Silencing RRM2 inhibits multiple myeloma by targeting the Wnt/β-catenin signaling pathway

XIA LIU[1], JIAMIN PENG[2], YAYUN ZHOU[2], BEI XIE[2] and JIANCHAO WANG[3]

[1]Central Laboratory, The Second Affiliated Hospital of Zhejiang Chinese Medical University;
[2]The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310005;
[3]Department of Clinical Laboratory, Zhejiang Tongde Hospital of Zhejiang Province, Hangzhou, Zhejiang 310012, P.R. China

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Abstract. Ribonucleotide reductase M2 (RRM2) is one of the two subunits that comprise ribonucleotide reductase (RR), the enzyme that catalyzes the conversion of ribonucleotide 5'-diphosphates into 2'-deoxyribonucleotides, which are required for DNA synthesis. RRM2 is a stress response factor important for the development of several tumors. However, its role in multiple myeloma (MM) remains to be fully elucidated. The present study aimed to investigate the role of RRM2 in MM. The expression of RRM2 in patients with MM was analyzed using the Oncomine database. The results demonstrated that RRM2 expression was higher in MM compared with healthy subjects. Reverse transcription-quantitative polymerase chain reaction and western blot results revealed that RRM2 expression was decreased following transfection with a small interfering RNA targeting RRM2 into NCI-H929 cells. RR activity and Cell Counting Kit-8 assays demonstrated that RRM2 silencing reduced RR activity and inhibited cell proliferation. Annexin V-propidium iodide staining indicated that the percentage of apoptotic NCI-H929 cells was increased following RRM2 silencing compared with that in the control group. Increased phosphorylation of H2AX indicated that RRM2 silencing may activate the DNA-damage response pathway in NCI-H929 cells. Western blot analysis revealed that protein levels of the apoptosis-associated factor Bcl-2 were reduced, whereas Bax, cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase 1 were upregulated following RRM2 silencing compared with the control group. In addition, the results demonstrated that RRM2 silencing may inhibit target gene expression in the Wnt/β-catenin signaling pathway by increasing the phosphorylation of glucose synthase kinase 3β. These findings indicated that RRM2 may be involved in the proliferation and apoptosis of MM cells via the Wnt/β-catenin signaling pathway, suggesting that RRM2 may represent a novel therapeutic target for MM.

Correspondence to: Dr Xia Liu, Central Laboratory, The Second Affiliated Hospital of Zhejiang Chinese Medical University, 318 Chaowang Road, Hangzhou, Zhejiang 310005, P.R. China
E-mail: liuxia1986@zju.edu.cn

Professor Jianchao Wang, Department of Clinical Laboratory, Zhejiang Tongde Hospital of Zhejiang Province, 234 Gucui Road, Hangzhou, Zhejiang 310012, P.R. China
E-mail: xhwangjianchao@163.com

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Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the clonal proliferation of plasma cells in the bone marrow and the presence of monoclonal immunoglobulin in the blood and/or urine (1). MM accounts for ~13% of hematologic cancers and is the second most common hematological malignancy in adults worldwide (1,2). According to age-adjusted rates between 2009 and 2013, the number of new cases of myeloma was 6.5/100,000 people per year, whereas the mortality rate was 3.3/100,000 people per year (3). The estimated 5-year prevalence is ~230,000 patients worldwide (4). Although the introduction of novel agents (thalidomide, lenalidomide and bortezomib) into clinical application has remarkably improved response and survival rates in patients with MM, the disease remains largely incurable due to relapse and drug resistance (5). Therefore, developing novel therapeutic strategies for MM is urgently required.

Ribonucleotide reductase (RR) is a potential therapeutic target for cancer since it catalyzes the conversion of ribonucleoside 5'-diphosphates into 2'-deoxyribonucleoside 5'-triphosphates, which are necessary for DNA repair and replication (6). Human RR comprises two subunits: RRM1, and one of two RRM2 and p53R2 homologues (7). RRM1 protein levels are relatively stable across the cell cycle; by contrast, RRM2 is expressed during the late G1/early S phase, when DNA replication occurs. RRM2 is a dimer comprising two 44-kDa proteins, each of which contains a tyrosine free radical and non-heme iron (8). As the RRM2 subunit is the primary regulator of RR enzymatic activity, its effect on the biological activities of RR protein has been extensively studied (7,8). RRM2 has been reported to serve an active role in tumor progression and identified as a predictor of poor patient outcome for several types of cancer (9-11). RRM2 overexpression is associated with cancer cell invasiveness, metastasis,

tumorigenesis and poor patient outcome; it may serve as a prognostic biomarker for a number of types of cancer and it regulates several oncogenes that control malignancy (12-16). However, the significance of RRM2 in MM and the mechanisms underlying its regulation of biological functions remain unclear.

RNA interference (RNAi), which is induced by small interfering RNAs (siRNAs), is a strategy used to suppress the expression of individual genes with a high degree of specificity. RNAi has become the method of choice for regulating gene expression, as opposed to antisense ribozymes or DNAzyme technology (17). Lin *et al* (18) reported that the suppression of RRM2 expression using RNAi sensitizes colon cancer cells to DNA damage agents and RR inhibitors. Duxbury *et al* (19) demonstrated that siRRM2 enhances pancreatic adenocarcinoma cell chemosensitivity to gemcitabine. Wang *et al* (15) reported that siRRM2 increased human cervical cancer cell apoptosis and promoted cell cycle arrest at the G1 stage *in vitro*, and inhibited tumor formation *in vivo*. These reports suggest that further study of siRNA-mediated suppression of RRM2 as a cancer therapeutic may provide positive results. Therefore, the aim of the present study was to determine whether RRM2-specific siRNA suppression may be an effective strategy to inhibit MM cell proliferation *in vitro*.

The Wnt signaling pathway serves important roles in the regulation of various biological processes, including cell differentiation, cell proliferation, cell cycle and apoptosis. Activation of the Wnt/β-catenin signaling pathway contributes to the progression of human cancers including lung, colorectal and liver cancer, as well as MM. The canonical Wnt/β-catenin signaling pathway is involved in the pathogenesis of MM and regulates the differentiation, proliferation, apoptosis and migration of MM cells (20). Wnt responsiveness to MM plasma cells may be a major factor in the progression of MM (21). In addition, uncontrolled Wnt signaling may contribute to the defects in apoptosis that characterize MM (21). Therefore, the Wnt pathway was examined in the present study to explore the pathological mechanisms of MM.

The results of the present study demonstrated that RRM2 was expressed at high levels in MM, and that RRM2 knockdown inhibited RR activity and proliferation in MM cells. In addition, RRM2 silencing enhanced apoptosis and activated the DNA-damage response in MM cells. RRM2 downregulation may enhance apoptosis by targeting the Wnt/β-catenin signaling pathway. The present study aimed to provide a theoretical basis for the development of novel therapeutic targets for MM.

Materials and methods

Oncomine database analysis. Oncomine (oncomine.org/) was used to investigate the expression of RRM2 in MM, the search conditions set as follows: i) Gene: RRM2; ii) Analysis Type: cancer vs. normal analysis; iii) Cancer Type: MM; iv) Data Type: mRNA; v) Threshold: P=0001, Fold Change: 2, Gene Rank: Top 10%.

Cell culture. The MM cell lines RPMI-8226, U266, NCI-H929 and MM.1S were purchased from the Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin/streptomycin (Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C with 5% CO_2.

RNAi assay. NCI-H929 cells were plated at a density of $1x10^6$ cells/well in 6-cm dishes and incubated in complete RPMI-1640 medium without antibiotics at 37˚C for 24 h. Cells were transfected with RRM2 siRNA (siRRM2; 10 μM; cat. no. sc-36338; Santa Cruz Biotechnology, Inc.) using Lipofectamine® 3000 (Invitrogen, Thermo Fisher Scientific, Inc.). The RRM2 siRNA is a pool of three target-specific siRNAs (19-25 nucleotides long) designed to knock down RRM2 gene expression. The sequences of siRNA fragments were as follows: sc-36338A, sense 5'-CGAUGGCAU AGUAAAUGAAtt-3' and antisense 5'-UUCAUUUACUAU GCCAUCGtt-3'; sc-36338B, sense 5'-CACCAUGAAUUG UCCGUAAtt-3' and antisense 5'-UUACGGACAAUUCAU GGUGtt-3'; sc-36338C, sense 5'-CAAGGAGCUUCUUAA GUUAtt-3' and antisense 5'-UAACUUAAGAAGCUCCUU Gtt-3'. Cells transfected with scrambled siRNA (10 μM; cat. no. sc-44231; Santa Cruz Biotechnology, Inc.) and untreated cells were used as the siRNA control (siC) and blank groups, respectively. The sequences of control siRNA fragments were as follows: Sense: 5'-UUCUCCGAACGU GUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGG AGAATT-3'. Transfection was performed according to the manufacturer's protocol using Lipofectamine® 3000 reagent. Following co-culture for 12 h at 37˚C, the medium was replaced with culture media and the transfected cells were used for subsequent experiments.

Cell viability assay. The effect of RRM2 silencing on the proliferation of NCI-H929 cells was detected using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). NCI-H929 cells ($1x10^3$ cells/well) were seeded in 96-well plates and transfected with siRRM2 and siC. After 24 and 48 h of culture, 20 μl CCK-8 was added to each well and the plate was incubated for 4 h at 37˚C. Optical density (OD) at 450 nm was analyzed using a microplate reader (BioTek Instruments, Inc.). The experiment was performed in triplicate. Cell viability was calculated by comparing the OD of the transfected group with the blank group.

RR activity assay. RR activity was measured using a [^{3}H] cytidine diphosphate (CDP) reduction assay as previously described (22). Briefly, transfected and blank cells were lysed on ice in a low-salt homogenization buffer (10 mM HEPES; pH 7.2), and the supernatants were collected following centrifugation at 12,000 x g for 10 min at 4˚C. A total of 25 μg protein-containing supernatant was added to 100 μl reaction mixture, which contained 0.125 μmol/l [^{3}H] CDP, 50 mM HEPES (pH 7.2), 100 mM KCl, 6 mM dithiothreitol, 4 mM magnesium acetate, 2 mM ATP and 0.05 mM CDP. Following incubation at 37˚C for 15-30 min and dephosphorylation, samples were analyzed by high-performance liquid chromatography and liquid scintillation counting (22). RR activity was calculated as follows: RR activity (%) = dCDP/(CDP + dCDP) x100, where dCDP is the number of deoxyribonucleotides catalyzed from ribonucleotides by RR and CDP is the number of the

remaining ribonucleotide substrates in the enzyme reaction system.

Reverse transcription-quantitative PCR (RT-qPCR). Following 48 h of transfection, TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate total RNA according to the manufacturer's instructions. Purified RNA was reverse transcribed using oligonucleotide dT primers (Takara Biotechnology Co., Ltd.). The incubation conditions for RT was as follows: 37˚C for 15 min; 85˚C for 5 sec; and a hold at 4˚C. The reactions were performed using SYBR Green Mix kit (Roche Molecular Diagnostics) using the LightCycler® 480 (Roche Diagnostics). The following thermocycling conditions were used for the PCR: Denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and elongation at 60˚C for 1 min. Gene expression was normalized to the expression levels of β-actin. The relative expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method as follows: $\Delta\Delta Cq = \Delta Cq_{experimental\ group} - \Delta Cq_{control\ group}$, $\Delta Cq = Cq_{target\ gene} - Cq_{internal\ control}$; $2^{-\Delta\Delta Cq}$ is the relative transcript level of the target gene mRNA (23). Primer sequences were as follows: RRM2, forward 5'-ATCCGGATCCACTATGCT CTCCCTCCGTGT-3' and reverse 5'-GCTTAAGCTTATTTA GAAGTCAGCATCCAAG-3'; β-actin, forward 5'-TCCCTG GAGAAGAGCTACG-3' and reverse 5'-GTAGTTTCGTGG ATGCCACA-3'.

Western blot analysis. Cells were harvested and lysed in RIPA buffer (Beyotime Institute of Biotechnology) with protease inhibitor (Roche Diagnostics). Protein concentrations were determined using the bicinchoninic acid protein quantitation kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (20 μg) were separated by SDS-PAGE on 12% gel and transferred to PVDF membranes (EMD Millipore). Following blocking with 5% skimmed milk in TBS+0.1% Tween-20 (TBST), the membranes were incubated with primary antibodies against the following proteins: RRM2 (cat. no. sc-81850; 1:1,000; Santa Cruz Biotechnology, Inc.), Bcl-2 (cat. no. ab32124; 1:1,000; Abcam), Bax (cat. no. ab32503; 1:1,000; Abcam), caspase-3 (cat. no. ER30804; 1:1,000; Huabio, Inc.), cleaved caspase-3 (cat. no. ET1602-47; 1:1,000; Huabio, Inc.), poly(ADP-ribose) polymerase (PARP; cat. no. ET1608-56; 1:1,000; Huabio, Inc.), cleaved PARP (cat. no. ET1608-10; 1:1,000; Huabio, Inc.), p53 (cat. no. EM20603; 1:1,000; Huabio, Inc.), phosphorylated (p)-p53 (cat. no. ET1609-14; 1:1,000; Huabio, Inc.), glucose synthase kinase 3b (GSK-3β, cat.sc-81462; 1:1,000; Santa Cruz Biotechnology, Inc.), p-GSK-3β (cat. no. sc-373800; 1:1,000; Santa Cruz Biotechnology, Inc.), β-catenin (cat. no. ab32572; 1:1,000; Abcam), c-Myc (cat. no. ab32072; 1:1,000; Abcam), cyclin D1 (cat. no. ab16663; 1:1,000; Abcam) and β-actin (cat. no. M1210-2; 1:1,000; Huabio, Inc.) at 4˚C overnight. Following three washes with TBST, the PVDF membranes were incubated with horseradish peroxidase-conjugated antibodies rabbit anti-mouse immunoglobulin G (IgG; cat. no. HA1008;1:5,000, Hangzhou HuaAn Biotechnology Co., Ltd.) or goat anti-rabbit IgG (cat. no. HA1008; 1:5,000; Hangzhou HuaAn Biotechnology Co., Ltd.) for 1 h at room temperature. Enhanced chemiluminescence (ECL; Origene Technologies, Inc.) and GE ECL Start detection system reagent (GE Healthcare) were used to visualize the bands. The signals were analyzed using the Bio-Rad Gel Imaging System (Bio-Rad Laboratories, Inc.) and densitometry was performed using ImageJ version 1.8.0 software (National Institutes of Health).

Apoptosis assay. Following transfection for 48 h, an Annexin V-FITC/PI kit (Beyotime Institute of Biotechnology) was used to measure apoptosis according to the manufacturer's protocol. Briefly, 10^5 cells transfected with siRRM2 or siC or those treated with medium only were collected and washed with PBS. A total of 50 μl binding buffer, 5 μl Annexin V-FITC and 5 μl PI were added into the cell suspension and mixed at room temperature in the dark for 10 min. Apoptosis was measured and analyzed by flow cytometry (NovoCyte Flow Cytometer; ACEA Biosciences, Inc.) within 1 h. The flow cytometry data was analyzed using NovoExpress™ (version no. 1.2.1; ACEA Biosciences, Inc.)

Statistical analysis. Data are presented as the mean ± SD, and all experiments were repeated at least three times. One-way ANOVA followed by a Dunnett's post-hoc test was applied to compare differences between groups using GraphPad Prism 6 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

RRM2 is significantly upregulated in patients with MM. To determine the expression profile of RRM2 in patients with MM, the Oncomine (oncomine.org/) online collection of microarrays was used. Using the Agnelli Myeloma microarray dataset (24), RRM2 expression was higher in MM compared with that in the healthy subjects (Fig. 1A).

Construction of an in vitro RRM2 knockdown MM model. MM cell lines, RPMI-8226, U266, NCI-H929 and MM.1S, were used to examine the role of RRM2 in MM, and samples from healthy subjects were taken as the control cell lines (data not shown). The results demonstrated that RRM2 was highly expressed in these cell lines and no differences were observed among the cell lines (Fig. 1B). Based on this result, NCI-H929 cells were selected for further experiments. NCI-H929 cells were transfected with siRNA targeting RRM2; RT-qPCR results demonstrated that the mRNA expression of RRM2 was significantly decreased in the siRRM2 group compared with that in the siC group, demonstrating 74% interference efficacy ($P<0.05$; Fig. 1C). In addition, protein levels detected by western blotting were significantly reduced in the siRRM2 group compared with the siC group (Fig. 1D). These results confirmed that transfection with siRRM2 induced a significant decrease in RRM2 expression.

Reduced expression of RRM2 inhibits cell RR activity and proliferation in NCI-H929 cells. Following the construction and confirmation of the RRM2 knockdown model, RR activity was analyzed in the transfected cells; the results revealed that enzymatic activity was markedly decreased following downregulation of RRM2 expression compared with the siC group (Fig. 2A). The viability of transfected cells was investigated using CCK-8 assay, which demonstrated that downregulation

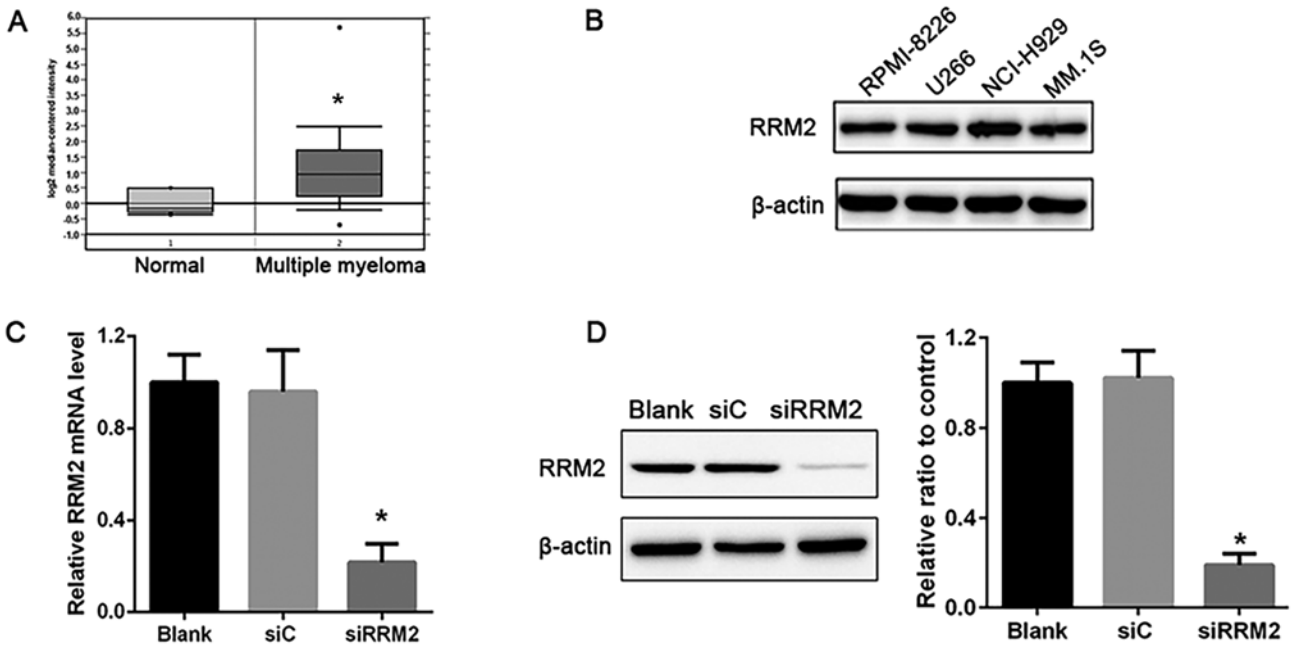

Figure 1. RRM2 is overexpressed in MM tissues and cells. (A) Oncomine data mining analysis of RRM2 mRNA levels in MM and non-MM tissues. (B) Western blot analysis of RRM2 protein expression in MM cell lines. (C) The relative levels of RRM2 mRNA following 48-h transfection with siRRM2 was lower compared with those in the siC group. (D) RRM2 protein expression was downregulated in NCI-H929 cells following 48-h exposure to siRRM2; β-actin was used as a loading control. The RR protein expression was showed by western blotting (left), and quantified by densitometry ratio of RRM1 and β-actin, the ratio was normalized to blank, which was set as 1 (right). *P<0.05 vs. siC. MM, multiple myeloma; RRM2, ribonucleotide reductase subunit M2; siC, control small interfering RNA; siRRM2, RRM2 small interfering RNA.

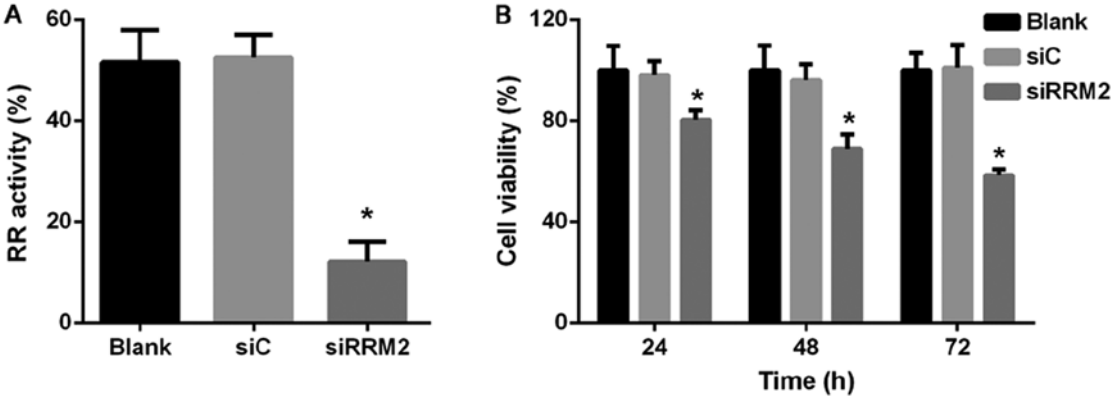

Figure 2. RRM2 interference inhibits the viability of NCI-H929 cells. (A) RR activity was evaluated in NCI-H929 cells at 48 h after transfection with siC or siRRM2; the results demonstrated decreased RR activity following RRM2 knockdown compared with siC. (B) Cell Counting Kit-8 assay demonstrated an inhibition of MM cell proliferation following downregulation of RRM2 compared with the siC group. Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. siC. RR, ribonucleotide reductase; RRM2, ribonucleotide reductase subunit M2; siC, control small interfering RNA; siRRM2, RRM2 small interfering RNA.

of RRM2 significantly inhibited cell proliferation in a time-dependent manner in NCI-H929 cells. Cell viability in the siRRM2 group was reduced to 80.4, 68.9 and 58.4% of siC group levels at 24, 48 and 72 h, respectively (P<0.05; Fig. 2B).

Reduced expression of RRM2 induces apoptosis in NCI-H929 cells. Although RRM2 has been demonstrated to be involved in cell cycle arrest and apoptosis in a number of cancer cells, its mechanism in MM cell apoptosis was unclear. In the present study, apoptosis induction upon knockdown of RRM2 was observed in NCI-H929 cells at 48 h. Following knockdown of RRM2 expression, the proportion of apoptotic cells was increased to 19.9%, whereas the apoptotic rate in the blank and siRNA control groups was 4.03 and 4.60%, respectively (P<0.05; Fig. 3A and B). These results suggested that reduced RRM2 expression may promote MM cell apoptosis.

Reduced expression of RRM2 activates the DNA-damage response in NCI-H929 cells. To determine whether the cytotoxicity induced upon RRM2 knockdown was associated with DNA damage, the phosphorylation level of histone H2AX at serine 139 (γ-H2AX) in NCI-H929 cells was evaluated. The results demonstrated that γ-H2AX levels were increased following siRRM2 transfection in NCI-H929 cells compared

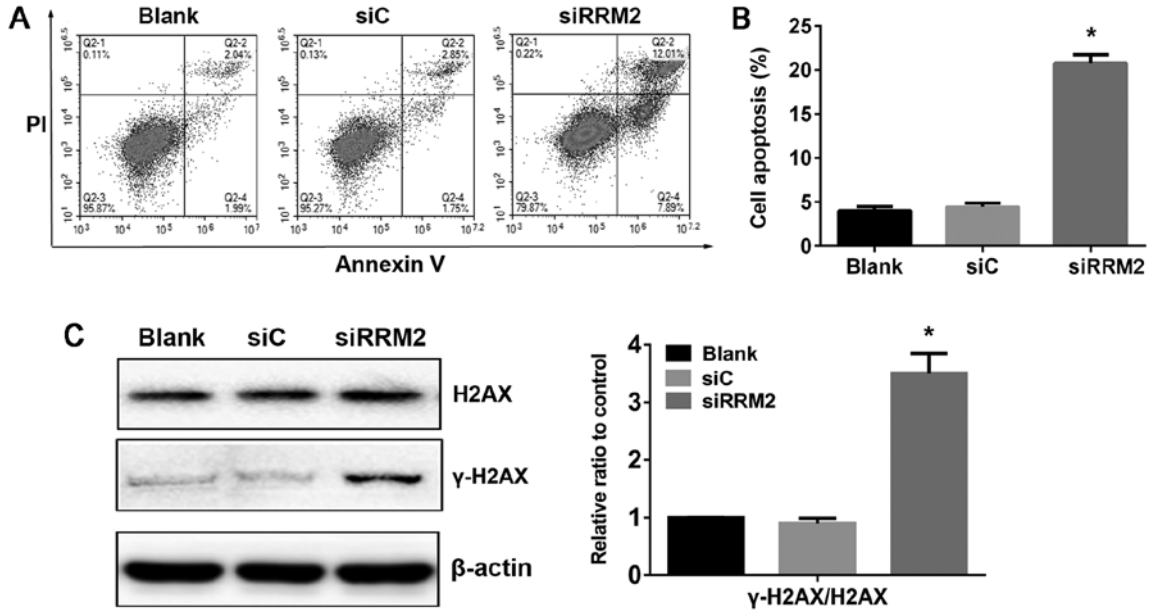

Figure 3. RRM2 downregulation promotes apoptosis and activates the DNA-damage response in multiple myeloma cells. (A and B) Flow cytometry results indicating the apoptotic rate in NCI-H929 cells, which was higher in the siRRM2 group compared with the siC group. (C) Downregulation of RRM2 significantly enhanced the expression of γ-H2AX compared with that in the siC group; β-actin was used as a loading control. Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. siC. RRM2, ribonucleotide reductase subunit M2; PI, propidium iodide; siC, control small interfering RNA; siRRM2, RRM2 small interfering RNA; H2AX, histone H2AX; γ-H2AX, phosphorylated H2AX (serine 139).

with siC (Fig. 3C). This result indicated that reduced expression of RRM2 may induce apoptosis with a profound increase in markers of DNA damage in NCI-H929 cells.

Reduced expression of RRM2 alters apoptosis-associated protein expression in NCI-H929 cells. To delineate the molecular mechanism of RRM2 in apoptosis in MM, proteins that are activated during the repair of DNA strand breaks and upon apoptosis were detected. The levels of apoptosis-associated proteins such as Bcl-2, Bax, caspase-3, and cleaved caspase-3 were evaluated; Bcl-2 levels were reduced in the siRRM2 transfection group compared with the siC group. By contrast, Bax and cleaved caspase-3 levels were increased following RRM2 knockdown compared with the siC group. Western blotting of PARP-1, which catalyzes single-strand DNA break repair, demonstrated that RRM2 knockdown induced an increase in cleaved PARP-1 compared with that in the siC group (Fig. 4A).

siRNA-mediated RRM2 knockdown suppresses Wnt/β-catenin signaling through a phosphorylated GSK-3β-dependent mechanism. To further investigate the molecular mechanism associated with the induction of apoptosis in NCI-H929 cells following RRM2 knockdown, the expression of downstream target genes of Wnt/β-catenin signaling pathway was examined. Total β-catenin levels were significantly reduced by siRNA-mediated RRM2 knockdown compared with the siC group in NCI-H929 cells. The downstream effectors of Wnt/β-catenin signaling, including cyclin D1 and c-Myc, which serve important roles in tumor progression, were also decreased following RRM2 knockdown compared with the siC group. Additionally, GSK-3β phosphorylation expression, which promotes β-catenin degradation, was upregulated compared with that in the siC and blank groups (Fig. 4B). These results suggested that inhibition of Wnt/β-catenin signaling through enhanced GSK-3β phosphorylation may be one of the underlying molecular mechanisms through which RRM2 knockdown induces apoptosis in MM cells.

Discussion

The pathogenesis of MM is affected by alterations, aberrations, and/or dysregulation of endocrine, genetic and metabolic factors (25). Advances in MM treatment include chemotherapy and autologous hematopoietic stem cell transplantation; however, the prognosis for patients with MM remains poor (26). Thus, discovery of effective therapeutic methods and novel molecular biomarkers for MM detection is urgently required.

The optimization of cellular dNTP concentration is crucial for high-fidelity DNA replication and repair (7). RR is an enzyme that is involved in cell proliferation by providing materials required for DNA synthesis. RRM2, which is a small subunit of RR, is overexpressed in a number of tumors and its overexpression is associated with tumor aggressiveness, poor prognosis and poor overall survival (12,13). A study by Li *et al* (27) reported that the suppression of RRM2 inhibits cell proliferation, induces cell cycle arrest and promotes apoptosis in human neuroblastoma cells. In addition, Li *et al* (28) demonstrated that RRM2 promotes the progression of human glioblastoma. RRM2 inhibitors, such as hydroxyurea, GIT-2040 and tripine, are viable treatment options, either as a monotherapy or in combination with cancer chemotherapy, based on the outcome of clinical trials in various cancer types, including myeloid leukemia, renal cell carcinoma, advanced non-small cell lung cancer and prostate cancer (29). These reports suggested that therapies targeting RRM2 may serve as useful strategies for controlling cancer progression.

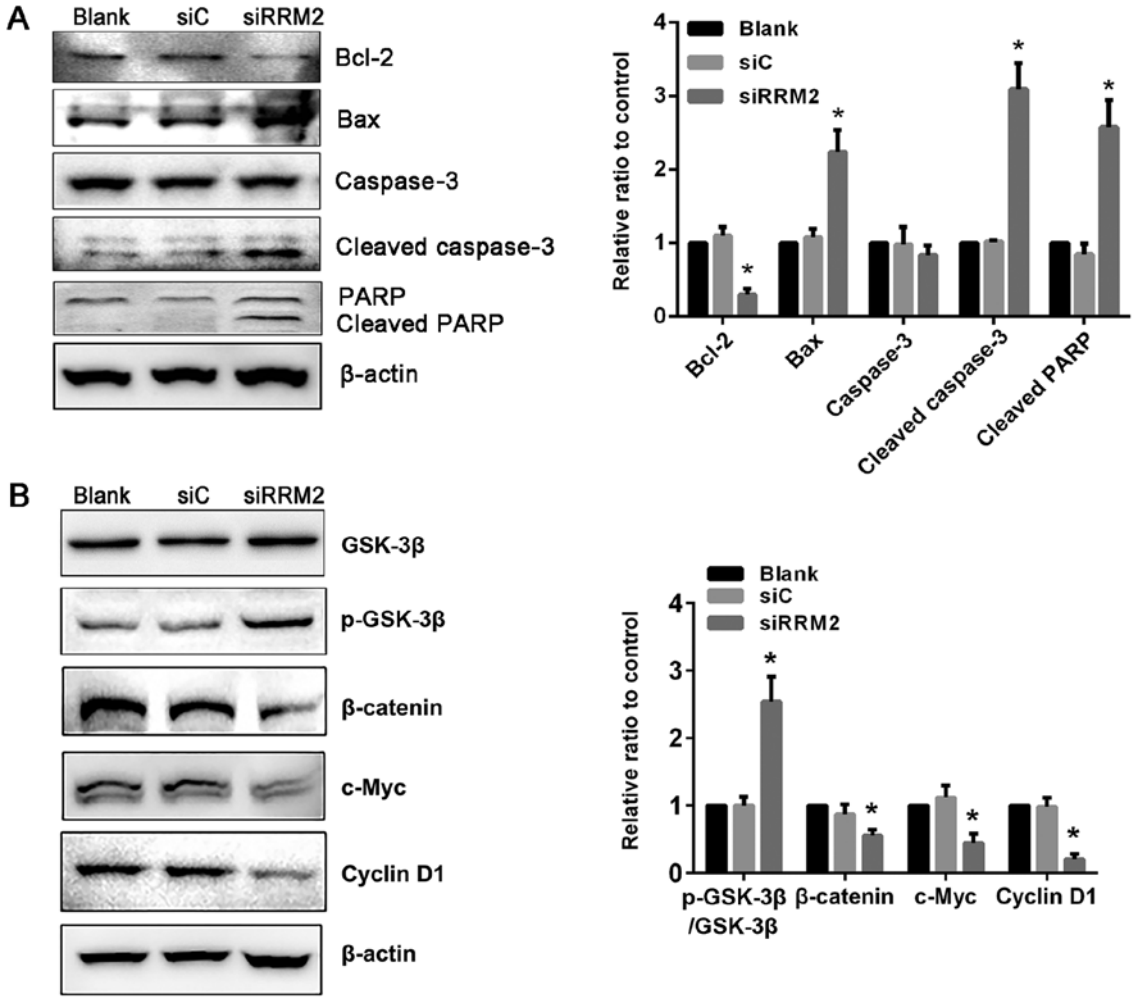

Figure 4. Downregulation of RRM2 alters the expression of apoptosis-related proteins and inhibits Wnt/β-catenin signaling through a p-GSK-3β-dependent mechanism. (A) Western blot analysis results demonstrated that RRM2 silencing reduced Bcl-2 expression, but increased the protein levels of Bax, cleaved caspase-3 and cleaved PARP. (B) Western blotting demonstrated that the protein expression levels of β-catenin and downstream targets of the Wnt/β-catenin signaling pathway were reduced following siRRM2 transfection compared with the siC group. By contrast, GSK-3β phosphorylation was increased; β-actin was used as a loading control. *P<0.05 vs. siC. RRM2, ribonucleotide reductase subunit M2; siC, control small interfering RNA; siRRM2, RRM2 small interfering RNA; PARP, poly(ADP-ribose) polymerase; p-, phosphorylated; GSK-3β, glucose synthase kinase 3β.

To the best of our knowledge, the role of RRM2 in MM has not been reported. Therefore, further studies on the mechanism associated with RRM2 in MM are required. In the present study, the overexpression of RRM2 in MM tumor tissues was demonstrated using the publicly accessible microarray database, Oncomine. The result indicated that RRM2 may serve an important role in the progression of MM. The effects of RRM2 on the NCI-H929 MM cell line proliferation and apoptosis were also examined. Apoptosis is involved in maintaining tissue homeostasis in multicellular organisms in various physiological and pathological situations (30). Resistance to apoptosis is an indication of human cancer and promotes its development and progression (31). Resistance to apoptosis is one of the leading causes of failure of leukemia therapy, as a number of anticancer treatments mediate apoptosis (32). The present study demonstrated that RRM2 downregulation led to reduced NCI-H929 cell proliferation and increased apoptosis. DNA damage is a characteristic of apoptosis, and γ-H2AX is best known for its role in DNA double-strand break repair (33). The present study revealed that RRM2 downregulation induced an increase in γ-H2AX levels, which suggested that it induced DNA damage in NCI-H929 cells. These results were further confirmed by the upregulation of Bax and downregulation of Bcl-2 protein expression following siRRM2 transfection. Caspase-3 is an important member of the caspase family and represents a key step in the induction of apoptosis (34); cells transfected with siRNA targeting RRM2 induced activation of caspase-3 and inactivation of downstream PARP, which a nuclear protein involved in DNA repair and cell apoptosis (35).

The induction of apoptosis by RRM2 knockdown in NCI-H929 cells may be a result of its inhibitory effect on the Wnt/β-catenin signaling pathway. This pathway has been described to manage embryonic development, and accumulating evidence indicates that abnormal activation promotes cancer progression through the modulation of downstream genes (36). Therefore, the Wnt/β-catenin signaling pathway is involved in cancer cell proliferation and apoptosis (37). In addition, it participates in the pathogenesis of MM by regulating MM cell differentiation, proliferation, apoptosis and migration (38). The present study demonstrated that RRM2 knockdown may activate the phosphorylation of GSK-3β and

reduce the expression of β-catenin. Downstream effectors of the Wnt/β-catenin pathway, c-Myc and cyclin D1, are associated with cancer cell proliferation and differentiation (36); in the present study, RRM2 knockdown significantly downregulated c-Myc and cyclin D1 expression levels. Therefore, the results of the present study demonstrated that downregulation of RRM2 may induce apoptosis through the suppression of the Wnt/β-catenin signaling pathway.

The present study revealed a possible role of RRM2 in MM, which may provide new insights for the diagnosis and treatment of MM. However, there were limitations to this study, for instance, the interactions between the RRM2 and Wnt/β-catenin signaling need to be investigated to determine the underlying role of RRM2 in MM development.

In conclusion, the results of the present study indicated that RRM2 upregulation occurred in MM tumors, and that RRM2 knockdown inhibited MM cell proliferation. In addition, RRM2 downregulation may promote apoptosis and activate DNA damage, possibly through the suppression of Wnt/β-catenin signaling via a p-GSK-3β-dependent mechanism. These results suggest that RRM2 may be involved in MM tumorigenesis, and may serve as a biomarker and potential therapeutic target for MM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

XL and JW designed the experiments. XL wrote the manuscript. JP and YZ performed the experiments. BX analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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