Ribonucleotide Reductase Small Subunit M2B Prognoses Better Survival in Colorectal Cancer

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Abstract

Ribonucleotide reductase subunit RRM2B (p53R2) has been reported to suppress invasion and metastasis in colorectal cancer (CRC). Here, we report that high levels of RRM2B expression are correlated with markedly better survival in CRC patients. In a fluorescence-labeled orthotopic mouse xenograft model, we confirmed that overexpression of RRM2B in nonmetastatic CRC cells prevented lung and/or liver metastasis, relative to control cells that did metastasize. Clinical outcome studies were conducted on a training set with 103 CRCs and a validation set with 220 CRCs. All participants underwent surgery with periodic follow-up to determine survivability. A newly developed specific RRM2B antibody was employed to carry out immunohistochemistry for determining RRM2B expression levels on tissue arrays. In the training set, the Kaplan–Meier and multivariate Cox analysis revealed that RRM2B is associated with better survival of CRCs, especially in stage IV patients (HR = 0.40, 95% CI = 0.18–0.86, P = 0.016). In the validation set, RRM2B was negatively related to tumor invasion (OR = 0.45, 95% CI = 0.19–0.99, P = 0.040) and lymph node involvement (OR = 0.48, 95% CI = 0.25–0.92, P = 0.026). Furthermore, elevated expression of RRM2B was associated with better prognosis in this set as determined by multivariate analyses (HR = 0.48, 95% CI = 0.26–0.91, P = 0.030). Further investigations revealed that RRM2B was correlated with better survival of CRCs with advanced stage III and IV tumors rather than earlier stage I and II tumors. Taken together, our findings establish that RRM2B suppresses invasiveness of cancer cells and that its expression is associated with a better survival prognosis for CRC patients. Cancer Res; 71(9):3202–13. ©2011 AACR.

Introduction

Ribonucleoside diphosphate reductase (RR) plays an essential role in converting ribonucleoside diphosphate (NDP) to 2’-deoxyribonucleoside diphosphate (dNDP; ref. 1). In humans, 1 large subunit (M1) and 2 small subunits (RRM2 and RRM2B) of RR have been identified (2, 3). The large subunit M1 (RRM1) contains substrate and allosteric effector sites that control the RR holoenzyme activity and substrate specificity (4–6). The RR small subunits form 2 equivalent dinuclear iron centers that stabilize the tyrosyl free radical required for the initiation of electron transferring during catalysis (7).

Two RR small subunits, RRM2 and RRM2B, have an 80% similarity in protein sequence (3). Using a synthetic heptapeptide that inhibits RR activity, RRM2B has been shown to bind to RRM1 through the same binding domain as RRM2 (8). Nevertheless, there are several different features that have been recognized in the 2 RR small subunits. An in vitro study showed that recombinant human RRM2 was able to oxidize a reactive oxygen species (ROS) indicator carboxy-H2DCFDA and generate ROS in the presence of mitochondrial extract (9). An increased RRM2 expression enhanced the sensitivity to H2O2 attack significantly in KB transfectants (9). The increase in oxidized ROS may activate the Ras/Raf signaling pathway in cancer cells (10). Gene transfer studies revealed that over-expressing the recombinant mouse RR subunit R2 (homologous to human RRM2) caused an increase in the membrane-associated Raf-1 expression (30%), mitogen-activated protein kinase (MAPK) 2 activity (70%), and Rac-1 activation (3-fold), remarkably elevating the metastatic potential of BALB/c 3T3 and NIH 3T3 cells (11). The RRM2 overexpression increases cellular invasiveness and matrix metalloproteinase (MMP) 9 expression in human cancer cells (12). Enhanced MMP-9 gene expression is associated with increased tumor growth and metastasis of solid tumor malignancies including colon cancer (13–16). Therefore, inhibiting RRM2 may reduce the proliferation and invasive ability of cancer cells. In contrast, in the presence of wild-type p53, RRM2B (also called p53R2) is

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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RRM2B is a DNA repair protein that has been linked to better survival of colorectal cancer patients. This study investigated the effect of RRM2B on tumor invasion and metastasis.

**Materials and Methods**

**Orthotopic xenograft mouse model construction**

The study design is displayed in Figure 1A. To generate RRM2B overexpression colon cancer transfectant, the expression plasmid of pEGFP-N1-RRM2B was confirmed after construction and sequencing. The expression plasmid was transfected into HCT-116 cells and the vector of pEGFP-N1 was transfected for a negative control. The stable clones were selected by G418 (1 mg/mL for selection and maintenance). Meanwhile, the cells were sorted by flow cytometry twice, based on green fluorescent protein (GFP) fluorescence to enhance the purity of transfectants to more than 90%. The expression of GFP-RRM2B level was examined by Western blot. The GFP signal of HCT-116/vector and HCT-116/RRM2B could also be detected by fluorescence microscopy. About $5 \times 10^5$ cells of RRM2B overexpression transfectant or vector control were implanted into cecum of 8- to 10-week-old female NSG mouse (NOD.Cg-Prkdclid L22mrm1yw/J or 5J from Jackson Laboratories) to generate an orthotopic xenograft mouse model. The animals were euthanized 3 weeks after xenotransplantation. Tumor growth and metastasis were observed under fluorescence microscopy. In addition, all liver and lung tumor samples were collected for pathologic examination to confirm our findings. Animal use and experiment protocol were approved by the Institutional Animal Care and Use Committee (IACUC) of COH. Sample size estimation was based on nQuery 6.01 software. A sample size of 6 per group would be needed for 74% power with a 1-sided $\alpha$ of 0.05. The $\chi^2$ analyses and Fisher’s exact tests were employed to determine the statistical significance.

**siRNA and transfection**

The RRM2B, RRM2, and scramble siRNA were purchased from Santa Cruz Biotechnology Inc. Briefly, $2 \times 10^5$ cells were seeded per well in 6-well culture plates filled with 2 mL antibiotic-free normal growth medium supplemented with FBS and then incubated at 37°C in a CO$_2$ incubator for 24 hours. A total of 7.2 μL of 10 μmol/L RRM2B, RRM2, or scramble siRNA was transfected into HT-29 or HCT-8 cells by using a transfection reagent. Cells were incubated in the transfection medium for 5 hours and then replaced with normal cell culture medium. The inhibition of RRM2 and RRM2B was measured using quantitative real-time PCR (qRT-PCR).

**In vitro invasion assay**

BD Matrigel invasion chambers were commercially available from BD Biosciences. In brief, the 8-mm porosity polycarbonate membrane was covered with 1 mL of medium that contained $1 \times 10^5$ cells per well. The plates were then incubated for 24 hours at 37°C in a 5% CO$_2$ incubator. Media was then removed, and noninvading cells were gently scraped off using a cell scraper. The filter was then washed twice with PBS and stained with 0.5% methylene blue for 4 hours. The cells that passed through the filters and adhered to the lower surface were counted by means of optical microscopy.

**Multiple tissue array and multiple tissue board construction**

All formalin-fixed, paraffin-embedded (FFPE) human colon and rectum tissue samples were obtained from surgery. For training set, tissue samples were reassembled and multiple tissue boards (MTB) were constructed. On MTBs, 1 slide includes 8 to 12 pieces of the tissue samples. Each piece was approximately $1 \times 10$ mm$^2$. As for validation set, samples were reassembled to construct multiple tissue arrays (MTA).
Figure 1. Construction of RRM2B overexpression transfectant for generation of xenograft mouse model. A, design of animal study: the pEGFP-RRM2B was used to construct the RRM2B expression plasmid. After confirmation by sequencing, the GFP-RRM2B expression plasmid and control plasmid were transfected into HCT-116 cells. The stable transfectant was selected by G418 (1 mg/mL). In addition, the transfectants were further sorted by flow cytometry on the basis of GFP fluorescence. After that, the transfectants were injected into the cecum of NSG mice to generate a xenograft mouse model. After 3 weeks, the mice were euthanized for examination. B, the expression of RRM2B was examined by Western blot. C, after G418 selection and flow cytometric sorting, the transfectants were visualized by GFP fluorescence (left). Invasion assay was employed to determine the invasive ability of HCT-116/vector and HCT-116/RRM2B. The optical and fluorescence images of invasion cells are displayed in middle and right, respectively. D, the siRNA of scramble, RRM2B, and RRM2 were transfected into HCT-8 and HT-29 cells. After an incubation period of 48 hours, total RNA from corresponding cells were extracted and used to measure RRM2B and RRM2 mRNA level by qRT-PCR after transfection. E, increase of invasion ability by inhibition of RRM2B in HCT-8 and HT-29 cells. FL, fluorescence microscopy.
Each MTA contained a maximum of 64 piece sections and each piece of tissue was approximately 0.8 × 0.8 mm². In total, 7 MTAs were stained at the same time to avoid bias yielded from different immunohistochemical (IHC) staining conditions. For each IHC staining, the multiple tissue check board included tumor and normal colorectal tissue samples as positive and negative controls. All MTAs and MBTs were stored at room temperature. The correlation test was conducted between expression of RRM2B and years of samples obtained. Correlation analysis result displayed storage time did not affect RRM2B levels in statistical significant (P > 0.05).

**Antibodies for IHC staining**

Because nonspecific targeting of commercial RRM2B antibody was reported (25), we developed new RRM2B antibody for IHC staining. Recombinant human RRM2B peptide (acetyl-PEPAGLQDERSC-amide; amino acids 4–18), a commercially produced rabbit polyclonal antibody by Rockland Immunochemicals was used against human RRM2B in these studies. The synthetic human RRM2B peptide (acetyl-PEPAGLQDERSC-amide; amino acids 4–18) was injected to immunize New Zealand white rabbit. Specific anti-RRM2 antibodies from serum were purified by immunoaffinity chromatography using human RRM2B peptide coupled to agarose beads. Clones were chosen for antibody production based on their activities on paraffin-embedded human tissues. Sensitivity was optimized on multiple tissue check board by limiting serial dilutions (1:1,000). To validate the efficacy of RRM2B antibody, qRT-PCR was used to correlate mRNA and protein levels in human tissue sections and cancer cell lines. The antibody for TP53 (1:400 dilution) IHC staining was commercially available from Santa Cruz Biotechnology Company. RRM2 antibody (1:5 dilution) was generated and selected by our laboratory. The IHC staining images of TP53 and RRM2 are displayed in Supplementary Figure S1.

**Quantitative IHC assays**

To avoid biases, the standards and conditions of immunohistochemistry for RRM2B expression determination were optimized on the training set (MTB) and validated on the validation set (MTA). Details of the deparaffinization protocol and immunohistochemistry were described in a previous publication (20). Briefly, after deparaffinization, the endogenous peroxidase activity was blocked using 3% H₂O₂. The slides were incubated with normal goat serum for 20 minutes and then applied with primary antibody for 20 minutes at room temperature. After 7 minutes of H₂O₂ treatment, the array slides were incubated with polymer horseradish peroxidase–labeled corresponding antibodies for 30 minutes. DAB (3,3'-diaminobenzidine; 0.05 g DAB and 100 mL 30% H₂O₂ in 100 mL PBS) was then applied for 5 and 10 minutes, respectively. Each slide was counterstained with Dako's hematoxylin. PBS was used as a negative control.

The RRM2B was heterogeneously expressed between and within tumors. In generally, RRM2B was predominantly cytoplasmic staining in immunohistochemistry; however, some nuclear staining was also observed. RRM2B expression was quantified using a visual grading system on the basis of the extent of staining. Only immunoreactivity in the cytoplasm was evaluated. To reduce the image reader bias, an automated imaging system was employed to obtain digital images of the stained sections for subsequent quantitative analyses. Each sample was scored by 2 independent investigators in a double-blind manner. Two independent investigators reviewed and scored the subcellular localization (e.g., cytoplasm vs. nucleus), staining intensity (e.g., integrated optical density), and/or percentage of stained cells (e.g., total area or percentage of cells positive) for each image. Discrepancies in scores were resolved after joint review by the readers.

**Design of outcome study**

As shown in Supplementary Figure S2, this is a retrospective study. The Institutional Review Board protocols were reviewed and approved by COH and the Second Affiliated Hospital of ZJU, respectively. A series of assessable 103 CRCs from COH were recruited as training set. Meanwhile, a series of 220 CRCs consecutive patients from Second Affiliated Hospital of ZJU were entered as the evaluation set. Eligible participants of the training (from COH) and validation (from ZJU) sets received surgical treatment between 1980–1985 and 1999–2004, respectively. Inclusion criteria were (i) CRC with pathologic diagnosis; (ii) informed consent or waiver; and (iii) receipt of at least 1 follow-up. Exclusion criteria were: (i) lack of pathologic diagnosis or tissue samples; (ii) failure to obtain consent for research; (iii) loss of follow-up within 3 months; (iv) less than 18 years of age; and (v) multiple cancers. The demographic distribution of participants is described in Supplementary Table S1.

Careful chart review was conducted and pathoclinical data were abstracted. Variables assessed included birth date, gender, date of diagnosis, date of operation, type of chemotherapy, date of chemotherapy, type of radiotherapy, date of radiotherapy, tumor node metastasis (TNM) stage, relapse/metastasis status, date of relapse/metastasis, date of last follow-up, and vital status at last follow-up. The above information was coded and entered into a CRC database. Double data entry and logic checks were used to reduce errors. In the training set (103 CRCs from COH), 41 cases have had adjuvant chemotherapy and 15 cases have had radiotherapy. In the validation set (220 CRCs from ZJU), 79 cases have had adjuvant chemotherapy. Radiotherapy is not applicable in training set. The participants in the training set include 94 Caucasian, 2 African-American, 6 Asian, and 1 unknown ethnicity. All CRCs in validation set are Chinese (Asian). All patients were followed up until June 2007 and details of their demographic and survival data were updated.

Sample size was calculated using parameter estimates obtained from a pilot study previously conducted at COH. Using nQuery Advisor 6.01 software, it was determined that a sample size of 200 patients would be needed for more than 80% power with a 2-sided α of 0.05.

The specificity of antibody was validated and efficiency was optimized by using samples from training set, and then it was applied in validation set to determine RRM2B expression levels. The database was created for survival analysis. All patients were periodically followed for survival; patients with
curative operations were also followed for recurrence-free survival. The follow-up period was calculated from the date of surgery until the date of last contact. Recurrence was defined as the time to initial tumor recurrence. Later metastasis or local recurrence was considered evidence of tumor relapse. Only deaths from CRC were considered as endpoint of disease survival.

**Data management and statistical analysis**

Microsoft Office Access and Excel were used to input data and create databases for CRCs. The missing cases were labeled with the appropriate "missing" code. The software of JMP 8.0 Software (SAS Institute) and GraphPad Prism 5.0 were used for statistical analysis and figure drawing. Group comparisons for continuous data were conducted using t tests for independent means or 1-way ANOVA. For categorical data, we employed \( \chi^2 \) analyses, Fisher's exact tests, or binomial tests of proportions. Multivariate logistic regression models were used to adjust for covariates on the ORs. Kaplan–Meier analysis and Cox hazard proportional model were applied to overall survival and progress-free survival analysis. Multivariate analyses and stratification were applied to reduce the confounding effects impact on the estimation of OR and HR.

**Results**

**Overexpression of RRM2B reduces the invasiveness of HCT-116 transfectant**

The colon cancer cell HCT-116 contains a low level of RRM2B, and its expression was elevated through gene transfection. The plasmid transfection and selection procedure are detailed in Materials and Methods (Fig. 1A). The vector pEGFP-N1 was used as a negative control. In Figure 1B, the Western blot analysis revealed that endogenous RRM2B could be barely detectable in both HCT-116/vector cells and HCT-116/RRM2B cells. In contrast, GFP-RRM2B was observed clearly in HCT-116/RRM2B but not in HCT-116-GFP cells.

Using fluorescence microscopy, the GFP signal could be detected in both HCT-116/vector and HCT-116/RRM2B cells (Fig. 1C, left). The invasion assay showed that the increase of RRM2B significantly decreased invasion ability from 34 (range: 13–53) per field to 20 (range: 4–8) per field (10 x) in HCT-116/RRM2B cells (test, \( P = 0.004 \); Fig. 1C, middle and right), which is congruent with the previous studies (20). Furthermore, siRNA was used to down-regulate RRM2B and confirm the above finding in HCT-8 and HT-29 CRC cells (Fig. 1D). Here, inhibition of RRM2B by siRNA resulting in the increase of RRM2B was observed on HT-29 cells. On the other hand, inhibition of RRM2 by siRNA also caused slightly increase of RRM2B in both HCT-8 and HT-29 cells. These results were compatible with our previous observation that RRM2B and RRM2 had opposite expression patterns in human normal tissues and many cancer cell lines (26). This is because RRM2B is expressed at G1 phase and RRM2 is at S phase, and they play different biological roles on DNA repair and DNA replication (3, 27). Nevertheless, inhibition of RRM2B caused an approximate 2-fold increase in the invasiveness of CRC cells (Fig. 1E).

**RRM2B is associated with better survival of CRC patients in a training set**

The immunohistochemistry was employed to determine the expression level of RRM2B in human samples. Because nonspecific targeting of commercial antibody has been reported (25), we developed and validated a new specific RRM2B antibody for IHC staining. In Western blot, only 1 band close to 42 KDa could be visualized by selected RRM2B antibody in HCT-116/vector cells (Fig. 1B), which represents endogenous RRM2B protein. To validate specificity of the anti-RRM2B antibody, a recombinant full-length RRM2B peptide was used as nonspecific blocking peptide. In

**Overexpression of RRM2B reduces the metastasis of HCT-116 cells in an orthotopic xenograft NSG mouse model**

In the subcutaneous xenograft model, the average tumor weight of HCT-116/RRM2B (0.41 g; range: 0.20–0.99) was smaller than HCT-116/vector (0.69 g; range: 0.25–0.61) at the day 14. However, it was not statistical significant due to insufficient of sample size (4 mice/group; Supplementary Fig. S3). In the orthotopic xenograft mouse model, the HCT-116/vector and HCT-116/RRM2B cells were collected and implanted into the cecum of NSG mice through microsurgical operation. Six HCT-116/vector mice and 6 HCT-116/RRM2B xenograft mice were successfully created. For a pilot study, one HCT-116/vector mouse was euthanized on day 14 to preobserve metastasis. All others were euthanized on day 21. The optical images of cecum, lung, and liver are displayed in the left column in Figure 2A and B. Under the fluorescence microscope, 3 of the 5 HCT-116/vector mice showed distinct green fluorescence in the local bowel and metastatic sites of peritoneum, mediastinum, lung, and liver (Fig. 2A, left and middle columns). However, of the six HCT-116/RRM2B mice, only 1 mouse suffered minor local invasion in bowel (Fig. 2B, top, left, and middle columns). All HCT-116/RRM2B mice showed no GFP fluorescence in liver or lung (Fig. 2B, middle and bottom, left and middle columns). Because the GFP-RRM2B fusion protein fluoresces weaker than the GFP protein, it may limit the detection of metastasis in the HCT-116-GFP/RRM2B xenograft mouse model. To avoid this possibility, all tissue samples from cecum (tumor), liver, and lung were collected and examined by pathologists; the hematoxylin and eosin (H&E) staining results are shown (Fig. 2A and B, right panel), which is consistent with the metastatic lesions observed on each organ using fluorescence microscopy. Furthermore, immunohistochemistry was applied to confirm the RRM2B expression in tumor samples (Fig. 2A and B, right top corner panels). Above findings revealed that RRM2B suppresses tumor invasion and distant organ metastasis in the CRC xenograft mouse model (Pearson test, \( P = 0.026 \); and Fisher's Exact test, \( P = 0.06 \)).
Figure 3A, left, RRM2B is dominant in normal colon epithelial cytoplasm. After application of 1 μg/mL RRM2B peptide as a preblocker, the IHC signal was blocked in the colon tissue sample (Fig. 3A, middle). However, the IHC signal could not be blocked by RRM2 peptide in the same sample (Fig. 3A, right). It was indicated that the antibody we used in this study could specifically recognize RRM2B rather than RRM2 in IHC staining. The RRM2B IHC staining standard is shown in Figure 3B.

In Figure 3C, it shows that the RRM2B expression was relatively lower in tumor tissue than that in the corresponding normal sections (Fig. 3C, left two panels), whereas the RRM2B level in metastatic colon cancer was much lower than that in the corresponding primary colon cancer (Fig. 3C, right two panels). These observations are compatible with our previous report, which found that RRM2B has a malignancy-suppressing potential of cancer cells.
On the basis of the distribution of RRM2 expression, we stratified negative (−) and weakly positive (+) as RRM2B (low), and positive (+++) and strong positive (+++) as RRM2 (high). Kaplan–Meier analysis illustrated that RRM2B (high) significantly associates with better survival in stage IV CRC patients (log-rank,  \( P = 0.038 \); Fig. 3D) but failed to show statistical significance in other stages. The multivariate Cox proportional hazard analysis suggested that RRM2B (high) is associated with better survival of CRC patients (HR = 0.70, 95% CI = 0.39–1.26,  \( P = 0.240 \)), especially in CRCs with stage IV (HR = 0.40, 95% CI = 0.18–0.86,  \( P = 0.016 \); Fig. 3E, Supplementary Table S2). These findings suggested that RRM2B may reduce the risk of death from CRC in advanced stage patients.

Overexpression of RRM2B is negatively related to TNM stage and is associated with better survival of CRC patients in a validation set

To validate the above findings, an outcome study was conducted on a validation set, which included 220 assessable CRCs collected from ZJU in China. In the validation set, the Kaplan–Meier analysis revealed that RRM2B, RRM2, and TNM stage significantly associates with better survival of CRC patients (log-rank,  \( P = 0.032 \); Fig. 4A) but RRM2 is related to poor overall survival (log-rank,  \( P = 0.003 \); Fig. 4B). Meanwhile, Cox hazard proportional analysis indicated that the HRs of RRM2B and RRM2 for overall survival were 0.56 (95% CI = 0.32–0.98,  \( P = 0.040 \)) and 2.07 (95% CI = 1.19–3.72,  \( P = 0.013 \)), respectively (Fig. 4A and B). To eliminate potentially confounding effects, a multivariate Cox proportional hazard model was employed for further analysis of the overall survival of CRC patients. Here, we recruited factors including RRM2, TP53, \( \beta \)-catenin, TNM stage, location, age, and gender as cofactors. In Figure 4C, the results from the analysis revealed that RRM2B, RRM2, and TNM stage indeed affected the overall survival of CRC patients significantly ( \( P < 0.05 \)). Nevertheless, RRM2B was significantly related to better survival of CRCs (HR = 0.48, 95% CI = 0.26–0.91,  \( P = 0.030 \)), whereas the factors of TNM stage and RRM2 were significantly related to worse survival. The adjusted HRs of RRM2 were 2.57 (95% CI = 1.38–4.96,  \( P = 0.001 \)) and TNM stage (III and IV vs. 0 and II) was 3.94 (95% CI = 2.13–7.75,  \( P < 0.001 \)). Furthermore, \( \beta \)-catenin, TP53, tumor location gender, and age did not significantly impact the overall survival of CRC patients in the validation set ( \( P > 0.05 \)). These findings confirmed that RRM2B plays an opposing role to RRM2 and is associated with better survival of CRC patients.

Stratification analysis for clarifying the invasiveness suppression of RRM2B in validation set

To avoid confounding effects, multivariate and stratification analysis was conducted for further validation. To exclude

### Table 1. Logistic analysis for expression of RRM2B and TNM stage of CRCs in validation set

<table>
<thead>
<tr>
<th>RRM2B</th>
<th>OR (95% CI)</th>
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<tr>
<td>RRM2B (low)</td>
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<td>Reference</td>
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<tr>
<td>RRM2B (high)</td>
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<td>0.45 (0.19–0.99)</td>
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</table>

NOTE: All information about age, tumor location, and TNM stages were derived at the time of first surgery. Information about tumor invasion, lymph node involvement, and distant organ metastasis were based on TNM stage from pathologic report.

*OR adjusted by sex, age at diagnosis tumor location, and RRM2.

**P < 0.05.
Figure 3. Validate efficiency of RRM2B antibody and optimize conditions of immunohistochemistry in training set. The rabbit against human RRM2B antibody (1:1,000 dilution) was employed to conduct IHC staining. A, left, normal colon epithelium with staining of RRM2B antibody only; middle, RRM2B antibody preblocked by recombinant RRM2B (1 mg/mL) peptide; right, RRM2B antibody preblocked by recombinant RRM2 (1 mg/mL) peptide. B, the standard of IHC scores for RRM2B. C, left 2 columns, IHC staining for normal colon epithelium (left) and cancer tissue (right) from the same patient; right 2 columns, IHC staining for primary colon cancer (left) and corresponding metastatic cancer (right). D, Kaplan–Meier analysis for overall survival with stage IV CRCs stratified by different RRM2B levels. E, multivariate Cox analysis for overall survival of CRCs with stage IV in training set. Detailed analysis result was displayed on Supplementary Table S2.
the effects from chemotherapy or radiotherapy, we analyzed those participants \((N = 139)\) without adjuvant therapies. For those participants, the adjusted HR of RRM2B is 0.51 (95% CI = 0.20–0.95, \(P = 0.035\)) adjusted by age and sex in validation set, which is compatible to the above result yielded from all cases.

Further stratification Cox analysis revealed that the RRM2B was shown to reduce the risk of death (HR = 0.53, 95% CI = 0.29–1.01, \(P = 0.053\)) and recurrence (HR = 0.63, 95% CI = 0.34–1.21, \(P = 0.165\)) in stage III and IV than in stage 0 and II (Fig. 5A), which is consistent with our findings from the training set. Nevertheless, RRM2 was related to poor overall and progress-free survival of CRC with either stage 0 and II or stage III and IV (Fig. 5B). Meanwhile, the Figure 5C and D further visualized that RRM2B is associated with better survival but RRM2 with poor survival in CRC patients with stage III and IV.

In Figure 5E, multivariate Cox analysis indicated that RRM2B is associated with better overall and progress-free survival in both colon and rectal cancer patients. In particular, the HR of RRM2B for overall survival in colon cancer was 0.39 (95% CI = 0.17–0.87, \(P = 0.023\)). On other hand, RRM2 is represented worse overall and progress-free survival of colon and rectal cancer (Fig. 5F). Notably, it reached to statistical significance in rectal cancer (HR of overall survival = 3.32, 95% CI = 1.54–7.72, \(P = 0.002\)). The Kaplan–Meier analysis visualized that RRM2B is significantly associated with better overall survival of colon cancer patients (log-rank \(P = 0.018\); Fig. 5G) and RRM2 is related to poor overall survival of rectal cancer significantly (log-rank \(P = 0.002\); Fig. 5H).

The above findings suggested that RRM2B appears related to better survival of CRC patients with later stage, whereas RRM2 seems related to poor prognosis of CRC patients with either early or late stages.

**Discussion**

The malignancy suppressing potential of RRM2B has been reported previously (20, 21). This study validated it using HCT-116, HT-29, and HCT-8 human CRC cell lines (Fig. 1C and E). As shown in Figure 2, RRM2B significantly suppressed invasion and metastasis of HCT-116 cell in a xenograft animal model (\(P = 0.026\)). In the clinical specimens study, RRM2B significantly related to better overall survival in stage IV CRC patients (HR = 0.40, 95% CI = 0.18–0.86, \(P = 0.016\)) of the training set. The result was validated as RRM2B negatively related to tumor invasion (OR = 0.45; 95% CI = 0.19–0.99, \(P = 0.040\)) and lymph node involvement (OR = 0.48; 95% CI = 0.25–0.92, \(P = 0.026\); Table 1) and positively associated with better overall survival (HR = 0.55, 95% CI = 0.32–0.98, \(P = 0.032\)), especially stage III and IV CRC patients (adjusted HR = 0.53, 95% CI = 0.28–1.01, \(P = 0.053\)), rather than early stage (HR = 1.02, 95% CI = 0.31–4.58, \(P = 0.165\); Fig. 5). The multivariate analysis indicated that the HR of RRM2B was 0.48 (95% CI = 0.26–0.92, \(P = 0.040\); Fig. 4C). The above findings suggest that the expression of RRM2B is associated with better prognosis in advanced stage CRC patients.

Several controversial results have been reported on RRM2B versus the survival of cancer patients. Okumura and colleagues...
Figure 5. Analysis for RRM2B/RRM2 and prognosis of CRCs by stratification. The Kaplan–Meier analysis was used to plot survival curve and multivariate Cox proportional hazard analysis was conducted to evaluate HR of RRM2B and RRM2. HRs were adjusted by sex and age at diagnosis. Stage IV CRCs were excluded in progression-free survival analysis. HRs of RRM2 and RRM2B were based on high expression versus low expression. With stratified by TNM stages, the multivariate Cox analyses for overall and progression-free survival of RRM2B and RRM2 are shown in A and B. The Kaplan–Meier analyses for RRM2B/RRM2 and overall survival of CRCs with stage III-IV are displayed in C and D. E, the multivariate Cox analyses for RRM2B and prognosis of CRCs stratified by tumor locations. F, RRM2 and prognosis of colon and rectal cancers. The Kaplan–Meier analyses are visualized on G and H for RRM2B and the overall survival of colon cancer and RRM2 and overall survival of rectal cancer, respectively. *, statistics significant on Cox analysis, \( P < 0.05 \).
(22, 28) and Uramoto and colleagues reported that RRM2B was related to poor prognosis of esophageal cancer or lung cancer patients (24). This might be caused by nonspecific targeting of anti-RRM2B antibodies obtained commercially. Many proteins were detected nonspecifically following the Western blot analysis using this antibody. The nonspecific targeting of commercially obtained anti-RRM2B antibodies was also reported by others (25). In our study, the specificity of the new RRM2B antibody has been verified, and efficacy of the antibody was optimized in IHC study. Those systematic and observable biases were taken into consideration and controlled. Moreover, our findings yielded from the training set were validated on the validation set with different racial and socioeconomic backgrounds, which confirmed that RRM2B plays a malignancy suppression role and is related to better survival of CRC patients.

The molecular mechanism through which RRM2B suppresses malignancy of cancer cells was reported in our previous publications (9, 29). Under a genotoxic stress, RRM2B was rapidly phosphorylated at its Ser-72 by ATM (ataxia telangiectasia mutated; ref. 30). RRM2B then dissociated from p21 and subsequently facilitated the accumulation of p21 in the nucleus in response to DNA damage–induced G1 arrest (29), which may help to repair DNA and prevent cells from accumulating mutations. On other hand, it was reported that RRM2B interacts with MAP/ERK kinase (MEK2)/MAPK and negatively regulates Ras/Raf-MEK/ERK (extracellular signal–regulated kinase) activity in lung cancer cells (31). Previous studies revealed that recombinant wild-type RRM2B lacks the ability to oxidize a ROS indicator but RRM2 can (9). In our previous study, we examined a series of RRM2B residues and found that mutants of RRM2B (Y331F) and RRM2B (Y285F) were able to oxidize carboxy-H2DCFDA like RRM2. This finding has been validated in Supplementary Figure S4A. To investigate whether the invasion of cancer cells was related to the pro-oxidant status, the expression plasmids of myc-RRM2B (wild type), RRM2B (Y331F), and RRM2B (Y285F) were constructed and transfected into HeLa and HCT-116 cells. Overexpression of RRM2B (wild type and mutants) could be detected by Western blot in HeLa and HCT-116 cells (Supplementary Fig. S4B). As with increased capability on oxidizing carboxy-H2DCFDA, overexpression of RRM2B (Y331F) and RRM2B (Y285F) also could significantly increase invasion ability in transfectants of RRM2B HeLa and HCT-116 cells (Supplementary Fig. S4C and D).

There are many genes and molecular events that relate to the prognosis of CRC (32, 33). RRM2B would be an eligible prognostic biomarker to predict survival and therapeutic response for in advanced CRCs. A further significance of this study has clarified that RRM2B and RRM2 played opposing biological roles on regulating pro-oxidant status and modulating invasion ability in cancer cells. The RR inhibitors, such as hydroxyurea, had been widely used for treatment of cancers. However, the low efficacy and side effects of current RR inhibitors significantly limited their application on many cancers including CRC. For most RR inhibitors, their low efficacy and side effects are caused by nonspecific targeting. Our previous and current studies showed that the invasion ability of cancer cells could be reduced by blocking RRM2 but it significantly increased by RRM2B siRNA (20). This phenomenon also has been observed by other research groups (12, 21). Therefore, understanding the different biological roles of RRM2B and RRM2 and their effects on malignancy may help to develop novel specific and efficient RR inhibitors that avoid resistance and minimize side effects in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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