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Overexpression of RRM2 in gastric cancer cell promotes their invasiveness via AKT/NF-κB signaling pathway

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The ribonucleotide reductase M2 subunit (RRM2) plays an active role in tumor progression and is frequently overexpressed in cancer. It plays a significant role in the regulation of cell invasiveness, cell migration and tumor metastasis. Elevated RRM2 expression has been reported to be associated with poor prognosis of gastric cancer. However, the molecular mechanisms of RRM2 in gastric cancer cells remain elusive. In our study, we found that RRM2 highly expressed in gastric cancer cells BGC823. RRM2 stimulation dose-dependently enhanced the invasion and migration of BGC823 cells. Furthermore, we found that the expressions of MMP-2 and MMP-9 in BGC823 cells were significantly increased after RRM2 stimulation. In addition, RRM2 time-dependently induced activation of AKT, IκBα, and NF-κB. These effects of RRM2 were prevented by AKT selective inhibitor GSK690693 as well as NF-κB selective inhibitor BAY117082. In conclusion, our findings establish a signaling role for RRM2 in gastric cancer cells and identify that the RRM2/AKT/NF-κB signaling pathway is essential for tumor invasiveness in gastric cancer cells. Thus, our data may provide knowledge for using RRM2 as a novel target for effective diagnosis and treatment of gastric cancer.

1. Introduction

Gastric cancer (GC) is the fourth most commonly diagnosed cancer and the second most common cause of cancer-related death worldwide, accounting for 8 % of cancer incidence and 10 % of cancer deaths (Guo et al. 2015). Even with advanced systematic therapy, the 5-year overall survival for patients with late-stage GC was no more than 20 %. Despite the therapeutic strategies have greatly developed, patients with advanced gastric cancer often suffer from invasion and metastasis, and the prognosis is still very poor. Determinants of cellular invasiveness represent a potential therapeutic target (Mello et al. 2015). Therefore, increasing efforts have been devoted to investigating the mechanisms of GC progression, especially the mechanisms of GC invasion.

Ribonucleotide reductase subunit M2 (RRM2), located in chromosome-2 p25-p24, is an enzyme of central importance in DNA synthesis. RRM2 itself is a dimer of two 44 kDa moieties, each containing a tyrosine free-radical and nonheme iron (Zhang et al. 2014). There is accumulating evidence that cells overexpressing RRM2 exhibit enhanced cellular invasiveness, suggesting its role as a prognostic factor and a possible therapeutic target for cancer therapy (Duxbury and Whang 2007). However, the mechanisms through which RRM2 affects the invasive phenotype have not been closely studied. Therefore, we examined these mechanisms in the context of human gastric cancer.

In this study, we identified that RRM2 was overexpressed in gastric cancer cells, and RRM2 contributed to gastric cancer cell invasion and migration. We further revealed that RRM2 regulated the expression of MMP-2 and MMP-9 and induced activation of AKT and NF-κB, which may contribute to gastric cancer cell invasion and migration enhanced by RRM2.

2. Investigations and results

2.1. Expression and secretion of RRM2 in gastric cancer cells

We first detected the expression of RRM2 in gastric cancer cells. Using real-time PCR, we found that RRM2 was markedly expressed in gastric cancer BGC823, MKN-45, SGC-7901 cells compared to human gastric epithelial cell line GES-1 cells at mRNA level (Fig. 1A). Of note, western blot revealed that RRM2 was overexpressed at protein level in gastric cancer cells (Fig.1B), and ELSIA assay showed that RRM2 secretion was also increased in all detected gastric cancer cells, suggesting a positive role of RRM2 in gastric cancer cells (Fig.1C).

2.2. RRM2 promotes gastric cancer cell invasion and migration

We then examined the influence of RRM2 on gastric cancer cells. BGC823 cells were exposed to different concentrations of RRM2 (10, 25, and 50 ng/ml) for 12 h to detect cell invasion and migration abilities. As shown in Fig. 2, we found that RRM2 dose-dependently induced the invasion and migration of BGC823 cells. Altogether, these data confirm that RRM2 contributes to the invasion and migration of gastric cancer cells.

2.3. RRM2 regulates the expressions of MMP-2 and MMP-9

Matrix metalloproteinases (MMPs) are important molecules involved in tumor invasion and metastasis. Using real-time PCR and ELISA assay, we here found that RRM2 dose dependently increased the mRNA and protein levels of MMP-2 and MMP-9 in BGC823 cells (Fig. 3).

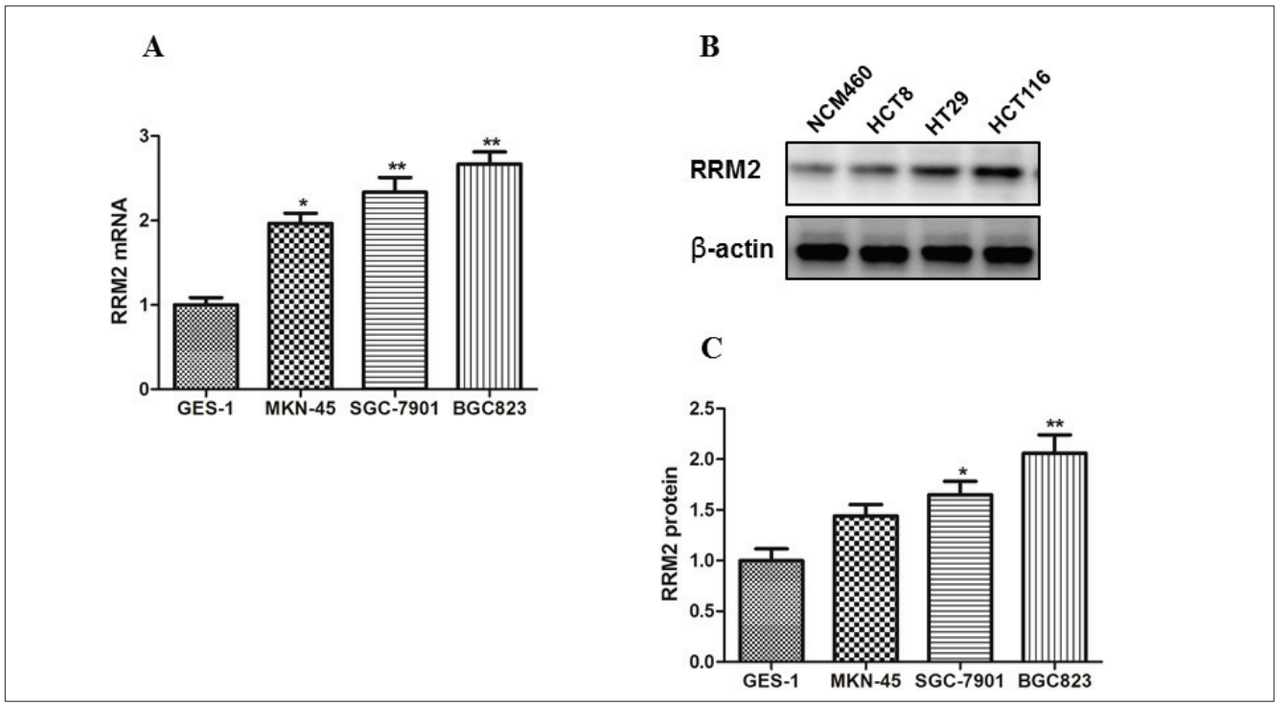

Fig. 1: The expression of RRM2 in gastric cancer cells. Real-time PCR was carried out to determine expression of RRM2 in gastric cancer BGC823, MKN-45, SGC-7901 cells and human gastric epithelial cell line GES-1 cells at mRNA level (A). Western blot was performed to determine expression of RRM2 in gastric cancer BGC823, MKN-45, SGC-7901 cells and human gastric epithelial cell line GES-1 cells at protein level (B). Results shown are representatives of three independent experiments, *p<0.05.

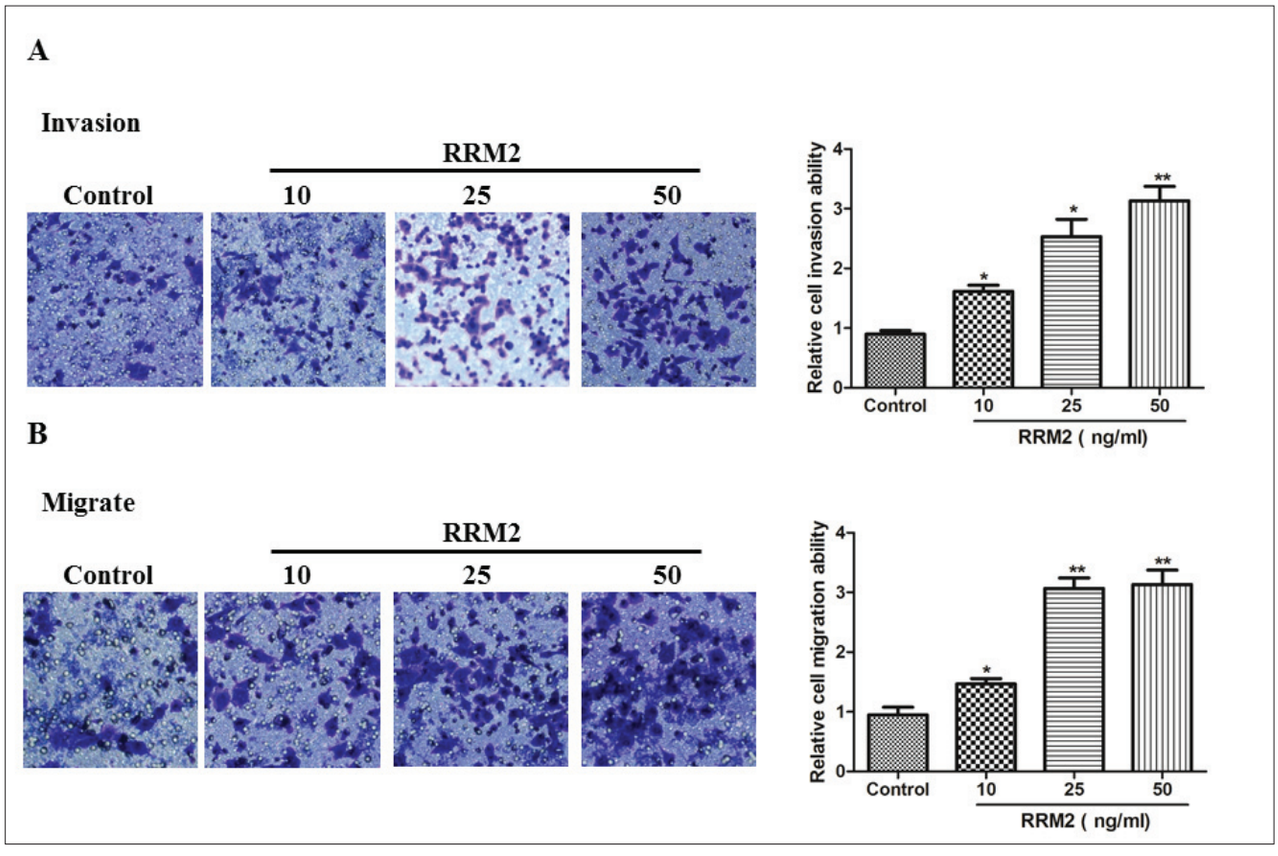

Fig. 2: Effects of RRM2 stimulation on BGC823 cells. BGC823 cells were treated with 10–100 ng/ml RRM2 for 12 h, invasion ability was measured by cell invasion assay (A). BGC823 cells were treated with 10-100 ng/ml RRM2 for 12 h, migration ability was measured by cell migration assay (B).

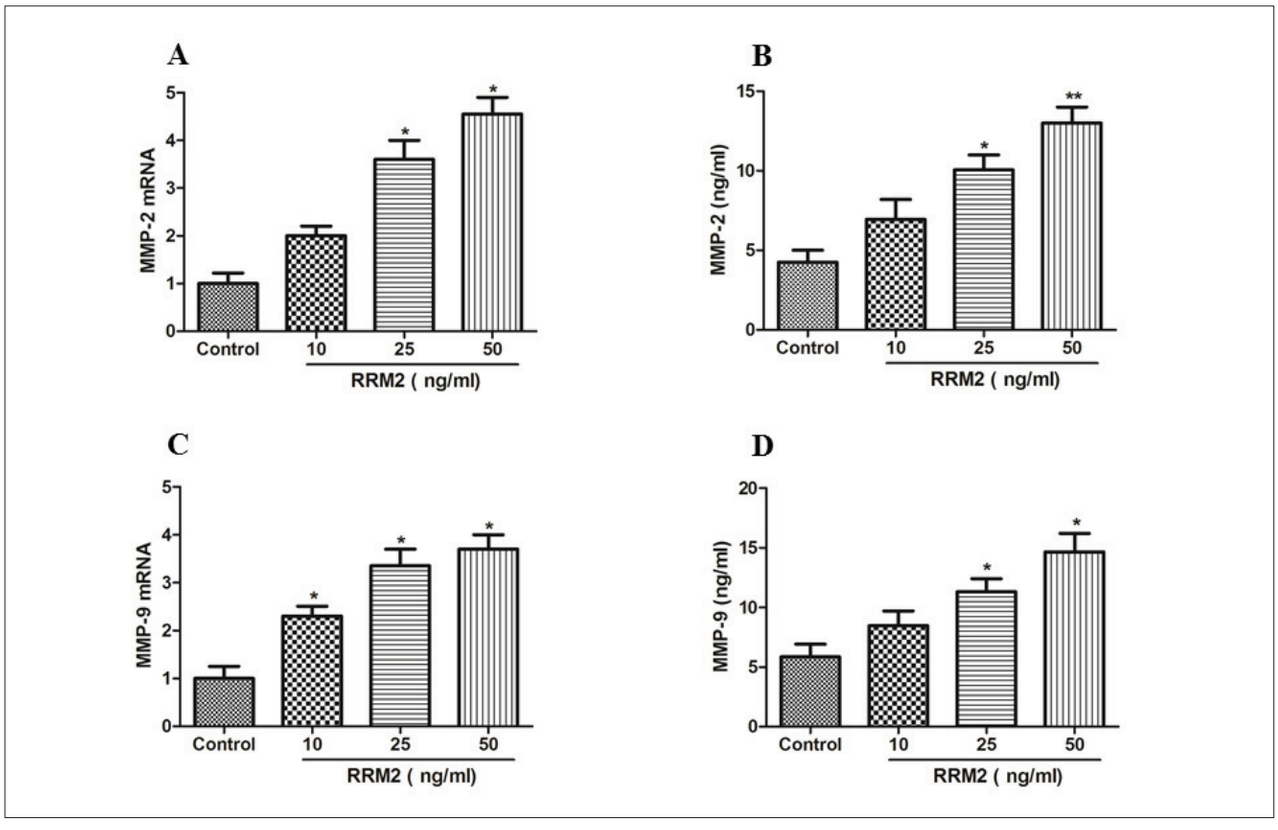

Fig. 3: Effects of RRM2 on MMP-2 and MMP-9 expression. BGC823 cells were treated with 10-50 ng/ml RRM2 for 12 h, the mRNA levels of MMP-2 and MMP-9 in BGC823 cells were measured by real-time PCR (A, B), and the protein levels of MMP-2 and MMP-9 in BGC823 cell supernatant were measured by ELISA assay (C, D).

2.4. RRM2 induces activation of AKT and NK-κB

To document the effect of RRM2 on AKT activation, we observed the phosphorylation of AKT by western blot after RRM2 stimulation. The results showed that activation of AKT was observed after RRM2 stimulation, in a time-dependent manner, and peak activation occurred at 30 min (Fig.4A). We further found that RRM2 stimulation resulted in phosphorylation of IκBα and NF-κB P65. We then explored the activity of NF-κB by NF-κB activity assay. The results showed that RRM2 time-dependently induced activation of NF-κB, and peak activation occurred after 30 min (Fig. 4B, 4C).

2.5. RRM2 affects gastric cancer cell invasion and migration via AKT/ NF-κB pathway

To determine the role of AKT and NF-κB activation in RRM2-mediated cell invasion and migration, GSK690693 (a selective inhibitor of AKT, 10 μM) was used to inhibit AKT activation, while BAY117082 (a selective inhibitor of NF-κB, 5 μM) was used to inhibit NF-κB activation. After pretreatment of BGC823 cells with GSK690693 and BAY117082 before RRM2 stimulation, NF-κB activity assay showed that GSK690693 suppressed activation of NF-κB, suggesting that RRM2 induces NF-κB activation via AKT pathway. We also found that BAY117082 attenuated activation of NF-κB in BGC823 cells (Fig. 5A). Further, using invasion assay and migration assay, we revealed that MMR2-induced cell invasion and migration of BGC823 cells were inhibited by GSK690693 and BAY117082. Together, these data indicate that RRM2 promotes gastric cancer cell invasion and migration through activation of AKT/NF-κB pathway.

3. Discussion

RRM2, a subunit of ribonucleotide reductase (RR), has been implicated to be an important mediator in tumor progression and development (Wang et al. 2012). It may have oncogenic effects including tumor development, metastasis and drug resistance (Kang et al. 2014). Recently, there has been reported that RRM2 overexpression has been shown to increase cellular invasiveness in human pancreas cancer cells (Wang et al. 2015). In this study, a major finding is identification of the RRM2 effects on gastric cancer cell invasion and migration. Our data revealed that RRM2 stimulation increased the expression of MMP-2 and MMP-9, *via* activating the AKT/NF-κB signaling pathway, which finally resulted in a significant increase in cell invasion and migration.

RRM2 expression positively correlated with malignancy of endometrial cancer. Lu et al. (2012) demonstrated the high expression of RRM2 in gastric cancer patients compared to the controls and RRM2 overexpression is positively correlated with invasion depth, poorly differentiated type, and tumor node metastasis stage. However, little is known about the exact role of RRM2 in gastric cancer. In this study, we found that RRM2 was markedly expressed in gastric cancer cells. Stimulation with RRM2 dose-dependently promoted the invasion and migration of gastric cancer BGC823 cells. In addition, knockdown of endogenous RRM2 inhibited the invasion and migration of BGC823 cells, whereas overexpression of RRM2 promoted the invasion and migration of BGC823 cells. It implies the important role of RRM2 in gastric cancer invasiveness from these results.

AKT, a serine/threonine kinase, is constitutively activated in astrocytoma and tightly correlates with poor outcome of patients with the disease (Qzes et al. 1999). Increased AKT activity promotes malignant behavior of invasive glioblastoma cells, and down-regulation of AKT importantly inhibits invasion of glioblastoma cells (Molina et al. 2010). Cumulative evidence has revealed that the AKT signaling pathway plays a crucial role in many cellular processes of cancer, including cell invasion, migration, and metastasis (Chin and Toker 2009). Here, we found that RRM2 time dependently induced activation of AKT, and the AKT pathway was a response to RRM2-mediated cell invasion and migration

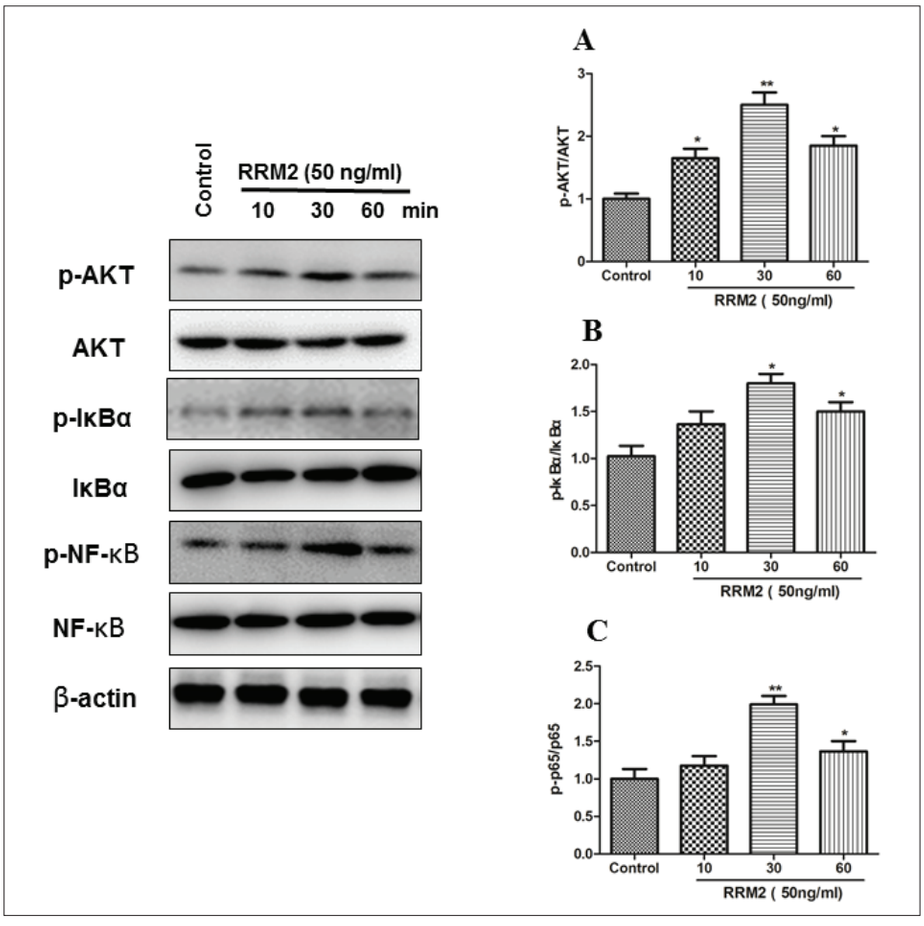

Fig. 4: Effects of RRM2 on activation of AKT/NF-κB signaling pathway in gastric cancer cells. BGC823 cells were treated with RRM2 of different times as indicated, the expressions of phosphor-AKT , AKT (A) phosphor-IκBα, IκBα (B) and phosphor-P65, P65 (C) were detected by western blot. Results shown are representatives of three independent experiments, *p<0.05.

of gastric cancer cells. NF-κB, a transcription factor, provides an increasingly complex view of the mechanism of signal-mediated transcriptional activation. One of the most distinctive features of the NF-κB signaling pathway is the rapidly deployed negative feedback loop that results in post-induction repression (Hayden and Ghosh 2008). This phenomenon results from the strong activation of the IκBα gene by NF-κB. Newly synthesized IκBα protein enters the nucleus and successfully competes with DNA for the NF-κB. The newly-formed IκBα-NF-κB complex is transported to the cytoplasm, where it resumes its status in the resting cell ready for future activation. After stimuli, IκBα is phosphorylated and separates from the p50-p65-IκBα heterotrimer. Then the p50-p65 heterodimer translocates to the nucleus and NF-κB is finally activated. In our study, we found that RRM2 could active IκBα and NF-κB (Zaragoza et al. 2006). In this study, we determined that RRM2 induced NF-κB activation *via* the AKT pathway. We found

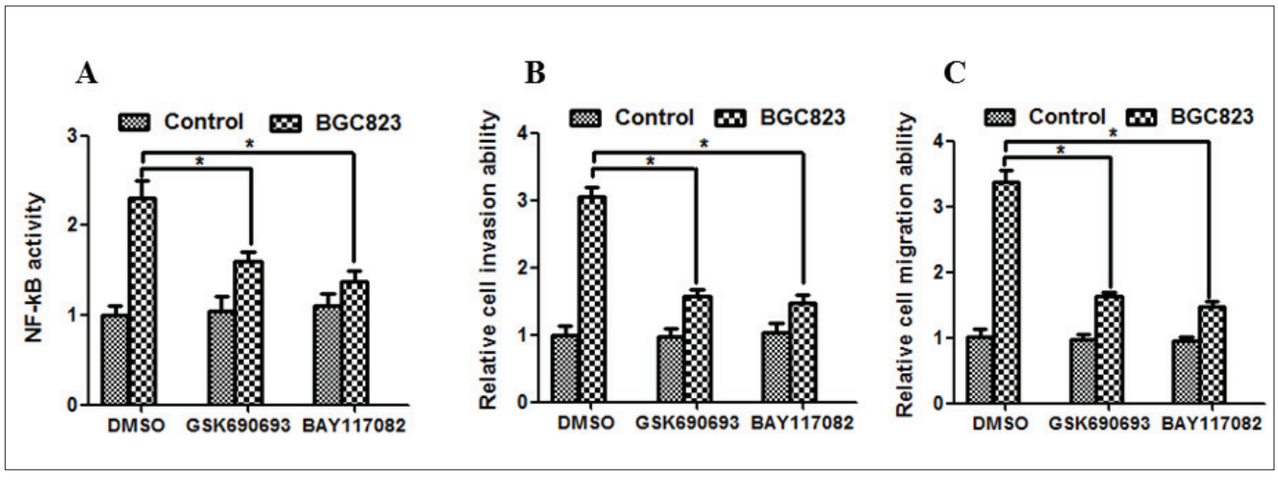

Fig. 5: Effects of AKT/NF-κB signaling pathway on RRM2-mediated cell invasion and migration. Cells were pretreated with GSK690693 and BAY117082 respectively and incubated with or without RRM2, and then
NF-κB activity was examined by NF-κB activity assay (A). Cells were pretreated with GSK690693 and BAY117082 respectively and then incubated with or without RRM2, cell invasion and migration abilities were determined by cell invasion and migration assays (B, C). Results shown are representatives of three independent experiments, *p<0.05.

that NF-κB, as a well-known actor in carcinogenesis and cancer development, was involved in RRM2-mediated gastric cancer cell invasion and migration. Moreover, we found that RRM2 increased the expression and secretion of MMP-2 and MMP-9 in gastric cancer cells.

In conclusion, the present study showed that RRM2 promotes gastric cancer cell invasion and migration through activation of AKT/NF-κB signaling pathway and regulates the expressions of MMP-2 and MMP-9. Therefore, RRM2 may act as a potential target for the diagnosis and therapy of gastric cancer. Targeting RRM2 and its downstream signaling intermediaries represents a rational approach for developing novel anticancer therapeutics.

4. Experimental

4.1. Chemicals and antibodies

RRM2 was purchased from ProSpec (Rehovot, Israel).GSK690693 (selective inhibitor of AKT) and BAY117082 (selective inhibitor of NF-κ B) were all obtained from Sigma (St Louis, MO, USA). Primary antibodies for RRM2, AKT, p-AKT, IκBα, p-IκBα, P65, p-P65 were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Cell culture

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA.). The cells were cultured in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS). All cells were incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C.

4.3. Quantitative real-time PCR

Total RNA samples were extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized through reverse transcription using random primer and M-MLV reverse transcriptase (Tiangen, Beijing, China). Real-time PCR was performed using SYBR
Green SuperMix (Invitrogen) and analyzed with an Applied Biosystems 7900 Real-Time PCR System. Primer sequences were as follows:
RRM2, 5'-GCGATTTAGCCAAGAAGTTCAGAT-3' (forward) and
5'-CCCAGTCTGCCTTCTTCTTGA-3' (reverse); MMP-2,
5'-CTTCTTCCCTCGCAAGCC-3'(forward) and
5'-ATGGATTCGAGAAAACCG-3' (reverse); MMP-9,
5'-ACGCAGACATCGTCATCC-3' (forward) and
5'-AACCGAGTTGAACCACG-3' (reverse); β-actin,
5'-CTGGGAGTGGGTGGAGGC-3' (forward) and
5'-TCAACTGGTCTCAAGTCAGTG-3' (reverse).

4.4. Western blot

Cells were washed twice with ice-cold PBS and lysed on ice and collected after centrifugation. Thirty micrograms of cell lysate lysate was separated on 12 % SDS-polyacrylamide (SDS-PAGE) gels. After electrophoresis, protein was transferred to the polyvinylidene difluoride (PVDF) membrane. After blocking for 1 h in Tris buffered saline (TBS) containing 5 % BSA, the membranes were incubated with primary antibody at 4 °C overnight. The membranes were further incubated for 1 h at room temperature with secondary antibodies and visualized using an enhanced chemiluminescence kit (Applygen Technologies Inc). West Pico Chemiluminescent (Pierce) was used as the substrate to visualize protein bands, which were quantified using densitometry image analysis software (Image Master VDS; Pharmacia Biotech). Normalization was made against β-actin expression.

4.5. Transwell invasion assay

Transwell invasion assay was performed with transwell inserts obtained from Costar (San Diego, CA, USA). Briefly, cells were treated with RRM2 of different concentrations and then were resuspended in RPMI-1640 media containing 1 % BSA into the upper chamber in which matrigel matrix was precoated. RPMI-1640 containing 20 % FBS was added to the lower chamber as a chemoattractant. Cells were allowed to invade for 12 h at 37 °C. Non-invading cells were removed with a cotton swab. Invading cells were fixed with 4 % neutral buffered formalin for 15 min at room temperature and further stained with crystal violet for 20 min. Finally, cells were photographed, and seven random fields were chosen for calculating the number of invading cells.

4.6. Transwell migration assay

Transwell migration assay was performed with transwell inserts obtained from Costar (San Diego, CA, USA). Cells were treated with RRM2 of different concentrations and then were resuspended in RPMI-1640 media containing 1 % BSA. 20,000 cells in 200 µl were added into the upper chamber, while RPMI-1640 containing 20 % FBS was added to the lower chamber. Cells were allowed to migrate for 12 h, and then the migrated cells were fixed and stained with crystal violet. Finally, cells were photographed, Tumor Biol. and seven random fields were chosen for calculating the number of cells.

4.7. ELISA assay

To detect MMP-2 and MMP-9 secretion, cells were stimulated with RRM2 for 16 h, and cell supernatant was collected and determined by ELISA kits (Danyelbiothech, Rehovot, Israel), following the manufacturer's instructions.

4.8. NF-κB activity assay

After co-transfecting with a luciferase reporter vector pGL4.32 (luc2P/NFkB-RE/Hygro) and a renilla luciferase-expressing vector that were obtained from Promega (Madison, WI, USA) using Lipofectamine 2000 (Invitrogen), cells were treated with RRM2 of different concentrations for indicated time, and then the luciferase activities of firefly and renilla were measured by the Dual Luciferase Reporter Gene Assay kit (Beyotime, Wuhan, China). Finally, the firefly luciferase activity was normalized to the renilla luciferase activity to identify NF-κB activation.

4.9. Statistical analysis

All results were expressed as mean±SEM from at least three independent experiments. The statistical significance of mean difference between two groups was determined using two-tail Student's t -test and P values of less than 0.05 and 0.01 were considered significant and highly significant, respectively.

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