

Emerging roles of the ribonucleotide reductase M2 in colorectal cancer and ultraviolet-induced DNA damage repair

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Annexin V/PI apoptosis kit. The motility and invasion of CRC cells were assessed by the Transwell chamber assay. Cells were irradiated with a 254 nm UV-C lamp to detect the UV sensitivity after RRM2 depletion.

RESULTS: Immunohistochemical staining revealed elevated RRM2 levels in CRC tissues. RRM2 overexpression was positively correlated with invasion depth ($P < 0.05$), poorly differentiated type ($P = 0.0051$), and tumor node metastasis stage ($P = 0.0015$). The expression of RRM2 in HCT116 cells was downregulated after transfection, and HCT116 cell proliferation was obviously suppressed compared to control groups ($P < 0.05$). In the invasion test, the number of cells that passed through the chambers in the RRM2-siRNA group was 81 ± 3 , which was lower than that in the negative control (289 ± 7) and blank control groups (301 ± 7.2). These differences were statistically significant ($P < 0.01$). Our data suggest that RRM2 overexpression may be associated with CRC progression. RRM2 silencing by siRNA may inhibit the hyperplasia and invasiveness of CRC cells, suggesting that RRM2 may play an important role in the infiltration and metastasis of CRC, which is a potential therapeutic strategy in CRC. In addition, RRM2 depletion increased UV sensitivity.

CONCLUSION: These findings suggest that RRM2 may be a facilitating factor in colorectal tumorigenesis and UV-induced DNA damage repair.

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Key words: Ribonucleotide reductase M2; Colorectal cancers; Tissue microarray; Ultraviolet irradiation; Carcinogenesis; Metabolic genes

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Abstract

AIM: To investigate the roles of the ribonucleotide reductase M2 (RRM2) subunit in colorectal cancer (CRC) and ultraviolet (UV)-induced DNA damage repair.

METHODS: Immunohistochemical staining of tissue microarray was performed to detect the expression of RRM2. Seven CRC cell lines were cultured and three human colon cancer cell lines, i.e., HCT116, SW480 and SW620, were used. Reverse transcription polymerase chain reaction and Western blotting were performed to determine the mRNA and protein expression levels of RRM2, respectively. Cell proliferation assay, cell cycle analysis were performed. Cell apoptosis was evaluated by double staining with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI) using

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INTRODUCTION

Colorectal cancer (CRC) is the leading cause of cancer-related death worldwide. In addition, the incidence and mortality rates of CRC are on the rise^[1]. CRC develops through a specific series of events, from the transformation of normal colonic epithelium to an adenomatous intermediate, and then ultimately adenocarcinoma^[2]; however, the molecular mechanism underlying CRC metastasis is still not fully understood. With evolving insights into the biology of colorectal neoplasia and advances in assay technology, molecular tests are now being developed as accurate, user friendly, safe, and affordable screening tools^[3]. For the accurate diagnosis and adequate treatment of CRC, the identification and understanding of the molecules responsible for cancer progression are critical.

Given that all cancer cells rely on changes in metabolism to support their growth and survival, targeting metabolism has the potential to affect cancers arising from many different tissues. Ribonucleotide reductase (RR) is one of these target metabolic enzymes for cancer therapy. RR transforms RNA building blocks to DNA building blocks by catalyzing the substitution of the 2' OH-group of a ribonucleotide with a hydrogen by a mechanism involving protein radicals^[4]. In humans, bacteria, and yeast, RR is a highly regulated enzyme in the deoxyribonucleotide synthesis pathway^[5]. Human RR is an enzymatic complex that consists of two nonidentical subunits, M1 and M2. M2 has two alternative subunits; namely, ribonucleotide reductase M2 (RRM2), which is involved in DNA replication (the focus of this paper), and p53R2, which is induced by p53 and is involved in supplying dNTPs for mitochondrial DNA replication and repair^[6]. In addition, RRM2 is the limiting factor of RR enzymatic complex catalytic activity, which is specifically expressed in the S phase^[7,8]. Therefore, RR plays an important role in the regulation of cell proliferation^[9-11]. *In vitro* studies have shown that aggressive tumor proliferation results in overamplification of RR^[12,13]. In some types of cancer, elevated RRM2 levels correlate with chemoresistance^[14], cellular invasiveness^[15], and poor patient outcome^[16], which suggests that RRM2 may contribute to malignant progression, and thus be a potential therapeutic target. Recent findings have established that p53R2 suppresses the invasiveness of cancer cells, and its expression is associated with a better survival prognosis for CRC patients^[17]; however, the function of RRM2 in CRC is unclear. Here, we demonstrate that RRM2 may play an

important role in the development of CRC and may contribute to the response to ultraviolet (UV) irradiation.

MATERIALS AND METHODS

Patients, tissue microarray and histological analysis

This study consisted of a tissue microarray (TMA, Shanghai Outdo Biotech, National Engineering Center for Biochip at Shanghai, China) containing 56 CRC tissues and 56 adjacent normal mucosa, which were laparoscopically resected at Shanghai Minimal Invasive Surgery Centre (Shanghai Jiao Tong University School of Medicine) between 2007 and 2009. In addition, another 78 specimens of CRC tissues were laparoscopic resected between 2011 and 2012. None of the patients received preoperative treatment such as radiation or chemotherapy. All research protocols in the present study were approved by the Institutional Review Board. Staging of the tumors was performed according to the tumor node metastasis classification updated by the American Joint Commission on Cancer and the World Health Organization^[18]. Pathological diagnoses were verified by two different pathologists. TMA blocks (5 μ mol/L) were sectioned. Preparation of sections from paraffin blocks was performed as previously described^[19]. Immunohistochemical and immunocytochemical analysis of RRM2 was performed with an anti-RRM2 antibody [R2 (E-16): SC-10846] (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States). Relative RRM2 expression and subcellular location were classified into 5 groups based on the intensity of RRM2 staining and its distribution between the cytoplasm and nucleus.

Cell lines and culture conditions

Seven human CRC cell lines were purchased from American Type Culture Collection (Manassas, VA, United States). The CRC cell lines, HCT116 and HT-29, were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, and nonessential amino acids. SW1116, SW620, SW480, LoVo and Colo205 cells were maintained in the same medium, except that McCoy's 5A medium was substituted with RPMI 1640 medium (Sigma Aldrich, St Louis, MO, United States). Cells were maintained at 37 °C in a 5% CO₂ incubator, and were grown according to standard protocols.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Reverse transcription was performed with oligo dT primers. Real-time polymerase chain reaction (PCR) was carried out in an Applied Biosystems 7500 System with Power SYBR Green PCR Master Mix (2 \times , Applied Biosystems, Warrington, United Kingdom). Relative levels of gene expression were determined with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control.

RNA interference

Transfection of cells with siRNAs targeting RRM2 was

carried out by mixing the siRNAs with diluted Lipofectamine™ 2000 at a final siRNA concentration of 20 nmol/L. The target sequences for siRNAs were as follows: 5'-AAACCCGAGGAGAGAUUUTT-3', 5'-GGAGC-GAUUUAGCCAAGAATT-3' and 5'-GCACUCU-AAUGAAGCAAUATT-3'. siRNAs targeting RRM2 and a negative control siRNA (5'-UUCUCCGAACGUGUCAC-GUTT-3') were purchased from Genepharma (Shanghai, China). Each experiment was performed in triplicate.

Lentiviral shRNA vector

The following sets of oligonucleotides were used to knockdown RRM2 expression; shRRM2-sense: CACCG GAGCGATTTAGCCAAGAAGTCACCACTTCTTGG CTAAATCGCTCC and shRRM2-antisense: AAAAGG AGCGATTTAGCCAAGAAGTGTTGACTTCTTGG CTAAATCGCTCC. Each set of oligonucleotides were annealed and cloned into pGPH1/Neo (Genepharma, Shanghai, China).

Western blot analysis

An anti-RRM2 antibody (0.2 µg/mL) or goat polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology; 0.2 µg/mL) was used as the primary antibody. Seven human CRC cell lines were resuspended in ice-cold NP-40 lysis buffer, and cell extracts from the colorectal mucosa were prepared by scraping mucosal epithelial cells into ice-cold NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EGTA, and 1% NP-40) containing protease inhibitors. The isolated protein was quantified by a commercially available modified Bradford assay (Bio-Rad Laboratories, Hercules, CA, United States).

Cell viability assay

Cells were seeded at 2.5×10^3 cells per well in 96-well plates and cultured for 120 h. Cells were counted using the CASY TT Cell Counter (Roche Diagnostics, Indianapolis, United States). Cell viability was determined by adding 10 µL of Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) to each well at 37 °C in a 5% CO₂ incubator. Each experiment was performed three times. 96-well plates were read using an Epoch Microplate Spectrophotometer (BioTek, Winooski, United States) with Gen5™ Microplate Data Analysis Software included.

Invasion assays

Motility and invasion capabilities *in vitro* were measured in a transwell chamber assay (Becton Dickinson Labware, Bedford, MA, United States). HCT116 cells (2.5×10^5) were seeded in McCoy's 5A medium without FBS, and then plated on each 8.0 µmol/L pore size membrane insert in 24-well plates. McCoy's 5A medium plus 20% FBS was plated in the bottom wells as chemoattractants. After 24 h, cells on the top side of the inserts were removed with a cotton swab. Cells that had migrated to the underside of the inserts were stained with 0.05% crystal violet for 1 h before rinsing with phosphate buffered saline (PBS) for 20 min. Cells on each insert were counted at a $20 \times$

magnification in 10 random fields of view. The invasion assay was done in the same way except that the 8.0-µmol/L pore size membrane insert was coated with Matrigel that was diluted in McCoy's 5A medium (1:8 dilution). The results were expressed as mean \pm SD.

Flow cytometric analysis

HCT116 cells after different treatments were rinsed twice with saline PBS. Cells were dissociated, followed by the addition of 5 µL Annexin V-FITC. Cells were then gently vortexed and incubated for 15 min at 4–8 °C in the dark. Propidium iodide (PI, 10 µL) was added to the tube for another 5 min at 4–8 °C in the dark. The labeled cells were analyzed using the BD FACS Vantage System (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) in accordance with the manufacturer's protocol. Gating was implemented on the basis of the staining profiles of the negative control.

UV irradiation

Cells were irradiated with a 254 nm UV-C lamp (UVP Inc., Upland, CA), and doses were measured with a UVX radiometer (Upland).

Statistical analysis

All continuous values were expressed as mean \pm SD and all experiments were repeated three times. The results were subjected to a nonparametric Mann-Whitney *U* test. A paired Student's test was also used to analyze the intra-group differences. All statistical analyses were done using Stat View 5.0 for Windows (SAS Institute Inc., Cary, NC, United States). Fisher's exact test and Cochran-Armitage test were applied to analyze the relationship between RRM2 immunoreactivity and clinicopathological features. Student's *t*-test was also used to test differences in cell viability assays. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Overexpression of RRM2 in CRC tissues and clinicopathologic significance of RRM2 expression in CRC patients

To examine RRM2 protein expression in normal mucosa and CRC tissues, we performed hRRM2 immunohistochemistry on the TMA. The normal human colon exhibits occasional RRM2⁺ cells located at the base of the crypt (six cases). In comparison, 38 of 56 tumor samples exhibited a substantial increase in overall RRM2 expression and in the number of RRM2⁺ cells. In the majority of these tumors, RRM2 was present in the cytoplasm (Figure 1). We set the cutoff point of RRM2 overexpression as 10%, because this is obviously higher than the positivity in normal colorectal mucosa, and because it was easy to determine positive or negative expression with this simple cutoff value. Among the 56 CRC tissues included in the TMA, RRM2 overexpression ($\geq 10\%$ cancer cells stained) was observed in 38 cases (67.9%). The

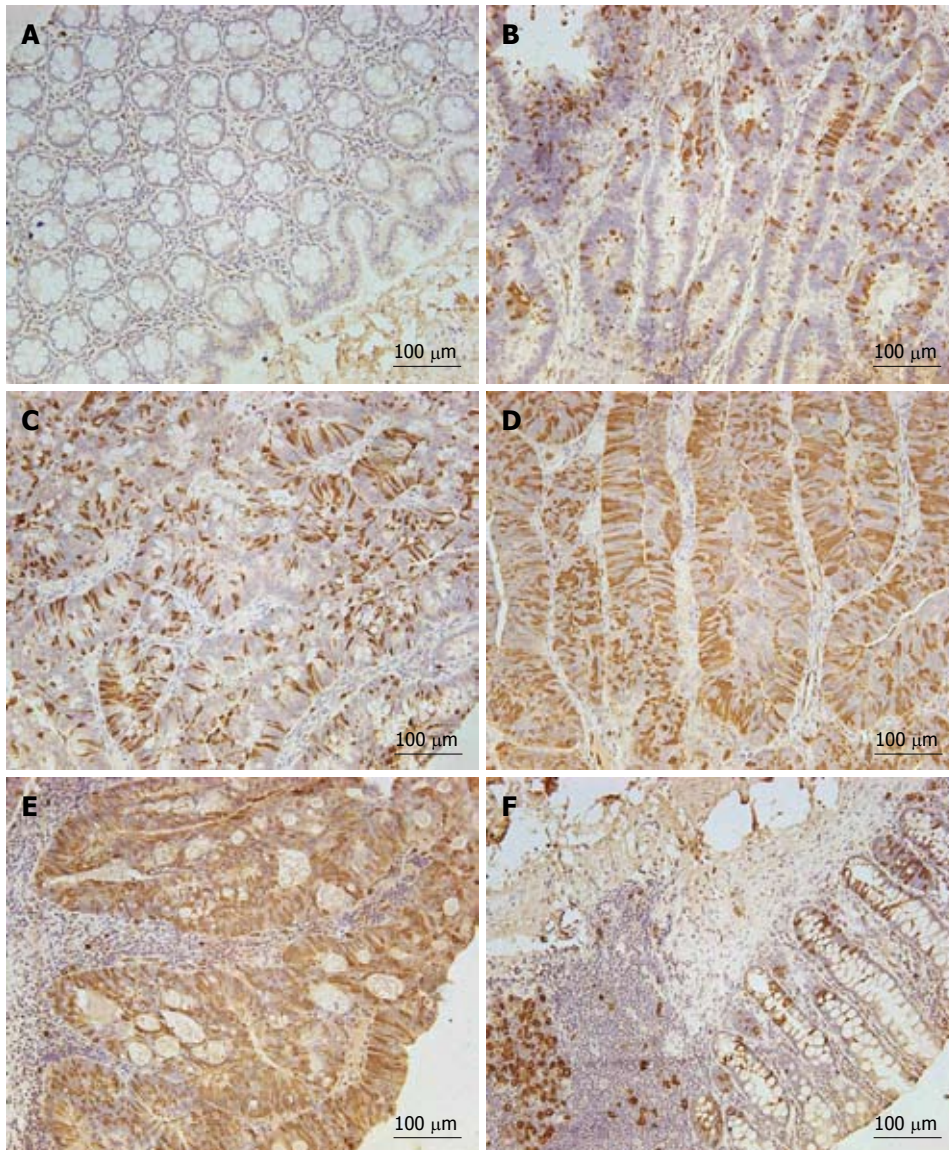


Figure 1 Immunohistochemical analysis of ribonucleotide reductase M2 expression in surgical specimens of tumor tissues or adjacent normal mucosa from patients with colon cancer. A: Normal colorectal mucosa without ribonucleotide reductase M2 (RRM2) expression. This case was considered RRM2-negative; B: Lower RRM2 expression in colorectal cancer (CRC); C-E: High RRM2 expression in CRC; F: Lymph node metastasis of CRC with RRM2 overexpression (original magnification $\times 20$).

relationship between RRM2 expression and clinicopathologic characteristics in CRC patients is summarized in Table 1. RRM2 overexpression was significantly positively correlated with invasion depth ($P = 0.0015$) and differentiation ($P = 0.0051$), although the frequency of RRM2 overexpression was lower in T4 tumors (20%) compared to T3 tumors (75%). However, no significant differences were observed with respect to gender, age, tumor size, or lymph node metastasis. To further elucidate the clinicopathologic significance of RRM2 overexpression in CRC, especially the correlation with invasion depth and poor differentiation, we examined RRM2 expression in another 78 CRC tissues. The results are summarized in Table 2 and RRM2 immunostaining was categorized further as score 0-3^[20]. These data are in accordance with previous studies showing correlation between RRM2 overexpres-

sion, invasion depth ($P < 0.0001$) and differentiation ($P < 0.0001$).

Suppression of RRM2 inhibits CRC cell growth and invasion

We next sought to determine whether RRM2 overexpression is functionally important for the *in vitro* proliferation of colonic cancer cell lines. RRM2 had the highest expression levels in HCT116 cells (Figure 2A and B). Then, HCT116 cells were transfected with three different siRNA sequences or a scrambled siRNA control. Quantitative PCR analysis of the HCT116 cell line demonstrated that the expression of RRM2 mRNA was reduced by three siRNA oligos segments to varying degrees (Figure 2C). We used the most efficient one to down-regulate RRM2 and evaluated cell growth; efficient knockdown of RRM2 protein levels after transfection was observed (Fig-

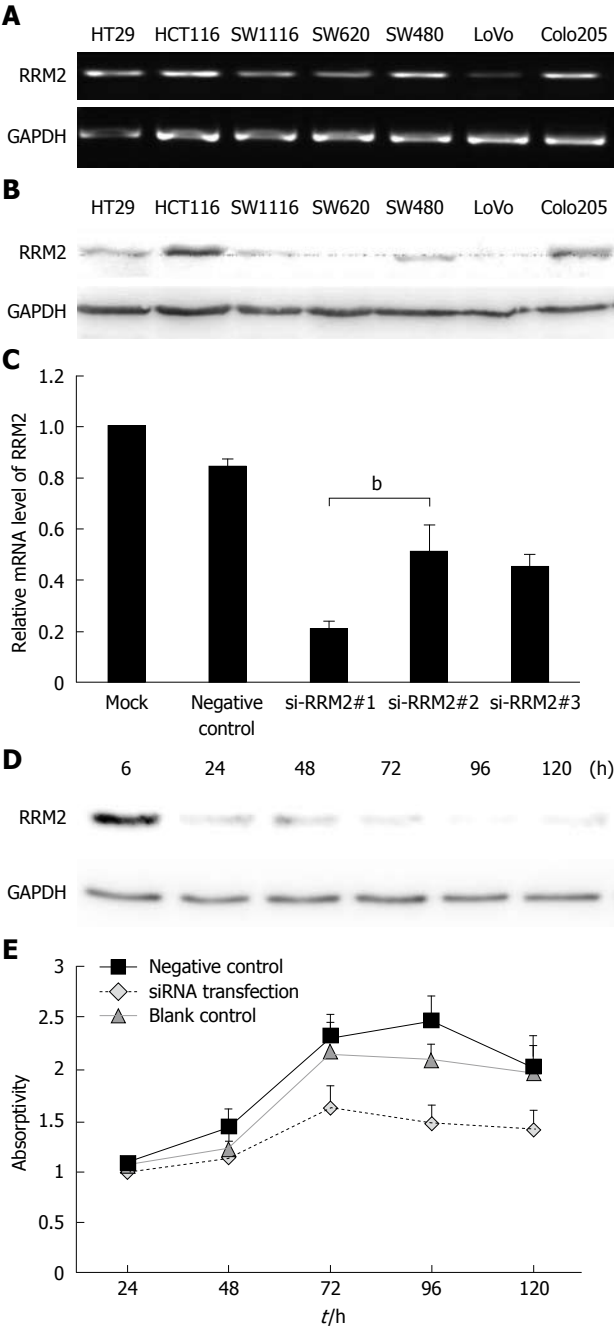


Figure 2 Expression of ribonucleotide reductase M2 in colorectal carcinoma cell lines and effect of ribonucleotide reductase M2 depletion on cell proliferation. **A:** Ribonucleotide reductase M2 (RRM2) expression in seven colorectal cancer (CRC) cell lines (PCR analysis); **B:** Expression of RRM2 protein in seven CRC cell lines (Western blotting); **C:** 48 h after siRNA transfection, relative mRNA level of HCT116 was determined (mock: lipofectamine only, negative control: lipofectamine + control siRNA), ^b $P < 0.01$ si-RRM2#1 vs si-RRM2#2; **D:** HCT116 cells were transfected with si-RRM2#1 and cultured for 120 h. Cells were lysed and RRM2 protein levels were detected by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase expression was used to control equal protein loading; **E:** At 24–120 h after siRNA transfection, the viability of HCT116 cells was determined by the CCK-8 assay. Analysis of cell growth in the presence or absence of RRM2 siRNAs revealed that blocking RRM2 protein synthesis significantly inhibited HCT116 cell growth. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

ure 2D). Analysis of cell growth in the presence or absence of the RRM2 siRNAs revealed that blocking RRM2

Table 1 Relationship between ribonucleotide reductase M2 gene expression in tumor and pathological parameters in patients n (%)

Clinical or pathologic feature	Total (n)	RRM2 ⁺	P value
All cases	56	38 (67.9)	
Gender			0.0908
Male	33	20 (60.6)	
Female	23	18 (78.3)	
Age (yr)			0.2094
≤ 64	29	19 (65.5)	
≥ 65	27	19 (70.4)	
Tumor stage (invasion depth)			0.0015
Tis/T1	3	0	
T2	16	6 (37.5)	
T3	32	24 (75.0)	
T4	5	1 (20.0)	
Tumor size (cm ² , average = 17.3)			0.0677
≤ 17	31	20	
> 17	25	11	
Lymph node metastasis (N)			0.2281
Absent	25	14 (56.0)	
1–3	21	16 (76.2)	
≥ 4	10	8 (80.0)	
Classification (differentiation)			0.0051
High-grade	5	1 (20.0)	
Mid-grade	45	35 (77.8)	
Low-grade	6	2 (33.3)	

RRM2: Ribonucleotide reductase M2.

Table 2 Relationship between ribonucleotide reductase M2 overexpression with invasion depth and poor differentiation

Pathologic feature	Total (n)	RRM2 ⁺				DF	P value
		Score 0	Score 1	Score 2	Score 3		
Tumor stage (invasion depth)						1	< 0.0001
Tis/T1	17	12	4	1	0		
T2	26	1	6	16	3		
T3	21	0	2	8	11		
T4	14	0	2	5	7		
Classification (differentiation)						1	< 0.0001
High	20	9	9	2	0		
Mid	39	4	3	20	12		
Low	19	0	2	8	9		

RRM2: Ribonucleotide reductase M2; DF: Degrees of freedom.

protein synthesis significantly inhibited CRC cell growth (Figure 2E). We used Annexin V/PI staining to identify the dead cells and then stratified them into categories of necrosis, early apoptosis, and both later apoptosis and necrosis. In each flow cytometry graph, later apoptotic or necrotic cells are shown in the upper-right quadrant. The total death rate 48 h after siRNA treatment showed a 2–3 fold increase. Flow cytometric analysis showed evidence of apoptosis when RRM2 functions were abrogated (Figure 3A and B). In the invasion assay, the number of cells that crossed through the chambers in the RRM2-siRNA group was 81 ± 3 , which was lower than that in the negative control (289 ± 7) and empty control groups (301 ± 7.2). These differences were statistically significant ($P < 0.01$) (Figure 3C and D).

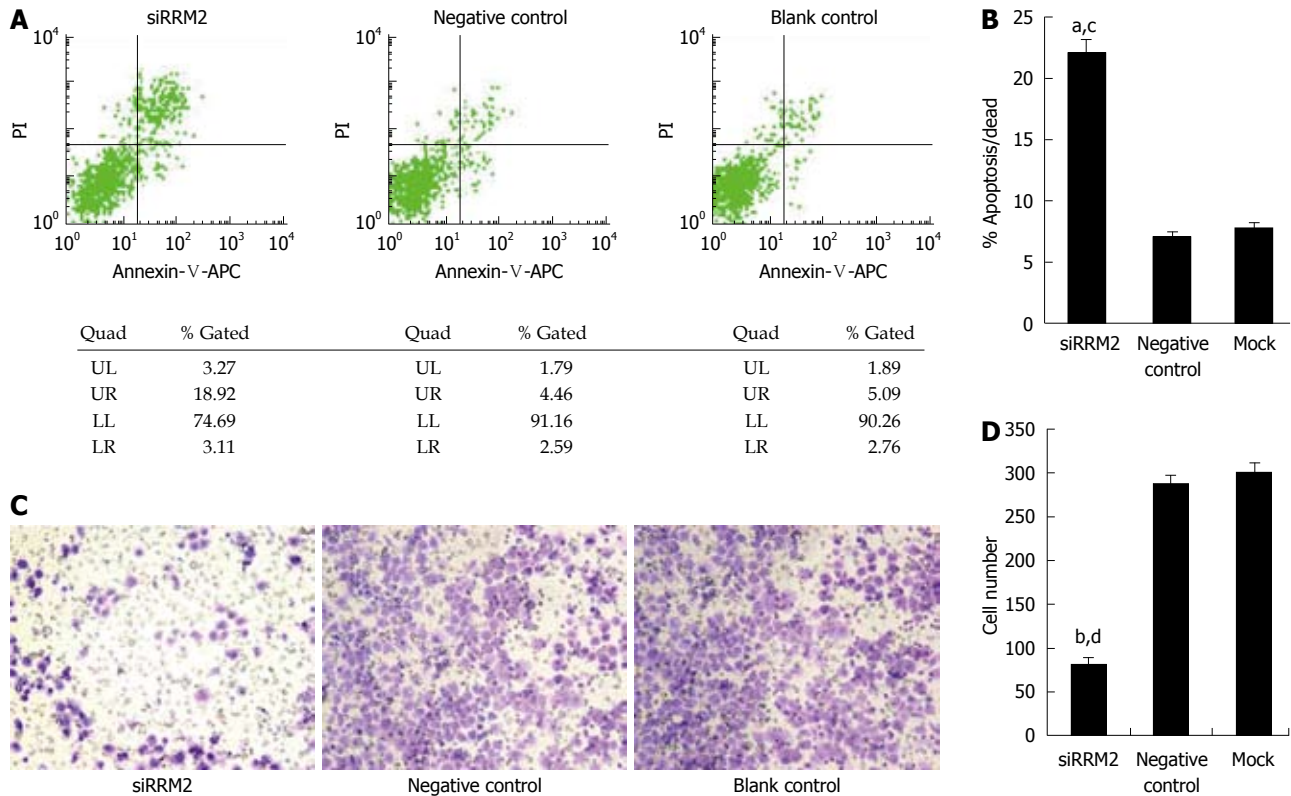


Figure 3 Effect of ribonucleotide reductase M2 on apoptosis and invasive capacity. A: Increased apoptosis following ribonucleotide reductase M2 (RRM2) mRNA inhibition by RNAi. Percentages of cells in the bottom left quadrant are live while cells to the right of the vertical line are apoptotic or dead; B: Quantifying bar graphs showing a significant increase in the percentage of apoptotic/dead cells following RRM2 mRNA knockdown with siRNA, ^a*P* < 0.05 vs negative control; ^c*P* < 0.05 vs blank control; C: Effect of RRM2 on invasive capacity of colorectal cancer cells. Invasion through the Matrigel-coated collagen insert after the indicated treatments is shown; D: Quantifying bar graphs showing a significant decrease in cell number following RRM2 mRNA knockdown with siRNA, ^b*P* < 0.01 vs negative control; ^d*P* < 0.01 vs blank control. Quad: Quadrant; UL: Upper left; UR: Upper right; LL: Lower left; LR: Lower right. PI: Propidium iodide.

Inhibition of hRRM2 by expression of antisense hRRM2 results in increased UV sensitivity of CRC cells

Four human CRC cell lines with low RRM2 expression, namely, SW1116 (p53 mutant-type), SW620 (mutated p53), HT29 (mutated p53), SW480 (p53 mutant-type), and LoVo (p53 wild-type), all proved to be more sensitive to UV irradiation in colony formation assays than HCT116 cells (p53 wild-type). HCT116 cells transfected with shRRM2 revealed significant RRM2 depletion, and its colony-forming ability under UV radiation was suppressed. At a dosage of 10 J/m², the colony-forming ability of the HCT116 cells transfected with RRM2 antisense was inhibited by 93.13%, whereas wild-type HCT116 cells maintained 51.28% viability, and SW620 cells (RRM2^{-/-}) maintained 32.40% viability (Figure 4A and B). In addition, the cell cycle distribution of the CRC cells did not significantly change at the various time points in the UV (-) panel. After a sublethal dose of UV radiation, an increased number of cells in the S-phase could be seen at 24-36 h (Figure 4C). Cellular morphology was observed by light microscopy. HCT116 cells with high levels of RRM2 expression, and SW480 cells, which do not express RRM2, were cultured under normal conditions or exposed to UV radiation for 48 h. Under these conditions, SW480 cells showed UV hypersensitivity, whereas the UV sensitivity of HCT116 cells with sup-

pressed RRM2 levels increased and cells went through necrosis. Thus, endogenous RRM2 functions to suppress hypersensitivity to UV radiation. Figure 5A gives a visual depiction of the morphological changes that occurred.

DISCUSSION

The importance of altered metabolism in the context of tumorigenesis has received renewed attention as metabolic changes entwined with the molecular hallmarks of cancer have been elucidated. Previous studies have shown that alterations in RR levels may significantly influence the biological properties of cells, including tumor promotion and tumor progression, suggesting that RR may be implicated in tumorigenesis. Thus, we were interested in determining the effect of RRM2 on tumor cell growth. The discovery of p53R2, an analog of RRM2 in mammalian cells, provided an interesting link between RR and cancer because it is a downstream target for the p53 tumor suppressor. Previous studies have shown that p53R2 is negatively related to tumor invasion and lymph node involvement. In addition, investigations have revealed that p53R2 correlates with a better survival of CRC with advanced grade III and grade IV tumors, rather than earlier grade I and grade II tumors^[18]. The genes for p53R2 and RRM2 are located on separate chromosomes.

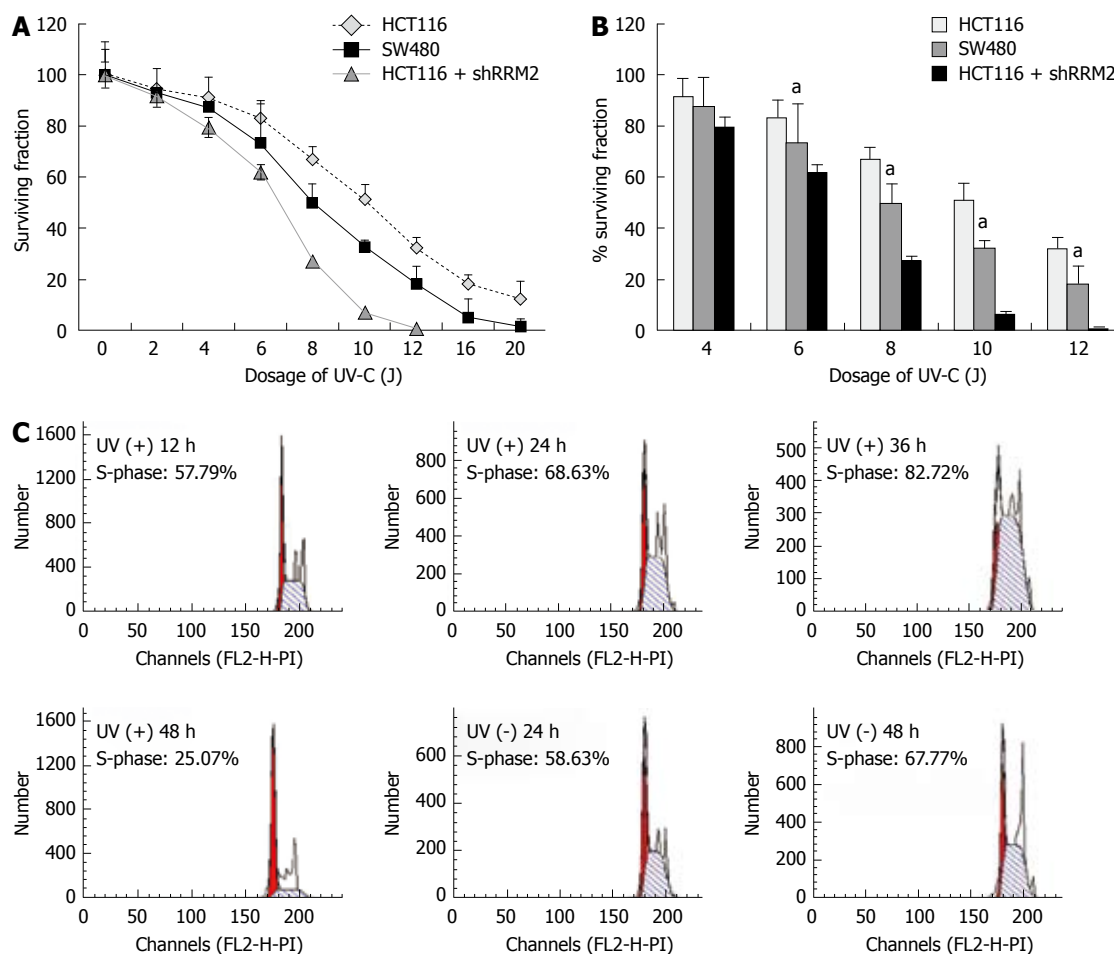


Figure 4 Inhibition of ribonucleotide reductase M2 by expression of antisense hRRM2 results in increased ultraviolet sensitivity of colorectal cancer cells. **A:** Ribonucleotide reductase M2 (RRM2)^{-/-} tumors and RRM2-depleted cells are hypersensitive to ultraviolet (UV)-C. SW620 cells, HCT116 cells (WT), and HCT116 cells (RRM2^{-/-}) were irradiated with increasing doses of UV-C and assayed for colony formation; **B:** Bar graph showing the quantification of the surviving fraction following 4–12 J irradiation. HCT116 cells transfected with shRRM2 revealed significant RRM2 depletion, and its colony-forming ability under UV radiation was suppressed, ^a*P* < 0.05 vs HCT116 group; **C:** Effects of colorectal cancer cell cycle irradiated with UV-C. After UV irradiation, an S-phase increase could be seen, starting from 12 h with a continued significant increase at 24 and 36 h.

p53R2 is 80%–90% identical to RRM2, but lacks its 33 amino-terminal residues, including a KEN box required for degradation during mitosis. p53R2 can substitute for RRM2 and form a highly active RR, which is believed to be active in DNA repair after DNA damage^[4]. However, the current knowledge about RRM2 function in human CRCs is still preliminary^[20,21]. Therefore, determining if RRM2 is essential for CRC progression may provide promising therapeutic opportunities.

Our findings showed that RRM2 expression is substantially upregulated in CRC using microarray analysis and RT-PCR. Moreover, RRM2 overexpression was significantly associated with invasion depth and differentiation, and clinical tissue specimens also showed that the expression levels of RRM2 may be associated with tumor stage (Figure 5B). These data suggest that RRM2 overexpression could be associated with CRC progression. Several previous reports demonstrated the frequent overexpression of the RRM2 protein and its possible role in other cancers^[20–23]. Recent observations showed that top 400 gene lists is produced by microarrays for each of the 13 cancer types and RRM2 appeared in nine lists of

significantly altered genes in cancers^[23], the results point to the value of RRM2 in normal to cancer transformation. RRM2 is upregulated in esophageal/gastric cancers and melanomas compared to pancreatic cancer^[24]. Other groups have also suggested that RRM2 may be a novel diagnostic marker and potential therapeutic target in bladder and gastric cancers. Furthermore, the studies suggested that RRM2 overexpression is associated with the male sex, the presence of muscularis propria invasion, the presence of Epstein-Barr virus, and expression of survivin, but not with age, histology, tumor size, lymph node metastasis, or expression of the phosphatase and tensin homolog, the phosphorylated signal transducer, and p53. Suppression of RRM2 synthesis inhibited the growth of gastric cancer cell lines *in vitro*^[20]. In addition, RRM2 is one of the putative tumor markers that is strongly expressed in the very early stages of premalignancy and preneoplasia of breast carcinomas^[25], and hepatocellular carcinoma cells are highly dependent on these genes for proliferation^[26]. Analyses also revealed that hRRM2 positively correlates with poor tumor differentiation of non-small cell lung cancer^[27]. It may be that

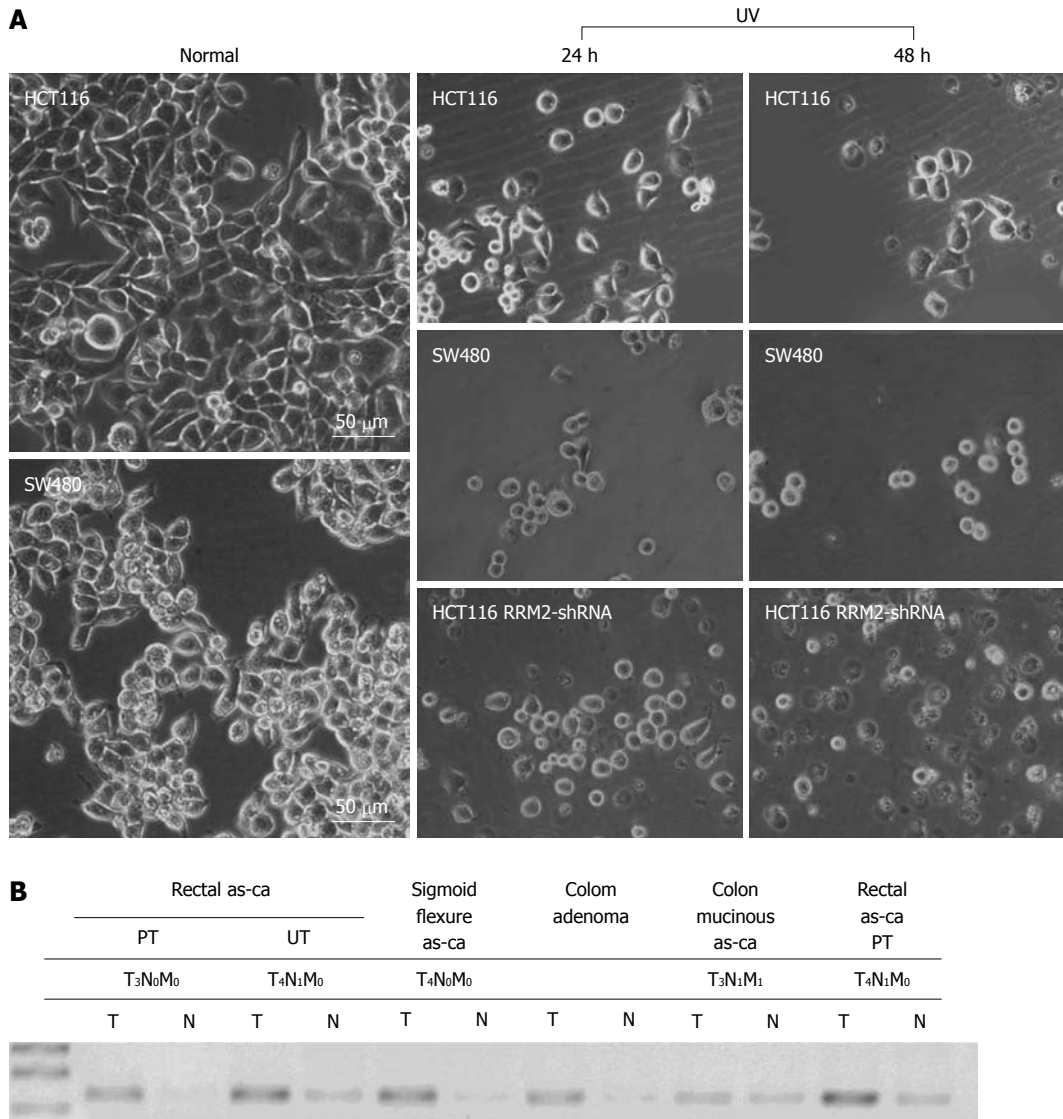


Figure 5 Light-microscopic photograph of cellular morphology. A: After 48 h of culture in normal conditions or exposed to ultraviolet (UV), HCT116 cells [high expression level of ribonucleotide reductase M2 (RRM2)] and SW480 cells (low expression level of RRM2) were photographed (20 × magnification). With UV-C irradiating, SW480 cells formed spheroids and HCT116 cells still adhered to each other and attached; B: Expression of RRM2 mRNA in clinical tissue specimens. Polymerase chain reaction analyses of 25 colorectal cancer patients (we show 6 patients) showed the expression levels of RRM2. PT: Protrude type; UT: Ulcerative type; as-ca: Adenocarcinoma.

the altered expression of metabolic enzymes or changes in the regulation of metabolic pathways also occur downstream of many oncogenes and tumor suppressor genes, and cancers with specific genetic lesions are dependent upon some of these metabolic changes. As was previously shown, RRM2 is upregulated in several cancers, and abrogating its expression reduces the proliferation of cancer cells, and triggered the development of RRM2-targeted siRNA-containing nanoparticles.

We also demonstrated that siRNA-mediated suppression of RRM2 synthesis inhibited the growth of the CRC cell lines investigated. In addition, abrogating its expression induced cell apoptosis. Therefore, it is likely that RRM2 overexpression is essential for the survival of CRC cells. Our finding is consistent with recent observations demonstrating that cells with altered RRM2 expression exhibit significantly reduced subcutaneous tumor

latency and increased tumor growth rates in syngeneic mice, and show markedly elevated metastatic potential in lung metastasis assays^[28]. Their studies demonstrate that RRM2 can participate in other critical cellular functions in addition to ribonucleotide reduction. For example, RRM2 overexpression may increase pancreatic cancer cell invasion in an NF- κ B-dependent manner, and RNA interference mediated silencing of RRM2 expression attenuated cell invasion and matrix metalloproteinase 9 activity^[16]. Another laboratory also reported that RRM2 overexpression plays an important role in tumor invasiveness^[29,30]. Our results are consistent with these previous reports, and suggest that targeting RRM2 has the potential for the treatment of CRC.

RRM2 is known to be involved in chemoresistance. The suppression of RRM2 can sensitize colon cancer cells to chemotherapeutic agents and significantly inhibit

the proliferation of melanoma cell lines with different oncogenic mutations with synergistic enhancement in combination with temozolomide^[31]. The relationship between RRM2 expression and chemotherapeutic effects in clinical settings has also been investigated. For instance, GTI-2040, an antisense oligonucleotide targeting RRM2, has been tested for the treatment of advanced metastatic solid tumors in clinical settings^[32]. Radiation is also a key component of carcinoma therapy. Unfortunately, it results in significant side effect in some patients. Thus, agents that radiosensitize CRC cells would be very useful. Here we show that RRM2 depletion decreased the surviving fraction of tumor cells in response to radiation and increased the sensitizer enhancement ratios. Recent observations suggest that targeting polo-like kinase 1 (PLK1) with small molecule inhibitors, in combination with radiation therapy, is a novel strategy in the treatment of some cancers^[33]. In addition, it has been hypothesized that there are some links between RRM2 and PLK1, because PLK1 was one of the most drastically downregulated genes following RRM2 silencing^[34]. Our data suggest RRM2 depletion increased UV sensitivity and make an argument for further exploring the role of RRM2 inhibition in CRC. It will be important to elucidate in detail the specific mechanisms by which RRM2 mediates tumor cell radiosensitivity.

In conclusion, we performed both functional and correlative studies on RRM2 in CRCs and provide strong evidence that RRM2 overexpression may be associated with CRC progression; thus suppressing its function may be a potential therapeutic strategy in CRC.

COMMENTS

Background

Previous studies have shown that alterations in ribonucleotide reductase (RR) levels may significantly influence the biological properties of cells, including tumor promotion and tumor progression, suggesting that RR may be implicated in tumorigenesis. Thus, the authors were interested in determining the effect of ribonucleotide reductase M2 (RRM2) on colorectal cancer (CRC) cells.

Research frontiers

Recently, metabolic genes have received increasing and specific attention due to their potential role in carcinogenesis. Overexpression of RRM2 has been revealed in breast cancer, renal cell carcinoma, prostate cancer and gastric cancer to elevated cellular proliferation and invasiveness.

Innovations and breakthroughs

Few studies have described the correlation between RRM2 and the development of CRC. And the possible mechanism by which RRM2 mediates CRC progression is unclear. It is well established that in some types of cancer, elevated RRM2 levels correlate with chemoresistance, but whether it may contribute to the response to ultraviolet (UV) irradiation is unclear. The results of this study suggest that RRM2 may be a facilitating factor in colorectal tumorigenesis and UV-induced DNA damage repair.

Applications

The data suggests that RRM2 subunit overexpression is associated with the CRC progression. RRM2 silencing may inhibit the hyperplasia and invasiveness of CRC cells. RRM2 may play an important role in the infiltration and metastasis of CRC and that suppression of its function could be a potential therapeutic strategy in CRC.

Terminology

RR is an essential enzyme required for the *de novo* conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, and it plays an impor-

tant role in DNA synthesis and repair. RR consists of two subunits, RRM1 and RRM2/p53R2.

Peer review

This is an interesting study investigating the importance of the hRRM2 in CRC and UV-induced DNA damage repair.

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