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Original Paper

Ribonucleotide reductase small subunit M2 serves as a prognostic biomarker and predicts poor survival of colorectal cancers

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Abstract

The overexpression of RRM2 [RR (ribonucleotide reductase) small subunit M2] dramatically enhances the ability of the cancer cell to proliferate and to invade. To investigate further the relevance of RRM2 and CRCs (colorectal cancers), we correlated the expression of RRM2 with the clinical outcome of CRCs. A retrospective outcome study was conducted on CRCs collected from the COH [(City of Hope) National Medical Center, 217 cases] and ZJU (Zhejiang University, 220 cases). IHC (immunohistochemistry) was employed to determine the protein expression level of RRM2, and quantitative real-time PCR was employed to validate. Multivariate logistic analysis indicated that the adjusted ORs (odds ratios) of RRM2-high for distant metastases were 2.06 [95% CI (confidence interval), 1.01–4.30] and 5.89 (95% CI, 1.51–39.13) in the COH and ZJU sets respectively. The Kaplan–Meier analysis displayed that high expression of RRM2 had a negative impact on the OS (overall survival) and PFS (progress-free survival) of CRC in both sets significantly. The multivariate Cox analysis further demonstrated that HRs (hazard ratios) of RRM2-high for OS were 1.88 (95% CI, 1.03–3.36) and 2.06 (95% CI, 1.10–4.00) in the COH and ZJU sets respectively. Stratification analysis demonstrated that the HR of RRM2 dramatically increased to 12.22 (95% CI, 1.62–258.31) in the MMR (mismatch repair) gene-deficient subgroup in the COH set. Meanwhile, a real-time study demonstrated that down-regulation of RRM2 by siRNA (small interfering RNA) could significantly and specifically reduce the cell growth and adhesion ability in HT-29 and HCT-8 cells. Therefore RRM2 is an independent prognostic factor and predicts poor survival of CRCs. It is also a potential predictor for identifying good responders to chemotherapy for CRCs.

Key words: adenocarcinoma, colon, metastasis, prognostic biomarker, rectum, ribonucleotide reductase, survival

INTRODUCTION

CRC (colorectal cancer) accounts for approx. 10% of all cancer deaths in the United States [1]. Improvements in early detection and treatment of CRC in the last few decades have resulted in decreasing overall mortality due to CRC, from 30.7 per 100 000 in 1990 to 20.5 per 100 000 in 2006 [1]. However, the 5-year survival rate for patients with metastatic CRC remains approximately 10%

Abbreviations: 5-FU, fluorouracil; CI, confidence interval; COH, City of Hope; CRC, colorectal cancer; dNTP, deoxyribonucleoside triphosphate; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; IHC, immunohistochemistry; MLH1, mutL homologue 1; MMP, matrix metalloproteinase; MMR, mismatch repair; MTA, multiple tissue array; MTB, multiple tissue board; OR, odds ratio; OS, overall survival; PFS, progress-free survival; ROS, reactive oxygen species; RR, ribonucleotide reductase; RRM2, RR small subunit M2; siRNA, small interfering RNA; TNM, tumour node metastasis; TS, thymidylate synthase; ZJU, Zhejiang University.

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[2]. This high mortality is partially attributable to liver metastasis, which accounts for 40–50% of CRC patients. Therefore it is critical for CRC to develop reliable prognostic biomarkers with capability of predicting metastasis. There are numerous genes that have been reported to be associated with the tumorigenesis of CRC [3], but only few biomarkers are applied for clinical use in guiding adjuvant CRC treatment [4].

RR (ribonucleotide reductase) is a time-limited and unique enzyme that converts NDP (ribonucleoside diphosphate) to dNDP (2′-deoxyribonucleoside diphosphate) [5], which is essential for DNA replication and cell proliferation. Given the critical role of RR in cell growth, there has been considerable interest in RR as a therapeutic target for cancer chemotherapy. In humans, one large subunit (M1) and two small subunits (RRM2 and RRM2B) of RR have been identified [6,7]. The large subunit M1 (RRM1) contains substrate and allosteric effector sites that control RR holoenzyme activity and substrate specificity [8–10]. RRM1 has been associated with better prognosis in early stage non-small cell lung cancers [11]. The tumour suppression ability of RR small subunit M2B (RRM2B) has been demonstrated in our previous publications [12,13]. The other small subunit, RRM2 has an 80% similarity to RRM2B. Unlike RRM2B, RRM2 has been reported to be overexpressed in a cancerous section in comparison with adjacent normal CRC tissue [14,15]. It may have oncogenic effects including tumour development, metastasis and drug resistance [16,17]. Recently, RRM2 overexpression has been shown to increase cellular invasiveness and MMP-9 (matrix metalloproteinase-9) expression in human pancreas cancer cells [18]. Enhanced gene expression of MMP-9 is associated with increased tumour growth and metastasic spread of solid tumour malignancies [19]. In gastric cancer, increase in RRM2 has been reported to be associated with increased aggressiveness [20]. Therefore we hypothesized that the RRM2 might enhance the metastasis of cancers through modulating its proliferation and invasive ability. Nevertheless, it needs to be validated in a large scale population.

It was reported that the activity of RR (EC 1.17.4.1) in rats growing hepatoma was almost 7000-fold higher than that in the corresponding normal liver tissue [21]. Therefore RR is considered as one of the critical anticancer targets. The RR inhibitors had been widely used for the treatment of leukaemia and solid tumour [22]. However, the efficacy of RR inhibitors was limited by its drug resistance and side effects [23]. Mostly, the side effects are caused by non-specific targeting of those RR inhibitors. Unlike RRM2, the RR large subunit M1 (R1 in mouse) exhibits a malignancy-suppressing potential, and is related to the good survival rate in early stage lung cancer [11]. Of interest, another RR small subunit, RRM2B (p53R2), has similar biological features to RRM1 and functions in metastasis-suppression in CRC [13]. Therefore understanding the different biological roles of RR subunits in cancer aggressiveness might help us to develop specific and efficient RR inhibitors to avoid drug resistance and side effects. In the present study, we tested the proliferation and adhesion ability changes of colon cancer cells using specific siRNA (small interfering RNA). Meanwhile, we conducted an outcome study on 217 CRCs collected from COH (City of Hope) and validated our findings on 220 CRCs from ZJU (Zhejiang University).

MATERIALS AND METHODS

Patients

All CRC patients' collection was based on approved Institutional Review Board protocols, and was under the regulation of COH and ZJU respectively. Patients gave informed consent. For the COH set, we collected 217 assessable CRCs from participants who received surgical treatment at COH between 1980 and 2004. Meanwhile, we also collected 220 cases of assessable CRCs from the Second Affiliated Hospital of ZJU as the ZJU set from participants who had surgical operations between 1999 and 2004. Inclusion criteria were: (i) CRC primary cancer; (ii) CRC with pathological diagnosis; (iii) informed consent or waiver of consent; (iv) age 18 or older; and (v) receipt of at least one follow-up within 5 years. Exclusion criteria were: (i) failure to get consent; (ii) multiple-cancer patients; and (iii) lost follow-up. The demographic distribution of participants is described in Table 1. In the COH set, 88 cases had adjuvant chemotherapy, and 42 cases had radiotherapy. In the ZJU set, 79 cases had adjuvant chemotherapy and radiotherapy is not applicable. The 5-FU (fluorouracil)/leucovorin combination is the basic protocol for adjuvant chemotherapy at COH. The initial course of 5-FU (450 mg/m^2) was given intravenously daily for 5 days; after 3 weeks, maintenance therapy was started with weekly intravenous doses, 450 mg/m^2 for 48 weeks. Leucovorin (20 mg/m^2 for low-dose; and 500 mg/m^2 for high-dose) was added to each cycle. Meanwhile, other chemotherapeutic agents such as Irinotecan (camptosar, 125 mg/m^2, weekly) or oxaliplatin (85 mg/m^2) also might have been added to regimens. Based on the response of patients, the chemotherapeutic agents' selection and dose were adjusted. Xeloda (capcitabine, 1250 mg/m^2, twice daily) replaced 5-FU in chemotherapy regimens after 2004. The participants in the COH set included 182 Caucasians, five African–Americans, 13 Asians and 17 unknowns. All CRCs in the ZJU set were Chinese (Asian). All patients were followed up until June 2007 and details of their demographic and survival data were updated.

Outcome study design

All eligible CRC patients were identified using a cancer registry. Careful chart review was conducted and pathoclinical data were extracted. Variables assessed included age at diagnosis, gender, date of diagnosis, date of surgery, date and type of chemotherapy, date of radiotherapy, TNM (tumour node metastasis) stage, relapse/metastasis status, date of relapse/metastasis, and date 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 for error reduction.

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 approx. 80% power with a two-sided α of 0.05.

All patients were periodically followed for the date of recurrence and death. The follow-up period was calculated from the date of surgery until the date of last contact. Recurrence was defined as the time from remission to initial tumour recurrence. Metastasis or local recurrence was considered evidence of tumour

Table 1 Pathoclinical features of CRCs and the IHC score of RRM2

	COH set ($n=217$)			ZJU set ($n=220$)		
Parameter	**Cases**	**RRM2-high (n)***	***P* value**	**Cases**	**RRM2-high (n)***	***P* value**
Age (years)						
<40	8	5 (62.5%)		16	7 (43.8%)	
40–49	17	9 (52.9%)		35	22 (62.9%)	
50–59	49	22 (44.9%)		52	27 (51.9%)	
60–69	65	22 (33.9%)		60	26 (43.3%)	
70–79	58	22 (37.9%)		44	24 (54.6%)	
>80	20	6 (30.0%)	0.38	13	9 (69.2%)	0.35
Gender						
Male	111	44 (41.5%)		126	61 (48.4%)	
Female	106	42 (37.8%)	0.58	94	54 (57.5%)	0.19
Location of tumour						
Colon						
Proximal†	100	38 (38.0%)		62	28 (45.1%)	
Distal ‡	67	28 (41.8%)		57	36 (63.1%)	
Rectum	50	20 (40.0%)	0.88	100	51 (51.0%)	0.16
TNM stages						
I	17	5 (29.4%)		46	22 (47.8%)	
II	134	49 (36.6%)		68	34 (50.0%)	
III	23	9 (39.1%)		92	47 (51.1%)	
IV	42	23 (54.8%)	0.15	14	12 (85.7%)	0.06
Tumour invasion§						
Within serosa	187	73 (39.0%)		154	60 (39.0%)	
Adjacent organ	23	8 (34.8%)	0.69	65	34 (52.3%)	0.2
Lymph node (LN)§						
No LN involved	162	61 (37.7%)		115	57 (49.6%)	
One or more LN (+)	55	25 (45.5%)	0.31	104	57 (54.8%)	0.44
Distant metastasis						
No	175	63 (36.0%)		206	103 (50.0%)	
Yes	42	23 (54.8%)	0.03	14	12 (85.7%)	0.01
Tumour grade§						
Well differentiated	23	6 (26.1%)		–	–	–
Moderately differentiated	170	71 (41.8%)		–	–	–
Poorly differentiated	19	7 (36.8%)	0.47	–	–	–
Adjuvant chemotherapy						
No	129	40 (31.0%)		139	77 (55.4%)	
Yes	88	46 (52.3%)	<0.01	79	36 (45.6%)	0.16
Radiotherapy						
No	175	65 (37.1%)		–	–	–
Yes	42	21 (50.0%)	0.05	–	–	–

*RRM2-high includes RRM2 CY+ (cytoplasm+) or NU+ (nuclear positive).
†Proximal colon includes hepatic flexure, transverse, cecum, appendix, ascending and splenic flexure.
‡Distal colon includes descending and sigmoid of colon.
§The missing cases were not included in the analysis.

relapse. Only deaths from CRC were considered as the endpoint of disease-specific survival.

MTB (multiple tissue board) and MTA (multiple tissue array) construction

For samples of the COH set, all are FFPE (formalin-fixed paraffin-embedded) human colon and rectum tissue sections that were re-assembled to construct MTBs from samples collected from COH. Each MTB contained 8–12 pieces of sections, with each piece approx. 1 mm×10 mm. As for the validation set, samples were re-assembled to construct MTAs. Each MTA contained a maximum of 64 piece sections and each piece of tissue was approx. 0.8 mm×0.8 mm. The tumour blocks also contained both tumour and normal colorectal tissue samples as positive and negative controls for each IHC (immunohistochemistry) staining. The MTBs and MTAs were stored at room temperature (25 °C). To

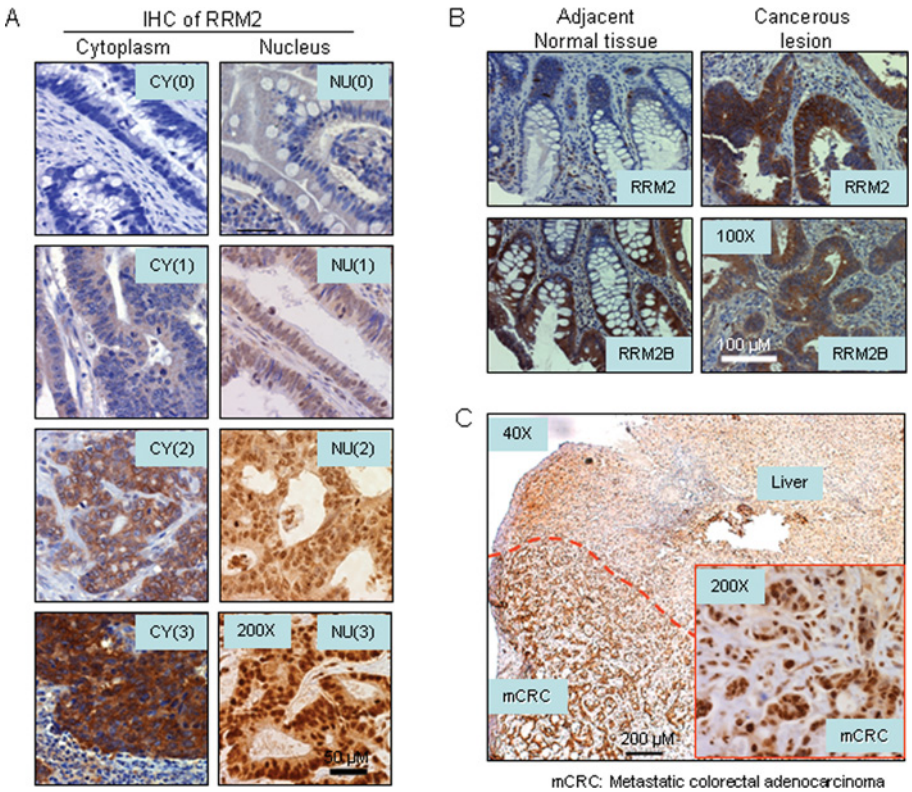

Figure 1 **Expression RRM2 at normal, primary adenocarcinoma and metastasis carcinoma of colon**
(**A**) The standard of IHC staining for RRM2 scoring: the left-hand panel displays the cytoplasmic RRM2 score of 0, 1, 2 and 3; and the right-hand panel shows the standard of nuclear RRM2 scoring. (**B**) The upper panel shows the increase of RRM2 in colon adenocarcinoma (right) in comparison with adjacent normal colon epithelium (left) with IHC staining. The RRM2B staining is displayed in the lower panel as a reference. (**C**) Nuclear accumulation of RRM2 in metastatic liver lesions colon adenocarcinoma.

determine whether the storage time affected the quality of IHC staining, we conducted a cross-tabulation between overall RRM2 score and year of diagnosis with the COH set. Both likelihood ratio ($P = 0.126$) and Pearson ($P = 0.372$) tests yielded results indicating that quality was not reduced due to increased storage time of samples.

Quantitative IHC staining and score

Protocol details of the deparaffinization and IHC is described in a previous publication [13]. A mouse monoclonal antibody against human RRM2, commercially produced by Convance using recombinant human RRM2 peptide [24], was used in FFPE samples in this study. To validate the efficacy of the RRM2 antibody, quantitative real-time PCR was used to correlate mRNA and protein expression levels in human tissue sections and cancer cell lines [24]. Since RRM2 has an 80% similarity with RRM2B (p53R2), the specificity of RRM2 antibody was validated by a parallel test on 217 CRC samples. All samples stain intensities of RRM2 and RRM2B antibodies were scored. The agreement analysis indicated $\kappa = -0.02$, and symmetry disagreement analysis showed Bowker $\chi^2 = 17.7$ ($P < 0.01$), suggesting that the selected monoclonal antibody against RRM2 does not cross-react with RRM2B.

The antibody for β-catenin (1:400 dilutions) IHC staining was commercially available from Santa Cruz Biotechnology Company. RRM2B antibody (1:100 dilutions) was generated and selected by our laboratory [12]. The antibody against hMLH1 (mutL homologue 1) (1:3 dilution), hMSH2 (1:50 dilution) and hMSH6 (1:50 dilution) were obtained from BD Pharmingen, Calbiochem and BD Transduction Laboratories respectively.

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. Based on the cytoplasm and nucleus score criterion of RRM2 displayed in Figure 1A, the subcellular localization (cytoplasm against nucleus), staining intensity (integrated absorption) and/or percentage of stained cells (the total area or percentage of cells positive) were scored for each image. Scores were organized by a rating scale as such: negative (0), weak positive (1), positive (2) and strong positive (3) (Figure 1A). Discrepancies in scores were resolved after joint review by the investigators.

Since previous studies suggested that RRM2 is a cytoplasmic protein, only cytoplasmic staining was considered in our previous study [2,13]. However, an intensive investigation revealed that RRM2 could only be seen on the nucleus in approximately 20% (44/217) of CRCs. The typical nuclear RRM2 (positive or

strong positive) could be seen in colon cancer cells from metastatic liver lesions. Further analysis also indicated that nuclear RRM2 significantly related to TNM stage (Supplementary Table S1 at http://www.clinsci.org/cs/124/cs1240567add.htm). Therefore we took both cytoplasmic and nuclear staining of RRM2 into consideration in this study. On the basis of the distribution of IHC staining, we considered cytoplasmic scores 2 and 3 as CY + (cytoplasm +), cytoplasmic scores 0 and 1 as CY − (cytoplasm −). For nuclear staining, nuclear scores 1, 2 or 3 were regarded as NU + (nucleus +) and nuclear score 0 as NU − (nucleus −). For overall RRM2 score, we defined RRM2-high as either RRM2 CY + or NU + , and RRM2-low as RRM2 CY − or NU − . The hMLH1, hMSH2 and hMSH6 were scored as positive and negative based on staining of nucleus (Supplementary Figure S1 at http://www.clinsci.org/cs/124/cs1240567add.htm).

The RRM2 siRNA and transfection assay

The RRM2, RRM2B and scrambled siRNA were purchased from Santa Cruz Biotechnology Inc. Briefly, 2×10^5 cells were seeded per well in six-well culture plates filled with 2 ml antibiotic-free normal growth medium supplemented with FBS (fetal bovine serum), and then incubated at 37 °C in a CO_2 incubator for 24 h. The siRNA (7.2 μl 10 μM) of RRM2, RRM2B or scrambled was transfected into HT-29 or HCT8 cells by using a transfection reagent (Lipofectamine™). Cells were incubated in the transfection medium for 5 h, then, replaced with the normal cell culture medium. The inhibition of RRM2 and RRM2B was measured using RT (reverse transcription)–PCR and Western blot.

qRT-PCR (quantitative RT–PCR)

The qRT-PCR protocol was described previously [26]. In summary, after microdissection, total RNA was extracted from tissue samples using the Qiagen RNeasy Mini Kit. cDNA was synthesized using the Superscript III first-strand cDNA synthesis kit (Invitrogen). qRT-PCR was carried out in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The PCR protocol was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The following primers were used: RRM2, 5′-GCGATTTAGCCAAGAAGTTCAGAT-3′ (forward) and 5′-CCCAGTCTGCCTTCTTCTTGA-3′ (reverse); β-actin, 5′-ATCTGGCACCACACCTTCTACAA-3′(forward) and 5′-GTACATGGC TGGGGTGTTGAAG- 3′ (reverse).

Real-time cell proliferation assay

The ACEA Biosciences RT-CES™ was used to monitor cell growth in real time [27]. This system uses microelectronic cell sensor arrays that are integrated into the bottom of microtitre plates. The electrode resistance was measured every 30 min. To quantify cell status based on the measured cell electrode impedance, the cell index (CI) parameter was derived according to the equation:

$$\mathrm{CI} = \max_{i=1,\ldots,N}\left(\frac{R\mathrm{cell}(fi)}{Rb(fi)} - 1\right)$$

where $R\mathrm{cell}(f)$ and $Rb(f)$ are the frequency-dependent electrode resistances (a component of impedance) in the presence or absence of cells, respectively. N is the number of the frequency points at which the impedance is measured. CI is a relative value to indicate how many cells attached to the electrodes. The slope of the CI curve reflects the growth speed of cells

Real-time cell adhesion assay

ACEA Biosciences E-plates® were coated with 5 μg/ml fibronectin (Sigma) before being applied in the adhesion assay. After transfection of siRNA and an incubation period of 48 h, approx. 2×10^4 corresponding cells in 100 μl of medium were seeded on to fibronectin-coated plates. The adhesion and spreading of cells were monitored using an ACEA Biosciences RT-CES™ system [28].

Data management and statistical analysis

The database was constructed by using MS-Access, and data were analysed using the JMP Statistical Discovery Software version 8.0 (SAS Institute). Group comparisons for continuous data were performed using Student t tests for independent means or one-way ANOVA. For categorical data, we employed χ^2 analyses, Fisher's exact tests or binomial tests of proportions. Multivariate logistic regression models were used to adjust for covariate effects on the OR (odds ratio). Kaplan–Meier analysis and Cox hazard proportional model were applied for OS (overall survival) and PFS (progress-free survival) analysis. In PFS analysis, patients with metastatic CRC not completely resected were excluded. Multivariate analyses and stratification were applied to reduce the confounders' impact on estimation of the OR and HR (hazard ratio). Missing data were coded and excluded from analyses.

RESULTS

Expression of RRM2 is associated with the progression of CRC

To determine the protein expression of RRM2 in the tissue specimens, we used IHC techniques on FFPE tissues. IHC of RRM2B, 80% similarity to RRM2, was employed as control in the same patient. Using the previously well-characterized RRM2 antibody, as described in our previous study [24], we localized the expression of RRM2 in clinically annotated colonic tissues and cancer from two separate patient cohorts. Although overall expression of RRM2 in normal colon tissue is low, RRM2 expression decreases with differentiation of the colonocyte along the crypt–villi axis in normal colon tissue [13]. It showed the opposite pattern of IHC staining for RRM2B (Figure 1B, left column). In addition, protein expression of RRM2 was dramatically higher in the colon cancer than in the adjacent normal colon, but RRM2B decreased slightly (Figure 1B, right column). High levels of RRM2 expression was noted in hepatic metastases from CRC. In particular, the increase in RRM2 expression in metastatic CRC localizes to the nucleus (Figure 1C). However, our previous study demonstrated that the RRM2B significantly decreases in liver metastatic CRC [12].

To investigate whether RRM2 expression was related to clinical factors such as TNM stage and histopathological features, uni- and multi-variate analyses were conducted on COH and ZJU datasets. Higher expression of RRM2 was significantly related to

Table 2 Non-conditional logistic analysis for RRM2 and stages of CRCs
OR indicates the relative risk of RRM2 (high against low). Adjusted OR, adjusted by age gender and tumour location. *$P < 0.05$ in the logistic analysis.

	COH set ($n = 217$)		ZJU set ($N = 220$)	
Parameter	**OR (95% CI)**	**Adjusted OR (95% CI)**	**OR (95% CI)***	**Adjusted OR (95% CI)**
Tumour invasion				
Within serosa	Reference	Reference	Reference	Reference
Adjacent organ	1.20 (0.50–3.11)	1.19 (0.48–3.13)	0.81 (0.44–1.50)	0.91 (0.47–1.74)
Lymph node				
Not involved	Reference	Reference	Reference	Reference
Involved	1.38 (0.74–2.56)	1.29 (0.68–2.44)	0.81 (0.47–1.37)	0.83 (0.48–1.45)
Distant metastasis				
No	Reference	Reference	Reference	Reference
Yes	2.15 (1.09–4.29)*	2.06 (1.01–4.30)*	5.99 (1.58–39.16)*	5.89 (1.51–39.13)*

distant metastases in both COH and ZJU CRC datasets on univariate analysis (Table 1), ($P < 0.05$). After adjusting for age and gender the non-conditional multivariate logistic regression validated RRM2 as a significant independent factor in increased risk of distant organ metastases in both datasets ($P < 0.05$). RRM2 was significantly associated with adjuvant chemotherapy in univariate analysis in the COH set ($P < 0.01$). Since RRM2 and chemotherapy were associated with aggressiveness of CRCs, we believed the statistical relationship between RRM2 and chemotherapy was caused by confounder effect. The adjusted OR were 2.06 (95% CI, 1.01–4.30) and 5.89 (95% CI, 1.51–39.13) in the COH and ZJU sets, respectively (Table 2). Among the clinical factors evaluated, including age, gender, tumour location, pathologic grade, tumour invasion and lymph node involvement, only RRM2 expression was a factor associated with metastases in CRC on multivariate analysis ($P > 0.05$).

RRM2 is a negative prognostic factor in CRC

We reasoned that if RRM2 expression increases association with distant metastases, then RRM2 may have an impact on the PFS and OS of CRC patients. Therefore further survival analysis was conducted to address this hypothesis. In the COH set, the median follow-up time of the participants was 61 months (inter-quartile range: 23–111 months); 75 patients died from CRC disease progression, eight died of other malignancies, and 32 died of unrelated reasons. During follow-up, 99 of the 217 patients developed local and/or distant recurrences. In ZJU set, the median follow-up time was 49 months (inter-quartile range, 25–71 months); 23 cases died from CRC disease progression and one died for an unrelated reason; 59 of the 220 cases developed local and/or distant recurrences.

Using Kaplan–Meier analysis, all participants of the COH set were stratified as: cytoplasmic negative against cytoplasmic positive (Figures 2A and 2B) or nuclear negative against nuclear positive (Figures 2C and 2D). It was visualized that the RRM2 on cytoplasm and nucleus were significantly related to poor OS of CRCs in the COH set (Figures 2A and 2C) (log-rank $P < 0.05$). In PFS analysis, both cytoplasm and nuclear RRM2 predicted poor survival, and the nuclear RRM2 positive reached statistical significance in COH set (Figure 2D). Similar results also could be seen in the ZJU set. Therefore the RRM2 signals from cytoplasm and nucleus were taken into consideration for further analysis. We re-categorized RRM2_Nu positive or RRM2_Cy positive as RRM2-high, and RRM2_Nu negative and RRM2_CY negative as RRM2-low. In the ZJU set, RRM2-high was significantly related to poor OS (Figure 2E) (log-rank $P = 0.013$), and eventually related to poor PFS (Figure 2F) (log-rank $P = 0.062$). The multivariate Cox analysis further indicated that RRM2 had a significant impact on OS of CRC (Figure 2G). A further multivariate Cox proportional hazard analysis suggested higher expression of RRM2 significantly increased the risk of death from CRC (Figure 2H), which was consistent with our finding in the COH set. The adjusted HRs of RRM2 for OS in COH and ZJU sets were 1.88 (95% CI, 1.03–3.36) and 2.06 (95% CI, 1.10–4.00) respectively. The validation of these findings through two independent data sets confirmed the prognostic potential of RRM2 expression in CRC.

Stratification analysis for RRM2 and prognosis of CRC

To avoid effects of confounders, the survival analyses for RRM2 are stratified by TNM stage and tumour location (Table 3). We determined the HRs, adjusted for age, sex, chemotherapy and radiotherapy, using multivariate Cox proportional hazard analysis. Although RRM2 expression was not associated with death and recurrence in the COH set ($P > 0.05$) when stratified by AJCC (American Joint Committee on Cancer) staging, RRM2 expression was associated with higher risk of death and recurrence in early (Stages I–II) and later (Stages III–IV) of CRC in the ZJU set. The expression of RRM2 increased the HR for death to 3.16 (95% CI, 1.40–7.50) in Stages III–IV CRCs and recurrence to 3.36 (95% CI, 1.03–12.00) in Stages 0–II CRC. In addition, RRM2 expression increased the HR of OS and PFS in both colon and rectal cancers of both the COH and ZJU sets when stratified by tumour location. In the COH set, RRM2 expression resulted in an increase in the HR of recurrence in colon cancer [HR = 2.25 (95% CI, 1.29–3.82)], especially in proximal colon cancer [HR = 2.35 (95% CI, 1.14–4.67)]. In the ZJU set, RRM2 significantly increased the risk of death in rectal cancer [HR = 3.34 (95% CI, 1.42–8.49)] and risk of recurrence in colon cancer [HR = 2.90 (95% CI, 1.13–7.93)].

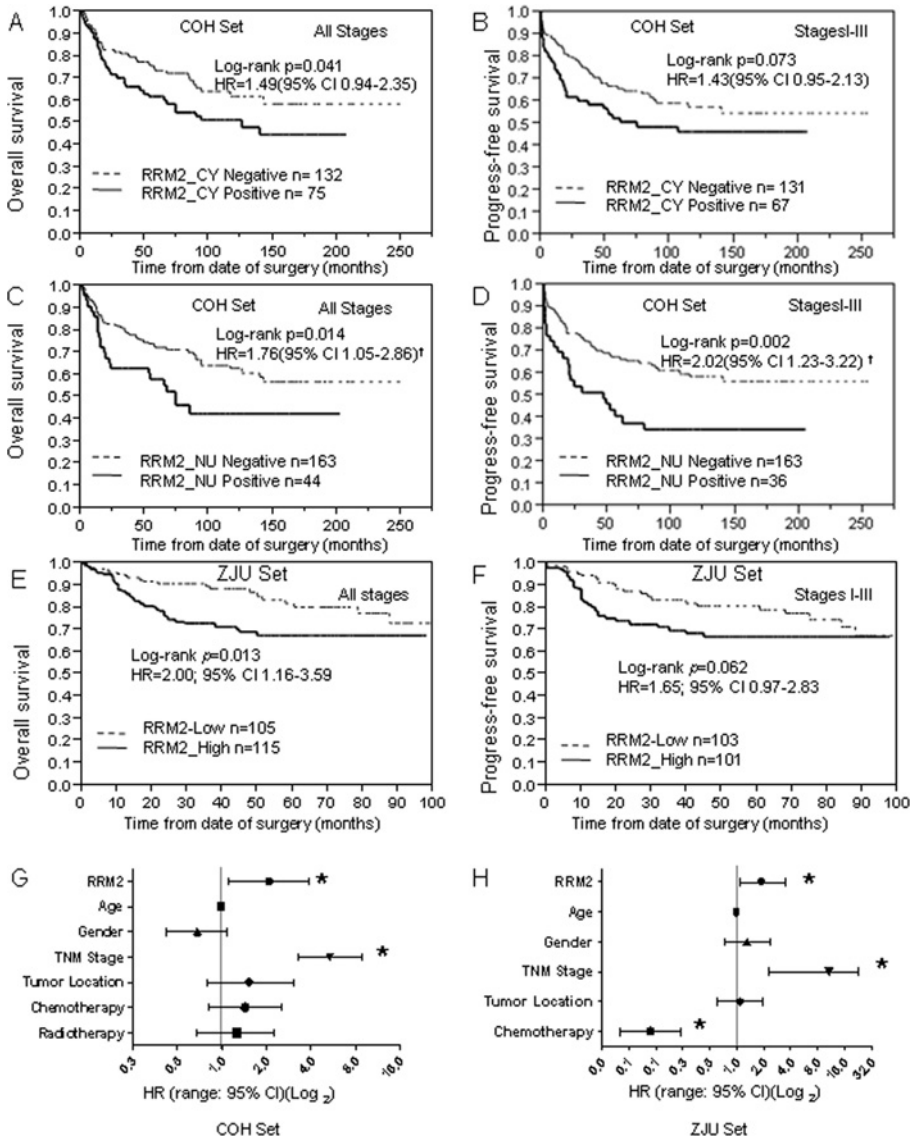

Figure 2 **Overexpression of RRM2 is associated with poor prognosis of CRCs**

The Kaplan–Meier analysis on RRM2 and survival of CRC in (**A–D**) the COH set and (**E** and **F**) for the ZJU set. For Kaplan–Meier analysis, the results of OS (**A**, **C** and **E**) and PSF (**B**, **D** and **F**) are shown. Analysis of cytoplasmic RRM2 (**A** and **B**) and nuclear RRM2 (**C** and **D**) and survival of CRCs from the COH set. (**E** and **F**) Overall RRM2 levels and outcome of CRC from the ZJU set. †$P < 0.05$ in a univariate analysis. Multivariate Cox analysis for OS of CRC in the COH (**G**) and ZJU (**H**) sets respectively. In Cox analysis, these factors included RRM2 (high against low), age (per unit), gender (male against female), TNM stage (stages III–IV against stages 0–II), tumour location (rectum compared with colon), chemotherapy (yes against no) and radiotherapy (yes against no). Radiotherapy is not applicable to the ZJU set and is not included. *$P < 0.05$ in the multivariate Cox model.

The antibodies against hMLH1, hMSH2 and hMSH6 were used to do IHC staining on CRC tissue samples. It was reported that malfunction of any MMR (mismatch repair) gene could cause the MMR defect [29]. Here, we defined at least one of above MMR proteins undetectable (negative) as MMR-deficient. In the COH set, 27 out of 118 CRCs (22.3%) with TNM stage II were regarded as MMR-deficient. The stratification analysis revealed that the HRs of RRM2-high for OS were 12.22 (95% CI, 1.62-256-8.31) and 1.27 (95% CI, 0.49–3.27) in MMR-deficient and MMR non-deficient subgroups, respectively (Table 3). The increased HR for PSF in the subgroup of MMR-deficient [HR = 4.93 (95% CI, 1.11–26.79)] also could be observed. In the ZJU set, 36 out of 195 CRCs (18.5%) were MMR-deficient. The increase in HR of RRM2-high for OS and PSF also could be seen, but it failed to reach statistical significance ($P > 0.05$) (Table 3). It was suggested that the RRM2 might potentially have an impact on poor survival of CRC more significantly in the MMR-deficient subgroup, but this needs further validation.

In Table 3, it is also indicated that the RRM2 had an impact on survival of CRC in subgroups either with or without chemotherapy, on both COH and ZJU sets. In the COH set, the high RRM2

Table 3 Stratification analysis for RRM2 and survival of CRCs
Note: multivariate Cox proportional hazard analysis was conducted to evaluate HR of RRM2 (high against low). HR was adjusted by sex and age. ‖$P < 0.05$ statistically significant by Cox analysis.

	COH set (n = 217)			ZJU set (*n* = 220)		
Parameter	***n***	**HR (95% CI) of OS**	**HR (95% CI) of PFS***	***n***	**HR (95% CI) of OS**	**HR (95% CI)of PFS***
All participants	217	1.88 (1.03–3.36)‖	2.17 (1.27–3.62)‖	218	2.06 (1.10–4.00)‖	1.73 (0.95–3.16)
TNM stages*						
Stages I and II	143	1.34 (0.45–3.56)	1.63 (0.68–3.62)	114	3.38 (0.94–13.50)	3.36 (1.03–12.00)‖
Stage III and IV	65	0.81 (0.38–1.73)	0.91 (0.70–1.17)	104	3.16 (1.40–7.50)‖	1.96 (0.90–4.39)
Tumour location						
Colon	166	2.18 (1.08–3.99)‖	2.25 (1.29–3.82)‖	118	2.20 (0.79–6.37)	2.90 (1.13–7.93)
Proximal†	99	2.31 (0.99–5.24)	2.35 (1.14–4.67)‖	62	1.63 (0.46–5.68)	1.74 (0.52–5.81)
Distal‡	67	2.28 (0.86–5.66)	2.10 (0.80–5.23)	56	1.71 (0.15–21.02)	7.34 (1.10–74.80)
Rectum	48	0.68 (0.27–1.49)	3.12 (0.30–30.22)	99	3.34 (1.42–8.49)‖	1.93 (0.82–4.62)
MMR gene§						
Deficient	27	12.22 (1.62–258.31)‖	4.93 (1.11–26.79)‖	36	7.39 (0.34–5783.88)	2.14 (0.32–21.16)
Non-deficient	91	1.27 (0.49–3.27)	1.75 (0.72–4.36)	159	1.73 (0.89–3.49)	1.34 (0.73–2.53)
Chemotherapy						
Yes	88	2.50 (1.09–5.60)‖	2.75 (1.25–5.88)‖	79	0.95 (0.55–1.56)	0.95 (0.62–1.42)
No	120	1.50 (0.58–3.60)	1.68 (0.77–3.42)	139	2.65 (1.08–7.11)‖	1.49 (0.63–3.78)

*The stage IV CRCs were excluded in PFS analysis.
†Proximal colon includes hepatic flexure, transverse, cecum, appendix, ascending and splenic flexure.
‡Distal colon includes descending and sigmoid of colon.
§MMR deficient: at least one of the MMR genes (hMLH1, hMSH2 and hMSH6) is not detectable in IHC staining. MMR genes were detected on 118 CRCs (stage II only) in the COH set and 195 CRCs in the ZJU set.

expression had a significant impact on the OS [HR = 2.50 (95% CI, 1.09–5.60)] and PSF [HR = 2.75 (95% CI, 1.25–5.88)] in the subgroup with chemotherapy. RRM2 expression increased the risk of death in CRC patients in the ZJU dataset and an increase in RRM2 was also related to the outcome of CRCs, and it had a significant impact on OS of CRC in the subgroup without chemotherapy in the ZJU set [HR = 2.65 (95% CI, 1.08–7.11)].

Therefore the above findings indicate that RRM2 is an independent factor having a negative impact on the OS and PSF of CRC in different TNM stages, tumour locations. Reasons for those that failed to reach statistical significance included insufficient samples size in the subgroups and differences in socioeconomic background.

siRNA inhibition of RRM2 decreases cell proliferation and adhesion in colon cancer cells

Based on the above findings, we hypothesized that inhibition of RRM2 expression level may not only decrease the proliferative ability but also reduce the metastatic potential of CRC. To test this hypothesis in a cell-based system, we attenuated RRM2 expression by using two short interfering RNA. These studies were conducted in two colon cancer cell lines, HCT-8 and HT-29 cells. The colon cancer cells were transfected with siRNA directed against RRM2. To ensure specificity of the siRNA, siRNA against RRM2B and scrambled siRNA were also transfected into cells as negative controls. After transfection, expression of RRM2 and RRM2B were reduced to 40 and 30%, respectively, in comparison with scrambled siRNA in HCT-8 cells (Figure 3A). Likewise, the siRNA transfection studies in HT-29 cells resulted in similar findings. The reduction of RRM2 or RRM2B by siRNA was also confirmed by Western blot analysis (Figure 3B). The real-time cell proliferation assay demonstrated that the slope of cell growth was significantly reduced by RRM2 siRNA in HT-29 as well as HCT-8 cells (Figure 3C). This phenomenon also could be seen in KB and PC3 cells [13,26,30].

Cell adhesion is another feature related to cancer metastasis. To investigate whether RRM2 plays a role in modulating cell adhesion in CRC, we developed a real-time cell adhesion assay based on the RT-CES® system. The CI was applied to indicate how many cells attached to the bottom of well. The adhesion and spreading of the cells were monitored continually every 3 min using the RT-CES system for a period of 1–8 h depending on the experiment. Since the doubling time of HCT-8 and HT-29 is more than 50 h, the cell growth would not likely affect adhesion ability in an 8 h observation period. In Figure 3(D), the adhesion ability was reduced to less 50% by RRM2 siRNA in comparison with scrambled siRNA in the HCT-8 cancer cell at a time point of 6 h. The p53R2 siRNA reduced slightly the adhesion of HCT-8 but not significantly.

DISCUSSION

The results of the present study demonstrated that expression of RRM2 is associated with progression of CRC (Figure 1). In particular, RRM2 expression is significantly related to distant organ metastasis in two clinical datasets, the COH set [OR = 2.06 (95% CI, 1.01–4.30)] and the ZJU set [OR = 5.89 (95% CI, 1.51–39.13)]. Kaplan–Meier analysis confirms that RRM2 expression has an negative impact on survival in patients with CRC

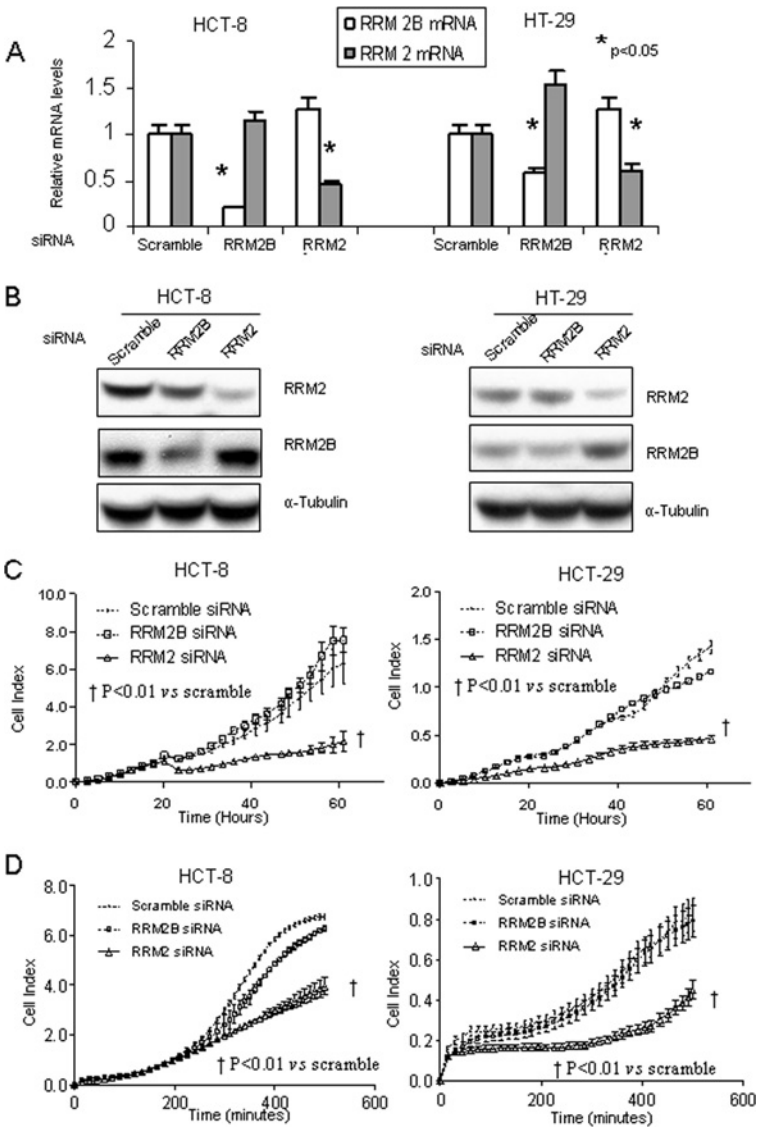

Figure 3 Inhibition of RRM2 by siRNA causes dynamic reduction of cell proliferation and invasion ability in colon cancer cells

siRNA was used to reduce RRM2 and RRM2B in HCT-8 and HT-29 cells. Approximately 1×10^5 cells/well were seeded in six-well plates. RRM2, RRM2B or scrambled siRNA, were transfected into HCT-8 and HT-29 cell by using a transfection reagent. After an incubation period of 48 h, total RNA and lysate from corresponding cells was extracted and used to measure mRNA and protein level, respectively. (**A**) mRNA levels of RRM2 and RRM2B determined by qRT-PCR. Each sample was measured for three times. $*P < 0.05$ compared with the corresponding scrambled siRNA sample. (**B**) Inhibition of RRM2 and RRM2B protein levels by siRNA was examined by Western blot. (**C**) After transfection for 24 h, approximately 5000 cells were seeded into the wells of an RT-ACE plate. Triplicate experiments were conducted for each sample. The real-time cell growth of HCT-8 and HT-29 cells was measured using an ACEA Biosciences real-time growth monitor. (**D**) The ACEA Biosciences plate was pre-coated with 5 μg/ml fibronectin. Approximately 2×10^4 cells were seed into wells in triplicate. Inhibition of adhesion by RRM2 siRNA in HCT-8 and HT-29 cells was determined using an ACEA Biosciences real-time monitor.

(Figure 2). In multivariate Cox analysis, the expression of RRM2 significantly increased the risk of death from CRC [COH set, HR = 1.88 (95% CI, 1.03–3.36); ZJU set, HR = 2.06 (95% CI, 1.10–4.00)] and is an independent poor prognostic factor of OS and DFS in multivariate analysis. It was reported that the mRNA expression level of RRM2 predicts poor survival in patients with lung adenocarcinoma who undergo treatment with docetaxel and gemcitabine [31]. These findings were confirmed in two sets of CRC patients with different race and socio-economic background.

Previous studies suggested that human RRM2 localized only to the cytoplasm forming the holoenzyme with the large and small subunits of RR [5]. Using a well-validated antibody directed against RRM2 and used previously in other publications [12,24],

we visualized RRM2 expression in the nucleus in approximately 20% of CRC. It was demonstrated that dNTPs (deoxyribonucleoside triphosphates) may be synthesized in the cytoplasm before diffusing into the nucleus for DNA incorporation [32]. However, the cell fractionation and fluorescence labelling studies demonstrated that RRM2 may translocate into the nucleus under conditions of genotoxic stress or cell proliferation in KB and PC-3 cancer cell lines [33–35]. In budding yeast, the small subunits RNR2 and RNR4 redistribute from the nucleus to the cytoplasm under hydroxyurea and methyl methanesulfonate treatments [36]. However, the RR activity of cancer cell nuclear section could not be detected in a cell fractionation study, which indicated that the biological role of RRM2 nuclear localization is largely unknown. Based on the above evidence, we take both cytoplasmic and nuclear RRM2 into consideration in this study and demonstrate that both cytoplasmic and nuclear expression of RRM2 is significantly related to TNM stage and poor prognosis of CRC.

Up-regulation of RRM2 increases RR activity, which provides extra dNTPs in cancer cells. To keep the integrity of genome, the cyclin F could bind to RRM2 at G_2-phase and cause the RRM2 degradation to reduce the dNTP pool [37]. Expansion or imbalance in the dNTPs pool would increase the DNA mutation rates [5], which could potentially enhance the malignant potential of cancer cells. Our data also showed that HR of RRM2 markedly increased in MMR-deficient subgroup (Table 3). On other hand, an *in vitro* study demonstrated that recombinant RRM2 was able to oxidize a ROS (reactive oxygen species) indicator carboxy-H2DCFD and generate ROS in the presence of mitochondrial extract [38]. Overexpression of RRM2 by transfection of RRM2 in human KB cells significantly reduces peroxide removal capability [38]. The RRM2 increases oxidized ROS, which may activate the signalling pathway of Ras/Raf in cancers [39]. On other hand, RRM2 also could be induced by oncogenic KRAS [40]. Gene transfection studies reveal that the recombinant mouse RRR2 (homologous with human RRM2) overexpression causes an increase in membrane-associated Raf-1 expression (30%), MAPK-2 (mitogen-activated protein kinase-2) activity (70%) and Rac-1 activation (3-fold), resulting in markedly elevated metastatic potential in BALB/c 3T3 and NIH 3T3 cells [17]. Further observation has shown that the R2 protein (RRM2) is not only a rate-limiting component for ribonucleotide reduction, but is also capable of acting in cooperation with a variety of oncogenes to promote transformation and tumorigenesis [17]. Overexpression of RRM2 may markedly increase the MMP-9 expression and enhance the cell invasion ability in cancer cell lines [18]. Furthermore, overexpression of RRM2 in KB and PC-3 cells could induce the migration ability of HUVECs (human umbilical vein endothelial cells) [13]. Therefore dNTPs pool expansion, acceleration of cell proliferation, and improvement of metastasis ability may partly explain why RRM2 increases the aggressiveness and causes poor survival in CRCs.

On the basis of systematic reviews and literature search results regarding biomarkers for CRC prognosis, many genes have been shown to be prognostic biomarkers of CRC. Multi-gene signatures are being validated for use as prognostic tools. MSI (microsatellite instability) may help to select patients with stage II CRCs who would not benefit from adjuvant chemotherapy [41,42]. The updated data from recent studies revealed that mutations of KRAS/BRAF are highly predictive of non-response to EGFR (epidermal growth factor receptor) inhibitors for advanced stage CRC [43,44]. Since TS (thymidylate synthase) is the target of 5-FU, the benefit of 5-FU chemotherapy could be yielded in TS high-expression, rather than TS low-expression CRCs [45]. However, the above biomarkers are not sufficient to assure clinical outcome of CRCs. Therefore we need to discover further molecular biomarkers of impairment in this or other signalling pathways to identify responders more specifically for CRC patients. However, there remains the need to identify definitive predictive biomarkers for use in patients to determine who would benefit from additional therapy.

Our RRM2 inhibition study revealed that anti-RRM2 siRNA had significant anti-proliferative and adhesive ability in CRC cells (Figure 3), as well as invasion ability [12]. This finding has been confirmed in an animal study [46]. A previous study also demonstrated that the anchorage-independent growth of HCT116 could be impaired by reduction of RRM2 protein [40]. Moreover, silencing of RRM2 by siRNA could enhance the DNA damage and markedly sensitize HCT-116 cells to camptothecin, a topoisomerase I inhibitor [47]. However, inhibition of RRM2B (which has 80% similarity in peptide sequence to RRM2) by siRNA seems to enhance the invasion ability in colon cancer cells [12]. These findings implied that developing novel specific RRM2 inhibitors might not only inhibit cancer cell proliferation but also reduce metastasis potential of cancers.

Taken together, RRM2 is an independent prognostic biomarker and predictive biomarker in CRC. Given the mechanistic contribution of RRM2 to proliferation and to invasion, RRM2 has the potential to not only assist in the determination of outcome in patients, but may be a therapeutic target. There are ongoing studies evaluating RR as a therapeutic target. As such, further studies in prospectively collected datasets are needed to validate these findings and characterize this potential fit-for-purpose biomarker.

CLINICAL PERSPECTIVES

- Ribonucleoside diphosphate reductase (RR) is an important target for cancer therapy because it is a time-limited enzyme in providing dNTP for DNA synthesis.
- In the present study, we found that the high expression of RRM2 was associated with distant metastasis and poor survival in two independent CRC patient sets. Furthermore, an *in vitro* study revealed that specifically inhibiting RRM2 expression, but not RRM2B, significantly reduced cell proliferation, invasion and adhesion in colon cancer cell lines.
- Therefore RRM2 is a potential prognostic biomarker predicting metastasis and poor outcome of CRC, which may assist in optimizing protocols for cancer therapy in the future. In addition, understanding the biological functions of the RR subunits might aid the development of novel specific RR inhibitors for cancer treatment.

AUTHOR CONTRIBUTION

Xiyong Liu conceived the study design, performed the data collection and analysis, and wrote the paper; Hang Zhang conducted the

experiments and collected the data; Lily Lai created the CRC database at the COH and edited the paper. Xiaochen Wang collected tissue samples at the ZJU; Sofia Loera optimized the condition of IHC and conducted IHC staining for tissue samples; Lijun Xue prepared the tissue samples and validated the antibodies for IHC. Huiyin He conducted the IHC staining; Keqiang Zhang, Shuya Hu and Yasheng Huang figured out *in vitro* experiments. Rebecca Nelson conducted the statistical analysis; Bingsen Zhou worked on data collection; Lun Zhou provided follow-up data; Peiguo Chu scored the IHC results; Suzhan Zhang and Shu Zheng provided the tissue samples and collected the pathoclinical information from the patients. Yun Yen functioned as a leader in study design, analysed the data and wrote the paper.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Ribonucleotide reductase small subunit M2 serves as a prognostic biomarker and predicts poor survival of colorectal cancers

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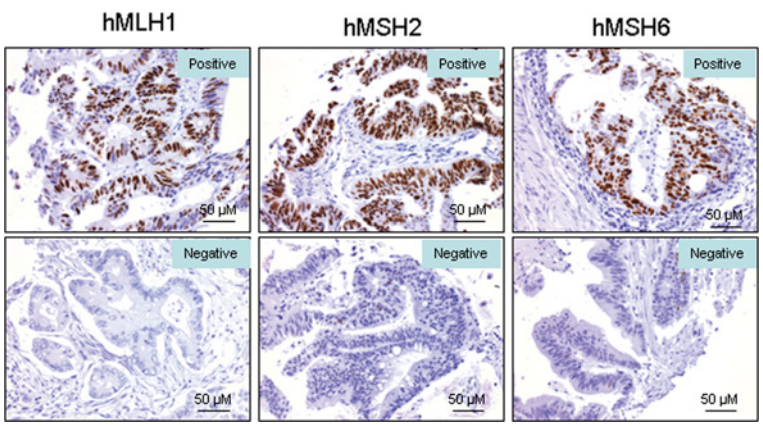

Figure S1 **IHC staining of mismatch repair genes' protein for CRCs**
Detail protocol of IHC was described in the Materials and methods section of the main paper. The IHC results of hMLH1, hMSH2 and hMSH6 are shown in left-hand, middle and right-hand panels respectively. The upper panel is the positive staining, and the lower is the negative staining.

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