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Original Article

RRM2 overexpression in glioblastoma enhances the proliferation and invasion of cancer cells

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Abstract: Glioblastoma multiforme (GBM) is the most devastating brain cancer, characterized by rapid growth rate and aggressive invasion into the surrounding normal brain tissue. Ribonucleotide reductase subunit M2 (RRM2) is an essential factor involved in DNA replication and repair. RRM2 overexpression has been implicated in the development and prognosis of a wide range of human tumors, including breast cancer, lung cancer, bladder cancer, ovarian cancer. However, the association between RRM2 and the progression of brain cancer remained largely unknown. In the current study, by analyzing microarray data, we identified for the first time that increased expression of RRM2 is highly correlated with the severity of GBM. GBM patients with high levels of RRM2 had a much lower survival rate than those with low RRM2 levels. Moreover, we also found that increased expression of RRM2 is essential for the proliferation of GBM cells, while knockdown of RRM2 arrested cancer cells at G1 phase, subjected them to apoptosis and therefore inhibited cell growth. In addition, silencing RRM2 also suppressed GBM cell invasion and migration by reducing the expression of metalloproteinase-2 (MMP-2) and MMP-9 through inhibiting p38 MAPK signaling pathway. Our result supported the oncogenic potential of RRM2 in GBM, and RRM2 silencing represented a promising therapeutic strategy by repressing cancer cell growth and metastasis.

Keywords: RRM2, glioblastoma, cell cycle arrest, apoptosis, proliferation, invasion

Introduction

Glioblastoma multiforme (GBM) is also known as grade IV astrocytoma. It is the most common and malignant brain cancer with rapidly reproducing tumor cells [1]. Moreover, GBMs are surrounded by a hypercellular zone, which contains highly invasive tumor cells infiltrating into nearby normal brain tissue and makes it impossible to entirely remove the tumor by surgery [2, 3]. Therefore, even after the most aggressive treatment, GBM tends to easily relapse in ~90% of the patients within 32~36 weeks post diagnosis [4, 5], and the median overall survival remains as low as 14.6 months, with no more than 10% sufferers stay alive at 5 years [6]. During the past several decades, many efforts have been made to evaluate new therapeutic approaches for GBM, however, none of them has proven to be curative due to the lack of fully understanding of pathological mechanism of the disease, and the treatment of GBM remains difficult.

Like many other types of cancer, deregulated cell cycle has been frequently identified in GBM cells, which contributes to cancer development and progression by accelerating cell division, disturbing genome stability and affecting apoptotic pathways. Ribonucleotide reductase (RNR) is an enzyme catalyzing the reduction of ribonucleoside diphosphate (rNDP) into deoxyribonucleoside diphosphate (dNDP), which is the rate limiting step in the biosynthesis of deoxyribonucleoside triphosphate (dNTP) and is therefore essential for DNA replication and repair [7]. RNR comprises two subunits, RRM1 and RRM2, of which RRM2 (Ribonucleotide reductase subunit M2) is the main regulator of RNR enzymatic activity [8]. Abnormally increased RRM2 expression has been reported to support rapid cell division by inducing dNTP accumulation and is

therefore involved in the development of a range of cancers, including breast cancer, non-small cell lung cancer (NSCLC), ovarian cancer, bladder cancer and colorectal cancer [9-12]. Besides, RRM2 overexpression is also related to poor prognosis in many cancers. For example, in estrogen-negative breast cancer patients, increased RRM2 mRNA levels were found to be correlated with worsened clinical outcome in a dose-dependent manner [13]. Cervical cancer patients with positive RRM2 expression tended to have a higher recurrent rate and lower survival than those with negative RRM2 expression [14]. Also, many studies have suggested RRM2 as a prognostic marker for NSCLC [15-17], and it is among the 12-gene set predictive of overall survival in NSCLC patients receiving adjuvant chemotherapy [18]. Moreover, growing evidences have also demonstrated the association of RRM2 overexpression with chemoresistance developed during cancer treatment. For example, RRM2 was involved in AKT-induced tamoxifen resistance in breast cancer, while inhibiting RRM2 expression could overturn the resistance and improve therapeutic efficacy [19]. Besides, the expression level of RRM2 also progressively increased during the development of gemcitabine resistance in pancreatic cancer, and the patients with low RRM2 tended to response greater to gemcitabine treatment [20].

All these evidences suggested the oncogenic activity of RRM2, and many efforts have been made to understand the crucial role of RRM2 in tumorigenesis and demonstrate the potential of RRM2 as a therapeutic target for cancer treatment. However, up to now, less attention has been placed on the association of RRM2 with malignant GBM. Although it has been reported that RRM2 inhibitors may enhance the cytotoxic effect of tamoxifen in malignant brain tumor cells [21], the underlying mechanism remains poorly understood. So in the current study, we, for the first time, explored the regulatory mechanism of RRM2 in the pathogenesis and prognosis of GBM.

Materials and methods

Cell culture and gene silencing

Human GBM cell lines, U251, was cultured in Dulbecco's modified Eagle's (DMEM) medium (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and maintained in a humidified atmosphere with 5% CO_2 at 37°C. Small interfering RNA (siRNA) targeting RRM2 (siRRM2) and non-targeting control (siNC) (Thermo Fisher Scientific, Waltham, MA, USA) were introduced into U251 cell with Lipofectamine 2000 reagent (Invitrogen).

Cell cycle distribution assay

siRRM2 and siNC-treated U251 cells were harvested and fixed in cold 70% ethanol at 4°C for 30 min. After wash twice with PBS, cells were treated with RNase and stained with 50 ug/ml propidium iodide (Sigma, St. Louis, MO, USA). Cell populations were analyzed in FACS Calibur Flow Cytometer. Each experiment was replicated three times.

Cell proliferation assay

U251 cells transfected with siRRM2 and siNC were seeded in 96-well plates at a density of 2×10^3 cells per well. Cell viability was detected using CyQUANT Cell Proliferation Assays Kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. All experiments were performed with five replicates.

Scratch wound assay

Cells were grown in 6-well plates to >90% confluence. Sterilized pipette tips were used to scratch a wound in cell monolayers. Cell migration was determined as the changes in the distance between wound edges. All experiments were repeated five times.

Transwell assay

Transwell invasion assays were performed with 24-well plates inserted with Matrigel-coated chambers (8-μm pores, Corning Incorporated, NY, USA). Cells cultured in FBS-free DMEM media were placed in the upper chamber at a density of 2×10^4 cells per well. The lower chamber was filled with DMEM media supplemented with 10% FBS. After incubation at 37°C for indicated period, cells invaded into the Matrigel membrane were fixed with 2.5% glutaraldehyde, stained with 0.5% crystal violet and visualized under a microscopy. All experiments were repeated five times.

Western blot analysis

Cell lysates were harvest with RIPA cell lysis buffer. Protein extracts were loaded onto 12%

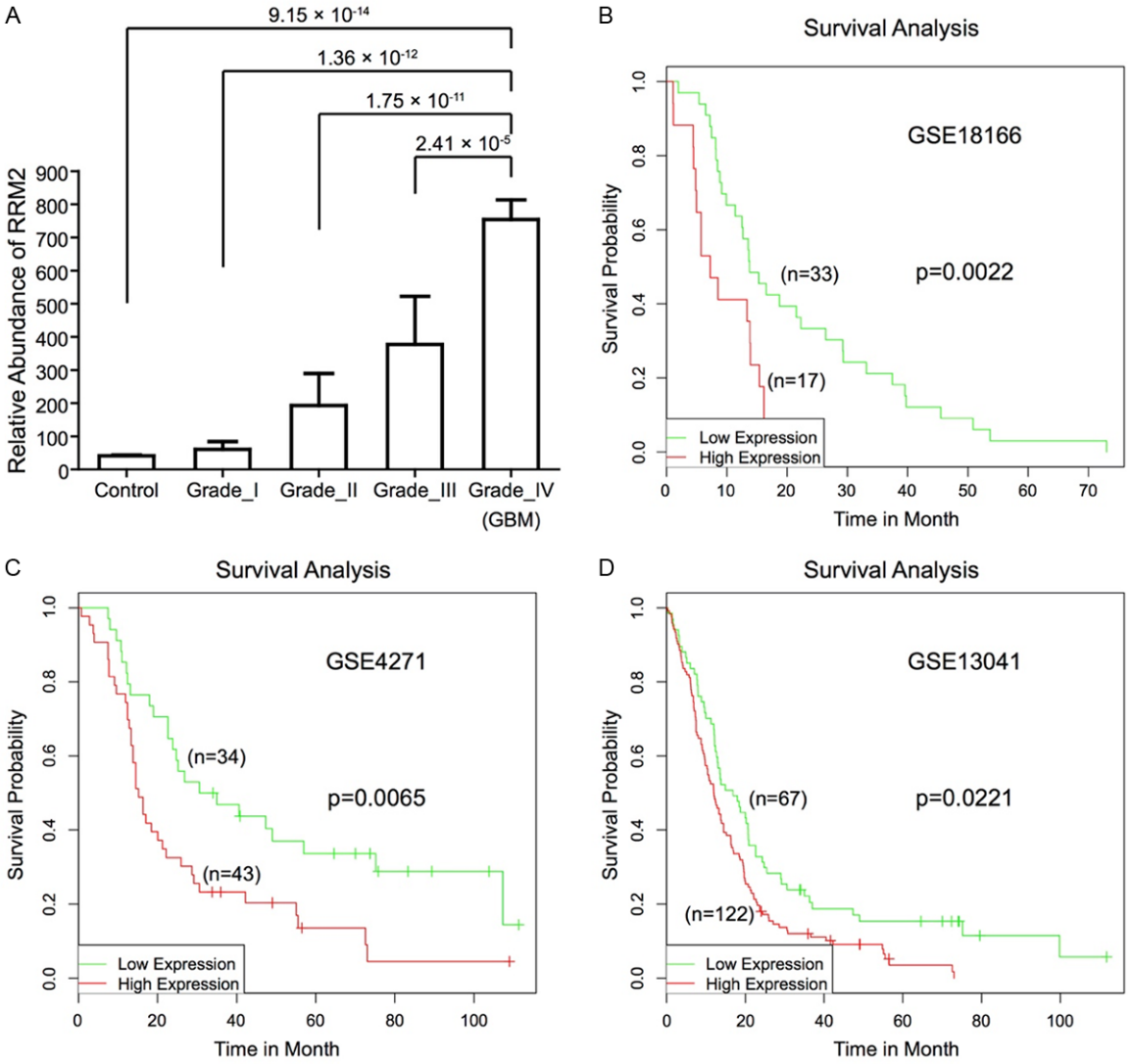

Figure 1. RRM2 is overexpressed in GBM and is predictive of poor prognosis of GBM patients. A. Relative abundance of RRM2 mRNA in normal brain (control), grade I astrocytoma (Grade_I), grade II astrocytoma (Grade_II), grade III astrocytoma (Grade_III) and grade IV astrocytoma (GBM). B-D. High level of RRM2 was associated with short survival in three different dataset documented in DRUGSURV Database.

SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane and immunoblotted with antibodies against RRM1, RRM2, MMP-2, MMP-9, Tubulin (Abcam, Cambridge, MA, USA), PARP, p38, phospho-p38, ERK1/2, phospho-ERK1/2, JNK and phospho-JNK (Cell Signaling Technology, Danvers, MA, USA). Protein bands were visualized with enhanced chemiluminescence (ECL, Millipore, Bredford, USA).

Statistical analysis

Relative RRM2 mRNA levels from microarray data were analyzed by one-way ANOVA followed with Bonferroni post-hoc test. All the experiments were performed with 3~5 replications. Differences between siRRM2 and siNC cells were analyzed with Student's t-test.

Results

RRM2 was overexpressed in GBM and associated with the prognosis of GBM patients

We first studied RRM2 mRNA levels with public available microarray dataset GSE16011, which contained gene expression data from normal brain (control), pilocytic astrocytoma (grade I astrocytoma), diffuse astrocytoma (grade II

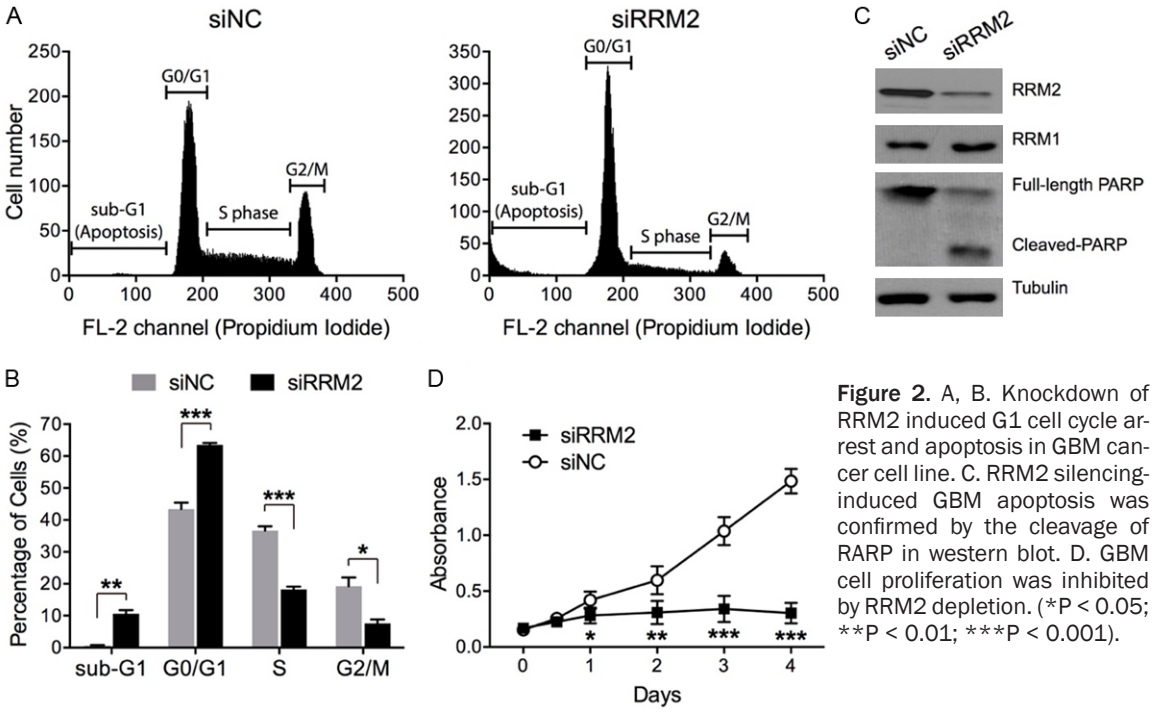

Figure 2. A, B. Knockdown of RRM2 induced G1 cell cycle arrest and apoptosis in GBM cancer cell line. C. RRM2 silencing-induced GBM apoptosis was confirmed by the cleavage of RARP in western blot. D. GBM cell proliferation was inhibited by RRM2 depletion. (*P < 0.05; **P < 0.01; ***P < 0.001).

Table 1. Cell cycle distribution in siRRM2 and siNC GBM cells

GBM cells	Cell cycle phases			
	sub-G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
siNC	0.66 ± 0.14	43.45 ± 1.93	36.65 ± 1.36	19.24 ± 2.71
siRRM2	10.66 ± 1.08	63.28 ± 0.97	18.25 ± 0.83	7.8 ± 0.96
Student's t-test	P < 0.01	P < 0.001	P < 0.001	P < 0.05

Data was expressed as mean ± SD from 3 replicates.

astrocytoma), anaplastic astrocytoma (grade III astrocytoma) and glioblastoma (GBM, grade IV astrocytoma). As shown in **Figure 1A**, RRM2 expression is gradually increased with tumor grades, and high-grade astrocytoma patients expressed a greater amount of RRM2 than those with low-grade tumors (One-way ANOVA, $P = 3.78 \times 10^{-27}$; Bonferroni post-hoc test, GBM vs control: $P = 9.15 \times 10^{-14}$; GBM vs Grade_I: $P = 1.36 \times 10^{-12}$; GBM vs Grade_II: $P = 1.75 \times 10^{-11}$; GBM vs Grade_III: $P = 2.41 \times 10^{-5}$).

Since high RRM2 level is predictive of poor prognosis in many cancer types [13-18], we then used DRUGSURV Database to study the association of RRM2 with the survival of GBM patients in three different microarray datasets (GSE18166, GSE4271 and GSE1304) [22]. For each dataset, patients with RRM2 expression higher than the average level were classified as high RRM2 group, while the others were in low RRM2 group, and the overall survival was then compared between the two groups. As a result, patients with high RRM2 expression had a significantly poorer survival outcome than the low RRM2 patients (**Figure 1B-D**). This pattern is evident among all three GBM datasets, suggesting the potential of RRM2 as prognostic marker for GBM.

Knockdown of RRM2 inhibited GBM cell growth by inducing cell cycle arrest and apoptosis

Above results suggested that high level of RRM2 is associated with the severity and the poor prognosis of GBM. Then, we ask if GBM cell proliferation could be inhibited by suppressing RRM2 expression. RRM2 is an important factor in cell cycle progression by providing dNTPs for DNA synthesis, and RRM2 expression is regulated in a cell-cycle dependent fashion, with the highest level at late G1/early S phase [7]. Therefore, we first used flow cytometry to evaluate cell cycle distribution of human GBM cell line U251 transfected with either

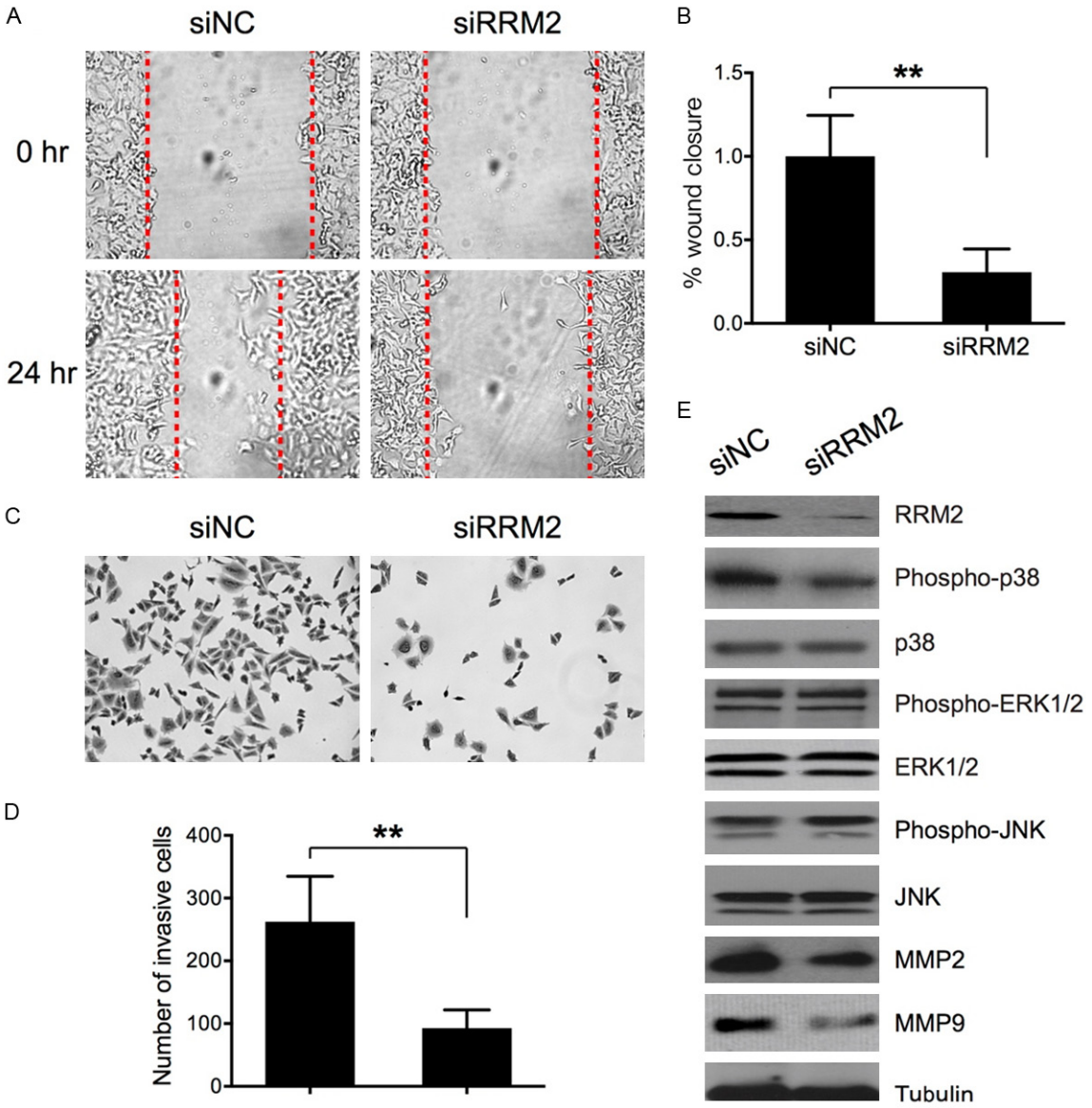

Figure 3. A, B. Scratch wound healing assay show that knockdown of RRM2 inhibited GBM cell migration. C, D. Transwell assay show that knockdown of RRM2 inhibited GBM cell invasion. E. Knockdown of RRM2 inhibited the expression of MMP-2 and MMP-9 by deactivating p38 but not ERK1/2 and JNK. (*P < 0.05; **P < 0.01; ***P < 0.001).

RRM2 siRNA (siRRM2) or a non-targeting control siRNA (siNC). As compared with control cells, siRRM2-treated GBM cells had a significant accumulation of G0/G1 phase cell population and corresponding reduction of S and G2/M phase populations, indicating a remarkable G1 cell cycle arrest induced by RRM2 knockdown (**Figure 2A** and **2B**; **Table 1**, Student's t-test, $P < 0.001$). Moreover, significant increase of sub-G1 population was also observed following RRM2 depletion, indicting more GBM cells were undergoing apoptotic cell death (**Figure 2A** and **2B**; **Table 1**, Student's t-test, $P < 0.01$). Increased apoptosis was also verified by western blot, in which, cleaved PARP fragment was found to be accumulated in RRM2-silenced GBM cells (**Figure 2C**). Both cell cycle arrest and apoptosis are known to inhibit cancer cell proliferation, and our cell proliferation assay further confirmed that GBM cell growth was largely suppressed by RRM2 knockdown (**Figure 2D**, Student's t-test, 1 Day: $P < 0.05$; 2 Day: $P < 0.01$; 3 Day: $P < 0.001$; 4 Day: $P < 0.001$).

Knockdown of RRM2 suppressed the migration and invasion ability of GBM cells

As mentioned above, the extensive invasive feature is also a hallmark of malignant GBM, and contributes to the high propensity of tumor recurrence [2, 3]. We thus explored the influence of RRM2 on the migration and invasion ability of GBM cells. As revealed by wound healing assay (**Figure 3A** and **3B**), the wound gap in control cells was largely filled out within 24 hours, while knockdown of RRM2 inhibited GBM migration and largely slowed down gap closure (Student's t-test, $P < 0.01$). Besides, transwell assay also demonstrated that RRM2 knockdown significantly reduced the number of invading cells and inhibited the invasiveness of GBM cells (**Figure 3C** and **3D**, Student's t-test, $P < 0.01$). Moreover, we also found that RRM2 silencing was accompanied with reduced expression of metalloproteinase-2 (MMP-2) and MMP-9 and reduced phosphorylation of p38, while no big change was observed for ERK1/2 and JNK (**Figure 3E**). MMP-2 and MMP-9 are important regulators of cell motility, and their activities are known to be regulated by ERK1/2 pathway, p38 MAPK pathway and JNK pathway. According to our results, RRM2 may support GBM migration and invasion by enhancing MMP-2 and MMP-9 expression through p38 MAPK signaling pathway but not through ERK and JNK pathway.

Discussion

RRM2 overexpression has been implicated in the development and progression of many cancers, however, the oncogenic role of RRM2 in GBM has been poorly understood. In the current study, by analyzing public available microarray data, we provided the first evidence that increased RRM2 mRNA expression is associated with high grade astrocytoma (GBM) and is predictive of the poor prognosis of GBM patients. And we also found that knockdown of RRM2 expression can inhibit GBM cell proliferation by inducing G1 cell cycle arrest and apoptosis, and reduce GBM invasion by suppressing p38 MAPK signaling pathway and decreasing the expression of MMP-2 and MMP-9.

RRM2 is playing a crucial role in dNTP biosynthesis and involved in DNA replication and repair. In each cell cycle, RRM2 is highly expressed at late G1 and early S phase and degraded at late S phase [7], which is an important step to prepare enough dNTP for the following DNA synthesis in S phase and facilitate cell cycle progression. Given these, the high RRM2 expression identified in GBM may contribute to the fast replication of cancer cells, while knockdown of RRM2 prevented cells from entering S phase by blocking dNTP supply and therefore arrested cell cycle at G1 phase. Cell cycle arrest usually results in cell growth inhibition and sensitizes cell to apoptosis. In our study, we did observe an increased apoptosis and decreased cell proliferation in siRRM2-treated GBM cells, suggesting the therapeutic potential of RRM2 silencing in GBM treatment.

In addition to rapid growth rate, GBM is also characterized by aggressive invasion into the surrounding normal brain tissue. Previous studies have demonstrated that high levels of RRM2 supported the invasion ability of prostate and pancreatic cancer cell by enhancing the expression of MMP-9 [23, 24]. Therefore, in the current study, we also explored the role of RRM2 in GBM cell migration and invasion, and we found that RRM2 overexpression was also associated with the high levels of MMP-2 and MMP-9 in GBM cells, while knockdown of RRM2 suppressed MMP-2 and MMP-9 expression by deactivating p38 and therefore inhibited the invasiveness of cancer cells, indicating the importance of p38 MAPK signaling pathway in RRM2-mediated GBM cell migration and invasion.

In summary, our results demonstrated that RRM2 overexpression is involved in GBM cell proliferation and invasion and suggested the potency of RRM2 silencing as a promising strategy for GBM therapy.

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Disclosure of conflict of interest

None.

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