

# Ribonucleotide reductase subunit M1 assessed by quantitative double-fluorescence immunohistochemistry predicts the efficacy of gemcitabine in biliary tract carcinoma

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**Abstract.** Gemcitabine is a commonly used chemotherapeutic agent for advanced biliary tract carcinoma (BTC), although its efficacy is insufficient. Therefore, it is essential to establish new diagnostic methods, which can predict responders before the treatment. The aim of this study is to identify the most reliable chemoresistance marker to gemcitabine in BTC among the 4 molecules (hENT1, dCK, RRM1 and RRM2) involved in gemcitabine metabolism. The expression of 4 molecules were investigated in 5 BTC cell lines, and correlated with gemcitabine sensitivity. RRM1 protein was also assessed by quantitative double-fluorescence immunohistochemistry (qDFIHC) in 10 patients with unresectable or recurrent BTC who received gemcitabine-based chemotherapy. RRM1 and RRM2 protein strongly correlated with the IC<sub>50</sub> value for gemcitabine in BTC cell lines (R=0.935, 0.771, respectively). In addition, patients with low RRM1 were significantly more sensitive to gemcitabine (p=0.033), and their survival was significantly better than patients with high RRM1 (p=0.001). In conclusion, RRM1 particularly in protein level is a reliable marker for gemcitabine resistance in BTC. Furthermore, qDFIHC is a useful method for the assessment of RRM1 protein, in order to design a tailor-made chemotherapeutic regimen for BTC patients.

## Introduction

Biliary tract carcinoma (BTC), including gallbladder carcinoma and intra- and extra-hepatic bile duct carcinoma, is relatively

rare, but the incidence of BTC has increased markedly in Japan over the past several decades (1). Although a complete surgical resection is the only curative modality, most patients are not eligible for surgery because of the advanced stage of disease at diagnosis. Moreover, even patients who underwent a surgical resection, eventually have a recurrence of the disease (2-4). The outcome of patients with unresectable or recurrent BTC is dismal, and their median survival is usually <1 year. Therefore, most patients with unresectable or recurrent BTC are possible candidates for palliative chemotherapy. To date, only 1 randomized trial has demonstrated that combination chemotherapy may improve the survival and quality of life in patients with advanced BTC in comparison to the best supportive care (5). However, no standard chemotherapy regimen for advanced BTC has been established. Several phase II trials with new chemotherapeutic agents, such as gemcitabine (6-10), capecitabine (11), oxaliplatin (12), or S-1 (13,14) have demonstrated tumor response in ~15-35% of patients treated with single agents, and in 20-40% of patients treated with their combinations (15-24). Among them, gemcitabine has shown promising activity against advanced BTC, and has been commonly used for patients with unresectable or recurrent BTC in Japan.

Gemcitabine (2'-difluorodeoxycytidine, dFdC) is a specific analogue of deoxycytidine. It is a novel anticancer agent that has significant efficacy in several solid tumors, including BTC (25-29). Gemcitabine is transported into the cell mostly by human equilibrative nucleoside transporter-1 (hENT1) (30), phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form (dFdCMP) (31), and then is further phosphorylated to the active triphosphate form. The subsequent incorporation of gemcitabine triphosphate (dFdCTP) into DNA, which causes the addition of a single deoxynucleotide and chain termination, is a major mechanism underlying the cytotoxicity of gemcitabine (32). In addition, the diphosphate metabolite of gemcitabine (dFdCDP) is also reported to inhibit ribonucleotide reductase (RR), thereby causing a decrease in the normal deoxycytidine triphosphate (dCTP) pools, a decreased negative feedback of dCK, and the enhanced phosphorylation of gemcitabine (33-35).

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**Key words:** gemcitabine, chemoresistance, ribonucleotide reductase subunit M1, quantitative double-fluorescence immunohistochemistry, biliary tract carcinoma

Table I. Primer sequence and the fragment size of hENT1, dCK, RRM1 and RRM2.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size (bp)
hENT1	GCTGCTGTGCAACATTAAGC	AAAGGCAGCCATGAAGAAGA	87
dCK	TTCCTGAACCTGTTGCCAGAT	GAGACATTGTAAGTTCCTCAAATTCATC	72
RRM1	CCTGGGGCATTGCTATTTA	GCTGCTCTTCCTTTCCTGTG	93
RRM2	ATGAAAACCTTGGTGGAGCGATT	TGGCAATTGGAAGCCATAGA	78
$\beta$ -actin	CGAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	60

hENT1 is the major transporter of gemcitabine. Cells lacking hENT1 are highly resistant to gemcitabine (36,37), and pancreas cancer patients with hENT1-positive tumor tissue have significantly longer survival after gemcitabine chemotherapy than patients without detectable hENT1 (38). dCK plays a key role in the activation of gemcitabine, and its activity correlates with the drug sensitivity (39-41). Deficiency in the dCK activity has been considered to be one of the main mechanisms for the development of resistance to gemcitabine (42). Another factor in gemcitabine resistance is the over-expression of RR. RR is a holoenzyme consisting of dimerized RR subunit 1 and 2 (RRM1, RRM2) which is essential for DNA synthesis (43). Increased RRM1 expression and activity has been shown to be a marker for gemcitabine resistance *in vitro* (44,45).

Thus, gemcitabine has a complex pathway of metabolism, and there are many factors that can contribute to either gemcitabine cytotoxicity or chemoresistance. A gene expression analysis may therefore help the management of cancer patients treating with gemcitabine, thus allowing the selection of subjects responding to the treatment. The present study assessed the expression pattern of 4 key molecules (hENT1, dCK, RRM1 and RRM2) involved in gemcitabine metabolism in BTC, and identify the most useful chemoresistance marker to gemcitabine among the 4 key molecules. We also quantitatively analyzed a gemcitabine resistance marker in clinical samples using quantitative double-fluorescence immunohistochemistry (qDFIHC), which was originally established in our laboratory (46).

## Materials and methods

**Cell lines and culture.** Three human gall bladder carcinoma cell lines (GB-d1, GBK-1 and KMG-C) and two human bile duct carcinoma cell lines (TFK-1 and HBDC) were used. GB-d1 was provided from Dr T. Date (Kyushu University, Fukuoka, Japan). GBK-1 was provided from Dr H. Egami (Kumamoto University, Kumamoto, Japan). KMG-C was provided from Dr H. Yano (Kurume, Japan). TFK-1 was from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). HBDC was established in our laboratory (47). The cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100  $\mu$ g/ml kanamycin (Meiji, Tokyo, Japan) and incubated at 37°C in a humidified atmosphere containing 20% O<sub>2</sub> and 5% CO<sub>2</sub> in air.

**Clinical samples.** Ten tissue samples were obtained from unresectable or recurrent BTC patients who underwent gemcitabine-based chemotherapy at the Department of Surgery, Saga University Hospital (Saga, Japan) from April 2005 to March 2008. Patients with pathologically confirmed unresectable, metastatic or recurrent BTC, and who had at least 1 measurable lesion (>2 cm in longer axis) according to the Response Evaluation Criteria in Solid Tumors (RECIST, National Cancer Institute, Cancer Therapy Evaluation Program), were considered eligible. Informed consent for the use of these specimens was obtained from all patients, and the study protocol was approved by the Ethics Committee of Saga University, Faculty of Medicine.

**Total RNA extraction and quantitative reverse transcription-polymerase chain reaction.** Total RNA was extracted from each cell line using an Isogen RNA extraction kit (Nippon Gene, Osaka, Japan) according to the manufacturer's instructions. For each cell line, 1  $\mu$ g of RNA was converted into cDNA using a ReverTra Ace (Toyobo, Osaka, Japan) reverse transcription reaction kit. The cDNA was used as a template for polymerase chain reaction (PCR). Quantitative reverse transcription (RT)-PCR was performed on a Light-Cycler<sup>TM</sup> instrument system (Roche, Mannheim, Germany) using the Light-Cycler-FastStart DNA Master<sup>TM</sup> SYBR Green I kit (Roche) according to the manufacturer's instructions. The primer sequences and the fragment size of each gene are shown in Table I. After a denaturing step at 95°C for 3 min, PCR amplification was performed with 50 cycles of 15 sec denaturing at 95°C, 5 sec annealing at 60°C and 10 sec extension at 72°C. We used a melting curve analysis to control for the specificity of the amplification products. The quantitative value was normalized by the  $\beta$ -actin expression which was used as an internal control. These experiments were all carried out in triplicate and the mean values were calculated.

**Chemotherapeutic agent.** Gemcitabine was provided from Eli Lilly and Co. (Indianapolis, IN, USA). It was dissolved in RPMI-1640 medium supplemented with 1% FBS and 100  $\mu$ g/ml kanamycin, and diluted to the required concentrations immediately before use.

**Drug sensitivity test.** The cytotoxic effect of gemcitabine treatment in BTC cell lines was assessed by MTT assay using a CellTiter 96<sup>TM</sup> non-radioactive cell proliferation assay kit

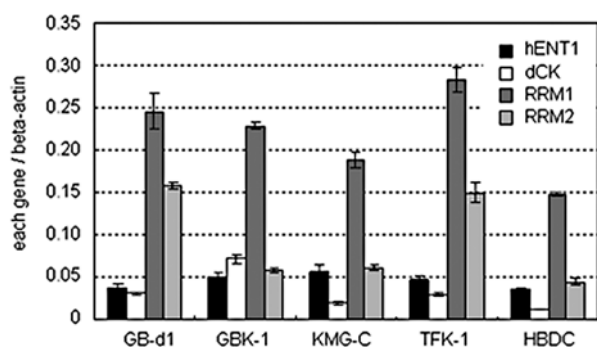


Figure 1. Expression levels of hENT1, dCK, RRM1 and RRM2 in 5 biliary tract carcinoma cell lines. The expression level of RRM1 was the highest among the 4 genes in all 5 cell lines. The quantitative value was normalized by  $\beta$ -actin expression used as an internal control. The data represent the mean  $\pm$  SD of three independent experiments.

(Promega, Madison, WI, USA). In brief,  $8 \times 10^3$  cells per well were seeded in triplicate onto 96-well plates and cultured for 24 h. The cells were exposed to an increasing concentration of gemcitabine, ranging from 0.1  $\mu$ M to 10 mM, for 72 h. The proliferation curves were then constructed by calculating the mean value of the optical density measurements at 590 nm using a 96-well plate reader (Immuno-mini NJ2300, Nalge Nunc International K.K., Tokyo, Japan).

**Western blot analysis.** Whole cell lysate from cultured cells was prepared using lysis buffer composed of 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% Triton X-100 and a protease inhibitor cocktail mix (Roche Diagnostics GmbH, Mannheim, Germany). The samples were dissolved in NuPage™ LDS sample buffer (Invitrogen, Carlsbad, CA, USA) and 1 M dithiothreitol (DTT), and heated for 5 min at 95°C. Aliquots containing 20  $\mu$ g of protein were subjected to NuPage 4-12% Bis-Tris Gel (Invitrogen) and electrophoretically transferred onto a Hybond™ nitrocellulose enhanced chemiluminescence membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) in transfer buffer. The membranes were blocked with 5% skim milk for 30 min, and incubated with primary antibodies for 2 h at room temperature. The primary antibodies used in the Western blot analysis were anti-RRM1 (1:500, Chemicon, Temecula, CA, USA), anti-RRM2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti- $\beta$ -actin (1:10000, Sigma). After incubation with the corresponding secondary antibodies, the signals were developed using an Amersham™ ECL Plus Western blotting detection system (GE healthcare, Buckinghamshire, UK). The digital images were acquired on a Lunimo-Image analyzer LAS3000 (Fujifilm, Tokyo, Japan), and the quantitative value of the signal intensity was calculated using a computer software Multi Gauge V3.1 (Fujifilm), and then it was normalized by the  $\beta$ -actin expression which was used as an internal control.

**Quantitative double-fluorescence immunohistochemistry (qDFIHC).** qDFIHC for RRM1 was performed according to the procedures described in previous studies with slight modifications (46,48). Briefly, paraffin-embedded samples were cut into 4- $\mu$ m sections, and these tissue sections were

deparaffinized in xylene and rehydrated in a graded series of ethanols. For antigen retrieval, the tissue sections were treated by microwave boiling in 1 mM EDTA (pH 8.0) for 10 min. The slides were then incubated with 5% skim milk for 10 min to block any nonspecific binding of the immunoreagents.

Next, the primary anti-RRM1 antibody (1:200, Chemicon) was placed onto the slides, and the slides were incubated at room temperature for 2 h. After washing in phosphate-buffered saline (PBS), the slides were incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibody (anti-mouse IgG, 1:100, Invitrogen) at room temperature for 30 min. The slides were then washed and incubated with the primary prediluted anti- $\beta$ -actin antibody (Gene Tex, San Antonio, TX, USA) at room temperature for 2 h. After washing, the slides were incubated with a Cy3 conjugated secondary antibody (anti-rabbit IgG, 1:200, Chemicon) for 30 min. After washing, the slides were mounted with mounting medium (FluoroGurd™, Bio-Rad Laboratories, Hercules, CA, USA) and then were subjected to Laser scanning microscopy. The digital images of qDFIHC were acquired on a confocal laser scanning microscope LSM5 Pascal (Carl Zeiss Microimaging, Jena, Germany), in which Cy3 (red color for  $\beta$ -actin) was acquired on channel 1, while FITC (green color for RRM1) was acquired on channel 2.

All of the digital images were acquired at  $\times 200$  magnification, where cancer foci avoiding non-cancerous cells could best be identified. Next, the digital images were analyzed using a computer software LSM image examiner. In each case, the ratio of the total fluorescence intensity of RRM1 (FITC) to  $\beta$ -actin (Cy3) was calculated, and the mean value of 3 fields was considered to be the quantification value.

**Statistical analysis.** The correlation between  $IC_{50}$  value for gemcitabine and the expression level of each gene or protein in the 5 BTC cell lines was assessed statistically by Pearson's correlation. Differences in the mean values were evaluated by the Student's t-test, and differences in the frequencies were analyzed with Fisher's exact test. The survival curve was calculated by the Kaplan-Meier method and compared by using the log-rank test.  $P < 0.05$  were considered to be statistically significant.

## Results

**mRNA expression of hENT1, dCK, RRM1 and RRM2.** Four genes (hENT1, dCK, RRM1 and RRM2) were selected as candidates for gemcitabine sensitive/resistance marker based on the metabolic pathway of gemcitabine. The expression level of each gene in 5 BTC cell lines was assessed by quantitative RT-PCR, and the result is shown in Fig. 1. In all cell lines, the expression level of RRM1 was the highest among the 4 genes, whereas the expression of hENT1 and dCK was at a low level in comparison to RR.

**Drug sensitivity to gemcitabine in the 5 biliary tract carcinoma cell lines.** The drug sensitivity to gemcitabine was assessed by MTT assay. The proliferation curves of 5 cell lines are shown in Fig. 2A. GB-d1 was the most sensitive to gemcitabine; in contrast, TFK-1 was the most resistant to gemcitabine. The  $IC_{50}$  value for gemcitabine in the 5 cell

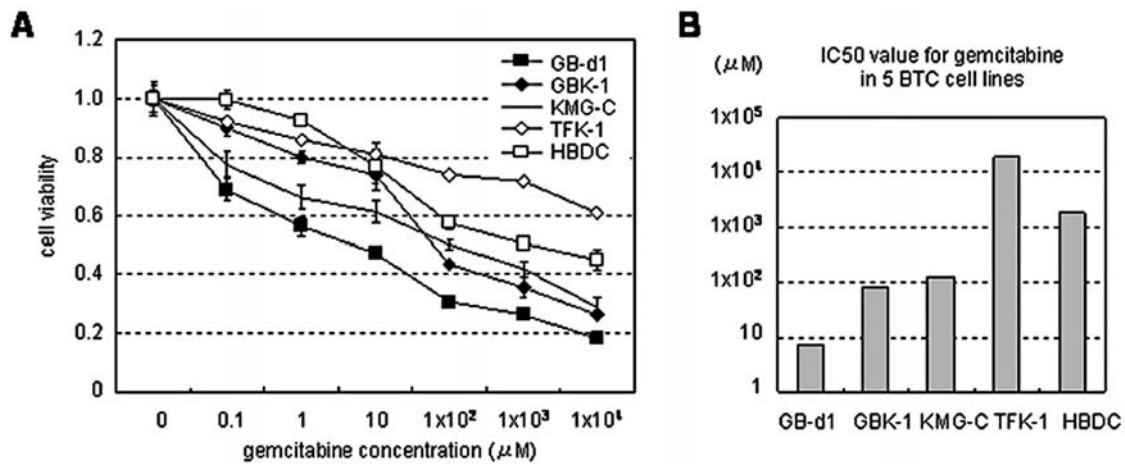


Figure 2. (A) The proliferation curves of 5 cell lines. A sample containing  $8 \times 10^3$  cells per well were seeded in triplicate onto 96-well plates. All cell lines were treated with 0.1  $\mu$ M–10 mM of gemcitabine for 72 h. The cell viability value was expressed relative to that of cells without treatment, and the data represent the mean  $\pm$  SD. (B) IC<sub>50</sub> value for gemcitabine in the 5 cell lines calculated from proliferation curves. The IC<sub>50</sub> value in TFK-1 was  $\sim$ 2,600-fold higher than in GB-d1 ( $1.91 \times 10^4$   $\mu$ M, 7.25  $\mu$ M, respectively).

Table II. Correlation between IC<sub>50</sub> and each four gene or their combination.

Gene or combination	R-value	R <sup>2</sup>	P-value
1/hENT1	-0.091	0.008	0.884
1/dCK	-0.114	0.013	0.885
RRM1	0.627	0.393	0.257
RRM2	0.524	0.275	0.365
1/(hENT1 x dCK)	-0.125	0.016	0.842
RRM1 x RRM2	0.621	0.386	0.264
(RRM1 x RRM2)/(hENT1 x dCK)	0.511	0.261	0.379

Table III. Correlation between IC<sub>50</sub> and RR protein.

Protein	R-value	R <sup>2</sup>	P-value
RRM1	0.935	0.874	0.020
RRM2	0.771	0.594	0.127

lines was calculated based on the result of the MTT assay, and the results are shown in Fig. 2B. The IC<sub>50</sub> value in TFK-1 was  $\sim$ 2,600-fold higher than in GB-d1.

*Correlation between the expression level of each gene and sensitivity to gemcitabine.* The correlation between the mRNA expression of each gene and IC<sub>50</sub> value for gemcitabine in the 5 cell lines is shown in Table II. The result shows a tendency for a positive correlation between the expression of RRM1 and the IC<sub>50</sub> value ( $R=0.627$ ), although this correlation was not statistically significant ( $p=0.257$ ). The expression of RRM2 also weakly correlated with the IC<sub>50</sub> ( $R=0.520$ ). However, the expression of hENT1 and dCK did not correlate with the gemcitabine sensitivity ( $R= -0.091, -0.114$ ,

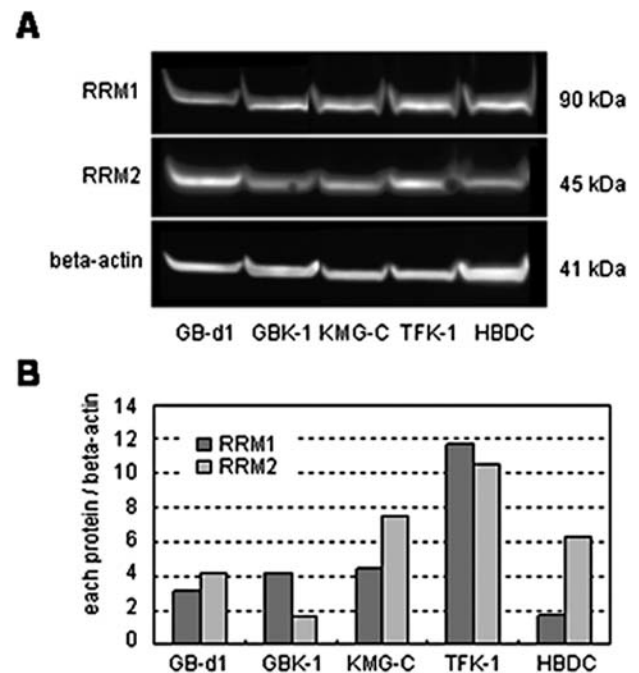


Figure 3. (A) A Western blot analysis of RRM1 and RRM2. Protein (20  $\mu$ g) per sample was loaded, and equal loading was confirmed by using  $\beta$ -actin as a control. The digital images were acquired on a Lunimo-Image analyzer LAS3000 to quantify the signal intensity. (B) Quantitative values of RRM1 and RRM2 protein adjusted by  $\beta$ -actin. The quantitative value was calculated using the Multi Gauge V3.1 computer software package.

respectively). Furthermore, the R-values of various combinations of 4 genes were not superior to those of a single RRM1 gene.

*Western blot analysis for RRM1 and RRM2.* Fig. 3A shows the expression of RRM1 and RRM2 protein in 5 BTC cell lines. The quantitative values of RRM1 and RRM2 protein adjusted by  $\beta$ -actin were calculated using digital images acquired on a LAS3000 (Fig. 3B). The correlation between the protein level of RR and the IC<sub>50</sub> value for gemcitabine in



Table IV. Clinicopathological features and the value of RRM1 quantified by qDFIHC.

Case	Age	Gender	Disease	Advanced/ recurrence	Histology	Regimen	Radiation	Response	Outcome	RRM1 (qDFIHC)	Survival (month)
1	51	Male	EBDC	Adv	Mod	GEM	+	SD	Dead	0.88	34.9
2	55	Male	BDC	Adv	Well	GEM+FT	+	PR	Dead	0.96	34.8
3	78	Female	IBDC	Adv	-	GEM+FT	-	SD	Dead	0.98	33.6
4	76	Male	IBDC	Rec	Well	GEM	-	SD	Dead	0.93	20.9
5	69	Female	EBDC	Adv	-	GEM	+	SD	Dead	0.93	20.2
6	76	Male	EBDC	Adv	Well	GEM	+	PR	Dead	0.98	18.2
7	77	Male	EBDC	Adv	-	GEM+S-1	+	SD	Dead	1.07	15.9
8	46	Female	EBDC	Rec	Well	S-1→GEM	-	PD	Dead	1.13	13.0
9	74	Female	GBC	Rec	Well	GEM+S-1	-	PD	Dead	0.99	9.3
10	66	Male	EBDC	Adv	Mod	GEM+FT	-	PD	Dead	0.99	5.5

IBDC, intra-hepatic bile duct carcinoma; Adv, advanced; FT, tegafur; PD, progressive disease; EBDC, extra-hepatic bile duct carcinoma; Rec, recurrence; PR, partial response; GBC, gall bladder carcinoma; GEM, gemcitabine; SD, stable disease.

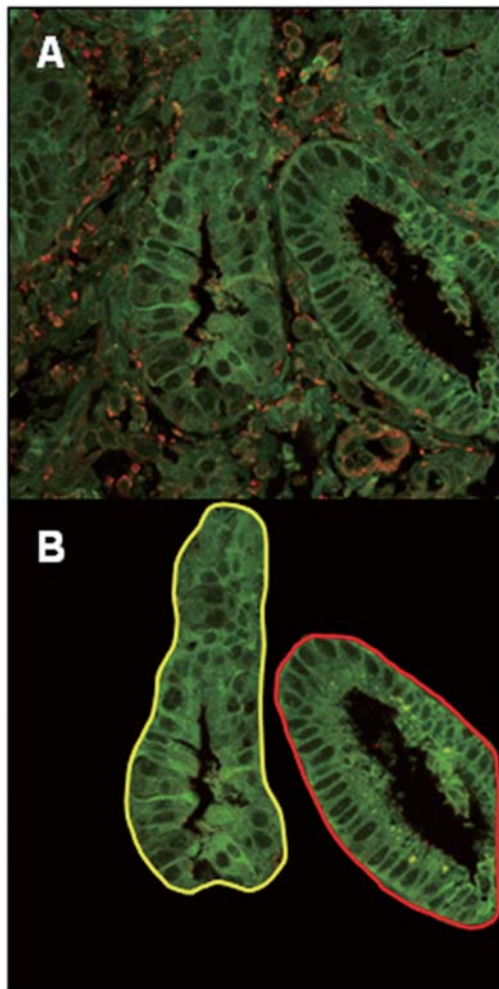


Figure 4. (A) Quantitative double-fluorescence immunohistochemistry for RRM1. The total fluorescence intensity of FITC and Cy3 was acquired on LSM5 Pascal to quantitatively evaluate the RRM1 expression in the field. The ratio of FITC for RRM1/Cy3 for  $\beta$ -actin was calculated and regarded as the relative RRM1 expression in each field, and then the mean value of 3 fields was considered to be the quantification value in each case. (B) RRM1 expression in cancer foci. The expression of RRM1 in stromal tissue was excluded like a microdissection in order to evaluate the cancer-specific expression of RRM1.

the 5 cell lines is shown in Table III. The results show a significantly strong correlation between RRM1 protein and the  $IC_{50}$  value for gemcitabine ( $R=0.935$ ,  $R^2=0.874$ ,  $p=0.020$ ). The expression of RRM2 protein also correlated with the  $IC_{50}$  value, although the correlation was weaker than for the expression of RRM1 and not statistically significant ( $R=0.771$ ,  $R^2=0.594$ ,  $p=0.127$ ).

**Quantification of RRM1 by qDFIHC in clinical samples.** The expression level of RRM1 protein in BTC patients was assessed by quantitative double-fluorescence immunohistochemistry (qDFIHC). We performed qDFIHC staining by FITC (green color) for RRM1 and by Cy3 (red color) for  $\beta$ -actin (Fig. 4A). As demonstrated in Fig. 4B, the expression of RRM1 protein in stromal tissue was excluded like a microdissection in order to evaluate the cancer-specific expression of RRM1.

**Correlation between clinical response to gemcitabine and expression of RRM1.** The qDFIHC was used to quantitatively determine the expression of RRM1 in 10 BTC patients. Table IV is a summary of patient characteristics and the values of RRM1 quantified by qDFIHC. All patients received gemcitabine-based chemotherapy with or without radiotherapy. The 10 patients, 6 males and 4 females, were aged from 46 to 78 years (mean:  $66.8 \pm 11.9$  years). Of the 10 patients, 3 were diagnosed with intrahepatic bile duct carcinoma, 6 with extra-hepatic bile duct carcinoma, and 1 patient had gall bladder carcinoma. The median follow-up period was 19.2 months (range: 5.5-34.9 months). All patients died from cancer during the follow-up period. The clinical response to chemotherapy was evaluated according to the RECIST criteria and classified into PR (partial response), SD (stable disease), and PD (progressive disease). Furthermore, the patients were divided into 2 groups as follows: disease control group (PR and SD patients) and non-control group (PD patients). Table V shows the relationship between patient characteristics and the response to gemcitabine. The expression level of RRM1 was divided into high and

Table V. Comparison between the effect of gemcitabine and clinicopathological features.

Factor	Control group <sup>a</sup> (n=7)	Non-control group <sup>b</sup> (n=3)	P-value
Age	68.9±11.3	62.0±14.4	NS
Gender			
Male	5	1	NS
Female	2	2	
Histology			
Well	3	2	NS
Mod	1	1	
Radiation			
+	5	0	NS
-	2	3	
RRM1			
High	1	3	0.033 <sup>c</sup>
(qDFIHC)			
Low	6	0	

<sup>a</sup>Control group, PR and SD patients; <sup>b</sup>Non-control group, PD patients; <sup>c</sup>Fisher's exact probability test.

low, with the mean value of all cases ( $0.984 \pm 0.072$ ) as a cut-off value. Although no significant differences were observed regarding age, gender, histology, and radiotherapy, the patients with a low RRM1 expression were significantly more sensitive to gemcitabine than those with a high RRM1 expression ( $p=0.033$ ).

**Patient survival according to the expression of RRM1.** The relationship between the patient survival and the expression level of RRM1 was statistically analyzed by Kaplan-Meier method. The survival of patients with a low RRM1 expression ( $n=6$ ) was significantly better than that of the patients with a high RRM1 expression ( $n=4$ ) ( $P=0.001$ ) (Fig. 5).

## Discussion

Biliary tract carcinoma (BTC) is one of the most aggressive human malignancies, in which only surgical resection offers the opportunity for cure (2-4). Unresectable and recurrent BTC patients have a very poor prognosis, and these patients will receive palliative chemotherapy. Gemcitabine has been commonly used as a chemotherapeutic agent for advanced BTC. However, the efficacy of this drug for advanced BTC is insufficient with a response rate of ~30% (6-10). Therefore, new strategies, such as combination with other drugs or a molecular approach, which allows the selection of responders, are necessary, in order to improve patient outcome.

Several molecules including ABCC5 (49), c-Src (50), BNIP3 (51) and focal adhesion kinase (FAS) (52) have been reported to be a gemcitabine sensitive/resistance marker.

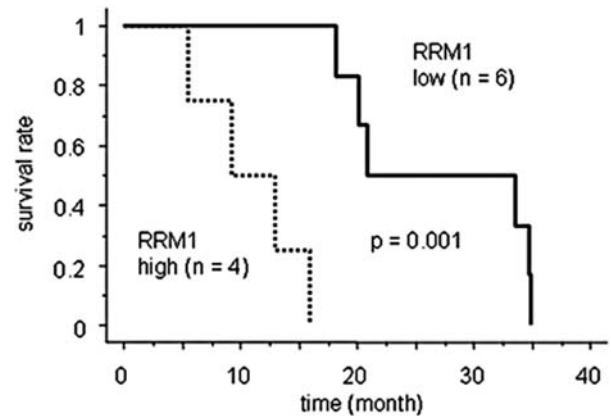


Figure 5. Kaplan-Meier estimates of the patients' survival according to the expression of RRM1. Patients with a low RRM1 expression showed a significantly longer survival than the patients with a high RRM1 expression.

Other *in vitro* studies have demonstrated that the expression of 4 molecules (hENT1, dCK, RRM1 and RRM2), which are involved in gemcitabine metabolism, can determine the efficacy of gemcitabine (36,37,42,44,45). Furthermore, two clinical studies have demonstrated that the assessment of hENT1 and RRM1 is useful in order to predict the response to gemcitabine in pancreas and lung cancer, respectively (38,53). Our previous study has also indicated the importance of RRM1 as a possible chemoresistance marker for gemcitabine in BTC (48). However, despite the numerous investigations to elucidate the mechanism of gemcitabine efficacy, no reliable marker has yet been identified. Therefore, it is necessary to establish new diagnostic methods, which can accurately predict the response to gemcitabine, in order to perform tailored chemotherapy for BTC patients.

The present study initially investigated the expression of 4 candidate genes for gemcitabine sensitive/resistance markers in 5 BTC cell lines. The mRNA level of RRM1 correlated with the  $IC_{50}$  value for gemcitabine ( $R=0.627$ ) and RRM2 also showed a slight correlation with the  $IC_{50}$  value ( $R=0.520$ ) (Table II). Furthermore, both RRM1 and RRM2 protein were more strongly correlated with gemcitabine resistance than the mRNA levels, although the relationship between RRM2 protein and the  $IC_{50}$  value for gemcitabine was not statistically significant (Table III). In contrast, neither hENT1 nor dCK were correlated with gemcitabine sensitivity. The expression of hENT1 and dCK in these cell lines might be too low to determine the response to gemcitabine. dCK expression is usually down-regulated by increased RRM1 expression via negative feedback pathways. In addition, several transporters other than hENT1, such as hENT2, hCNT1 and hCNT3, also regulate the uptake of gemcitabine (30). García-Manteiga *et al* demonstrated that hCNT1 determines the sensitivity to gemcitabine in pancreatic cancer cells which overexpress hENT1 (54).

Another study demonstrated that the expression of hENT1, dCK, RRM1 and RRM2 is not correlated with gemcitabine sensitivity, although the ratio of the expression level of the 4 genes correlates with gemcitabine resistance in pancreatic cancer cells (55). It might be difficult to determine the sensitivity to gemcitabine with a single factor, since many

factors contribute to the regulation of gemcitabine efficacy. This study also assessed the various combinations of these 4 genes in order to investigate the useful predictive index of gemcitabine. However, the single expression of RRM1 showed the strongest correlation with the IC<sub>50</sub> value for gemcitabine (Table II). RRM1 particularly in protein level is, therefore, considered to be the most reliable marker for gemcitabine resistance in BTC cell lines.

The expression of RRM1 protein was also assessed by qDFIHC in 10 patients with unresectable or recurrent BTC, as a pilot study, in order to validate the role of RRM1 in resistance to gemcitabine. This method, reported by us in 2007 (46), is a novel technique, which allows the quantification of protein even in tiny formalin-fixed paraffin embedded samples such as biopsy specimens. Moreover, using this method, a target protein can be easily quantified with avoiding non-cancerous tissues by surrounding cancer foci on a digital image such as a microdissection (Fig. 4B). The reliability of the qDFIHC method was initially evaluated by comparison with Western blot analysis. The expression of RRM1 protein quantified by qDFIHC is almost the same as that of Western blot analysis in 5 BTC cell lines (R=0.868, P=0.035, data not shown). The result obtained from this method is, therefore, considered to be available as a quantitative value of RRM1 protein. The result of qDFIHC demonstrated that patients with low RRM1 were significantly more sensitive to gemcitabine than those with high RRM1 (Table V). In addition, the survival of patients with low RRM1 was significantly longer than those with high RRM1 (Fig. 5). A study using a larger number of patients will be necessary to increase the reliability of our data since only 10 patients were enrolled in the present study.

It is usually difficult to obtain large clinical samples, which is needed for a conventional protein expression assay, from unresectable or recurrent BTC patients. In contrast, qDFIHC requires only a small amount of cancer tissue. Therefore, biopsy samples, obtained by the endoscopic approach or the exploratory laparoscopy, are sufficient. Thus, the assessment of RRM1 in cancer tissue by qDFIHC might be useful for the selection of patients who will benefit from gemcitabine-based chemotherapy.

In conclusion, among the several molecules involved in the gemcitabine metabolism, RRM1 particularly the level of protein expression is the most reliable marker for gemcitabine resistance in BTC cell lines. Furthermore, qDFIHC, which allows the quantification of cancer-specific expression of a target protein even in the biopsy specimens, is useful for the assessment of RRM1 protein in order to design a tailor-made chemotherapeutic regimen for BTC patients.

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