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High Expression of Ribonucleotide Reductase Subunit M2 Correlates with Poor Prognosis of Hepatocellular Carcinoma

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See editorial on page 580.

Background/Aims: Ribonucleotide reductase subunit M2 (RRM2) catalyzes the production of deoxynucleotide triphosphates, which are necessary for DNA synthesis. RRM2 has been reported to play an active role in tumor progression, and elevated RRM2 levels have been correlated with poor prognosis for colorectal cancer patients. This study aimed to elucidate the prognostic significance of RRM2 protein expression in hepatocellular carcinoma after surgery. **Methods:** RRM2 protein expression was evaluated using immunohistochemistry in tumor tissues from 259 hepatocellular carcinoma patients who underwent curative hepatectomy. **Results:** High RRM2 expression was observed in 210 of 259 patients (81.1%) with hepatocellular carcinomas. High RRM2 expression was significantly associated with viral etiology (p=0.035) and liver cirrhosis (p=0.036). High RRM2 expression was correlated with early recurrence (p=0.004) but not with late recurrence (p=0.144). Logistic regression analysis revealed that high RRM2 expression (p=0.040) and intrahepatic metastasis (p<0.001) were independent predictors of early recurrence. High RRM2 expression unfavorably influenced both shorter recurrence-free survival (p=0.011) and shorter disease-specific survival (p=0.002) and was an independent predictor of shorter disease-specific survival (p=0.008). **Conclusions:** High RRM2 protein expression might be a useful marker for predicting early recurrence and may be a marker for poor prognosis of hepatocellular carcinoma after curative hepatectomy. **(Gut Liver 2014;8:662-668)**

Key Words: Ribonucleotide reductase subunit M2; Hepatocellular carcinoma; Survival

INTRODUCTION

The prognosis of hepatocellular carcinoma (HCC) remains grave as a result of a high rate of tumor recurrence even after surgical resection.[1-3] Predicting clinical outcomes for HCC patients is a challenge for clinicians. Although there are reports on histologic parameters for predicting HCC prognosis, molecular markers for HCC prognosis could provide additional information.[4] Cancer classification using prognostic markers can identify patients with a high risk of recurrence or poor prognosis.[5,6]

Ribonucleotide reductase subunit M2 (RRM2) catalyzes the production of deoxynucleotide triphosphates, which are necessary for DNA synthesis.[7] Inhibition of ribonucleotide reductase stops DNA synthesis and cell proliferation, and RRM2 has been considered a promising target for cancer therapy.[8] Suppression of RRM2 synthesis inhibited the growth of gastric cancer cell lines *in vitro*.[9] RRM2 depletion significantly reduced antiapoptotic protein Bcl-2 expression and RRM2 suppression led to increased Bcl-2 degradation.[10] RRM2 was reported to play an active role in tumor progression[11] and elevated RRM2 levels were correlated with poor prognosis for patients with colorectal cancer, pancreas adenocarcinoma, lung adenocarcinoma, and ovarian cancer.[12-15] RRM2 was overexpressed in human HCC.[16,17] Small-interfering RNA-mediated knockdown of RRM2 inhibited the proliferation of HCC cells and the growth of HCC xenografts transplanted into immunodeficient mice.[16] Recent study showed that positive immunoreactivity for RRM2 was found in 84% (16 of 19) of the HCCs.[16] However, the prognostic significance of RRM2 protein expression in HCC remains uncertain.

In the present study, we evaluated RRM2 protein expression by immunohistochemistry in order to identify a marker associated with tumor recurrence and to elucidate the prognostic significance of RRM2 in 259 HCC patients with long-term follow-up.

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MATERIALS AND METHODS

1. Study subjects

Primary HCC tissues were collected from 259 patients who were treated with curative hepatectomy at the Samsung Medical Center, Seoul, Korea between July 2000 and May 2006. We defined curative resection as the complete resection of all tumor nodules with clear microscopic resection margins and no residual tumors as indicated by a computed tomography scan 1 month after surgery. None of the patients had received preoperative chemotherapy. This study was approved by the Institutional Review Board of the Samsung Medical Center. Tumor differentiation was graded histologically using the Edmondson and Steiner criteria.[18] Microvascular invasion was considered present when at least one or more endothelial cells or the tunica media of the vessel surrounded a neoplastic cell group. Intrahepatic metastasis and multicentric occurrence were defined according to the criteria of the Liver Cancer Study Group of Japan.[19] Staging of the tumors was performed according to both the American Joint Committee on Cancer (AJCC)[20] and the Barcelona Clinic Liver Cancer (BCLC) staging classification.[21] Using 2 years as the cutoff, tumor recurrence was classified as either early recurrence or late recurrence.[22]

We followed up all patients by monitoring serum α-fetoprotein levels and three phase dynamic computed tomography scans every 3 months after surgery. Magnetic resonance imaging was used in order to confirm tumor recurrence in suspected case. The follow-up period for recurrence was at least 26 months and the median follow-up period was 118 months (range, 14.0 to 151.4 months) for survivors. Recurrence-free survival (RFS) was defined from the date of resection until the detection of both intrahepatic and extrahepatic recurrence. We chose disease-specific death (HCC-related death) as the clinical endpoint for survival analysis, defined as 1) tumor occupying more than 80% of the liver, 2) portal venous tumor thrombus proximal to the second bifurcation, 3) obstructive jaundice due to the tumor, 4) distant metastases, or 5) variceal hemorrhage with portal venous tumor thrombus proximal to the first bifurcation.[23] Disease-specific survival (DSS) was defined from the date of resection to the date of HCC-related death.

Histologic sections were examined by two pathologists and the representative tumor areas free from necrosis or hemorrhage were premarked in formalin-fixed paraffin-embedded blocks. Two 2.0-mm-diameter tissue cores were obtained from donor blocks and arranged in recipient paraffin blocks. Two cores of normal liver tissue from 12 patients with metastatic colonic carcinoma of the liver were included in each microarray block. Each tissue microarray block contained up to 60 tissue cores.

2. Immunohistochemical analysis

Immunohistochemistry for RRM2 was performed as described elsewhere.[24] Epitope retrieval was performed with 0.01 mol/L citrate buffer (pH 6.0) for 30 minutes in a pressure cooker. Sections were incubated with mouse monoclonal antibody to RRM2 (clone 1E1, 1:800; Abcam Corp., Cambridge, MA, USA) for 30 minutes at room temperature. In order to validate the concordance between the tissue microarrays and whole tumor sections, we further detected RRM2 expression in 40 corresponding whole tumor sections randomly chosen from the 259 cases. No immunoreactivity was observed in the tissue sections used as negative control, in which the primary antibody was replaced by isotype-matched irrelevant antibody. The positive control (human normal kidney) showed cytoplasmic RRM2 expression in the epithelial cells of convoluted tubules.

Immunohistochemical staining was assessed by two independent pathologists (C.K.P. and B.L.) who were blinded to clinical

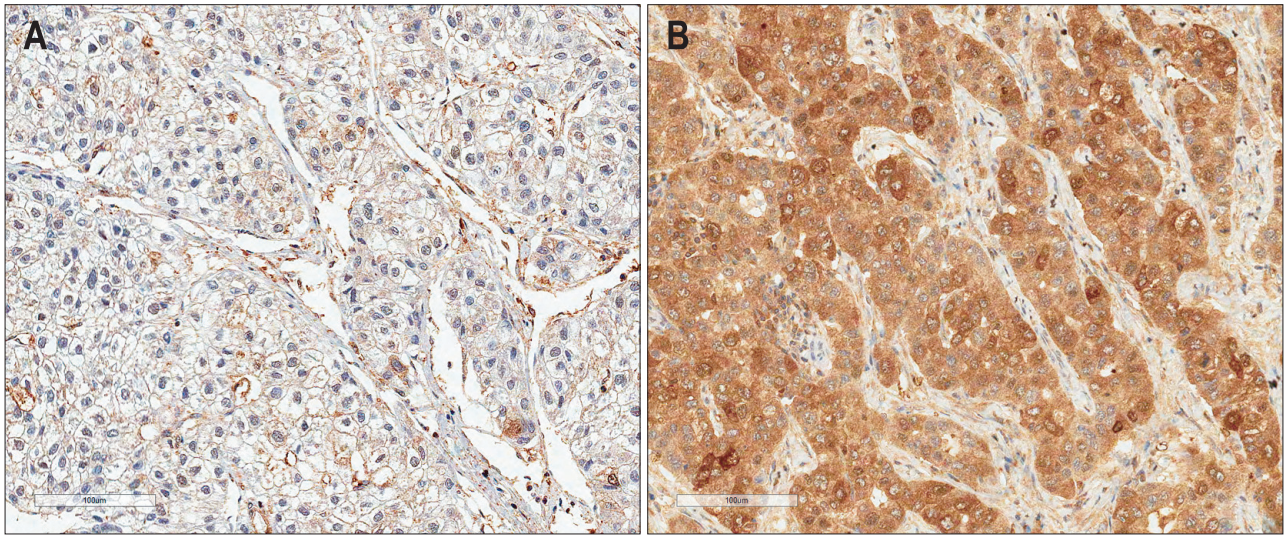

Fig. 1. Immunostaining of ribonucleotide reductase subunit M2 in hepatocellular carcinomas showing low expression (A) or high expression (B) (horseradish peroxidase stain, ×200).

details and any discrepancies were resolved by consensus. A nearly homogeneous immunostaining with moderate staining intensity was observed without any predominant expression pattern in whole tumor section of HCC. The percentage of stained tumor cells was scored from 0% to 100% and each sample was rated from 0 to 4 (0, 0%; 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, >75%). Duplicate tissue cores for each tumor showed high levels of homogeneity for proportion of stained cells. In cases of differences between duplicate tissue cores, the higher score was taken. The immunoreactivity of tumor was graded as low expression (0% to 50% stained tumor cells regardless of staining intensity) (Fig. 1A) or high expression (>50% stained tumor cells) (Fig. 1B).

3. Statistical analysis

All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). The correlation between RRM2 expression and clinicopathologic features was examined by the chi-square and Fisher exact tests. The logistic regression analysis was used for prediction of tumor recurrence. Survival curves were constructed using the Kaplan-Meier method and the differences in survival were evaluated using the log-rank test. The Cox regression hazard model was used to identify factors that were independently associated with survival. A multivariate analysis was performed including all parameters that were significantly associated with survival in a univariate analysis. A p-value of <0.05 was defined as being statistically significant.

RESULTS

1. Clinicopathologic characteristics of patients

The mean patient age was 52.2 years (range, 17 to 76 years), 211 patients were men, and 48 women. One hundred ninety-eight patients (76.4%) were infected with hepatitis B virus and 25 (9.7%) with hepatitis C virus. No viral marker was recognized in 36 patients (13.9%). Tumor recurrence was detected in 185 patients (71.4%), early recurrence in 140 patients (54.1%), late recurrence in 45 patients (17.4%), and 101 patients (39.0%) died of HCC. Nineteen of the 120 deaths in this study were due to non-HCC causes. Twelve of the 19 deaths were due to hepatic failure, five due to nonhepatic causes, and two due to unknown causes.

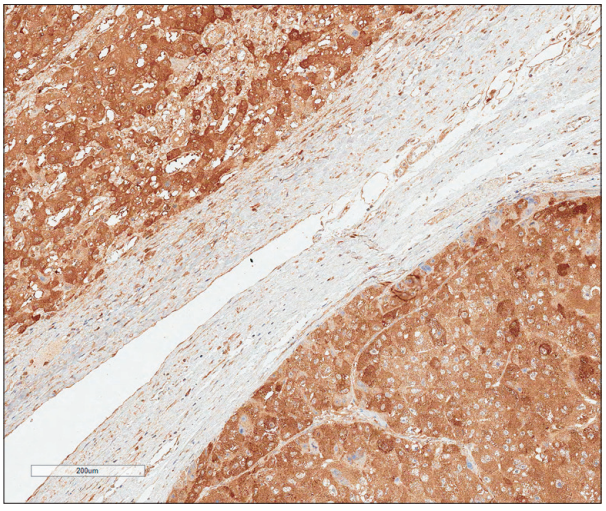

Fig. 2. Ribonucleotide reductase subunit M2 immunoreactivity in hepatocellular carcinomas (right lower part) was similar to that in normal hepatocytes (left upper part) (horseradish peroxidase stain, ×100).

2. RRM2 protein expression in HCC

RRM2 expression was observed in the cytoplasm of normal hepatocytes and bile ducts with moderate staining intensity. In HCC, immunoreactivity for RRM2 was similar to that of normal hepatocyte (Fig. 2). In a few cases membranous immunoreactivity was also found. High RRM2 protein expression was observed in 210 of the 259 HCCs (81.1%). High RRM2 expression was significantly associated with viral etiology (p=0.035) and liver cirrhosis (p=0.036). High RRM2 expression was correlated with the early recurrence (p=0.004), but not with the late recurrence (p=0.966) (Table 1).

3. Prediction of early recurrence in HCC

Univariate analysis revealed that early recurrence was significantly associated with larger tumor size (p=0.001), Edmondson grade III (p=0.003), microvascular invasion (p<0.001), major portal vein invasion (p=0.028), intrahepatic metastasis (p<0.001), higher AJCC T-stage (p<0.001), higher BCLC stage (p<0.001), viral etiology (p=0.009), and high RRM2 expression (p=0.003). Since AJCC T-stage and BCLC stage were associated with vascular invasion, we did not perform multiple analyses with these variables to avoid potential bias. On multivariate analysis, high RRM2 expression (p=0.040) and intrahepatic metastasis (p<0.001) were independent predictors of early recurrence (Table 2).

4. Correlation between RRM2 expression and clinical outcome

The RFS and DSS rates for 259 HCC patients were 40.9% and 76.4% at 3 years, 35.7% and 69.0% at 5 years, 29.9% and 64.3% at 7 years, and 28.8% and 58.5% at 9 years, respectively. Univariate analysis revealed that larger tumor size, Edmondson grade III, microvascular invasion, major portal vein invasion, intrahepatic metastasis, higher AJCC T-stage, higher BCLC stage, lower albumin level, and higher α-fetoprotein level showed unfavorable influences on both RFS and DSS. Viral etiology and liver cirrhosis showed unfavorable influences on RFS. High RRM2 expression showed an unfavorable influence on both RFS (p=0.011) and DSS (p=0.002) (Table 3). The 5-year RFS rate of the RRM2-high expression group was significantly lower than that of the RRM2-low expression group (31.4% vs 50.0%) (Fig. 3A). The median RFS of the RRM2-high expression group

Table 1. Correlations between Ribonucleotide Reductase Subunit M2 Expression and Clinicopathologic Features in 259 Patients with Hepatocellular Carcinomas

Variable	No.	High RRM2 expression, no. (%)	p-value	Variable	No.	High RRM2 expression, no. (%)	p-value
Age, yr				BCLC stage			
≤55	154	123 (79.9)	0.547	0–A	141	113 (80.1)	0.965
>55	105	87 (82.9)		B	104	85 (81.7)	
Gender				C	14	12 (85.7)	
Female	48	35 (72.9)	0.110	Albumin level, g/dL			
Male	211	175 (82.9)		>3.5	232	185 (79.7)	0.107
Tumor size, cm				≤3.5	27	25 (92.6)	
≤5.0	163	133 (81.6)	0.783	AFP level, ng/mL*			
>5.0	96	77 (80.2)		≤200	151	127 (84.1)	0.136
Edmondson grade				>200	98	75 (76.5)	
I	24	19 (79.2)	0.184	Etiology			
II	178	140 (78.7)		Nonviral	36	24 (66.7)	0.035
III	57	51 (89.5)		HBV	198	167 (84.3)	
Microvascular invasion				HCV	25	19 (76.0)	
(-)	114	87 (76.3)	0.083	Liver cirrhosis			
(+)	145	123 (84.8)		(-)	129	98 (76.0)	0.036
Major portal vein invasion				(+)	130	112 (86.2)	
(-)	247	200 (81.8)	1.000	Early recurrence (≤2 yr)			
(+)	12	10 (83.3)		(-)	119	87 (73.1)	0.004
Intrahepatic metastasis				(+)	140	123 (87.9)	
(-)	195	155 (79.5)	0.253	Late recurrence (>2 yr)			
(+)	64	55 (85.9)		(-)†	74	54 (73.0)	0.966
Multicentric occurrence				(+)	45	33 (73.3)	
(-)	247	202 (81.8)	0.249				
(+)	12	8 (66.7)					
AJCC T-stage							
1	110	86 (78.2)	0.550				
2	99	83 (83.8)					
3	44	35 (79.5)					
4	6	6 (100.0)					

RRM2, ribonucleotide reductase subunit M2; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus.
*Data for 10 patients were unavailable; †No early or late recurrence.

and RRM2-low expression group were 16.4±5.8 and 59.1±16.7 months, respectively. The 5-year DSS rate of the RRM2-high expression group was significantly lower than that of the RRM2-low expression group (64.3% vs 87.3%) (Fig. 3B). The mean DSS of the RRM2-high expression group and RRM2-low expression group were 97.7±8.3 and 129.5±13 months, respectively.

Multivariate analysis revealed that intrahepatic metastasis and lower albumin level were independent predictors of both shorter RFS and shorter DSS. High RRM2 expression was an independent predictor of shorter DSS (p=0.008). RRM2-high expression patients were more likely to suffer from disease-specific death than RRM2-low expression patients (hazard ratio, 2.73) (Table 4).

DISCUSSION

Overexpression of RRM2 significantly enhances the invasive potential of human cancer cells.[25] Increased invasiveness induced by RRM2 overexpression is associated with nuclear factor-κB activation and matrix metalloproteinase-9 appears to be an important effector of the enhanced invasiveness that

Table 2. Univariate and Multivariate Logistic Regression Models for Predicting Early Tumor Recurrence in 259 Patients with Hepatocellular Carcinomas

Variable	Univariate model			Multivariate model		
	Coefficient	OR (95% CI)	p-value	Coefficient	OR (95% CI)	p-value
Age (≤55 vs >55), yr	0.210	1.23 (0.75–2.03)	0.410	-	-	-
Gender (female vs male)	0.097	1.10 (0.59–2.07)	0.762	-	-	-
Tumor size (≤5.0 vs >5.0), cm	0.900	2.46 (1.45–4.17)	0.001	0.198	1.22 (0.62–2.41)	0.568
Edmondson grade (I+II vs III)	0.981	2.67 (1.41–5.06)	0.003	0.504	1.66 (0.78–3.52)	0.190
Microvascular invasion (no vs yes)	1.343	3.83 (2.28–6.43)	<0.001	0.430	1.54 (0.81–2.94)	0.192
Major portal vein invasion (no vs yes)	2.309	10.06 (1.28–79.13)	0.028	-0.188	0.83 (0.081–8.48)	0.874
Intrahepatic metastasis (no vs yes)	2.810	16.61 (6.38–43.21)	<0.001	2.408	11.11 (3.52–35.05)	<0.001
Multicentric occurrence (no vs yes)	0.555	1.74 (0.51–5.94)	0.375	-	-	-
AJCC T-stage (1 vs 2+3+4)	1.414	4.11 (2.44–6.94)	<0.001	-	-	-
BCLC stage (0+A vs B+C)	1.322	3.75 (2.24–6.32)	<0.001	-	-	-
Albumin level (>3.5 vs ≤3.5), g/dL	0.981	2.67 (1.09–6.55)	0.032	0.816	2.26 (0.83–6.19)	0.113
AFP level (≤200 vs >200), ng/mL*	0.444	1.56 (0.93–2.61)	0.092	-	-	-
Etiology (nonviral vs viral)	0.991	2.70 (1.28–5.66)	0.009	0.847	2.33 (0.99–5.53)	0.054
Liver cirrhosis (no vs yes)	0.357	1.43 (0.88–2.34)	0.154	-	-	-
RRM2 expression (low vs high)	0.979	2.66 (1.39–5.09)	0.003	0.808	2.24 (1.04–4.86)	0.040

OR, odds ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; RRM2, ribonucleotide reductase subunit M2.
*Data for 10 patients were unavailable.

Table 3. Univariate Analyses of Recurrence-Free Survival and Disease-Specific Survival in 259 Patients with Hepatocellular Carcinomas

Variable	Recurrence-free survival		Disease-specific survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (≤55 vs >55), yr	1.01 (0.76–1.36)	0.926	0.95 (0.64–1.41)	0.794
Gender (female vs male)	1.01 (0.70–1.45)	0.961	1.31 (0.77–2.20)	0.318
Tumor size (≤5.0 vs >5.0), cm	1.64 (1.22–2.21)	0.001	2.53 (1.71–3.74)	<0.001
Edmondson grade (I+II vs III)	1.95 (1.40–2.71)	<0.001	2.21 (1.45–3.37)	<0.001
Microvascular invasion (no vs yes)	2.15 (1.59–2.90)	<0.001	3.09 (1.99–4.81)	<0.001
Major portal vein invasion (no vs yes)	3.84 (2.07–7.09)	<0.001	6.43 (3.30–12.52)	<0.001
Intrahepatic metastasis (no vs yes)	4.38 (3.17–6.06)	<0.001	5.97 (3.99–8.92)	<0.001
Multicentric occurrence (no vs yes)	1.59 (0.86–2.92)	0.138	0.88 (0.32–2.39)	0.799
AJCC T-stage (1 vs 2+3+4)	2.24 (1.65–3.03)	<0.001	3.25 (2.06–5.11)	<0.001
BCLC stage (0+A vs B+C)	2.05 (1.53–2.74)	<0.001	3.45 (2.28–5.20)	<0.001
Albumin level (>3.5 vs ≤3.5), g/dL	1.89 (1.23–2.90)	0.004	2.47 (1.46–4.17)	0.001
AFP level (≤200 vs >200), ng/mL*	1.58 (1.18–2.13)	0.002	1.47 (0.99–2.19)	0.059
Etiology (nonviral vs viral)	2.06 (1.25–3.39)	0.005	1.64 (0.85–3.16)	0.137
Liver cirrhosis (no vs yes)	1.34 (1.00–1.79)	0.047	1.11 (0.75–1.64)	0.611
RRM2 expression (low vs high)	1.68 (1.13–2.49)	0.011	3.22 (1.56–6.63)	0.002

HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; RRM2, ribonucleotide reductase subunit M2.
*Data for 10 patients were unavailable.

RRM2 overexpression induced.[26] Levels of RRM2 modulate oncologically important intracellular signaling events that lead to changes in cellular invasiveness. Targeting RRM2 and its downstream signaling intermediaries represents a rational approach for developing novel anticancer therapeutics.[26] A molecular biomarker with prognostic value for survival outcomes after

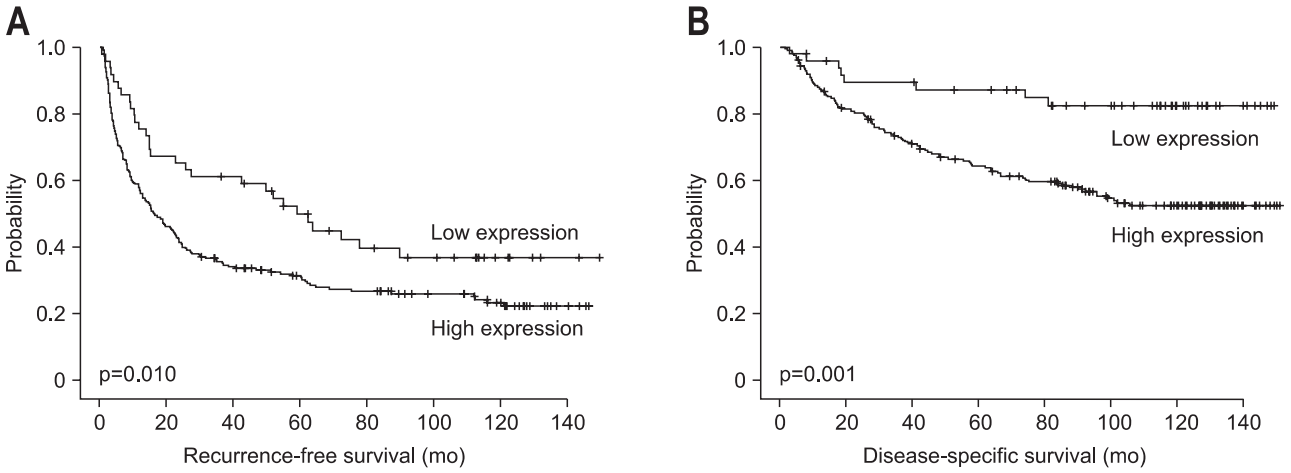

Fig. 3. Kaplan-Meier survival curves showing recurrence-free survival (A) and disease-specific survival (B) according to ribonucleotide reductase subunit M2 expression in 259 patients with hepatocellular carcinomas.

Table 4. Multivariate Analyses of Recurrence-Free Survival and Disease-Specific Survival in 259 Patients with Hepatocellular Carcinomas

Variable	Recurrence-free survival		Disease-specific survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Tumor size (≤5.0 vs >5.0), cm	1.16 (0.82–1.66)	0.407	1.38 (0.88–2.16)	0.163
Edmondson grade (I+II vs III)	1.16 (0.80–1.69)	0.427	1.09 (0.68–1.74)	0.721
Microvascular invasion (no vs yes)	1.35 (0.93–1.97)	0.114	1.47 (0.84–2.57)	0.173
Major portal vein invasion (no vs yes)	1.01 (0.50–2.03)	0.988	1.54 (0.75–3.19)	0.244
Intrahepatic metastasis (no vs yes)	3.07 (2.04–4.64)	<0.001	3.71 (2.21–6.23)	<0.001
Albumin level (>3.5 vs ≤3.5), g/dL	1.58 (1.01–2.48)	0.046	2.07 (1.20–3.56)	0.009
Etiology (nonviral vs viral)	1.59 (0.94–2.68)	0.082	-	-
Liver cirrhosis (no vs yes)	1.32 (0.96–1.82)	0.092	-	-
RRM2 expression (low vs high)	1.36 (0.90–2.05)	0.147	2.73 (1.30–5.70)	0.008

HR, hazard ratio; CI, confidence interval; RRM2, ribonucleotide reductase subunit M2.

surgery can guide further treatment decisions, such as selecting patients at high risk or recurrence.

In the current study, we elucidated the prognostic significance of RRM2 protein expression in a large cohort of HCC patients with long-term follow-up and showed that high RRM2 expression was significantly associated with viral etiology and liver cirrhosis. There were no correlations between high RRM2 expression and tumor aggressiveness, such as tumor size, microvascular invasion, major portal vein invasion, intrahepatic metastasis, AJCC T-stage, or BCLC stage. This is at variance with previous reports using cancer cell lines.[9,11,25,26] We suspect that the role of RRM2 might be different in between human cancer tissue and cancer cell line. Using logistic regression analysis, we found that high RRM2 expression and intrahepatic metastasis were independent predictors of early recurrence, suggesting that RRM2 might facilitate the dissemination of tumor cells and clinicians might consider more aggressive treatments for patients with high RRM2 expression in order to prevent recurrence. Moreover, high RRM2 expression showed an unfavorable influence on both RFS and DSS, and was an independent predictor of shorter DSS. Recent studies reported that high RRM2 expression was independent poor prognostic factor of RFS and overall survival in patients with resected pancreas adenocarcinoma or colorectal cancer.[12,13] Our results suggest that RRM2 is a potential new marker for predicting the prognosis of HCC after curative hepatectomy and clinicians should possibly have more rigorous follow-up after surgical resection for those patients with poor prognosis identified in this study.

In summary, our data show, for the first time, that high RRM2 protein expression might be a useful marker for predicting early recurrence and a marker for poor prognosis of HCC after curative hepatectomy. Further studies are needed to determine the value of RRM2 as a prognostic predictor in HCCs.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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