

MBD2 Correlates with a Poor Prognosis and Tumor Progression in Renal Cell Carcinoma

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Purpose: DNA methylation plays an important role in regulating gene expression. Methyl-CpG-binding domain (MBD) proteins recognize and bind to methylated DNA, which mediate gene silencing by the interaction with deacetylases and histone methyltransferases. MBD2 has been reported in various human cancers; however, its clinical implication and potential regulatory role in renal cell carcinoma (RCC) have not been elaborated.

Materials and Methods: In the study, we estimated the expression and prognostic value of *MBD2* in RCC cell lines and tissues by Western blotting and immunohistochemistry. The associations of *MBD2* expression and pathological characters and survival in RCC patients were performed using χ^2 and Kaplan–Meier survival analysis, respectively. Univariate and multivariable Cox regression analyses suggested the independent predictors in RCC prognosis. The functional role of *MBD2* in RCC progression was assessed by in vitro cell experiments. In addition, we identified the *MBD2*-mediated alterations of protein-related proliferation and EMT markers in RCC cells after *MBD2* overexpression and knockdown.

Results: We found that the protein levels of *MBD2* were upregulated in RCC cells and tissues. High *MBD2* expression was related to TNM stage and predicted poorer survival in RCC. Enforced expression of *MBD2* significantly promoted the proliferation, cycle progress, invasion and migration of RCC cells in vitro. However, downregulating *MBD2* remarkably weakened the above cell functions. Mechanistically, the promotive effect of *MBD2* overexpression may be regulated by its effects on *p21*, *p53* and *Cyclin D1* expression and EMT process.

Conclusion: These results indicated that *MBD2* confers an oncogenic function in the malignant progression of RCC. *MBD2* could be served as a meaningful prognostic biomarker and a latent therapeutic target in RCC patients.

Keywords: MBD2, RCC, prognosis

Introduction

RCC is one of the most common malignant tumors in the urinary system.¹ It is one of the few tumors with a higher incidence in males than in females and tends to increase worldwide.² Due to the concealed onset of RCC and the absence of typical clinical symptoms in the early stage, about 30% of patients have metastasis and malignant progression at the time of RCC diagnosis,^{3,4} and postoperative recurrence occurs in 20%-30% of patients.⁵ Typical RCC triad (low back pain, hematuria, abdominal mass) occurs or does not occur in advanced patients, among those who are insensitive to radiotherapy and conventional chemotherapy and have no significant clinical effects on treatments. Therefore, further understanding of the tumorigenesis of RCC is an unmet clinical need for identifying reliable prognostic biomarkers and therapeutic targets for RCC patients.

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DNA methylation regulates the transcriptional activity of DNA without permanent changes of nucleotide sequences. Epigenetic information of DNA methylation is interpreted and translated by recruiting “reader” molecules known as methyl-binding proteins (MBPs).^{6–8} MBPs binds to methylated DNA, namely CpG dinucleotides, and can organically link the DNA methylation with histone modification.^{6–9} Methyl-CpG-binding domain (MBD)-containing proteins belong to the first family of MBPs. Among these members, MBD2 evinces the deepest affinity with methylated DNA, which mimics chromatin remodeling or modification factors, alters the structure of chromatin and interferes with the binding of transcription factors to target genes following its binding to methylated DNA, subsequently inhibiting gene transcription concomitant with the alterations of histone conformation.^{6,10–12} *MBD2* is identified as a multiexon gene and serves as a transcriptional repressor/activator depending on the cellular context.¹³ *MBD2* protein is composed of 411 amino acids, among which ranging from 145 to 213 amino acids is the methyl-binding domain, which can bind to the specific methylation sequences of target genes.

Aberrant DNA methylation modifications are frequently detected in various tumors, and the main mechanisms for DNA methylation-involved tumorigenesis are that methylation levels of the promoter region of anti-oncogene are elevated, which promotes the key anti-oncogene silencing and drives tumorigenesis.^{12,14–16} *MBD2* has been reported in multiple human malignancies, including gastric cancer,¹⁷ breast cancer,^{18,19} colorectal cancer,²⁰ glioblastoma,^{21,22} hilar cholangiocarcinoma,²³ hepatocellular carcinoma,^{24,25} chronic myeloid leukemia²⁶ and prostate cancer.²⁷ Previous studies confirmed that *MBD2* mediates the transcriptional repression of tumor suppressor genes, such as *hTERT*,²⁸ *GSTP1*,²⁹ *BAIL*,²¹ *p14^{ARF}*/*p16^{INK4a}*,³⁰ and *14-3-3sigma*,²⁷ which supports the pivotal role of *MBD2* in abnormal epigenetic regulation of tumors. *MBD2* is recruited to the promoter of *BAIL* considered as an important tumor suppressor of antiangiogenic activity and maintains its epigenetic silencing to promote the carcinogenesis and tumor progression of glioblastoma.²¹ miR-520b impedes glucose metabolism, invasion, angiogenesis and chemosensitivity of glioma cells by directly targeting *MBD2*.²² *MBD2* deletion induces the activation of JAK2/STAT3 signaling pathway and inhibits tumor growth in chronic myeloid leukemia.²⁶ Increased *MBD2* is proposed as an independent prognostic factor for overall (OS) and disease-free survival (DFS) for hepatocellular carcinoma patients.²⁴ LOC105369748 upregulates

MBD2 through binding to miR-5095, which conveys oncogenic signals to accelerate the carcinogenesis and progression of hepatocellular carcinoma.²⁵ All above evidence indicates an oncogenic role of *MBD2* in the initiation and progression of cancers. However, the pathophysiologic function and regulatory mechanism of *MBD2* in RCC remain largely unknown.

In the present report, we sought to ascertain the clinical and prognostic significance of *MBD2* in RCC tissues and discovered that highly expressed *MBD2* was associated with the pathological characteristics and prognosis of RCC patients. Furthermore, effects of *MBD2* on proliferation, cell cycle, migration and invasion were investigated in RCC cells, which provided evidence that enforced expression of *MBD2* promoted cell proliferation, cycle progression, migration and invasion in vitro. Mechanistically, *MBD2* regulated proteins related to cell proliferation and EMT markers. Taken together, we reported that *MBD2* may be an independent prognostic indicator of RCC, which suggested the therapeutic potential of targeting *MBD2* for RCC patients.

Materials and Methods

Patients and Tissue Samples

A total of 109 pairs of RCC tissue samples and matched normal renal tissues were collected from the patients who received surgical resection of primary RCC at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China) from December 2013 to November 2015. All patients selected in the study were histopathological diagnoses with RCC and had never been subjected to radiotherapy and/or chemotherapy preoperatively. The detailed clinical and pathologic information of RCC patients were recorded and stored completely. The clinicopathological parameters included age, gender, Fuhrman grade, TNM stage. All RCC patients were termly followed up from 1 to 65 months to assess post-operative survival, and follow-up information was obtained by reviewing the well-documented medical records. After collection, all fresh tissues were quickly stored in liquid nitrogen and frozen at -80°C for subsequent experiments. The study protocol involving human specimens was approved by the Review Board of the Affiliated Hospital of Xuzhou Medical University. Written informed consent was collected from all participants prior to the study.

Immunohistochemistry Staining and Evaluation

Immunohistochemical (IHC) analysis was performed according to our previously described methods.³¹ Briefly, formalin-fixed, paraffin-embedded RCC tissues were cut into 4 μ m sections. After 2 h baking, the section was deparaffinized with dimethylbenzene and rehydrated in graded ethanol solutions, respectively. Then, the section was heated at 95°C for 5 min in a citrate buffer (pH = 6.0) for antigen retrieval. For blocking endogenous peroxidase activity, the slide was washed with PBS twice and then treated with 3% hydrogen peroxide for 15 min. After the incubation of normal goat serum, the section was incubated with the anti-MBD2 (Abcam, Cambridge, MA, US) for 12 h at 4°C. Then, the slide was incubated with appropriate secondary antibody for 1 h at room temperature. The detection of antigenic staining was conducted by 3, 3'-diaminobenzidine (DAB; Beyotime Biotechnology, Shanghai, China) and counterstained with hematoxylin (Beyotime Biotechnology). Finally, the slide was dehydrated and sealed.

Two independent pathologists assessed the IHC staining in each section by light microscopic examination. Staining intensity was assigned as follows: 0 (-); 1 (+); 2 (++); 3 (+++) and positively stained area was assigned as follows: 1 (0–25%); 2 (26–50%); 3 (51–75%); 4 (76–100%). Staining pattern of each section was scored according to the immunoreactive score (IRS) determined by the product of staining intensity and positively stained area. All specimens were categorized into low MBD2 expression (IRS < 6) and high MBD2 expression (IRS \geq 6).

Cell Culture

Human RCC cell lines including 786-O, ACHN, 769-P, Caki-1 and Caki-2 and normal human renal tubular epithelial cell HK-2 were supplied by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured according to our previously described methods.³¹

Transfection

Small interference RNAs (siRNAs) targeting human *MBD2* (siMBD2) and negative controls (siCon) were from GenePharma (Shanghai, China). The siRNA sense sequences were 5'-UUACUAGGCAUCAUCUUUCUU-3' for siMBD2 and 5'-GCCUUGGCAGCCUAGGCGA-3'

for siCon.³² siRNAs transfection in RCC cells was performed using siLentFect™ Lipid Reagent (Bio-Rad, Hercules, CA, USA). For the overexpression of *MBD2*, human *MBD2* plasmid was subcloned into the pcDNA3.1 vector (GenePharma). pcDNA3.1-MBD2 plasmids and empty vectors were transfected into the RCC cells using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Cells were cultured for 24 h or 48 h after transfection and then used for the following experiments.

Western Blotting

Cells were lysed in the cold RIPA buffer (Beyotime Biotechnology) with Phenylmethylsulfonyl fluoride (PMSF), and protein contents were determined by the BCA assay (BCA; Beyotime Biotechnology). Equal amount of protein was separated by 10% SDS-PAGE gel and then electrophoretically transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat skim milk for 1 h and then probed overnight at 4°C with primary antibodies: MBD2 (Abcam), E-cadherin (Abcam), p21 (Abcam), p53 (Abcam), N-cadherin (Abcam), Vimentin (Abcam), Cyclin D1 (Abcam) and GAPDH (Zhongshan biotech, Beijing, China). The following day, membrane was incubated with secondary antibodies. Finally, the signal of protein was visualized by an enhanced chemiluminescence detection system.

Cell Proliferation Assay

Cell proliferation was measured by the Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan). Transfected 786-O and ACHN cells were seeded into 96-well plates at a density of 2×10^3 cells and incubated at 37°C. After the inoculation for indicated times, 10 μ L CCK-8 reagent was added into wells containing 100 μ L of serum-free medium. The absorbance at 450 nm (OD 450) of cells was measured by a microplate reader.

Transwell Assay

Transwell assay was used to assess the cell invasion and migration ability using transwell chamber (BD Bioscience, USA). For invasion assay, matrigel was pre-coated in the upper chamber. 1×10^5 transfected cells in 100 μ L of medium without FBS were seeded into the upper chamber, while the medium with 10% FBS (600 μ L) was added into the bottom of the chambers. After 24 h, cells on the membrane filters were fixed in cold methanol, and noninvasive cells were softly erased

with a cotton swab. Finally, cells adhered to the lower membrane were stained with the crystal violet, calculated and photographed using a bright field microscopy at 200x magnification.

Cell Cycle Assay

Transfected RCC cells were harvested, washed, then fixed in 70% ice-cold ethanol for 12 h. Cells were then resuspended with DNA-staining solution for 30 min at room temperature in the dark. Finally, samples were detected using a FACScan flow cytometer (BD Biosciences). The distribution of cell cycle was determined using the CellQuest software program (BD Biosciences).

Statistical Analysis

All statistical analyses were determined using the SPSS 22.0. Data were presented as means \pm SD. The statistical significance of different groups was compared by *t*-test or one-way ANOVA. The χ^2 test was employed to assess the association between MBD2 expression and clinicopathological features of RCC, and the Kaplan–Meier method and Log rank test were used to estimate the OS and DFS. The significance of MBD2 expression in RCC patients was examined by the univariate and multivariate Cox regression analyses. *P*-value <0.05 was considered statistically significant.

Results

MBD2 is Highly Expressed in RCC

Initially, *MBD2* expression was detected in RCC cell lines and tissues using Western blotting. Results showed that *MBD2* significantly elevated in the RCC cell lines including 786-O, ACHN, 769-P, Caki-1 and Caki-2 compared with normal human renal tubular epithelial cell HK-2 (Figure 1A, $P < 0.001$). Next, we analyzed *MBD2* expression through excavating the online server GEPIA based on TCGA and GTEx data (<http://gepia.cancer-pku.cn/detail.php?gene=MBD2>) and found that *MBD2* was frequently overexpressed in the RCC tissues compared with adjacent non-tumor tissues (Figure 1B). We also detected the relative expression of *MBD2* between RCC tissues and tumor-adjacent tissues by available frozen specimens randomly selected from 109 cases of RCC. Comparative analysis revealed that the protein levels of *MBD2* were higher in RCC tissues than in matched normal tissues (Figure 1C, $P < 0.05$). To further investigate the *MBD2* expression in RCC tissues, IHC staining was performed with 109 tissue specimens. Strong staining of *MBD2* protein was frequently observed in RCC tissues, while weak or negative staining was more prone to be shown in adjacent non-tumor tissues. Representative illustrations for *MBD2* staining of both RCC and normal tissues are shown in Figure 1D and Supplementary Figure 1A. According to the

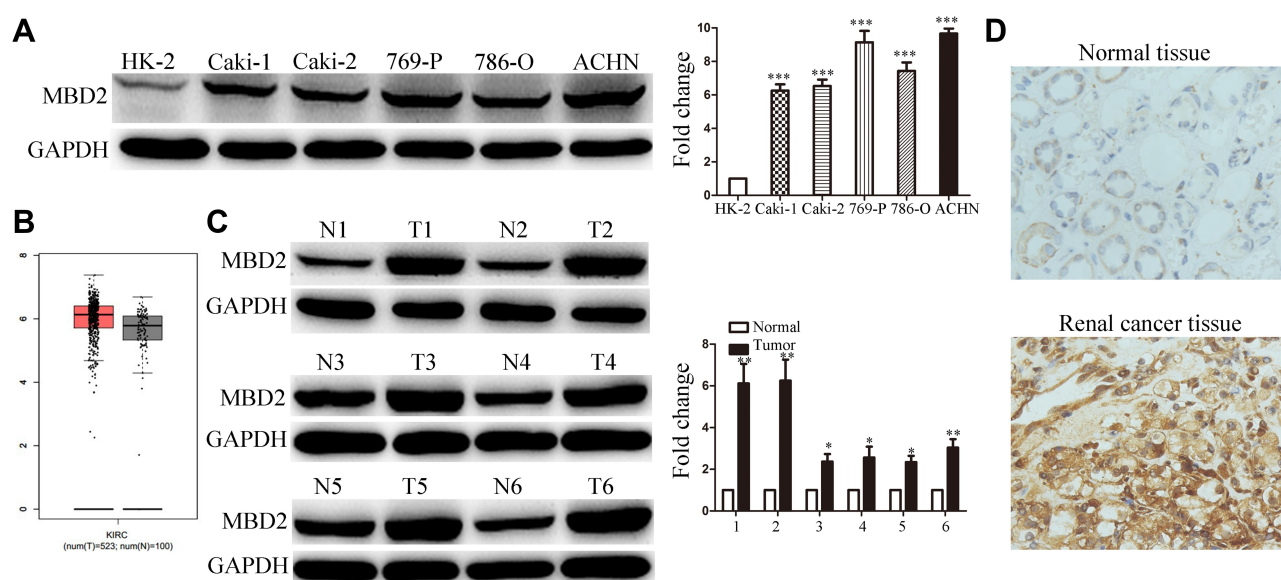


Figure 1 MBD2 expression is upregulated in RCC cells and tissues. (A) The protein expression of *MBD2* was determined in the several human RCC cell lines (Caki-1, Caki-2, 769-P, 786-O and ACHN) and human normal tubular epithelial cell line (HK-2) by Western blotting. (B) The expression levels of *MBD2* in KIRC (Kidney renal clear cell carcinoma; (T) and adjacent normal (N) tissues from TCGA and GTEx data were analyzed by excavating the online server GEPIA (<http://gepia.cancer-pku.cn/detail.php?gene=MBD2>). (C) *MBD2* protein levels in 6 representative pairs of RCC (T) and adjacent normal (N) tissues were presented in the immunoblot. (D) The representative IHC staining of *MBD2* in RCC and normal renal tissues. Original magnification, $\times 400$. Data represents the means \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

quantitative analysis of *MBD2* staining, RCC specimens were divided into two categories: low *MBD2* expression (IRS: 0–4) and high *MBD2* expression (IRS: 6–12). Sixty-six of 109 (60.6%) RCC samples were exhibited high *MBD2* expression; however, the high expression in tumor-adjacent tissues was merely 17 of 109 (15.6%). In addition, *MBD2* staining was detected in both cytoplasm and nucleus. Taken together, these results suggested *MBD2* was elevated in RCC and may be associated with RCC progression.

MBD2 is Correlated with Poor Prognosis in RCC

The relationship between *MBD2* expression and clinicopathological findings was presented (Table 1). Elevated *MBD2* expression was significantly correlated with T stage ($P = 0.044$), distant metastasis ($P = 0.016$) and TNM stage ($P = 0.011$), whereas no compelling correlation was found in age, gender, Fuhrman grade and lymph node involvement.

For further understanding the effect of *MBD2* expression on the clinical outcomes of RCC patients, we initially effectuated the survival curves by online server GEPIA, which revealed that high *MBD2* expression predicted the better OS (Figure 2A, $P < 0.001$) and DFS (Figure 2B, $P < 0.05$). Nevertheless, we performed the Kaplan–Meier survival curves and Log rank test from the 109 RCC samples, and results indicated that the RCC patients with high *MBD2* expression had poorer 5-year OS and DFS after radical nephrectomy than these with low *MBD2* expression (Figure 2C for OS, $P < 0.001$; Figure 2D for DFS, $P < 0.001$). We also conducted univariate and multivariate analyses through a Cox proportional hazard model to explore the prognostic factors in RCC patients. Univariate analysis uncovered that T stage, lymph node involvement, TNM stage and *MBD2* expression were identified as prognostic factors for OS and DFS (Tables 2 and 3), while age, Fuhrman grade and distant metastasis were not associated with the OS and DFS. Moreover, further multivariate analysis confirmed that increased *MBD2* expression was an independent risk factor for the OS and DFS in RCC patients (Table 2, HR: 3.004, 95% CI: 1.519–4.149, $P < 0.001$ for OS; Table 3, HR: 2.511, 95% CI: 1.519–4.149, $P < 0.001$ for DFS). Collectively, our results indicated that *MBD2* is involved in RCC progression and may be proposed as a valuable prognostic marker for RCC patients.

The Effects of MBD2 on the Proliferation of RCC Cells

Given that increased *MBD2* expression was related to malignant progression and poorer prognostic, we hypothesized that *MBD2* function as an oncogene in RCC cells. To decipher this, we performed the gain-of-function and loss-of-function experiments. Initially, 768-O and ACHN cells were transfected with *MBD2* plasmid or siRNA *MBD2*, and the transfection efficiency of *MBD2* overexpression and knockdown are shown Figure 3A ($P < 0.01$). CCK-8 assays were utilized to determine the RCC cell activity in vitro. Enforced *MBD2* expression accelerated the cellular growth in 768-O cells (Figure 3B, $P < 0.01$). Inversely, the blockade of *MBD2* expression restrained the proliferative ability of ACHN cells (Figure 3C, $P < 0.01$). Meanwhile, cell cycle assay was applied to analyze the *MBD2*-mediated cell cycle profiles. *MBD2* amplification compelled the cycle transition from G1 phase to S phase in 768-O cells (Figure 3D, Supplementary Figure 1B). *MBD2* silencing significantly decelerated cycle transition and increased G1 phase populations in ACHN cells (Figure 3E, Supplementary Figure 1B).

Malignant proliferation is an important characteristic of tumor cells, which is concomitant with the disorders of cell cycle-related proteins and signaling pathways. Previous studies have reported the promotive proliferation role of *MBD2* glioma cells.²² Western blotting revealed that *MBD2* potentiated the *Cyclin D1* expression concomitant with the inhibition of *p53* and *p21* in 768-O cells (Figure 3F, $P < 0.01$). Inverse results were observed in the ACHN cells with *MBD2* silencing (Figure 3G, $P < 0.05$). Overall, *MBD2* promoted the expression of cyclin D1 and suppressed the expression of *p53* and *p21*, leading to the proliferation of RCC cells.

The Effects of MBD2 on Migration and Invasion of RCC Cells

IHC results confirmed the potential oncogene role of *MBD2* and its overexpression was associated with aggressive phenotypes in RCC. We next investigated the *MBD2*-mediated the migration and invasion of RCC cells through quantification analysis of transwell assay. Results indicated that the 768-O cells transfected with *MBD2* plasmid obviously increased the cell migratory and invasive capabilities (Figure 4A, $P < 0.01$). The *MBD2* silencing conferred by siRNA effectively impaired the number of invaded ACHN cells (Figure 4B, $P < 0.001$).

Table I The Correlations Between Expression and Clinicopathologic Characteristics in RCC Patients

Variables	Total (n=109)	MBD2 Expression		
		Low (%)	High (%)	P-value
Diagnostic category				
Matched normal tissues	109	92 (84.4)	17 (15.6)	P < 0.001
RCC tissues	109	43 (39.4)	66 (60.6)	
Gender				
Male	54	18 (33.3)	36 (66.7)	0.195
Female	55	25 (45.5)	30 (54.5)	
Age, years				
≤ 60	49	22 (44.9)	27 (55.1)	0.293
> 60	60	21 (35.0)	39 (65.0)	
Fuhrman grade				
G1+G2	63	22 (34.9)	41 (65.1)	0.258
G3	46	21 (45.7)	25 (54.3)	
T stage				
T1-T2	74	34 (45.9)	40 (54.1)	0.044
T3-T4	35	9 (25.7)	26 (74.3)	
Lymph node involvement				
N0	73	33 (45.2)	40 (54.8)	0.080
N1	36	10 (27.8)	26 (72.2)	
Distant metastasis				
M0	58	29 (50.9)	28 (49.1)	0.016
M1	51	14 (28.0)	36 (72.0)	
TNM stage				
I-II	57	29 (50.9)	28 (49.1)	0.011
III-IV	52	14 (26.9)	38 (73.1)	

Note: P-values < 0.05 are indicated in bold.

Tumor cells are characterized by the local infiltration and distant metastasis. EMT is a process in which polar epithelial cells transform into motile mesenchymal cells and acquire the ability to migration and invasion. We explored the alterations of EMT markers in RCC cells after *MBD2* treatment. Western blotting showed that the elevated expression of *MBD2* was accompanied by an increase in mesenchymal markers, N-cadherin and Vimentin, whereas a decline in epithelial marker, E-cadherin (Figure 4C, $P < 0.01$). Abating expression of *MBD2* totally produced an opposite effect (Figure 4D, $P < 0.01$). Together, *MBD2* potentiated the migration and invasion of RCC cells and induced EMT.

Discussion

DNA methylation is widely existed in bacteria, plants and mammal, which is involved in maintaining the stability of genome and chromosome structure, gene

imprinting, X chromosome inactivation and gene silencing and is closely related to the occurrence and development of cell carcinogenesis and cancer.^{6,9,13,33–35} DNA methylation can directly or indirectly affect the gene transcription and expression without the alterations of DNA sequences and gene products. Researches show that abnormal DNA methylation is involved in tumorigenesis by affecting the structure of chromatin and the expression of tumor suppressor genes and oncogenes.^{33,36} The abnormal methylation of tumor-related genes exists in almost all human tumors. In cancers, the hypermethylation of CpG islands in the promoter region of tumor suppressor genes, as a universal inactivation mechanism, mediates the silencing of tumor suppressor genes, which is involved in the carcinogenesis and tumor progression.^{10,16,21,35}

MBD family plays an important role in the processes of aberrant DNA methylation related to tumors. As a member

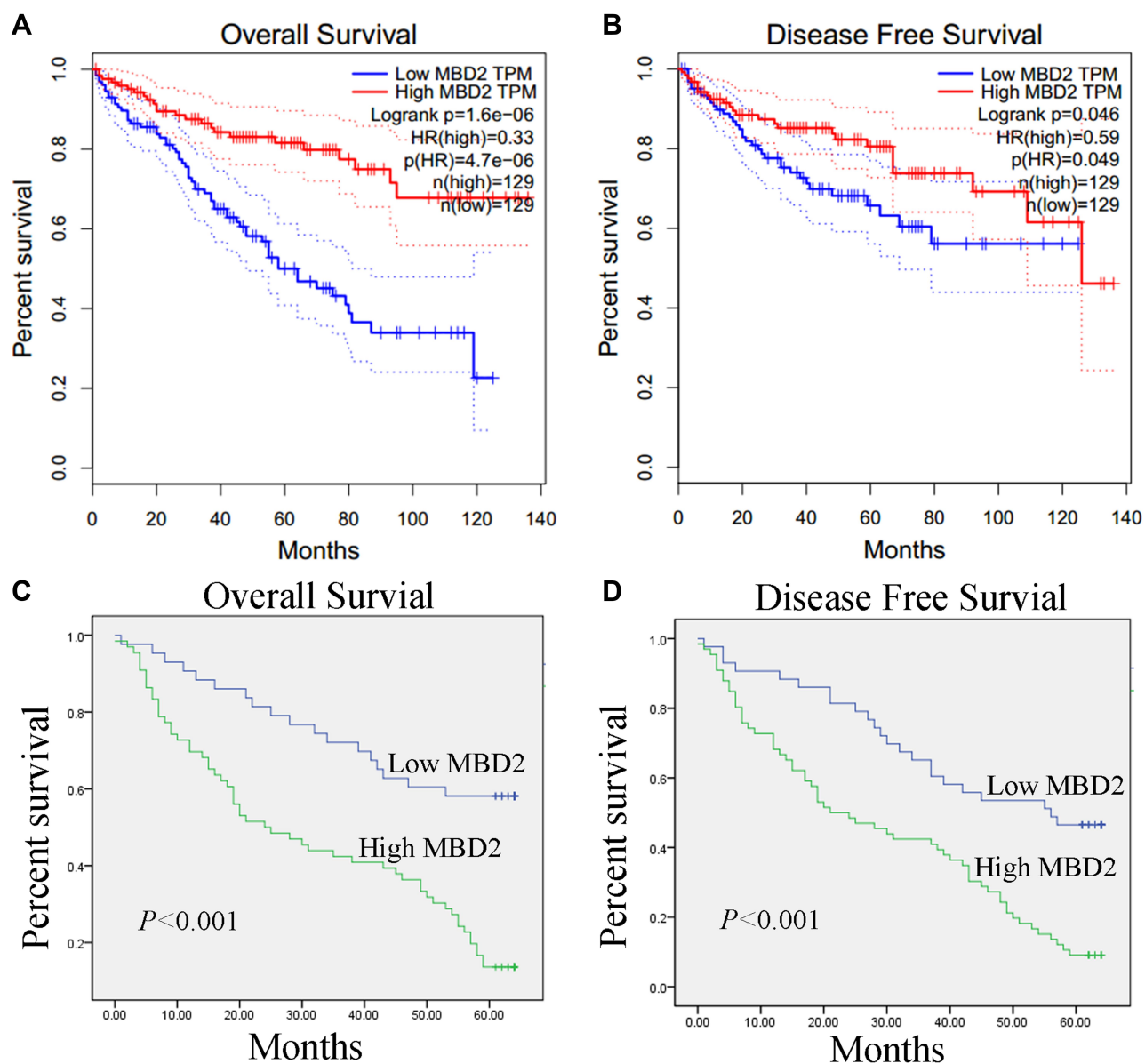


Figure 2 MBD2 expression is associated with poor survival in RCC patients. **(A and B)** The high expression of *MBD2* predicts the poor overall and disease-free survival in RCC patients through analyzing data from GEPIA. **(C and D)** The Kaplan–Meier analysis of overall and disease-free for 109 RCC patients was shown based on quantitative IHC staining of *MBD2*.

of MBD family, the *MBD2*, a methylation-dependent transcriptional repressor, is characterized by specifically recognizing and binding to methylated DNA and inhibits gene transcription through recruiting co-repressors like the NuRD complex to methylated CpG islands.^{12,37} Abundant evidence has demonstrated that *MBD2* mediates the transcriptional repression of tumor suppressor genes, such as *hTERT*,²⁸ *GSTP1*,²⁹ *BAIL*,²¹ *p14^{ARF}*/*p16^{INK4a}*,³⁰ *14-3-3sigma*.²⁷ Amplified *MBD2* is detected in glioblastoma^{21,22} and hepatocellular carcinoma.³⁸ The *MBD2*-mediated epigenetic silencing of *BAIL* drives

tumor growth in glioblastoma.²¹ *MBD2* deletion induces the inactivation of JAK2/STAT3 pathway and suppresses cell proliferation capacity in chronic myeloid leukemia.²⁶ *MBD2^{-/-}* is protective against the carcinogenesis and tumor progression and significantly suppresses the intestinal tumorigenesis in *Apc^{Min/+}* mice.³⁹ Devailly et al reported that *MBD2* is enriched in the methylated regions of target genes and associated with the transcriptional silencing during malignant transformation of human mammary cells.¹⁸ However, the expression pattern and functional roles of *MBD2* in RCC pathogenesis remain yet reported.

Table 2 Univariate and Multivariate Cox Analyses of Prognostic Markers for OS in RCC Patients

Prognostic Variables	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (Male vs Female)	1.019	0.647–1.605	0.934			
Age, years (≤ 60 vs > 60)	1.081	0.685–1.705	0.739			
Fuhrman grade (G1+G2 vs G3)	1.182	0.749–1.866	0.472			
T stage (T1+T2 vs T3+T4)	2.245	1.409–3.575	0.001	1.264	0.635–2.514	0.505
Lymph node involvement (N0 vs N1)	2.393	1.502–3.813	P < 0.001	2.340	1.022–5.360	0.044
Distant metastasis (M0 vs M1)	1.275	0.798–2.037	0.309			
TNM stage (I–II vs III–IV)	1.767	1.120–2.787	0.014	0.706	0.330–1.509	0.369
MBD2 expression (Low vs High)	3.194	1.872–5.451	P < 0.001	3.004	1.739–5.190	P < 0.001

Note: P-values < 0.05 are indicated in bold.

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

Table 3 Univariate and Multivariate Cox Analysis of Prognostic Markers for DFS in RCC Patients

Prognostic Variables	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (Male vs Female)	1.044	0.678–1.605	0.846			
Age, years (≤ 60 vs > 60)	0.897	0.583–1.382	0.623			
Fuhrman grade (G1+G2 vs G3)	1.192	0.773–1.838	0.427			
T stage (T1+T2 vs T3+T4)	2.243	1.433–3.511	P < 0.001	1.401	0.693–2.833	0.347
Lymph node involvement (N0 vs N1)	2.325	1.489–3.632	P < 0.001	1.893	0.858–4.176	0.114
Distant metastasis (M0 vs M1)	1.380	0.886–2.148	0.154			
TNM stage (I–II vs III–IV)	1.759	1.141–2.712	0.011	0.765	0.380–1.540	0.453
MBD2 expression (Low vs High)	2.741	1.683–4.463	P < 0.001	2.511	1.519–4.149	P < 0.001

Note: P-values < 0.05 are indicated in bold.

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

In order to illuminate the biological and clinical significance of *MBD2*, we firstly detected the protein levels of *MBD2* in RCC cells and tissues. Western blotting showed that increased *MBD2* was found in the RCC cell lines compared with normal renal epithelial cell HK-2. And high expression profiles of *MBD2* were shown in the RCC tissues when compared with adjacent normal tissues. In addition, we performed the IHC staining with 109 RCC specimens, which supported the increased *MBD2* in RCC. Higher expression of *MBD2* was significantly associated with T stage, distant metastasis, and TNM stage. Survival analysis revealed that *MBD2* expression was negatively correlated with the OS and DFS of RCC patients, and both univariate and multivariate analysis demonstrated the role of *MBD2* as an independent prognosis predictor for RCC patients. In accordance with this, Liu et al reported that the upregulated *MBD2* expression, as a candidate prognostic factor in hepatocellular carcinoma, was associated with poor OS and DFS, along with the clinicopathological

characteristics including tumor size, vascular invasion and BCLC stage. Increased *MBD2* is associated with malignant progression of glioma tissues.^{21,22} In contrast, several studies have yielded the conflicting results as to the *MBD2* expression in cancer and matched normal tissues.^{17,40}

We studied the biological effects of *MBD2* gene in RCC cells via interfering and overexpressing *MBD2*. Our results suggested that *MBD2* contributed to RCC cells malignancy. Forced expression of *MBD2* apparently potentiated the proliferation, migration and invasion of RCC cells through CCK-8 and transwell assays. Meanwhile, *MBD2* expedited the cell cycle progression from G1 phase to S phase. On the contrary, *MBD2* depletion impeded cell proliferation, migration and invasion and decelerated cell cycle progression. Western blotting indicated that the aberrant expression of *MBD2* augmented the Cyclin D1 level and impaired the *p53* and *p21* levels in RCC cells. *CyclinD1* activates and binds to the cell cycle-dependent kinase 4/6 (CDK4/6) to form

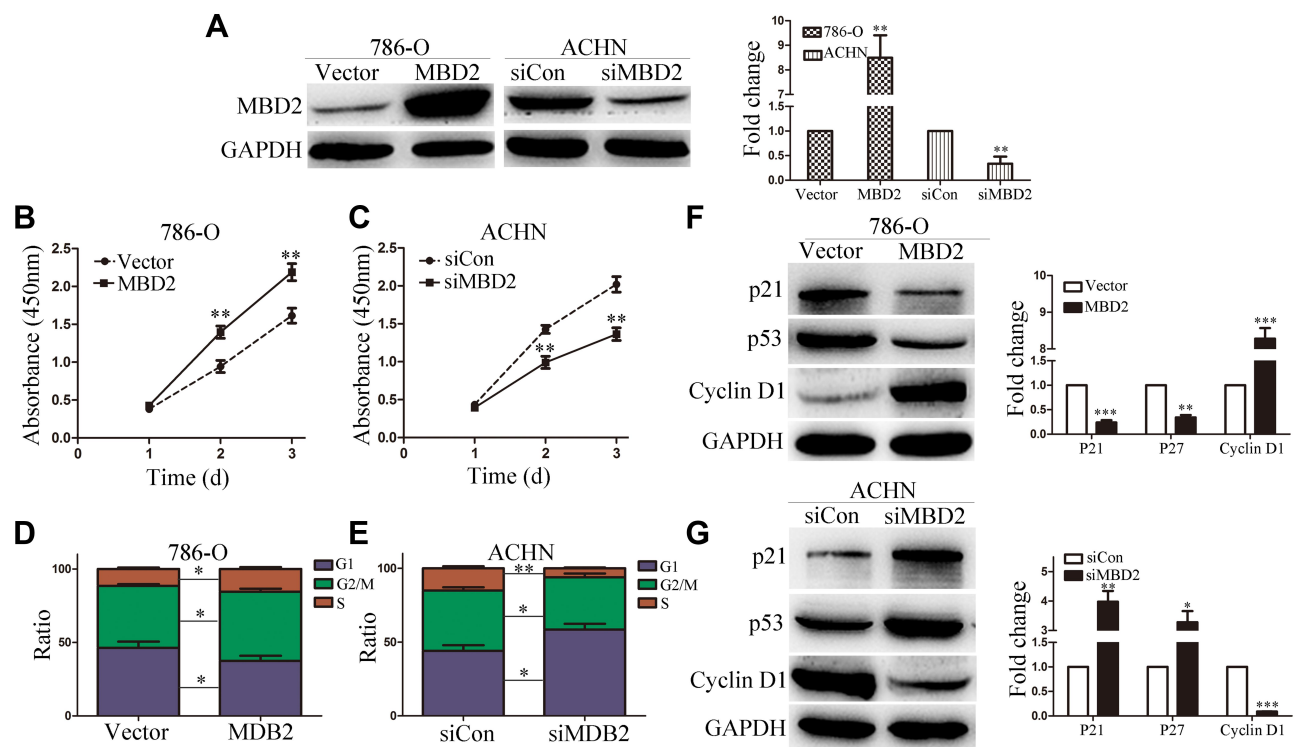


Figure 3 MBD2 promotes the proliferation of RCC cells. **(A)** The protein expression of *MBD2* was determined by Western blotting in the 786-O and ACHN cells transfected with *MBD2* plasmid or siRNA. **(B and C)** The effects of *MBD2* overexpression or knockdown on 786-O and ACHN cells as performed by CCK-8 assays. **(D and E)** Cell cycle distributions were analyzed by flow cytometry in 786-O and ACHN cells, and the percentage of cell population were presented in the histograms. **(F and G)** The Western blot analysis of the expression of *P21*, *P27* and *Cyclin D1* in 786-O and ACHN cells. Data represents the means \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

a kinase complex with *Cyclin D1* as a regulatory subunit and CDK4/6 as catalytic subunit, which enables cells to enter the S phase through G1/S regulatory point.⁴¹ Tumor suppressor gene *p21* is a broad-spectrum inhibitor of cell CDK and a negative regulator of cell cycle, which can prevent the cells from passing through the G1-S phase. *p53* bind to the promoter region of *p21* to activate its transcription, preventing the transition from G1 to S phase and arresting the cell to G1 phase.⁴² Therefore, *MBD2* may regulate the proliferation ability of RCC cells partly through *Cyclin D1*, *p53* and *p21*. Moreover, *MBD2* increased the mesenchymal markers expression and decreased the epithelial marker (E-cadherin) expression in RCC cells. EMT characterized by the deletion of epithelial cell phenotypes and the appearance of mesenchymal cell phenotypes works as a decisive role in the tumorigenesis, invasion and metastasis.^{43,44} EMT enhances the migration and invasion of cancer cells by deleting the expression of connective molecules, potentiating the adhesion and motor ability.^{43,44} *MBD2* may regulate the migration and invasion partly via EMT

molecules, E-cadherin, N-cadherin and Vimentin. Together, *MBD2* conferred on cells tumor malignant properties, promoted the cell proliferation, cycle progress, migration and invasion and regulates the proliferation and metastasis-related proteins in RCC, which is consistent with the previous reports of supporting cancer-promoting role of *MBD2* in glioma,²² hepatocellular carcinoma,²⁴ chronic myeloid leukemia.²⁶ The exact molecular mechanisms of *MBD2* affecting the proliferation, migration and invasion of RCC cells need to be further explored.

Conclusion

In conclusion, the present study provides evidence for the connection between *MBD2* and RCC. We conclude that *MBD2* shows a high expression in both RCC tissues and cell lines, is associated with a poor prognosis of RCC patients and might be an independent prognostic factor for RCC patients. *MBD2* acts as an oncogene in RCC cells by promoting the cell growth, cycle progress, migration and invasion. Further researches are still in demand to

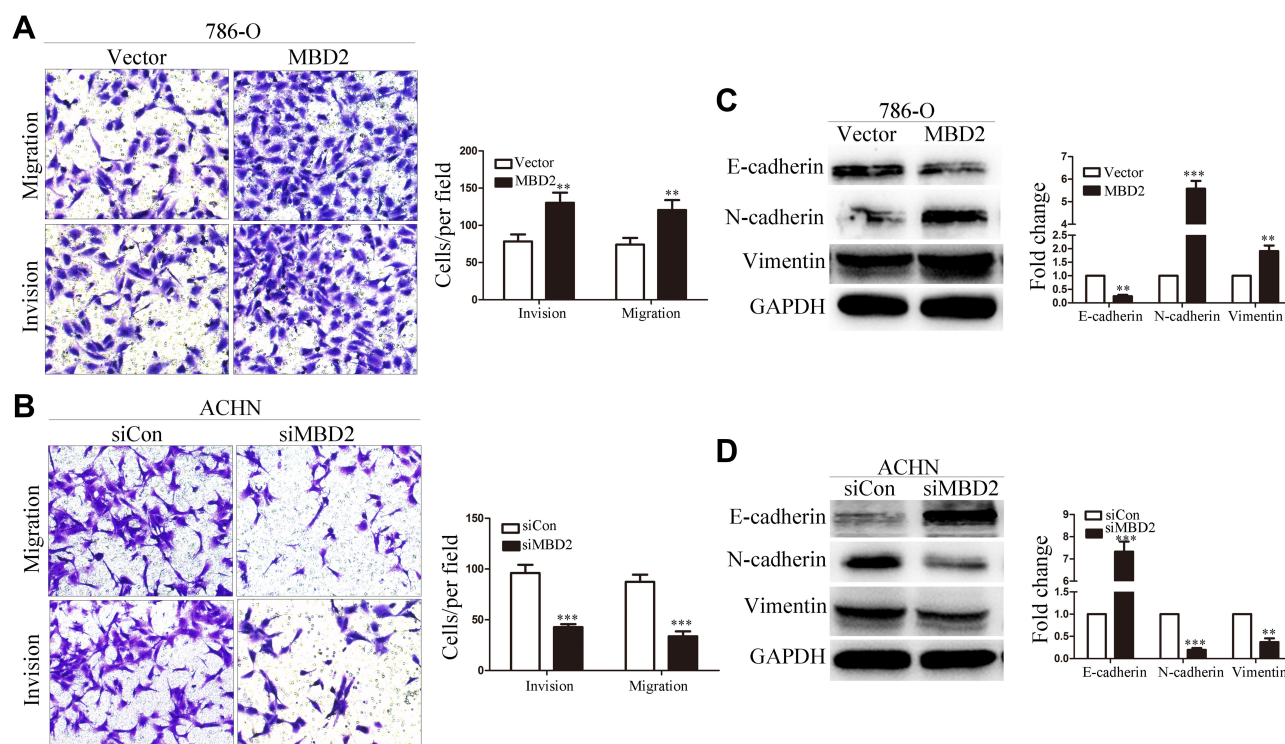


Figure 4 MBD2 promotes the migration and invasion of RCC cells. **(A and B)** The effect of MBD2 on cell migration and invasion was examined in 786-O and ACHN cells after MBD2 was re-expressed or knocked down, respectively. **(C and D)** The Western blot analysis for the protein expression of indicated EMT molecules (E-cadherin, N-cadherin and Vimentin) in 786-O and ACHN cells. Data represents the means \pm SD. ** $P < 0.01$; *** $P < 0.001$.

clarify the precise molecular mechanism of MBD2 in RCC. Therefore, targeting MBD2 would be considered as a potential therapeutic avenue for RCC patients.

Author Contributions

Junnian Zheng was involved in the study design. Liantao Li was a major contributor in writing the manuscript. Li Na, Liantao Li and Nianli Liu analyzed the patient data. Li Na and Fuchun Huo conducted the in vitro experiments. All authors approved the final manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Funding

The present study was financially supported by the Jiangsu Provincial Medical Youth Talent (QNRC2016773), Jiangsu Provincial Science and Technology Program (BK20161157), Six Talent Peaks Project in Jiangsu Province (WSN-119), Post graduate Research & Practice Innovation Program of Jiangsu Province (KYCX18_2185), the Jiangsu Provincial Medical Innovation Team under Grant CXTDA2017034.

Disclosure

The authors report no conflicts of interest in this work.

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