tertiary medical center. It turned out that the median age, gender, treatment, and the survival of these patients were not statistically different from those in the previous large studies of patients with resectable pancreatic adenocarcinoma [13, 14]. The results from univariate analyses demonstrate common histopathological prognostic factors for resectable pancreatic adenocarcinoma [15, 16], including tumor size, stage, number of positive lymph nodes, the presence of lymphovascular invasion or perineural invasion, and the absence of adjuvant therapy.

In addition to the traditional morphological markers, molecular markers associated with the prognosis have also been extensively studied; especially those involved in the transport and

Table 3. Multivariable Analysis for Overall Survival and Progression-free Survival in the Entire Cohort

	OS			PFS		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
Number of positive lymph nodes						
per 1 node increase	1.1	1.06-1.2	0.0003	NA	NA	NA
Perineural invasion						
yes/no	1.9	1.2-3.2	0.009	2.9	1.5-5.4	0.0009
Adjuvant therapy						
yes/no	0.6	0.3-0.9	0.02	0.4	0.2-0.8	0.01

NA: not applicable

Table 4. Univariate Analysis for Overall Survival and Progression-Free Survival in Patients Treated with Adjuvant Gemcitabine

	Overall Survival (n =44)			Progression-Free Survival (n = 33)		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
RRM2 expression						
positive/negative	0.8	0.4-1.8	0.6	1.5	0.6-3.8	0.4
T stage						
T2/T1	2.7	0.8-9.5	0.1	1.1	0.2-5.0	0.9
T3/T1	3.6	1.04-12.3	0.04	1.1	0.2-4.8	0.9
Tumor size						
per 1 cm increase	1.3	0.9-1.7	0.1	0.9	0.6-1.4	0.6
Number of positive lymph nodes						
per 1 node increase	1.2	1.1-1.4	0.005	1.1	0.9-1.3	0.3
Overall stage						
IIA/I	1.7	0.6-4.8	0.3	0.6	0.2-2.3	0.5
IIB/I	2.0	0.8-5.1	0.1	1.6	0.6-4.5	0.4
Lymphovascular invasion						
yes/no	0.8	0.4-1.5	0.5	1.4	0.6-3.4	0.4
Perineural invasion						
yes/no	1.6	0.8-3.2	0.2	5.5	1.6-18.9	0.007
Gender						
female/male	1.1	0.6-2.1	0.7	0.7	0.3-1.8	0.5
Age						
per 10-y increase	0.96	0.7-1.4	0.8	0.6	0.4-1.01	0.05
N stage						
N1/N0, Nx	1.5	0.8-2.8	0.2	2.1	0.9-4.8	0.1
Differentiation						
poor/moderate	1.7	0.8-3.5	0.2	1.5	0.5-4.3	0.4
Surgical margin						
positive/negative	1.5	0.7-2.9	0.3	1.2	0.4-3.2	0.8

metabolism of gemcitabine [7], the mainstay chemotherapeutic agent of pancreatic adenocarcinoma [5]. RRM1, the ribonucleotide reductase large subunit for substrate and allosteric regulator bindings [8] has been established as the prognostic and predictive factor of survival in patients with various types of cancers including pancreatic adenocarcinoma treated with gemcitabine [12]. RRM2, the ribonucleotide reductase small subunit, was believed to be biochemically unrelated to the enzyme overall activity and substrate specificity as a protein radical provider [8]. However, in preclinical studies, it was associated with tumorigenesis, invasiveness and gemcitabine resistance [10, 11].

Currently, contradictory evidence of prognostic value and predictive value of RRM2 exists. Gio-

Table 5. Multivariable Analysis for Overall Survival and Progression-free Survival in Patients Treated with Adjuvant Gemcitabine

	OS			PFS		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
Number of positive lymph nodes						
per 1 node increase	1.2	1.1-1.4	0.005	NA	NA	NA
Perineural invasion						
yes/no	NA	NA	NA	5.5	1.6-18.9	0.007

NA: not applicable

vannetti and coworkers [13] stratified 67 patients with pancreatic adenocarcinoma treated with gemcitabine into two or three equally divided groups based on RRM2 mRNA level. No significant correlations between RRM2 expression and survival were found. However, this conclusion was challenged by a Japanese study of 31 patients. Itoi and coworkers [17] reported that RRM2 mRNA level of fine needle aspiration biopsy specimen was a key predictive marker of survival and response in gemcitabine-treated patients. This was further supported by the Fujita study also from Japan [14], where 70 patients with early stage pancreatic adenocarcinoma were included and 40 patients were treated with gemcitabine. Recursive partitioning analysis was used to stratify patients based on the RRM2 mRNA level in their tumors. Decreased RRM2 mRNA level was associated with increased OS and PFS in both the entire cohort and the subpopulation who received gemcitabine.

The disease stage of the patients in our study was similar to those in the Fujita study [14]. However, RRM2 protein expression determined by immunohistochemistry appeared to be neither prognostic nor predictive in both the entire cohort of 117 patients with resectable pancreatic adenocarcinoma and 44 patients subsequently treated with adjuvant gemcitabine after tumor resection. Our results were more consistent with the conclusions of the Giovannetti study [13].

Several possible explanations were proposed for the apparent disagreements. First, different quantification methods were used to examine RRM2 expression. All the previous studies used QRT-PCR for RRM2 mRNA quantification with only one reference gene either β-actin or glyceraldehyde-3-phosphate dehydrogenase [13, 14, 17]. However, further analyses with Norm-Finder (v19) and geNorm (v3.5) packages demonstrated that β-actin was not as stable as the two reference genes (RPL13A and HMBS) we used in pancreatic cancer (unpublished data). In addition, compared to immunohistochemistry, RNA-based quantification methods often suffer from the availability reduction of target mRNA to the cDNA probe due to cross-linking proteins and RNA caused by the fixation process. Thereby we chose to measure RRM2 protein expression with pancreatic-specific tissue array and immunohistochemistry using an anti-RRM2 antibody, which is known to work reliably on both Hollande's and formalin-fixed paraffin-embedded specimens. Second, these studies involved patients with different stages of pancreatic adenocarcinoma. A significant proportion of patients in the Giovannetti [13] and the Itoi studies [17] contained unresectable disease. Most of the patients studied in the Fujita study [14] had stage II disease although patients with stage III and IV disease were also included. As demonstrated in clinical practice, patients with resectable or unresectable pancreatic adenocarcinoma have drastically distinct response to therapy as well as prognosis [2]. Thereby, it is reasonable to suspect different RRM2 protein expression and its roles in the different stages of pancreatic adenocarcinoma. In this study, we exclusively evaluated patients with stage I and II disease that were all resectable to eliminate this possible issue that was probably encountered in previously reported studies. Third, different methods were used to choose the cut-off value for RRM2 stratification [13, 14]. The cut-off values from methods such as RPA and dichotomization based on median are dependent on the patient population included in a specific study. The dichotomization method based on the presence or absence of chemoluminescence in our study was independent to patient population and can be readily translated into clinical practice. Last, genotypic variations between Japanese population [14, 17] and Caucasian patients in the Gio-

vannetti [13] and our studies may also need to be considered.

Our study has several limitations. First, as a retrospective study, complete demographic and histopathological data, therapeutic details, and even outcome data were not available for all patients. This information was collected in a time span of 8 years. Thus it is unlikely to completely eliminate all potential selection bias and confounders. Second, interobserver variability is an intrinsic problem associated with the interpretation of the signal intensity in immunostaining. In the current study, central review process and dichotomization simply based on the presence or absence of RRM2 protein expression greatly reduced the interobserver discrepancy to a lesser extent.

This study demonstrated that RRM2 protein expression level determined by immunohistochemistry on paraffin-embedded pancreatic adenocarcinoma tissue is not prognostic of survival in the entire cohort of patients with resectable pancreatic adenocarcinoma. In addition, RRM2 protein expression level does not predict the treatment benefit of adjuvant gemcitabine in patients with resectable pancreatic adenocarcinoma.

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