European Review for Medical and Pharmacological Sciences 2020; 24: 4729-4737

Knockdown of HMGB2 inhibits proliferation and invasion of renal tumor cells *via* the p-38MAPK pathway

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Abstract. – OBJECTIVE: To investigate the function of HMGB2 in renal tumor ACHN cells in vitro and in vivo and to study the underlying molecular mechanisms.

PATIENTS AND METHODS: Kaplan-Meier analysis was used to study the relationship between expression of HMGB2 and prognosis of renal tumor. MTT assay was employed to examine cell proliferation and flow cytometry analysis was used to study the role of HMGB2 in cell apoptosis in ACHN cells. Transwell assays were used to explore the migration and invasion of ACHN cells. The effect of HMGB2 on tumor growth was investigated in vivo. Western blot was performed to evaluate the expression levels of p-JNK, p-ERK and p-p38MAPK.

RESULTS: HMGB2 was upregulated in renal tumor and correlated with worse overall survival in renal tumor patients. Down-regulation of HMGB2 suppressed ACHN cells proliferation, invasion and migration *in vitro*. Moreover, down-regulation of HMGB2 inhibited tumor growth *in vivo* and HMGB2 exerts the oncogene function partly via the inhibition of p-p38MAPK activation.

CONCLUSIONS: Our results provide novel insights into neuropathic pain and help to explore therapeutic targets in the treatment.

Key Words:
HMGB2, Renal tumor cells, MAPK pathway.

Introduction

Renal tumor is one of the common urinary system tumors, and surgery is considered the only effective treatment for primary kidney tumor. In recent years, renal tumor has been increasingly common and has a poor prognosis, for the drug resistant gene, insensitivity of radiotherapy or chemotherapy[1,2]. It is significant to research the molecular mechanism and the targeted therapy to specific gene for the improvement of clinical prognosis[3,4]. The high mobility group box protein 2 (HMGB2) belongs to the HMGB protein family, which comprises numbers of non-histone nuclear proteins that play important roles in cells[5]. The HMGB family consists of HMGB1, HMGB2, HMGB3 and HMGB4. And that HMGB2 is eighty percent homologous to HMGB1 and possesses indistinguishable biological features[6-8]. Campana et al[9] showed that HMGB1 acted as an oncogene and played a significant part in tumor cell viability, cell proliferation, as well as cell metastasis. The expression of HMGB1/RAGE proteins in renal cancer increased and was closely related to the clinical prognosis of patients[10]. However, compared with HMGB1, relatively little is known regarding the biological function of HMGB2.

In the present study, we performed RNA interference (RNAi) to knock down the expression of HMGB2 in ACHN cells to investigate the effects of HMGB2 inhibition on proliferation, apoptosis, migration, invasion, and further test the phosphorylation levels of JNK, ERK and p38MAPK in the downstream MAPK pathway. This study aims to explore the mechanism of HMGB2 inhibiting the biological behavior of ACHN cells, and provide a reliable basis for HMGB2 targeted therapy for renal cancer.

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Patients and Methods

Patients and Clinical Samples

A total of 15 metastatic and 15 non-metastatic tissues were collected from patients who underwent surgery for renal tumor at People's Hospital of Yichun City. Two independent genitourinary pathologists confirmed diagnoses. No anticancer treatments had been conducted before surgery in the study. These tissues were frozen in liquid nitrogen immediately after surgery and subsequently stored at −80°C until analyzed. Tumor staging was assessed in accordance with the 2009 TNM system and criteria of WHO. Overall survival (OS) was defined as the interval between the dates of surgery and death or date of last contact. Written informed consent was obtained from all individual participants included in the study. This investigation was approved by the Ethics Committee of People's Hospital of Yichun City. 15 paired samples were tested by RNA sequencing. The data were compared to determine differentially expressed HMGB2. The differentially expressed HMGB2 were identified with the Bioconductor R package limma. The *p*-value was adjusted for multiple testing using the false discovery rated (FDR) method (FDR<0.05), and |log2 fold change|≥2.0 and adjusted *p*-value < 0.05 was considered to indicate a statistically significant difference.

Microarray Analysis

A processed HMGB2 expression data in renal tumor from TCGA database was acquired from FRKM database and analyzed with the limma package of R. Overall survival (OS) was defined as the interval between the dates of surgery and death or date of last contact.

Cell Lines and shRNA Transfection

Renal tumor cell lines (including 786-O, Caki-1,769-P, OS-RC-2, ACHN) and normal kidney epithelial cells (HK2) were purchased from the Type Culture Collection (Shanghai, China). ACHN cells were previously preserved in our lab. All cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), and incubated in a humidified incubator containing 5% CO_2 at 37°C. The cells were divided into four groups, blank control group (blank), empty vector transfected group (shRNA-NC), HMGB2 transfected group (shRNA-HMGB2), and HMGB2 re-expression group (HMGB2). ACHN cells in logarithmic growth phase were collected, digested and transferred into a 6-well culture plate with a concentration of 5*10^4/mL. Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was used for cell transfection according to the manufacturer's instructions. The short hairpin RNA (shRNA) against HMGB2 (shRNA-HMGB2) and negative control shRNA (shRNA-NC) were designed and synthesized by Jikai Company (Shanghai). The following shRNA sequences were used: shRNA-HMGB2, 5'-CACCGTCTGCTAAAGAGAAGGAAATTCAAGAGATTTCCTTTCTCTTTAGCA-GACTTTTTTG-3; shRNA-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; HMGB2, 5'-GTCCGAATTCACCACCATGGGCAAAGGAGATCCTAA-3' (forward) and 5'-CGCCGCGGCCGCTTATTCATCATCATCATCTT-3' (reverse). The blank control group was transfected with liposomes.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol® kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was employed to extract total RNA, according to the manufacturer's protocol. The optical density (OD) at 260 nm and 280 nm was recorded with an ultraviolet/visible spectrophotometer, and the concentration and purity of the total RNA were measured. Reverse transcription was performed using PrimeScript RT reagent Kit (TaKaRa, Dalian, Liaoning, China). Quantitative real-time PCR analyses were performed by Applied Biosystems (Foster City, CA, USA, 7500 system) using SYBR Premix Ex Taq™ (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primers were as follows: the upstream and downstream primer sequences of HMGB2 gene were 5'-TATGGCAAAAGCGGACAAGG-3' and 5'-CTTCGCAACATCACCAATGGA-3'(196 bp); the upstream and downstream primer sequences of GAPDH gene, 5 '-GAGAGGGAA ATCGTG CGTG AC-3 ' and 5 ' -CATCTGCTGGAAGGTGGACA-3'(452 bp). The relative expression level of HMGB2 was calculated using the $2^{-\Delta\Delta CT}$ method. qRT-PCR assay was performed three times for each sample. The cDNA obtained by RT was stored at -20°C. PCR program was as follows: pre-denaturation at 95°C for 5 min and a total of 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec following extension at 72°C for 30 sec.

Cell Viability by MTT Assay

MTT assay was used to examined cell viability after knocking down of HMGB2. In the Eagle's minimum essential medium (Eagle's MEM) containing 10% FBS, U251 and U-87MG cells were collected and suspended and then seeded in the 96-well plates with a density of 2,000 cells/well in 200 μL. Then, they were incubated in an incubator (37°C and 5% CO_2) 24 hours later, 100 μL MTT solution (5 mg/mL) was added to each well, and then incubated at 37°C and 5% CO_2 for another 4 hours. Then, we removed the medium and added 150 μL dimethyl sulfoxide (DMSO) to each well. Then, we shook plates warmly on shaker for 10 min to dissolve the crystals entirely. A wavelength of 570 nm was used to examine the OD value.

Cell Apoptosis by Flow Cytometry

ShRNA-HMGB2, shRNA-NC and liposome cells after transfection for 48 h were collected to the final concentration of 1 × 10^6/ml and rinsed twice with phosphate-buffered saline (PBS). The Annexin V-FITC Apoptosis Kit (BD Bioscience, San José, CA, USA) was used to assay the apoptosis ratio.

Cell Invasion Assay

The invasive ability of HMGB2 on ACHN cells were determined using transwell assay (8-μm pore size polycarbonate filter) coated with Matrigel (Corning, MA, USA). Briefly, each group (ShRNA-HMGB2 group, shRNA-NC group and blank control group of cells (5×10^4 cells) were suspended in 100 μL serum-free 1640 medium, and then inoculated in the upper chamber. Then, 600 μL RPMI-1640 medium containing 10% FBS were added into the lower chamber and then cells were cultured in 37°C and 5% CO_2 for another 24 hours. After that, cells on the surface of the upper chamber were removed using sterile cotton swab and cells that had invaded the lower surface of the basement membrane were stained with 4% paraformaldehyde for 10 min and following stained with 0.1% crystal violet. Stained cells were counted in five randomly selected fields under a light microscope (magnification, x40).

Western Blot Analysis

ShRNA-HMGB2, shRNA-NC and liposome cells after transfection for 48 h were collected. Radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haimen, Jiangsu, China) was used to extract cell protein. The protein concentration was examined with the bicinchoninic acid (BCA, Beyotime, Haimen, Jiangsu, China). Then, 20 μg of each protein was added to per lane of 10% sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). After the proteins were separated, they were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Company, Bedford, MA, USA). The membranes were then blocked with 5% non-fat milk for 2 h at room temperature and incubated with primary antibodies against HMGB2, phospho-ERK1/2 (1:1000), phospho- JNK1/2/3 (1:1000), p-p38MAPK (1:1000, Cell Signaling Technology, Danvers, MA, USA), and β-actin antibody (1:200) overnight at 4°C. Followed by incubating with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 1 hour at room temperature. Enhanced chemiluminescence (ECL) reagent was used to detect the signal on the membrane under Las4000 Luminescent *Imaging Analyzer (*Bio-Rad, Hercules, CA, USA*).*

Subcutaneous Tumor Model and Gene Therapy

All animal experimental procedures were carried out according to the Institutional Animal Care and Use Committee Protocol Guidelines of People's Hospital of Yichun City. Six-week-old female immune-deficient nude mice (BALB/c-nu) were maintained at the laboratory animal facility (People's Hospital of Yichun City, Yichun, China), and were housed individually in microisolator ventilated cages with free access to water and food. Two mice were injected subcutaneously with 1x10^8 ACHN cells in 50 μl of PBS pre-mixed with 50 μl of Matrigel matrix. When the tumor size reached 5 mm in length, they were surgically removed, cut into 1-2 mm^3 pieces, and reseeded individually into other mice. The mice were randomly assigned as shRNA-HMGB2 group, shRNA-NC group and blank control group at the time tumor size was about 5 mm in length. The tumor volume was measured every three days with a caliper, calculated by the following formula: volume = (length * width)²/ 2.

Statistical Analysis

Data are expressed as the mean ± standard deviation (mean ± S.E.). Differences between the two groups were analyzed using a Student's *t*-test. The significance between different samples was analyzed by one-way ANOVA or Tukey's multiple comparison test. Repetitive measurement data

were analyzed by repeated measure of ANOVA. Analysis of OS followed the Kaplan-Meier method and comparisons were made using Log-rank tests. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered to indicate a statistically significant difference.

Results

HMGB2 Was Upregulated In Human Renal Tumor Tissues and Predicted Poor Prognosis

In an attempt to investigate whether HMGB2 was involved in renal tumor, HMGB2 protein expression in 15 paired renal tumor samples and adjacent non-tumor tissue were analyzed. As shown by Figure 1A, the HMGB2 of renal tumor samples was markedly higher than pair-matched normal tissues. The expression level of HMGB2 was then assessed in normal kidney epithelial cells (HK2) and in five renal tumor cell lines (786-O, Caki-1, 769-P, OS-RC-2, ACHN). The data showed that HMGB2 expression significantly increased in renal tumor cell lines compared with HK-2 cells (Figure 1B).

A processed HMGB2 expression data in renal tumor from TCGA database was acquired from FRKM database. We analyzed the expression of HMGB2 in 70 paired renal cancer tissues and their corresponding noncancerous tissues. The preliminary screening showed HMGB2 was significantly upregulated in these 70 paired renal tissues (Figure 1C). HMGB2 expression in renal tumor tissues was then dichotomized as low group (n = 397) or high group (n = 131) determined by the median value of HMGB2 (of which is 25.48). Kaplan-Meier analysis and log-rank test were performed to investigate the effects of HMGB2 expression and clinicopathological factors on OS in renal tumor patients. The results showed that patients with high expression level of HMGB2 had reduced OS compared to those with low HMGB2 expression level (Figure 1D). Correlation regression analysis indicated that expression level of HMGB2 was in a significant positive correlation with the TNM stage ($p = 0.039$, Figure 1E).

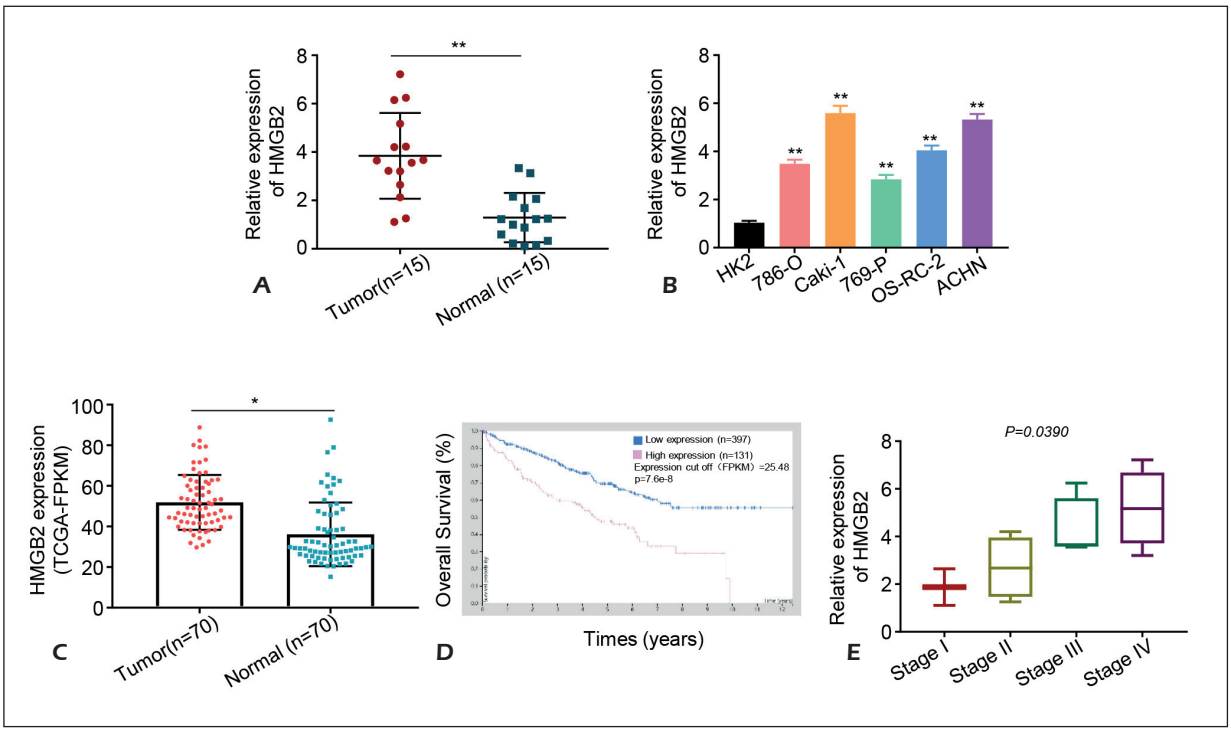

Figure 1. HMGB2 was upregulated in renal tumor and correlated with worse overall survival in renal tumor patients. **A**, Expression of HMGB2 was significantly higher in renal tumor tissues compared with the pair-matched adjacent normal tissues (**$p<0.01$). **B**, The level of HMGB2 was elevated in renal tumor cell lines than in HK-2 cells line (**$p<0.01$). **C**, Relative expression of HMGB2 in 70 pairs of renal tumor tissues and their normal tissues in TCGA database (*$p<0.05$). **D**, Kaplan-Meier overall survival curves of renal tumor patients according to the expression level of HMGB2 in TCGA database ($p\leq0.01$, log-rank). **E**, Correlation regression analysis between the expression of HMGB2 and TNM stage of patients ($p<0.05$).

HMGB2 Knockdown Inhibits Renal Tumor Cell Proliferation

We knock-down the expression of HMGB2 by transfecting specific shRNA-HMGB2 into ACHN cells. There were three groups including the shRNA-HMGB2 group, shRNA-NC group and blank control group. At 48 h, five fields of sight were randomly selected, and transfection efficiency (number of fluorescent cells/total number of cells) was determined using a fluorescence microscope. The highest transfection efficiency of HMGB2-shRNA transfecting into ACHN cells was 65% at 48 h examined by real-time PCR (RT-PCR) assay. The results revealed that the expression level of HMGB2 mRNA in ACHN cells was significantly down-regulated in shRNA-HMGB2 group (7.686±1.833 *vs.* 0.9143±0.2268, $p<0.05$; 7.983±0.7731 *vs.* 0.9143±0.2268, $p<0.01$, Figure 2A), compared to shRNA-NC group and blank control group. But there was scarcely any difference between the shRNA-NC group and blank control group (7.686±1.833 *vs.* 7.983±0.7731, $p>0.05$, Figure 2A). Thus, the expression of HMGB2 mRNA decreased significantly in ACHN cells transfected by shRNA-HMGB2.

To further study the effects of knockdown of the HMGB2 on ACHN cells proliferation, we transfected cells with shRNA-HMGB2 and then examined cell viability using MTT assay at 24, 36, 48, 72, and 96 hours. Compared with the shRNA-NC and liposome group, the viability of ACHN cells ($p<0.01$) was significantly inhibited in shRNA-HMGB2 cells time dependently (Figure 2B). With the transfection time went on, the peak proliferation inhibition efficiency was at 72 h. There was scarcely any significant difference between the shRNA-NC and blank control groups ($p>0.05$).

Flow cytometry assay was used to examined cell apoptosis in shRNA-HMGB2, shRNA-NC and liposome ACHN cells after transfecting for 48 h. Results showed that the shRNA-HMGB2 group

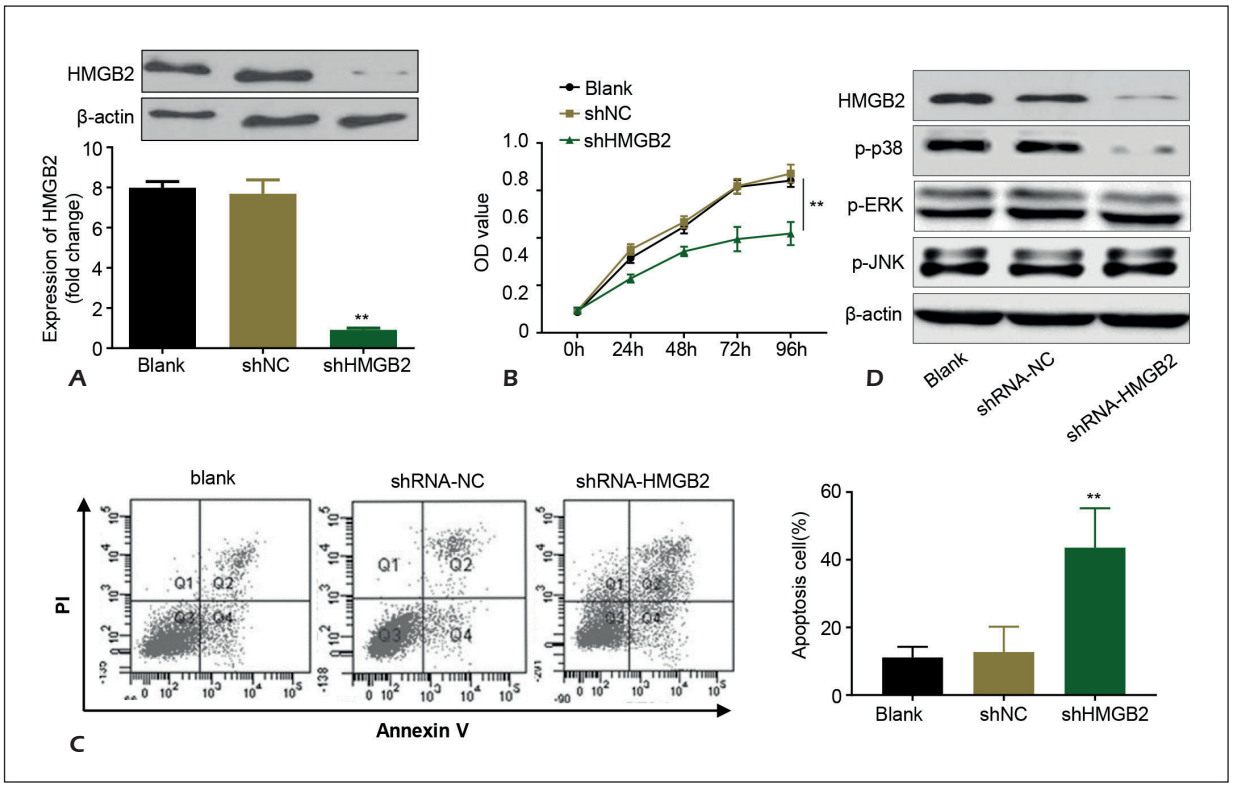

Figure 2. HMGB2 knockdown inhibits renal tumor cell proliferation. **A**, The mRNA and protein level of of HMGB2 was detected by RT-PCR and Western blot assay (**$p<0.01$). **B**, ACHN cells were transfected with shRNA-HMGB2, shRNA-NC and liposome for 24, 48, 72 and 96 h, and the cell viability was measured by MTT Assay (**$p<0.01$). **C**, ACHN cells were transfected with shRNA-HMGB2, shRNA-NC and liposome for 48 h and the apoptosis was measured by flow cytometry. A representative gating strategy for apoptosis was analyzed by flow cytometry. The frequency of the apoptosis cells in shRNA-HMGB2, shRNA-NC, and blank control groups. **D**, ACHN cells were transfected with shRNA-NC, sh-HMGB2 and liposome for 48 h, p-ERK, p-JNK and p-p38MAPK expression level was examined by Western blot assay.

had significantly larger proportion of apoptotic ACHN cells than the shRNA-NC group (12.7±2.83 *vs.* 43.57±4.412, $p<0.01$, Figure 2C). Therefore, the inhibition of HMGB2 may promote the apoptosis of ACHN cells. Additionally, Western blot assay was performed to examine the expression of MAPK for further studying the molecular mechanisms of HMGB2. As shown in Figure 2D, p-p38MAPK protein levels were downregulated in ACHN cell lines with low-expression of HMGB2. There was no difference between p-ERK, and p-JNK protein levels. The result showed that there existed scarcely any significant difference in the protein expressions of p-JNK, p-ERK and p-p38 MAPK between the shRNA-NC and blank control groups.

HMGB2 Knockdown Inhibits Renal Tumor Cell Invasion and Migration

Migration and invasion are important biological behavior of malignant tumors[11]. We performed the transwell assay to detect the effect of HMGB2 on the invasion and migration. The invasive and migratory ability of shRNA-HMGB2, shRNA-NC and liposome cells were determined by the transferring number of cells. As a result, the average invading cells treated by HMGB2 shRNA-infection were much more than those of non-infected and blank control cells (Figure 3). No significant differences were found between non-infected treatment and blank control treatment. The invasive effect of shRNA-HMGB2, shRNA-NC and liposome on cells was determined by the transferring number of cells. As a result, the average invading cells treated by HMGB2 shRNA-infection were much fewer than those of non-infected and blank control cells (16.0%±2.68% *vs.* 39.0%±4.95%, $p<0.05$; 16.0%±2.68% *vs.* 40.13%±5.35%, $p<0.05$, Figure 3A). Similarly, the average migrating cells treated by of HMGB2 shRNA-infection were much fewer than those of non-infected and blank control cells (26.1%±6.55% *vs.* 66.80%±6.161%, $p<0.05$, 26.1%±6.55% *vs.* 62.60±7.662%, $p<0.05$, Figure 3B). HMGB2 knockdown inhibited the invasion and migration abilities of the ACHN cells.

HMGB2 Knockdown Inhibits Growth of In Vivo Xenograft Tumors

The above experiments *in vitro* corroborated that the suppression effect of HMGB2 knockdown on tumor growth of ACHN cells. Furthermore, we investigated the effect of HMGB1 knockdown on xenograft tumor growth *in vivo*. The volume and weight of xenograft tumor in shRNA-HMGB2 group exhibited a significant reduction compared with shRNA-NC and blank control groups (Figures 4A and 4B). The data indicated HMGB2 knockdown may suppress the *in vivo* tumor growth of human renal tumor cells.

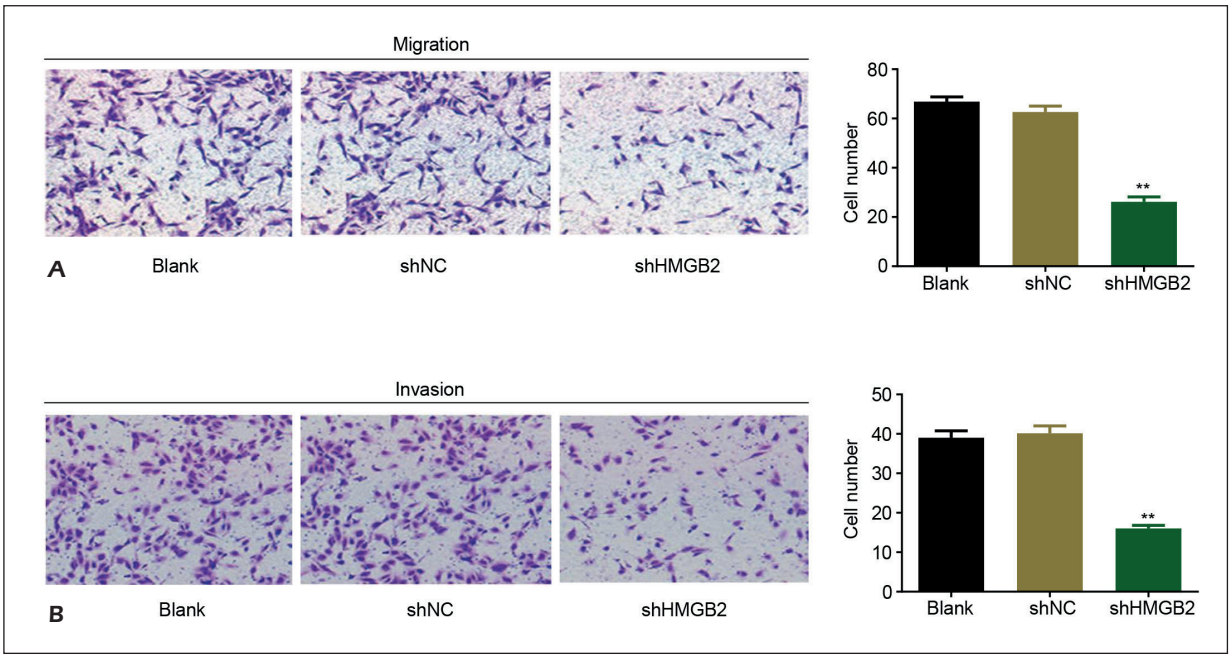

Figure 3. HMGB2 knockout inhibited ACH cell invasion and migration. **A**, The number of invaded cells in each high-power field (magnification: X200) was recorded (**$p<0.01$). **B**, The number of migrated cells in each high-power field (magnification: X200) was recorded (**$p<0.01$).

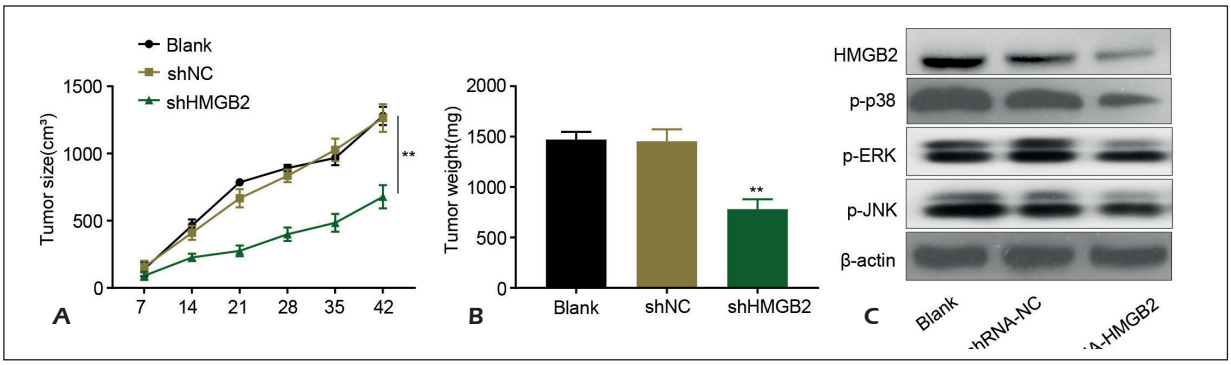

Figure 4. The effects of HMGB2 knockdown in tumor growth in vivo. **A**, The average volume of tumors in shRNA-HMGB2 group was significantly smaller than tumors in the NC group and blank control ones (**p<0.01). **B**, The average weight of tumors in shRNA-HMGB2 group was significantly lighter than tumors in the NC group and blank control groups (**p<0.01). **C**, The p-ERK, p-JNK and p-p38MAPK expression was detected by Western blot in xenograft tumor.

Western blot assay was also performed to examine the expression of MAPK in xenograft tumor. As shown in Figure 4C, p-p38MAPK protein levels were downregulated in shRNA-HMGB2 group. There was also no difference between p-ERK, and p-JNK protein levels. There was no significant difference in the protein levels of p-JNK, p-ERK and p-p38 MAPK between the shRNA-NC and blank control groups.

Discussion

It was found that HMGB2 was upregulated in renal tumor tissues and cell lines using RNA sequencing technique. We also identified that high expression was closely correlated to the aggressive clinicopathologic features and unfavorable prognosis in renal tumor patients.

In this study, a lot of function experiments were performed to clarify the function of HMGB2 in ACHN cells, showing that knockdown of HMGB2 by shRNA inhibits cell proliferative activities and invasive potential, and reduced xenograft tumor growth. RT-PCR assay exhibited that the expression level of HMGB2 was decreased significantly in ACHN cells transfected by shRNA-HMGB2 and the peak transfection efficiency was at 24 h. MTT assay exhibited that down-regulation of the HMGB2 on ACHN cells inhibited proliferation, and the peak inhibition efficiency was at 72 h. In conclusion, down-regulation of HMGB2 in ACHN cells could induce the apoptosis of tumor cells, suggesting that HMGB2 is an important molecular target of renal tumor cells[12].

The invasive and metastatic behaviors are the main biological characteristics of the malignant tumors, and also the major cause of treatment failure or death in cancer patients[13,14]. The transwell invasion assay can mimic the tumor invasion environment and reflect the ability of the cell to penetrate the extracellular matrix[15]. Transwell assay results showed that the ACHN cells transfected with shRNA-HMGB2 significantly decreased cell migration and invasion compared to shRNA-NC and the blank control group, which indicate that inhibition of HMGB2 inhibited migration and invasion of ACHN cells. Knockdown of HMGB2 expression significantly decreased proliferation and invasiveness in human gastric cancer cells and hepatocellular carcinoma cells[16,17], which consistent with our study.

To further explore the main mechanism for the effect of HMGB2 on the behavior of ACHN, we examine the level of MAPK. The MAPK family includes three main members of ERK, JNK and p38[18]. MAPK plays an important part in the development of tumors that clarified in previous studies[19,20]. Corona et al[21] showed that the MEK inhibitor PD98059 inhibited ERK phosphorylation and the level of cyclin D1 (Cyclin D1) in colon cancer cells, resulting in inhibiting the proliferation of tumor cells. McCubrey et al[22] found Ras mutations result in abnormal activation of downstream MEK and ERK, in various malignant tumors such as melanoma, ovarian cancer, and colon cancer, which caused abnormal proliferation of tumor cells. The activation of MAPK signaling pathway plays an important role in the tumorigenesis, progression and invasion of renal cell carcinoma, and the depression of MAPK activation promotes apoptosis in renal cancer cells[23,24]. In present study, Western blot assay showed the decrease level of HMGB2 protein and p-p38MAPK

protein in shRNA-HMGB2 transfecting ACHN cells. When HMGB2 was re-expressed in ACHN cells by transgenic technology, the p38MAPK protein level also returned to normal, which indicated that p38MAPK is an important downstream of HMGB2. Since there is no significant difference between expression levels of p-JNK and p-ERK, indicating HMGB2 did not activate proteins of JNK and ERK. Uzgare et al[25] found p38 activation tissues was significantly higher in early highly differentiated prostate cancer than normal tissues, while ERK and JNK activation was significantly increased in the study of transgenic models for prostate cancer, which indicated the relative activation and roles of p38, ERK and JNK in different tissues may be different[25-28]. In present study, the inhibition of HMGB2 induced the decrease of downstream p-p38MAPK protein level but did not affect the activation of JNK and ERK, suggesting that p-p38MAPK is an important target of HMGB2.

Conclusions

HMGB2 is up-regulated in renal tumor tissues and cells and its overexpression is associated with poor prognosis in patients. Inhibition of HMGB2 can inhibit ACHN cell viability, which may cause the inhibition of downstream p38MAPK. The present study showed that HMGB2 is a hypothetical drug target for renal cancer. Further *in vitro* and animal studies are needed to verify this theory and provide a reliable basis for HMGB2 targeted therapy for renal cancer.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Funding

The study was supported by National Natural Science Foundation of China (81660105).

Ethics approval and consent to participate

This study was conducted in accordance with the Helsinki Declaration II and it was approved by the People's Hospital of Yichun City.

Authors' contributions section

Zhi-Hong He, Hui Xiao, Fang Guo and Xiao-Xiong Hu contributed to the study design. Zhi-Hong He, Hui Xiao, Fang Guo, Zi-Yun Luo and Jian-Wei Yi conducted the literature search. Zhi-Hong He, Hui Xiao, Fang Guo, Xiao-Xiong Hu and Zi-Yun Luo acquired the data. All authors wrote the article. Jian-Wei Yi performed data analysis. All authors gave the final approval of the version to be submitted. All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare that they have no conflict of interests.

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