Ribonucleotide reductase small subunit M2B prognoses better survival in colorectal cancer

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Running title: RRM2B relates to better survival of colorectal cancer

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Abbreviations:

RR: ribonucleotide reductase;
RRM1: human ribonucleotide reductase large subunit M1;
RRM2: human ribonucleotide reductase small subunit M2;
RRM2B: a TP53 dependent human ribonucleotide reductase small subunit R2 (p53R2);
CRC: colorectal cancer;
FFPE: formalin-fixed, paraffin-embedded tissue block;
IHC: immunohistochemistry;
MTA: multiple tissue array;
MTB: multiple tissue board;
siRNA: short interference RNA;
dNTP: deoxyribonucleoside triphosphate;
NDP: ribonucleoside diphosphate;
OR: odds ratio;
HR: hazard ratio;
95% CI: 95% confidence interval;
OS: overall survival;
PFS: progress-free survival.
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 is correlated with markedly better survival in CRC patients. In a fluorescence-labeled orthotopic mouse xenograft model, we confirmed that overexpression of RRM2B in non-metastatic 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 perform immunohistochemistry (IHC) 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 (Hazard ratio, HR=0.40; 95% CI 0.18-0.86, p=0.016). In the validation set, RRM2B was negatively related to tumor invasion (odds ratio, 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). Further, 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-IV tumors rather than earlier stage I-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.
Keywords: Ribonucleotide Reductase, colorectum, adenocarcinoma, survival, metastasis
Introduction

Ribonucleoside diphosphate reductase (RR) plays an essential role in converting ribonucleoside diphosphate (NDP) to 2'-deoxyribonucleoside diphosphate (dNDP) (1). In humans, one large subunit (M1) and two 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 two equivalent di-nuclear iron centers that stabilize the tyrosal 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 two RR small subunits. An in vitro study demonstrated 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 H$_2$O$_2$ 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 overexpressing the recombinant mouse RR subunit R2 (homologous to human RRM2) caused an increase in the membrane-associated Raf-1 expression (30%), MAPK-2 activity (70%) and Rac-1 activation (3-fold), remarkably elevating the metastatic potential of BALB/c 3T3 and NIH 3T3 cells (11).
overexpression increases cellular invasiveness and 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 induced and protects against mutagenesis under genotoxic stress (17). The disruption of the p53-RRM2B DNA repair pathway was associated with colon tumorigenesis in ulcerative colitis (18). When γ-irradiated, inhibiting the RRM2B expression by siRNA causes a three-fold increase of mutation rate in TK6 cells (19). Our previous studies revealed that RRM2B is negatively related to cancer cell invasion and colorectal cancer metastasis (20). It was also reported that inhibiting RRM2B significantly enhances the invasive potential of various human cancer cells such as the head and neck cancer cell line KB, HSC-3 and Ca9-22 and the prostate cancer cell line PC-3 (20, 21). Therefore, RRM2B may play an opposite role when compared to RRM2 in suppressing the aggressiveness of cancer cells.

According to these findings, it is implied that RRM2B may function as a protective factor to prevent cells from undergoing malignant transformation, cell invasion and metastasis. Yet some outcome studies did not show the protective effect of RRM2B for lung and esophageal cancers (22, 23). After analyzing the protein expression of RRM2B in paraffin-embedded tumor samples from 130 well-characterized non-small cell lung cancer (NSCLC) patients, the expression level of RRM2B had a limited impact on the survival of lung cancer patients ($P>0.05$)(24). Moreover, positive RRM2B expression significantly correlated with poor survivability ($p<0.05$)(22). The results from these human tissue studies seemed to contrast the findings from mechanism-based studies.
Intriguingly, it was recently reported that commercially available anti-RRM2B antibodies may not recognize target proteins selectively, indicative of their non-specifically binding nature (25). It prompted us to re-examine whether RRM2B is correlated with a better or worse prognosis.

To address whether RRM2B have invasion-suppressing ability on colorectal cancer (CRC), we first generated the fluorescence labeled RRM2B-overexpressing transfectant HCT-116/RRM2B. We have demonstrated that RRM2B significantly reduced the distant metastasis of CRC in a xenograft mouse model. In addition, we also developed and validated a new RRM2B antibody to determine the RRM2B expression level on tissue samples for 103 CRCs from City of Hope and 220 CRCs from Zhejiang University. We conducted an outcome study to further validate that RRM2B is significantly related to better survival of CRC, which is compatible to those findings based on in vitro cell and in vivo animal model experiments. These findings suggested that RRM2B might be a potential prognostic biomarker to predict outcome for CRCs, and also revealed that RR small subunits, RRM2B and RRM2, played opposite roles in tumor invasiveness.
Materials and Methods

Orthotopic xenograft mouse model construction

The study design is displayed in Fig. 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 (1mg/ml for selection and maintenance). Meanwhile, the cells were sorted by Flow Cytometry twice, based on 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 be also detected by fluorescence microscopy. About $5 \times 10^5$ cells of RRM2B overexpression transfectant or vector control were implanted into cecum of 8-10 weeks old female NSG mouse (NOD.Cg-Prkdc<sup>scid</sup> I<sup>2rg<sup>tm1Wjl</sup>/SzJ from Jackson Laboratories) to generate an orthotopic xenograft mouse model. The animals were euthanized three weeks after xenotransplantation. Tumor growth and metastasis were observed under fluorescence microscopy. In addition, all liver and lung tumor samples were collected for pathological examination to confirm our findings. Animal use and experiment protocol were approved by the Institutional Animal Care and Use Committee (IACUC) of City of Hope. Sample size estimation was based on nQuery 6.01 software. A sample size of six per group would be needed for 74% power with a one-sided, $\alpha$ of 0.05. The chi-square 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. (Santa Cruz, CA). Briefly, 2×10^5 cells were seeded per well in 6-well culture plates filled with 2ml antibiotic-free normal growth medium supplemented with FBS, then incubated at 37°C in a CO2 incubator for 24 hours. 7.2μl of 10 μM 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, then, replaced with normal cell culture medium. The inhibition of RRM2 and RRM2B was measured using qRT-PCR.

**In vitro invasion assay**

BD Matrigel™ invasion chambers were commercially available from BD Biosciences (San Jose, CA). In brief, the 8-mM porosity polycarbonate membrane was covered with 1 ml of medium that contained 1x10^5 cells per well. The plates were then incubated for 24 hours at 37°C in a 5% CO2 incubator. Media was then removed, and non-invading 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 (MTA) and multiple tissue board (MTB) 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, one slide includes 8-12 pieces of the tissue samples. Each piece was approximately 1 mm × 10mm. As for validation set, samples were re-assembled to construct multiple tissue arrays (MTAs). Each MTA contained a maximum of 64 piece sections and each piece of tissue was approximately...
0.8mm × 0.8 mm. In total 7 MTAs were stained at the same time to avoid bias yielded from different 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 MTBs 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 immunohistochemistry staining**

Since non-specific targeting of commercial RRM2B antibody was reported (25), we developed new RRM2B antibody for IHC staining. Recombinant human RRM2B peptide (Acetyl-PERPEAAGLDQDERSC-amide; amino acids 4-18), a commercially produced rabbit polyclonal antibody by Rockland Immunochemicals (Gilbertsville, PA) was used against human RRM2B in these studies. The synthetic human RRM2B peptide (Acetyl-PERPEAAGLDQDERSC-amide; amino acids 4-18) was injected to immunize New Zealand white rabbit. Specific anti-RRM2B 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 (1:1000). To validate the efficacy of RRM2B antibody, quantitative real-time 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 Supplement Fig. 1.

**Quantitative immunohistochemistry (IHC) assays**

To avoid biases, the standards and conditions of IHC for RRM2B expression determination were optimized on the training set (multiple tissue board, MTB) and validated on the validation set (multiple tissue array, MTA). Details of the de-paraffinization protocol and IHC were described in a previous publication (20). Briefly, after de-paraffinization, 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 hydrogen peroxide treatment, the array slides were incubated with polymer horseradish peroxidase labeled corresponding antibodies for 30 minutes. DAB (0.05 g DAB and 100 ml 30% H₂O₂ in 100ml 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 IHC; however some nuclear staining was also observed. RRM2B expression was quantified using a visual grading system based on 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 two 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 Supplement Fig.2, this is a retrospective study. The IRB protocols were reviewed and approved by City of Hope (COH) and the Second Affiliated Hospital of Zhejiang University (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 were received surgical treatment between 1980-1985 and 1999-2004 respectively. Inclusion criteria were: (i) CRC with pathological diagnosis; (ii) informed consent or waiver; and (iii) receipt of at least one follow-up. Exclusion criteria were: (i) lack of pathological 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 Supplement Table 1.

Careful chart review was conducted and pathoclinical data was 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, 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. 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 City of Hope. 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 two-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, Cary, NC) and GraphPad Prism 5.0 were used for statistical analysis and figure drawing. Group comparisons for continuous data were performed using t-tests for independent means or one-way analyses of variance. For categorical data, we employed chi-square analyses, Fisher’s exact tests, or binomial tests.
of proportions. Multivariate Logistic regression models were used to adjust for covariate effects on the odds ratio (OR). Kaplan-Meier analysis and COX hazard proportional model were applied to overall survival (OS) and progress-free survival (PFS) analysis. Multivariate analyses and stratification were applied to reduce the confounding effects impact on the estimation of OR and hazard ratio (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 the Materials and Methods (Fig. 1A). The vector pEGFP-N1 was used as a negative control. In Fig. 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 panel). The invasion assay demonstrated that the increase of RRM2B significantly decreased invasion ability from 34 (range 13-53) per view-field (10×) in HCT-116/vector to 6(range 4-8) per view field (10×) in HCT-116/RRM2B cells (t-test, $p=0.004$) (Fig. 1C, middle & right panels), 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 RRM2 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). Yet, inhibition of RRM2 by siRNA significantly decreased the invasion by these cells (Fig 1D&E). It was suggested that the expression level of RRM2B negatively correlates with the invasiveness of CRC cells.

**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 gram, range 0.20-0.99) was smaller than HCT-116/Vector (0.69 gram, range 0.25-0.61) at the Day 14. However, it was not statistical significant due to insufficient of sample size (4 mice per group) (Supplement Fig 3.). 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 six HCT-116/RRM2B xenograft mice were successfully created. For a pilot study, one HCT-116/vector mouse was euthanized on Day 14 to pre-observe metastasis. All others were euthanized on Day 21. The optical images of cecum, lung and liver are displayed in the left column in Fig. 2A & 2B. Under the fluorescence microscope, three of the five HCT-116/vector mice showed distinct green fluorescence in the local bowel and metastatic sites of peritoneum, mediastinum, lung and liver (Fig. 2A, left & middle columns). However, out of the six HCT-116/RRM2B mice, only one mouse suffered minor local invasion in bowel (Fig. 2B, upper panel, left & middle column). All HCT-116/RRM2B mice showed no GFP fluorescence in liver or lung (Fig. 2B, middle and lower panels, left & middle columns). Since 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 H&E staining results are shown (Fig 2A & 2B right column), which is consistent with the metastatic lesions observed on each organ using fluorescence microscopy. Furthermore, IHC was applied to confirm the RRM2B expression in tumor samples (Fig. 2A & 2B, corner). 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, \( p=0.06 \)).

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

The IHC was employed to determine the expression level of RRM2B in human samples. Since non-specific targeting of commercial antibody has been reported (25), we developed and validated a new specific RRM2B antibody for IHC staining. In Western blot, only one band close to 42 KDa could be visualized by selected RRM2B antibody in HCT-116/vector cells (Figure 1B), which represents endogenous RRM2B protein. To validate specificity of the anti-RRM2B antibody, a recombinant full-length RRM2B peptide was employed to block antigen-antibody interaction in the IHC study. In addition, a recombinant full-length RRM2 (a homologue of RRM2B) was used as non-specific blocking peptide. In Fig. 3A, *left*, RRM2B is dominant in normal colon epithelial cytoplasm. After application of 1\( \mu \)g/ml RRM2B peptide as a pre-blocker, 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 Fig. 3B. In Fig. 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); while, 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.

Based on the distribution of RRM2 expression, we stratified negative (-) and weakly positive (±) as RRM2B (low), and positive (+) and strong positive (++) as RRM2B (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, Supplement Table 2). 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 median follow-up time was 49 months and the longest was 99 months. By the end of the observation period, 54 patients died from colorectal cancer progression and related reasons, 71 had a local recurrence or distant metastasis.
To investigate the relevance between RRM2B and TNM stages, a multivariate non-conditional logistic analysis was performed. Results revealed that the adjusted odd ratios (OR) of RRM2B were 0.45 (95% CI 0.19-0.99, \( p=0.040 \)), 0.48 (95% CI 0.25-0.92, \( p=0.026 \)) and 0.53 (95% CI 0.15-1.94, \( p=0.289 \)) for tumor invasion, lymph node involvement, and distant metastasis, respectively (Table 1). RRM2, an oncofetal protein with 80% similarity to RRM2B, was employed as a positive control in the validation study. It indicated that RRM2 is significantly associated with the distant metastasis of CRCs (\( p=0.010 \)) (Supplement Table 3). It was suggested that RRM2B played opposite roles of RRM2 in the invasiveness and metastasis of CRCs.

To further investigate whether RRM2B is related to the survival of CRC patients, the Kaplan-Meier and COX analyses were conducted on the validation set. It illustrated that RRM2B is significantly associated with better survival of CRC patients (Log-rank \( p=0.032 \)) (Fig. 4A) but RRM2 is related to poor OS (Log-rank \( p=0.013 \)) (Fig. 4B). Meanwhile, COX hazard proportional analysis indicated that the hazard proportional ratios (HRs) of RRM2B and RRM2 for OS 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 & 4B). 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 co-factors. In Fig. 4C, the results from the analysis revealed that RRM2B, RRM2 and TNM stage indeed affected the overall survival of CRC patients significantly \( (p<0.05) \). 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 was 2.57 (95% CI 1.38-4.96, \( p = 0.001 \)), and TNM stage (III-IV vs 0-II) was 3.94 (95% CI 2.13-7.75, \( p < 0.001 \)). Nevertheless, \( \beta \)-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 the effects from chemotherapy or radiotherapy, we analyzed those participants \( (N = 139) \) without adjuvant therapies. For those patients, 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-IV than in stage 0-II (Fig 5A), which is consistent with our findings from the training set. Nevertheless, RRM2 was related to poor OS and PFS of CRC with either stage 0-II or stage III-IV (Fig. 5B). Meanwhile, the Fig 5 C & D further visualized that RRM2B is associated with better survival, but RRM2 with poor survival in CRC patients with stage III-IV.

In Fig. 5E, multivariate COX analysis indicated that RRM2B is associated with better OS and PFS in both colon and rectal cancer patients. In particularly, the HR of RRM2B for OS in colon cancer was 0.39 (95% CI 0.17-0.87, \( p = 0.023 \)). On other hand, RRM2 is
represented worse OS and PFS of colon and rectal cancer (Fig 5F). Notably, it reached to statistical significance in rectal cancer (HR of OS is 3.32, 95% CI 1.54-7.72, \( p=0.002 \)). The Kaplan-Meier analysis visualized that RRM2B is significantly associated with better OS of colon cancer patients (Log-rank \( p=0.018 \)) (Fig. 5G); and RRM2 is related to poor OS of rectal cancer significantly (Log-rank \( p=0.002<0.05 \)) (Fig. 5H).

The above findings suggested RRM2B appears related to better survival of CRC patients with later stage, while 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 & 1E). As shown in Fig. 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-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 Uramoto reported that RRM2B was related to poor prognosis of esophageal cancer or lung cancer patients (22, 24, 28). 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 non-specific 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 social-economic 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 (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 MEK2–MAPK and negatively regulates Ras/Raf-MEK-ERK activity in lung cancer cells (31). Previous studies revealed that recombinant wild type RRM2B lacks the ability to oxidize a reactive oxygen species (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 Supplement Fig. 4A. To investigate whether the invasion of cancer cells was related to the prooxidant 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 (Supplement Fig. 4B). As with increased capability on oxidizing carboxy-H2DCFDA, overexpression of
RRM2B (Y331F) and RRM2B (Y285F) also could significantly increase invasion ability in HeLa and HCT-116 cells (Supplement Fig. 4 C&D). Meanwhile, the expression of MMP-9, an invasion related protein, was also escalated in transfectants of RRM2B (Y331F) and RRM2B (Y285F), but not RRM2B (Wt) (Supplement Fig 4B). It is suggested that the different properties between RRM2B and RRM2 on regulating prooxidant status of cancer cells might be one of the important mechanisms involved in modulating invasiveness. Taken together, the role of RRM2B in DNA repair and regulation on prooxidant status might be, at least, part of the mechanisms to explain why RRM2B suppresses invasiveness in cancers (Supplement 5).

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 is clarified that RRM2B and RRM2 played opposing biological roles on regulating prooxidant 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 colorectal cancer. For most RR inhibitors, their low efficacy and side effects are caused by nonspecific targeting. Our previous and current studies demonstrated 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.
Acknowledgement:

Grant support: This project was partially supported by NIH/NCI R01 CA127541.

We thank Lucy Brown and her colleagues from her core facility for assisting with the flow cytometry analysis. We also appreciated Mariko Lee helping with the digital image capturing in the Microscope Core Lab, and Dr. Frank Un and Mansze Kong with their assistance on the English editing.
Reference

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

<table>
<thead>
<tr>
<th></th>
<th>RRM2B OR (95% CI)</th>
<th>Adjusted OR* (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within Muscuris Propria</td>
<td>11 : 43</td>
<td>0.51 (0.24-1.04)</td>
</tr>
<tr>
<td>Out Muscuris Propria</td>
<td>55 : 110</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29 : 86</td>
<td>0.61 (0.34-1.09)</td>
</tr>
<tr>
<td>Yes</td>
<td>37 : 67</td>
<td></td>
</tr>
<tr>
<td>Distant Metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>61 : 145</td>
<td>0.76 (0.25-2.55)</td>
</tr>
<tr>
<td>Yes</td>
<td>5 : 9</td>
<td></td>
</tr>
</tbody>
</table>

Note: all information about age, tumor location, 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 pathological report.

* OR: odd ratio, adjusted by sex, age at diagnosis, tumor location, and RRM2.
† p<0.05
Figure Legends

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 (1mg/ml). In addition, the transfectants were further sorted by Flow Cytometry based on GFP fluorescence. After that, the transfectants were injected into the cecum of NSG mice to generate a xenograft mouse model. After three weeks, the mice were euthanized for examination. B, The expression of RRM2B was examined by Western blot. C, After G418 selection and Flow Cytometry sorting, the transfectants were visualized by GPF fluorescence (left panel). 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 panels 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.

Figure 2. Overexpression of RRM2B reduces tumor invasion and metastasis ability in xenograft mouse model. The images of optical, fluorescence and H&E staining are displayed in the left middle and right columns respectively. Meanwhile, the images of
cecum (tumor), lung and liver are displayed in the upper, middle and lower panels, respectively in either (A) or (B). The IHC staining of RRM2B for tumors are displayed on the right upper corner of H&E images. A, Examination of tumor invasion and metastasis potential of HCT-116/vector in orthotopic xenograft mouse model. B, Determination of the malignancy of HCT-116/RRM2B in the orthotopic xenograft mouse model.

Figure 3. Validate efficiency of RRM2B antibody and optimize conditions of Immunohistochemistry (IHC) in training set. The rabbit against human RRM2B antibody (1:1000 dilute) was employed to conduct IHC staining. A, left: normal colon epithelium with staining of RRM2B antibody only; middle: RRM2B antibody pre-blocked by recombinant RRM2B (1μg/ml) peptide; right: RRM2B antibody pre-blocked by recombinant RRM2 (1μg/ml) peptide. B, The standard of IHC scores for RRM2B. C, left two columns: IHC staining for normal colon epithelium (left) and cancer tissue (right) from the same patient; Right two columns: IHC staining for primary colon cancer (left) and corresponding metastatic cancer (right). D, Kaplan-Meier analysis for OS with stage IV CRCs stratified by different RRM2B levels. E, Multivariate COX analysis for OS of CRCs with stage IV in training set. Detail analysis result was displayed on Supplement Table 2.

Figure 4. Expression of RRM2B/RRM2 and overall survival of CRC in validation set. The Kaplan-Meier curve plots for RRM2B and RRM2 are displayed on (A) and (B), respectively. C, multivariate COX analysis for OS of CRCs on validation set.
Multivariate COX proportional hazard analysis was conducted to evaluate hazard ratio of RRM2B for overall survival of CRCs. The HRs of RRM2/RRM2B were based on high-expression vs low expression; β-catenin was nuclear positive vs negative; TP53 was mutation vs wild type; TNM stage was stage III-IV vs 0-II; Tumor location was rectum vs colon, gender was male vs female; age was based on per unit changes. * p<0.05

**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 hazard ratio of RRM2B and RRM2. HRs were adjusted by sex and age at diagnosis. Stage IV CRCs were excluded in PFS analysis. HRs of RRM2 and RRM2B were based on high-expression vs low-expression. With stratified by TNM stages, the multivariate COX analyses for OS and PFS of RRM2B and RRM2 are shown in (A) and (B). The Kaplan-Meier analyses for RRM2B/RRM2 and OS of CRCs with stage III-IV are displayed in (C) and (D). The (E) displays the multivariate COX analyses for RRM2B and prognosis of CRCs stratified by tumor locations; and (F) shows RRM2 and prognosis of colon and rectal cancers. The Kaplan-Meier analyses are visualized on (G) and (H) for RRM2B and the OS of colon cancer and RRM2 and OS of rectal cancer, respectively. * Statistics significant on COX analysis, p<0.05.
Figure 1.

A Expression Plasmid Construct

HCT-116 Colon cancer Cell line
Gene transfection
Selected by GFP signal using flow cytometry
Sorted by GFP signal
Selected by G418 (1mg/ml)
Check Expression of RRM2B
Check Purity by Flow Cytometry
Inject 0.5 million cells into cecum of NSG mouse
Check cecum liver & lung by FL and Pathologists

B Seeding control
Fluorescence

C Invasive cancer cell
Optical
Fluorescence

D * p<0.05; compared with scramble siRNA

E Invasion assay

* p<0.05; compared with scramble siRNA

1- Scramble siRNA; 2- RRM2B siRNA; 3- RRM2 siRNA
Figure 2.

A. HCT-116/vector

<table>
<thead>
<tr>
<th>Optical</th>
<th>GFP-Fluorescence</th>
<th>H&amp; E Staining</th>
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<tbody>
<tr>
<td>Cecum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
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<tr>
<td>Liver</td>
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B. HCT-116/RRM2B

<table>
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<th>Optical</th>
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<td>Liver</td>
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Figure 3

A. Antibody against RRM2B only, pre-blocked by rRRM2B peptide, pre-blocked by rRRM2 peptide.

B. Negative (-), weak positive (±), positive (+), strong positive (++).

C. Normal colon, colon cancer, primary colon cancer, metastatic colon cancer.

D. Log-Rank P=0.038, HR=0.42 (95% CI 0.20-0.91). Median survival time: RRM2B(high) 14.5 months, RRM2B(low) 7.0 months.

E. Stage IV (n=40): RRM2B, Gender, Age, Location, Chemotherapy, Radiotherapy.

Cases at risk: RRM2B(low) 21, 4, 4, 1, 0, 0; RRM2B(high) 19, 6, 3, 2, 2, 1.
Figure 4

**RRM2B**

A

Log-rank p=0.032
HR=0.56 (95% CI 0.32-0.98)

RRM2B(low) n=66
RRM2B(high) n=154

Cases at risk
RRM2B(low) 66 51 34 17 7 1
RRM2B(high) 154 138 97 70 33 1

**RRM2**

B

Log-rank p=0.013
HR=2.07 (95% CI 1.19-3.27)

RRM2 (low) n=105
RRM2(high) n=115

Cases at risk
RRM2(low) 104 94 74 48 27 1
RRM2(high) 116 95 57 39 13 1

**C**

Multivariate COX analysis

- RRM2B
- RRM2
- β-Catenin
- TP53
- TNM stage
- Tumor location
- Gender
- Age

HR (range: 95% CI) (Log 2)
Figure 5

A

OS

RRM2B

PFS

Stage 0, I & II

Stage III & IV

Adjusted HR (Range: 95% CI)(Log 2)

0.125

0.25

0.5

1

2

4

8

B

OS

RRM2

PFS

Stage 0, I & II

Stage III & IV

Adjusted HR (Range: 95% CI)(Log 2)

0.125

0.25

0.5

1

2

4

8

C

Overall Survival

Stage III-IV

Log-rank p=0.05
HR=0.53 (95% CI 0.28-1.01)

RRM2B(low) n=37
RRM2B(high) n=69

Cases at risk
RRM2B(low) 37 25 12 7 2 1
RRM2B(high) 69 54 34 26 11 1

Time from date of surgery (Months)

D

Overall Survival

Stage III-IV

Log-rank p=0.05
HR=1.95 (95% CI 1.03-3.91)

RRM2(low) n=47
RRM2(high) n=59

Cases at risk
RRM2B(low) 47 39 23 17 9 1
RRM2B(high) 59 40 23 16 5 1

Time from date of surgery (Months)

E

OS

RRM2B

PFS

Colon

Rectum

Adjusted HR (Range: 95% CI)(Log 2)

0.125

0.25

0.5

1

2

4

8

F

OS

RRM2

PFS

Colon

Rectum

Adjusted HR (Range: 95% CI)(Log 2)

0.125

0.25

0.5

1

2

4

8

G

Overall Survival

Colon

Log-rank p=0.018
HR=0.39 (95% CI 0.17-0.87)

RRM2B(low) n=37
RRM2B(high) n=81

Cases at risk
RRM2B(low) 39 28 20 10 5 1
RRM2B(high) 81 75 56 38 16 2

Time from date of surgery (Months)

H

Overall Survival

Rectum

Log-rank p=0.002
HR=3.32 (95% CI 1.54-7.72)

RRM2(low) n=49
RRM2(high) n=51

Cases at risk
RRM2B(low) 49 47 37 26 16 1
RRM2B(high) 51 43 20 15 6 1

Time from date of surgery (Months)
Ribonucleotide reductase small subunit M2B prognoses better survival in colorectal cancer

Xiyong Liu, Lily Lai, Xiaochen Wang, et al.

Cancer Res Published OnlineFirst March 17, 2011.

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