

KRAS-mediated Up-regulation of RRM2 Expression Is Essential for the Proliferation of Colorectal Cancer Cell Lines

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Abstract. *Background:* We previously investigated the mRNA expression of colorectal cancer cell lines via a microarray analysis and found several genes that were significantly up-regulated by oncogenic KRAS under serum-starved conditions. Of these genes, we focused on ribonucleotide reductase M2 (RRM2), which was reported to be associated with DNA synthesis. *Materials and Methods:* Cell proliferation and colony formation assays were performed using HCT116 cells transfected with lentiviral RRM2-shRNAs. *Results:* Under serum-starved conditions, the expression level of RRM2 protein increased in HCT116 cells compared to HKe3 cells (HCT116 cells with a disruption in oncogenic KRAS), and the re-expression of KRAS in HKe3 cells induced the expression of RRM2. Both the cell proliferation under serum-depleted conditions and the anchorage-independent growth were impaired by the reduction of RRM2 protein expression. *Conclusion:* RRM2 represents a novel therapeutic target, thus highlighting the potential utility of RRM2 inhibitors in colorectal cancer with oncogenic KRAS.

KRAS mutations are frequently observed in colorectal cancer (CRC) and the precise oncogenic mechanism of KRAS *in vivo* has been intensively studied; however, KRAS-targeted therapy has not been clinically developed, and patients with CRC bearing oncogenic KRAS do not benefit from cetuximab, a monoclonal antibody against the epidermal growth factor receptor. We previously established HKe3 cells,

human CRC HCT116 cells disrupted at oncogenic KRAS (1), and the analyses using the HKe3 cells have contributed to the understanding of tumor development through oncogenic KRAS signaling both *in vitro* and *in vivo* (1-6).

In these studies, we reported several genes that were significantly up-regulated by oncogenic KRAS in CRC cell lines under serum-starved conditions (2, 7, 8). Of these genes, we now focus on ribonucleotide reductase M2 (RRM2) (2). Ribonucleotide reductase (RNR) plays an essential role in catalyzing the conversion of ribonucleotide 5'-diphosphates to their 2'-deoxynucleotides, which are required for DNA synthesis and repair (9, 10). Human RNR consists of two subunits, RRM1 and RRM2. Many studies have demonstrated that increased RNR is involved in both tumor progression and malignancy (11-13). The increased expression and activity of RNR have also been reported to be determinants of gemcitabine chemoresistance in pancreatic adenocarcinoma cell lines and mammary adenocarcinoma cells (14, 15); however, the clinical studies of RNR inhibitors, including hydroxyurea and triapine, failed show repression of tumor progression in patients with pancreatic adenocarcinoma and non-small cell lung cancer (16-18).

In this study, we addressed the correlation between oncogenic KRAS and RRM2 with regard to the regulation of its protein expression, and further addressed the precise function of RRM2 in CRC growth under stress conditions.

Materials and Methods

Cell culture. Human CRC HCT116 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 cells, HKe3 cells and e3-MKRas#9 cells were cultured as described previously (19). For the serum-starved condition, cells were washed twice with phosphate-buffered saline (PBS) and cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) for 24 hours.

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All cell lines used were confirmed to be *Mycoplasma*-free, as determined using the MycoAlert system (Lonza, Verviers, Belgium). Cell morphology was regularly checked to ensure the absence of cross-contamination of cell lines.

Western blotting analysis. Proteins were extracted from CRC cells grown in DMEM supplemented with 10% fetal calf serum (FCS) or in serum-free media as described previously (19) and Western blotting analyses were performed using anti-RRM2 antibodies (Abnova, Taipei, Taiwan) and an anti-actin antibody (A2066; Sigma-Aldrich, St Louis, MO, USA) as described previously (20). Actin intensity was used as a loading control.

Generation of lentivirus vectors expressing RRM2-shRNA. The short hairpin interfering RNA (shRNA) targeting luciferase was used as a control. For RRM2 knockdown, shRNAs were designed as described below. The shRNA duplexes used were: RRM2 shRNA #1 top, 5'-GAT CCC GCG ATG CCT TGT GTC AAG AAT TCA AGA GAT TCT TGA CAC AAG GCA TCG TTT TTT CCA AC-3' and RRM2 shRNA #1 bottom, 5'-TCG AGT TGG AAA AAA CGA TGC CTT GTG TCA AGA ATC TCT TGA ATT CTT GAC ACA AGG CAT CGC GG-3'; RRM2 shRNA #2 top, 5'-GAT CCC GCC ATC GGA GGA GAG AGT AAT TCA AGA GAT TAC TCT CTC CTC CGA TGG TTT TTT CCA AC-3' and RRM2 shRNA #2 bottom, 5'-TCG AGT TGG AAA AAA CCA TCG GAG GAG AGA GTA ATC TCT TGA ATT ACT CTC TCC TCC GAT GGC GG-3'. The shRNA expression vectors were constructed as described below.

The human U6 promoter (Gene bank accession #M14486 gene sequence 65-329) was inserted into *Clal* and *Sall* sites of the pLenti6/V5-Dest (Invitrogen, Carlsbad, CA, USA), and then U6-term was inserted into the *Sall* and *MluI* sites to form pLenti6-U6+term. The resulting pLenti6-U6+term was then cleaved with *BsmBI* to form a cloning site for double-stranded synthetic oligonucleotide DNA.

The stably transfected clones exhibited a reduction of the RRM2 protein. The shRNA expression vectors were transfected into 293FT cells to produce packaged lentivirus. The lentivirus particles were packaged using the ViraPower Lentiviral Expression System (Invitrogen). The HCT116 cells were then infected with lentivirus RRM2 shRNAs to obtain stably transfected clones, and serial dilution was performed to obtain the subclones of HCT116 cells expressing RRM2 shRNAs.

Proliferation assay. A total of 20,000 cells were cultured in 200 μ l/well of medium with 10% FCS or 1% FCS (serum-depleted condition). The time course-dependent cell proliferation was measured by counting the number of cells using a hemocytometer after 24 hours and 48 hours.

Colony formation assay. A total of 2,000 cells were seeded onto non-adhesive dishes coated with hydrophilic polymers (Hydrocell; Cell Seed, Tokyo, Japan) using a normal medium containing 1.3% methyl serlose (Wako, Osaka, Japan). After incubation for 5 days, the colonies were analyzed using a Biorevo BZ-9000 inverted-phase microscope (Keyence, Osaka, Japan). A total of 20 of the colonies were measured, and the average of the maximum diameter was calculated.

Statistical analysis. The data are presented as the means \pm standard deviation from triplicate assays. The statistical analyses were performed using unpaired Student's *t*-test. Differences at $p < 0.05$ were considered to be statistically significant.

Results

The RRM2 protein is upregulated by oncogenic KRAS under serum-starved conditions. To confirm the expression levels of the RRM2 protein in the colorectal cancer cell lines, a Western blot analysis was performed. An anti-RRM2 antibody was used to detect the product of approximately 44 kDa. In HCT116 cells, the expression level of the RRM2 protein was increased by 1.95-fold in DMEM supplemented with 10% FCS and further increased by 4.55-fold under the serum-starved condition compared with the expression level in HKe3 cells (Figure 1A). Furthermore, the increased expression of the RRM2 protein was similarly observed in both the HCT116 and e3-MKRas#9 cell lines (HKe3-derived stable transfectants expressing oncogenic KRAS) cells under the serum-starved condition, thus suggesting that oncogenic KRAS regulates the expression of RRM2 protein, especially under the serum-starved condition (Figure 1B).

The decrease in RRM2 expression affects the proliferation of cells under serum-depleted conditions. To determine the biological significance of RRM2 in tumorigenesis, we first established stably transfected clones exhibiting a reduction of the RRM2 protein using lentivirus RRM2-shRNAs. A Western blot analysis showed that the expression levels of RRM2 proteins in the HCT116 cells expressing the RRM2-shRNA #1 and #2 were decreased by 1.16- and 1.89-fold compared with those in HCT116 cells transfected with the control-shRNA #1 and #2 (Figure 2A). Serial dilution was then performed to obtain each subclone, showing a further decrease in the RRM2 expression. Subclones were obtained from HCT116 cells with RRM2 shRNA #1 and #2 cells, and the expression levels of each subclone (#1-1, #1-2, #1-3, #2-1 and #2-2) were examined. The expression levels of RRM2 in #1-1, #1-2, #1-3 and #2-1 cells decreased by 3.5-, 1.05-, 1.58- and 5.0-fold compared to that in #2-2 cells, respectively (Figure 2B).

To address whether RRM2 knockdown affects the proliferation of HCT116 cells, a cell proliferation assay was performed using the #2-2 cells (as a control), #1-3 cells (a clone with a low expression level of RRM2) and #2-1 cells (a clone without RRM2 expression). In DMEM supplemented with 10% FCS, the relative growth of #1-3 and #2-1 cells grown for 24 hours were not significantly different, whereas the relative growth rates of the #2-1 cells grown for 48 hours were significantly lower ($p < 0.05$ Figure 2C). In the serum-depleted condition (1% FCS), the relative growth rates of #1-3 and #2-1 cells were significantly decreased in cells grown for 24 hours ($p < 0.001$ Figure 2D) and 48 hours ($p < 0.0001$ Figure 2D) compared with control cells, respectively. The relative growth rates of the #2-1 cells significantly decreased ($p < 0.05$ Figure 2D) compared with #1-3 cells when the cells were grown under serum-depleted conditions (1% FCS) for

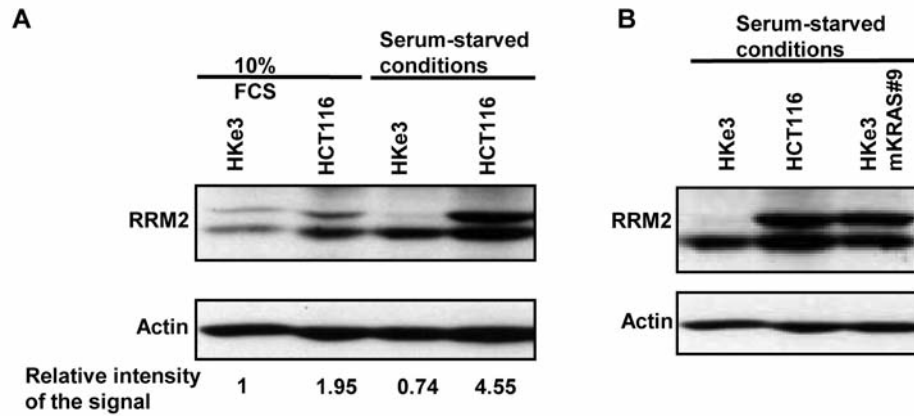


Figure 1. The RRM2 protein is upregulated by oncogenic KRAS under serum-starved conditions. A: Western blotting analysis of RRM2 in HCT116 and HKe3 cells in DMEM supplemented with 10% FCS, and under serum-starved conditions. The relative intensities of the signals were normalized to the signal for HKe3 cells in DMEM supplemented with 10% FCS. B: Western blotting analysis of RRM2 protein expression in HKe3, HCT116 and HKe3 mKRAS#9 cells (HKe3-derived activated KRAS-expressing cells) under serum-starved conditions.

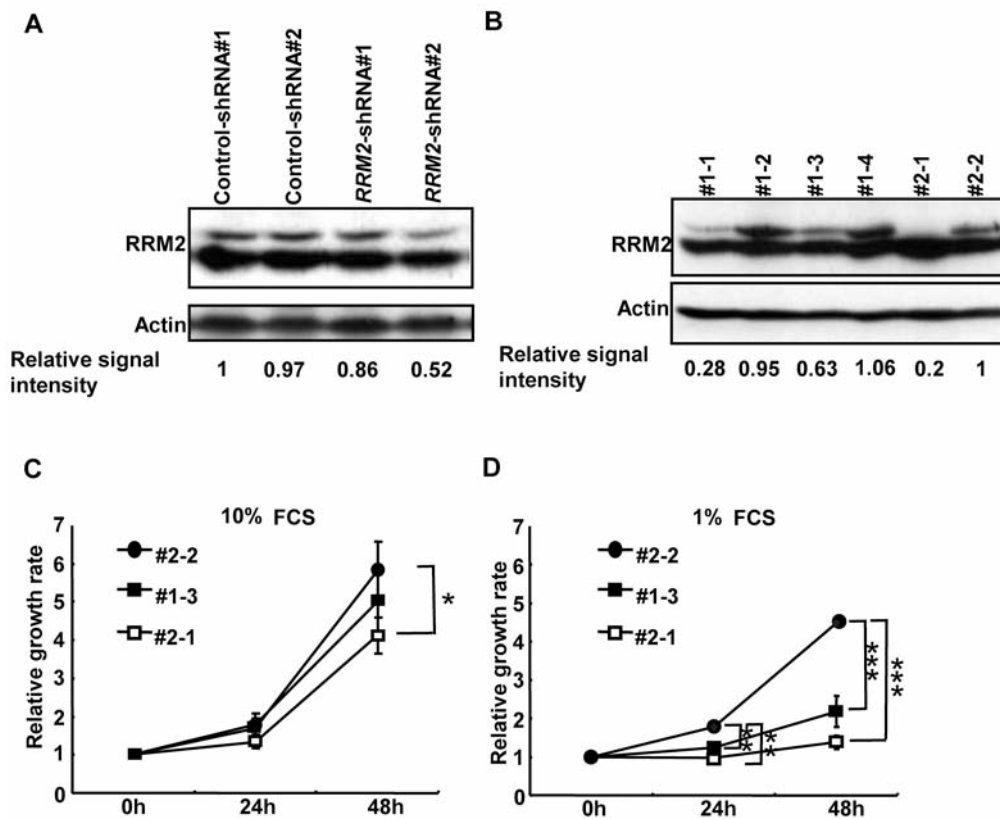


Figure 2. The reduction of RRM2 expression affects the proliferation of cells under serum-depleted conditions. A: Western blotting analysis of RRM2 in HCT116 cells transfected with the control-shRNA#1 and #2, and RRM2-shRNA#1 and #2 under serum-depleted conditions. The relative intensities of the signals for each cell line were normalized to the signal for HCT116 cells transfected with control-shRNA#1 under serum-depleted conditions. B: Western blotting analysis of RRM2 expression in the subclones from HCT116 cells transfected with RRM2 shRNAs. The relative intensities of the signals for each cell were normalized to the signal for the #2-2 cells. C and D: The cell growth rate of subclones cultured in DMEM supplemented with 10% FCS (C), or cultured under the serum-depleted condition (1% FCS; D). The relative growth rates at each time point were compared with that at 0 hour. * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

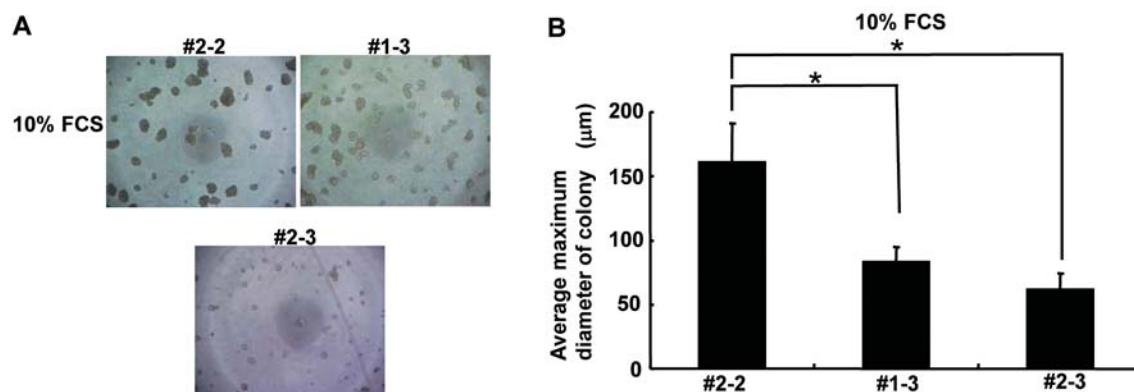


Figure 3. The reduction of RRM2 expression affects anchorage-independent growth. A: The morphology of colonies of #2-2, #1-3, and #2-2 cells. B: The average maximum diameters of colonies of #2-2, #1-3, and #2-2 cells. * $p < 0.0001$.

48 hours. Together, these results indicate that the cell growth rate for HCT116 cells was suppressed in a dose-dependent manner based on the extent of RRM2 protein reduction.

The reduction of RRM2 expression affects anchorage-independent growth. To assess the role of RRM2 in anchorage-independent growth, the colony formation assay was performed in subclones of HCT116 cells transfected with RRM2-shRNAs. Morphologically, #1-3 cells (the clone with a low expression of RRM2) formed smaller colonies, and #2-1 cells (the clone without RRM2 expression) produced the smallest colonies compared with control cells (Figure 3A), suggesting that anchorage-independent growth was impaired by the reduction of the RRM2 protein level. The average maximum diameter of the colonies of #1-3 and #2-1 cells was significantly reduced, by 1.39- and 2.78-fold, compared with that of #2-2 cells ($p < 0.0001$, Figure 3B). Similar results were obtained under another, less severe, serum-depleted condition (5% FCS) (data not shown). These results suggest that RRM2 plays a critical role in anchorage-independent growth.

Discussion

In this study, we found that RRM2 is up-regulated by oncogenic KRAS and that its expression correlates with CRC cell growth under stress conditions, such as serum withdrawal, and the loss of cell anchorage, both of which have been reported to be associated with cell stress (21, 22).

The expression of certain genes that directly control the rate of key metabolic pathways, including nucleotide synthesis, are drastically altered at different stages of tumor progression (23). These alterations are generally considered to be an adaptation of tumor cells to their microenvironment. For example, most tumors are exposed to hypoxia and poor nutritional conditions because of insufficient vascularization of the tumor (24-26), and thus many studies have reported

that serum-depleted conditions and/or hypoxia appear to induce the activity of various carcinogenesis-related factors (27-31). We previously reported that cells with oncogenic KRAS, and its downstream effector epiregulin, show a relatively high proliferative capacity under serum-starved conditions and also exhibit anchorage-independent growth (1, 7). These results suggest that oncogenic KRAS precisely regulates the expression of genes associated with stress conditions, and that it also appears to regulate the expression of RRM2, which may play a critical role in DNA synthesis.

The precise mechanisms connecting KRAS signaling and RRM2 expression under stress conditions are unclear, however, RRM2 is a novel candidate therapeutic target, thus highlighting the potential future utility of RRM2 inhibitors in CRC with oncogenic KRAS.

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