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microRNA-505 negatively regulates HMGB1 to suppress cell proliferation in renal cell carcinoma

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Abstract

microRNAs have been recognized to regulate a wide range of biology of renal cell carcinoma (RCC). Although miR-505 has been reported to play as a suppressor in several human tumors, the physiological function of miR-505 in RCC still remain unknown. Therefore, the role of miR-505 and relevant regulatory mechanisms were investigated in RCC in this study. Quantitative real-time polymerase chain reaction was conducted to detect the expression of miR-505 and high mobility group box 1 (HMGB1) in both RCC tissues and cell lines. Immunohistochemical staining was used to assess the correlation between HMGB1 expression and PCNA expression in RCC tissues. Subsequently, the effects of miR-505 on proliferation were determined in vitro using cell counting kit-8 proliferation assays and 5-ethynyl-2′-deoxyuridine incorporation. The molecular mechanism underlying the relevance between miR-505 and HMGB1 was confirmed by luciferase assay. Xenograft tumor formation was used to reflect the proliferative capacity of miR-505 in vivo experiments. Overall, a relatively lower miR-505 and higher HMGB1 expression in RCC specimens and cell lines were found. HMGB1 was verified as a direct target of miR-505 by luciferase assay. In vitro, overexpression of miR-505 negatively regulates HMGB1 to suppress the proliferation in Caki-1; meanwhile, knock-down of miR-505 negatively regulates HMGB1 to promote the proliferation in 769P. In addition, in vivo overexpression of miR-505 could inhibit tumor cell proliferation in RCC by xenograft tumor formation. Therefore, miR-505, as a tumor suppressor, negatively regulated HMGB1 to suppress the proliferation in RCC, and might serve as a novel therapeutic target for RCC clinical treatment.

KEYWORDS

HMGB1, miRNA-505, proliferation, renal cell carcinoma

1 | INTRODUCTION

Renal cell carcinoma (RCC), as a common lethal malignancy, accounts for approximately 5% of all adult malignant neoplasms and is definitely one of the top 10 most frequent cancer worldwide (Capitanio et al., 2018; Siegel, Miller, & Jemal, 2018). Meanwhile, it is also the most frequent cause of cancer-related mortality, with 65,340 new cases and 14,970 deaths in 2018 in the United States (Siegel et al., 2018). On the basis of different biological characteristics and therapies, RCC can be divided into different histological subtypes, including clear cell RCC,

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papillary RCC, and chromophobe RCC and so on (Capitanio et al., 2018; Patel et al., 2015). Due to the resistance to chemotherapy and radiotherapy, RCC easily progresses to malignant potential (Buti et al., 2013; De Meerleer et al., 2014). Nevertheless, 20–30% of RCC patients at the time of diagnosis had still locally advanced or developed metastases, and approximately 30–50% of RCC patients would develop advanced RCC following surgical resection of the primary tumor (Diamond et al., 2015; Pike et al., 2018). Currently, surgical resection, as the main therapeutic methods for patients with RCC, has a good curative effect in cases of local RCC (Klatte & Stewart, 2018; J. R. Li et al., 2018). However, the proliferation of tumor cells is one of the major challenges in the clinical treatment of RCC (Akhtar, Al-Bozom, & Al hussain, 2018). Consequently, the identification of novel molecules that inhibit the proliferation of RCC cells is imminent (Ciccarese et al., 2015). Therefore, understanding the detailed molecular mechanism of RCC progression is crucial to identify effective biomarkers for the early effective interventions and diagnosis of RCC.

microRNA (miRNA) is considered as a class of evolutionary conserved small noncoding RNA molecules containing 18–25 nucleotides, which are important posttranscriptional regulators of the gene expression through binding to the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs; He, Chen, & Shi, 2018; Ran, Liang, Deng, & Wu, 2017). A wide range of biological processes is concerned, such as cell proliferation, apoptosis, invasion, and migration, which have been recognized to be regulated by miRNAs (Morais, Dias, Teixeira, & Medeiros, 2017; Xing & He, 2016). Thus, the deregulated expression of miRNA is closely related to the occurrence and development of tumors (Mukhadi, Hull, Mbita, & Dlamini, 2015). The proliferation of cancer cells is an important stage of malignant tumor progression, especially in advanced tumors (Milosevic et al., 2015). Growing evidence has suggested that miRNAs are responsible for the proliferation of RCC involving regulation of downstream target genes (Morais et al., 2017; Ran et al., 2017). Therefore, miRNAs could be regarded as oncogenes or tumor suppressor genes according to their multiple target mRNAs.

In this study, we identified miR-505 as a tumor suppressor by directly binding to the 3′-UTR of high mobility group box 1 (HMGB1) in RCC cell lines. HMGB1, a highly conserved chromatin-binding protein implicated in diverse biological processes, has been documented to promote RCC cells in vitro and in vivo (Q. Chen, Guan, Zuo, Wang, & Yin, 2016). Recently, a number of published studies have indicated that HMGB1 contributed to carcinogenesis, and upregulation of HMGB1 expression could promote the development of various cancer types, including gastric cancer, ovarian cancer, and lung cancer (C. Zhang, Ge, Hu, Yang, & Zhang, 2013; J. Zhang, Kou, Zhu, Chen, & Li, 2014; W. Zhang, Tian, & Hao, 2014). HMGB1 is always overexpressed in RCC and promotes malignant progression (Kargı et al., 2016). Accordingly, we hypothesized whether miR-505/HMGB1 axis was involved in the development of RCC.

Recently, some studies have shown that miR-505 acted as a tumor suppressor in glioblastoma, colorectal cancer, and so on (Y. Chen, Bian, & Zhang, 2018; Liu et al., 2018; C. Zhang, Yang, Fu, & Liu, 2018). Nevertheless, the possible association between miR-505 and RCC was not clear. Hence, the expression level of miR-505 in RCC tissue samples was analyzed compared with paracancerous tissue samples, and its potential biomedical functions on RCC cell proliferation was explored to confirm whether miR-505 was a new molecular biomarker of RCC.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Forty-two pairs of RCC tissues and paracancerous tissues were collected from patients diagnosed with RCC at the Department of Urology, Nanjing First Hospital, Nanjing Medical University and Department of Urology, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University (China) between 2015 and 2018. Each pair of RCC and paracancerous tissue was from the same patient after pathological examination. Then, the tissues fragments were immediately transferred into liquid nitrogen and stored at −80°C before use. This study was approved by the Ethics Committee of Nanjing Medical University.

2.2 | The Cancer Genome Atlas (TCGA) data acquisition

The genomic alteration data on patients with RCC and corresponding clinicopathologic profiles was downloaded at TCGA (https://cancergenome.nih.gov/), which as a freely opened public platform, was a source for abundant cancer-related data. This study complied with TCGA publication guidelines and policies (http://cancergenome.nih.gov/publications/publicationguidelines). Thus, the relevant expression level of miR-505 in RCC of TCGA database were determined.

2.3 | Cell lines and cell culture

The human renal cancer cell lines (Caki-1, Caki-2, ACHN, 769P, 786-O) and a normal human renal tubular epithelial cell (HK-2) were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in McCoy's 5A or Dulbecco's modified Eagle's medium or 1640 (Gibco-BRL, Gaithersburg) supplemented with 10% fetal bovine serum (10% FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured in a humidified air atmosphere at 37°C with 5% CO_2.

2.4 | Construction of stable cell lines and cell transfection

Selected human renal cancer cells Caki-1 and 769P were transfected with constructed lentiviral vectors (Lv2-pGLV-u6-puro; GenePharma, Shanghai, China). The empty lentiviral vector was taken as a control. Based on the protocols, cells were infected with lentiviruses and screened with puromycin. Caki-1 were transfected with miR-505 mimics while 769P were dealt with miR-505 inhibitor, NC and anti-NC were used as negative control.

In addition, selected cell lines were transfected with HMGB1 small interfering RNA (siRNA; GenePharma), negative control siRNA

(GenePharma), pcDNA3.1-HMGB1 (GenePharma), empty pcDNA3.1 plasmid (GenePharma). For plasmid and siRNA transfections, cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific, Shanghai, China) based on the protocols.

2.5 | Isolation of total RNA and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured cell lines and clinical samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Primescript RT Reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. miR-505 and HMGB1 mRNA levels were quantified by qRT-PCR with StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR® Premix Ex Taq™ Reagent (Takara). Data analysis was performed with ABI Step One Software version 2.1 and the relative mRNA level was calculated using $2^{-\Delta\Delta C_t}$ method.

The following primers were used for qRT-PCR reaction:
microRNA-505: forward: 5′-CGUCAACACUUGCUGGUUUCCU-3′,
reverse: 5′-GGGAGCCAGGAAGUAUUGAUGU-3′;
U6: forward: 5′-CTCGCTTCGGCAGCACA-3′,
reverse: 5′-AACGCTTCACGAATTTGCGT-3′;
HMGB1: forward: 5′-AATACGAAAAGGATATTGCGT-3′,
reverse: 5′-GCGCTAAACCAACTTAT-3′;
β-actin: forward: 5′-CCTGGCACCCAGCACAAT-3′,
reverse: 5′-GCTGATCCACATCTGCTGGAA- 3′.

2.6 | Cell proliferation assay

Cell proliferation was assessed using a cell counting kit-8 assay (CCK-8; Dojindo, Kyushu, Japan). Pretreated cells were seeded into a 96-well plate with 3×10^3 cells/well and cultured for 1, 2, 3, and 4 days. The absorbance was measured at 450 nm with a microplate reader after incubated at 37°C for 3 hr.

2.7 | 5-Ethynyl-2′-deoxyuridine (EdU) proliferation assay

To display the function of miR-505 on cell proliferative, the EdU proliferation assay (RiboBio, Nanjing, China) was carried out according to the manufacturer's instructions. Twenty-four hours after transfection, cells were incubated with 50 μM EdU for 2 hr. Then an Apollo staining and 4′,6-diamidino-2-phenylindole (DAPI) staining were performed according to the instructions to detect the EdU-positive cells (red cells) with a fluorescence microscope. The EdU incorporation rate was revealed as the ratio of EdU-positive to total DAPI-positive cells (blue cells).

2.8 | Bioinformatics analysis

The potential targets of miR-505 were predicted and analyzed using bioinformatics method. In this study, three public available algorithms including PicTar, TargetScan, miRWalk, and miRanda were utilized. The results indicated that 3′-UTR of HMGB1 bound to miR-505 with the high score, suggesting that HMGB1 might be a potential downstream target of miR-505.

2.9 | Luciferase report assay

The wild-type HMGB1 3′-UTR sequence or the mutant sequence of HMGB1 3′-UTR containing predicted binding sites of miRNA-505 were inserted into pGL3 promoter vector (Genscript, Nanjing, China). RCC cell line Caki-1 and 769P were seeded onto 24-well plates (5×10^5cells/well) the day before transfection, then the cells were cotransfected with luciferase reporter vectors (0.12 μg) and miR-505 mimic or negative control using Lipofectamine 3000 Reagent (Invitrogen). Luciferase reporter assay was conducted 48 hr after transfection by using a Luciferase Assay System according to the manufacturer's instructions.

2.10 | Western blot analysis

RCC cells were collected and lysed in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China). Total protein lysates extracted from tissues or cells were separated through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. Anti-beta actin monoclonal antibody was taken as the loading control. The membrane was blocked, incubated with 5% nonfat milk in mixture of Tris-buffered saline and Tween 20 for 2 hr at room temperature, and followed by immunostaining overnight at 4°C using rabbit polyclonal anti-HMGB1 antibody (Abcam, Cambridge, UK). Protein densitometric analysis was performed using the ImageJ software (Sun Microsystems, Santa Clara).

2.11 | Immunohistochemistry

The expression levels of HMGB1 and proliferating cell nuclear antigen (PCNA) were examined by immunohistochemical staining. Briefly, tissues were sliced into 3-μm sections and then deparaffinized. Subsequently, sections were incubated with rabbit monoclonal anti-HMGB1 (CST) and anti-PCNA (CST) antibodies at 4°C overnight. After washing three times with phosphate-buffered saline (Gibco-BRL, Gaithersburg), all sections were incubated with goat anti-rabbit IgG for 30 min, then were stained with the color reagent 3,3′-diaminobenzidine.

2.12 | In vivo experiments

The Animal Care and Use Committee approved the animal experiment that was performed in accordance with institutional guidelines. BAB/c nude mice, 5 weeks of age or older, were purchased from the animal center of Nanjing University (Nanjing, Jiangsu, China). Five mice in each group were injected with cells (1×10^7 suspended in 100 μl PBS) subcutaneously into the flank of each mouse. All of the mice were killed after inoculation of 6 weeks, then the metastatic nodes were examined

TABLE 1 Association of miR-505 and HMGB1 expression with clinicopathologic characteristics of renal cell carcinoma

		miR-505 expression			HMGB1 expression		
Parameters	**Number of cases**	**Low (%)**	**High (%)**	***p* Value**	**Low (%)**	**High (%)**	***p* Value**
Age (years)				0.339			0.204
<60	16	5	11		10	6	
≥60	26	12	14		11	15	
Gender				0.352			0.355
Male	21	8	13		12	9	
Female	21	11	10		9	12	
T stage				**0.011**			**0.031**
T1-T2	26	6	20		17	9	
T3-T4	16	10	6		5	11	
Lymph node metastasis				**0.036**			**0.044**
No	29	8	21		22	9	
Yes	13	8	5		6	9	
Distance metastasis				**0.047**			**0.031**
No	35	11	24		25	10	
Yes	7	5	2		2	5	

Bold represents P<0.05.

by necropsy and counted. The metastatic nude was monitored by the IVIS Lumina II system every 5 days. Then the renal tissues were fixed in 10% neutral phosphate-buffered formalin. The fixed samples were embedded in paraffin and stained with hematoxylin and eosin.

2.13 | Statistical analysis

All experiments were performed in triplicate independently and the χ^2-test was used to analyze the significant differences between the data shown in Table 1. Student's unpaired *t*-test was also adopted to detect the significant differences of other results. The data were presented as mean ± standard deviation (*SD*). All statistical analyses were performed using SPSS 22.0 software (IBM, Armonk) and $p < 0.05$ was considered to be statistically significant.

3 | RESULTS

3.1 | miR-505 expression is downregulated in RCC

To investigate the role of miR-505 in RCC, the expression level of miR-505 was significantly upregulated in RCC clinical tissues compared with marched paracancerous tissues ($p = 0.0096$) in TCGA database. Subsequently, we first detected the expression of miR-505 in 42 pairs of paired RCC tissues and paracancerous tissues. The results showed that miR-505 was significantly downregulated in paracancerous tissues compared with paracancerous tissues (Figure 1a). The expression levels of miR-505 in five RCC cell lines and the normal renal cell line HK-2 were analyzed by qRT-PCR. As shown in Figure 1b, all RCC cells showed significant downregulation of miR-505 compared with HK-2.

Then, χ^2-test was used to study the association between miR-505 expression level and clinicopathological features of renal cell carcinoma in 42 patients. The expression of miR-505 was negatively correlated with T stage ($p = 0.011$), lymph node metastasis ($p = 0.036$), and distance metastasis ($p = 0.047$). However, no significant correlation between the expression of miR-505 and other clinicopathological parameters was found (Table 1). Comparing to RCC tissues with lower expression of miR-505, the HMGB1 and PCNA expression levels was significantly decreased in miR-505 higher express RCC samples, which suggested that miR-505 might play an inhibitory role in RCC (Figure 1c,d).

3.2 | miR-505 inhibits proliferation in RCC cells

To further evaluate the role of miR-505 in RCC, Caki-1 cell (with lower expression of miR-505) and 769P cell (with higher expression of miR-505) were chosen for sequent research. Caki-1 and 769P cells were transfected with lentiviral constructs which designed to overexpress or knock-down miR-505 (Caki-1-miR-505, Caki-1-NC, 769P-anti-miR-505, and 769P-anti-NC). The transfection efficiency of the cell lines was validated by qRT-PCR, which showed that the expression of miR-505 was markedly increased in Caki-1 cells when transfected with miR-505 mimics, whereas miR-505 level was decreased in 769P cells after transfected with anti-miR-505 (Figure 2a).

CCk-8 and EdU proliferation assays were conducted to investigate the proliferation of RCC. The proliferation ability of Caki-1 cells (overexpression miR-505 group) could be suppressed by miR-505. On the contrary, the miR-505-inhibition group resulted in a markedly increased tendency of cell proliferation (Figure 2b,c), which suggested that miR-505 inhibited the proliferation of RCC cells.

3.3 | HMGB1 is a direct target of miR-505

To clarify the molecular mechanisms of miR-505 in mediating RCC proliferation, the miRNA target prediction software including TargetScan, miRDB, and microRNA.org were used to predict the candidate gene targets by intersecting outputs. From the

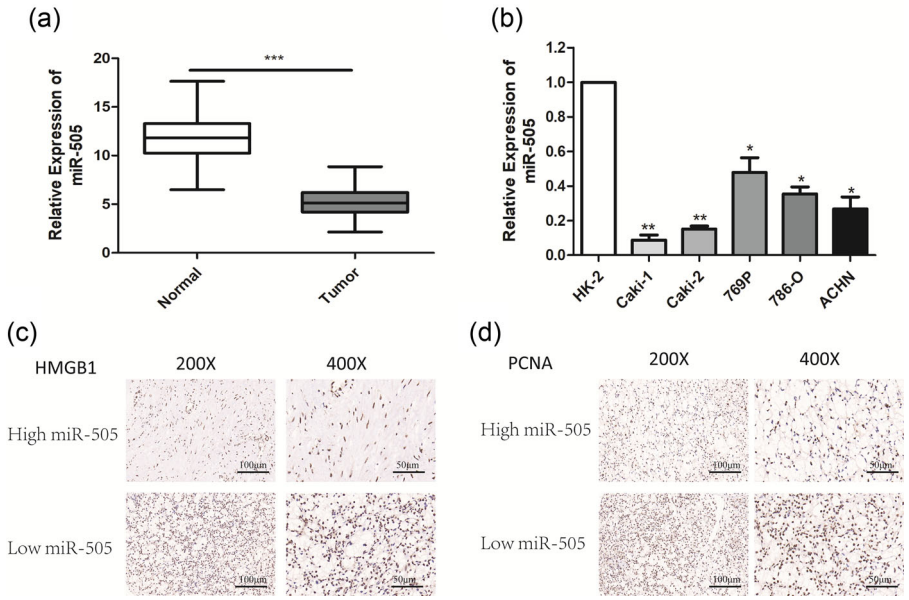

FIGURE 1 Expression of miR-505 in RCC clinical samples and cell lines. (a) The expression levels of miR-505 in 60 pairs of human RCC tissues and non-RCC tissues were explored using miRNA RT-PCR. (b) The expression levels of miR-505 in five renal cancer cell lines (Caki-1, Caki-2, ACHN, 769P, 786-O) and normal human renal tubular epithelial cell (HK-2). (c, d) Immunohistochemical staining against HMGB1 and PCNA collected from human RCC tissues showed a negative correlation between the miR-505 expression and both HMGB1 and PCNA. Data are represented as mean ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$. HMGB1: high mobility group box 1; miR: microRNA; PCNA: proliferating cell nuclear antigen; RT-PCR: real-time polymerase chain reaction; RCC: renal cell carcinoma [Color figure can be viewed at wileyonlinelibrary.com]

resultant list of 10 genes, we focused on the factors that are downregulated in RCC (Figure 3a). Of them, the bioinformatic software analysis suggested 3′-UTR of HMGB1 binds to miR-505 with the high score. Thus, this prediction was confirmed by a dual-luciferase reporter system, where miR-505 were cotransfected with luciferase reporter plasmids containing the 3′-UTR of HMGB1, or mutated HMGB1 (bearing deletions of the putative miR-505 target sites). As shown in Figure 3b, the cotransfection of miR-505 mimics suppressed the luciferase activity of the reporter (containing the wild-type HMGB1 -UTR sequence). However, miR-505 mimics did not affect the luciferase activity when the target cells were transfected with mutated HMGB1. These results suggested that HMGB1 might be a direct functional target of miR-505 in RCC.

The above results have demonstrated that miR-505 level was low expressed in RCC tissues compared with paracancerous tissues (Figure 1a). Then, we detected the expression level of HMGB1 in 42 paired tumor tissues and paracancerous tissues by qRT-PCR. Results suggested that HMGB1 was markedly upregulated in tumor tissues compared with paracancerous tissues (Figure 3c). Then, a two-tailed Pearson's correlation analysis was performed to evaluate the correlation between miR-505 and HMGB1. As shown in Figure 3e, the expression of miR-505 was negatively correlated with HMGB1 expression. Besides, the expression of HMGB1 was correlated with T stage ($p = 0.031$), lymph node metastasis ($p = 0.044$), and distance metastasis ($p = 0.031$). However, there was no significant correlation between the expression of HMGB1 and other clinicopathological parameters (Table 1).

3.4 | HMGB1 functions as an oncogene in RCC cells regulated by miR-505

To verify the role of HMGB1 in RCC, the expression level of HMGB1 was analyzed in RCC cell lines. A significantly higher HMGB1 expression in RCC cell lines (Caki-1, Caki-1, PC9, 786-O, and 769P) was observed, compared with the corresponding expression levels in HK-2 (Figure 3d). These results indicated HMGB1 might act as a carcinogenic gene in RCC cells. Then, we found that the mRNA and protein levels of HMGB1 were decreased after transfection with miR-505 mimics. Reversely, HMGB1 expression was increased after transfection with miR-505 inhibitor (Figure 3f). Collectedly, we demonstrated that the oncogene HMGB1 was a direct target gene of miR-505 and the expression level of HMGB1 was inhibited by miR-505.

3.5 | miR-505 suppresses proliferation in RCC cells by targeting HMGB1

To illuminate whether the proliferation induced by aberrant expression of miR-505 was in connection with the expression of HMGB1 in RCC cells, we conducted rescue assays. For upregulating HMGB1 expression, we used pcDNA3.1-HMGB1 in Caki-1 cell, while 769P cell was transfected with the small interfering RNA (siRNA) for downregulating HMGB1 expression. The expression of HMGB1 on both mRNA and protein levels in transfected cells were examined by qRT-PCR and western blot analysis, respectively. Compared with control group, miR-505 overexpression Caki-1 cell cotransfected with pcDNA3.1-HMGB1 exhibited an increased expression level of HMGB1. Consistently, relative

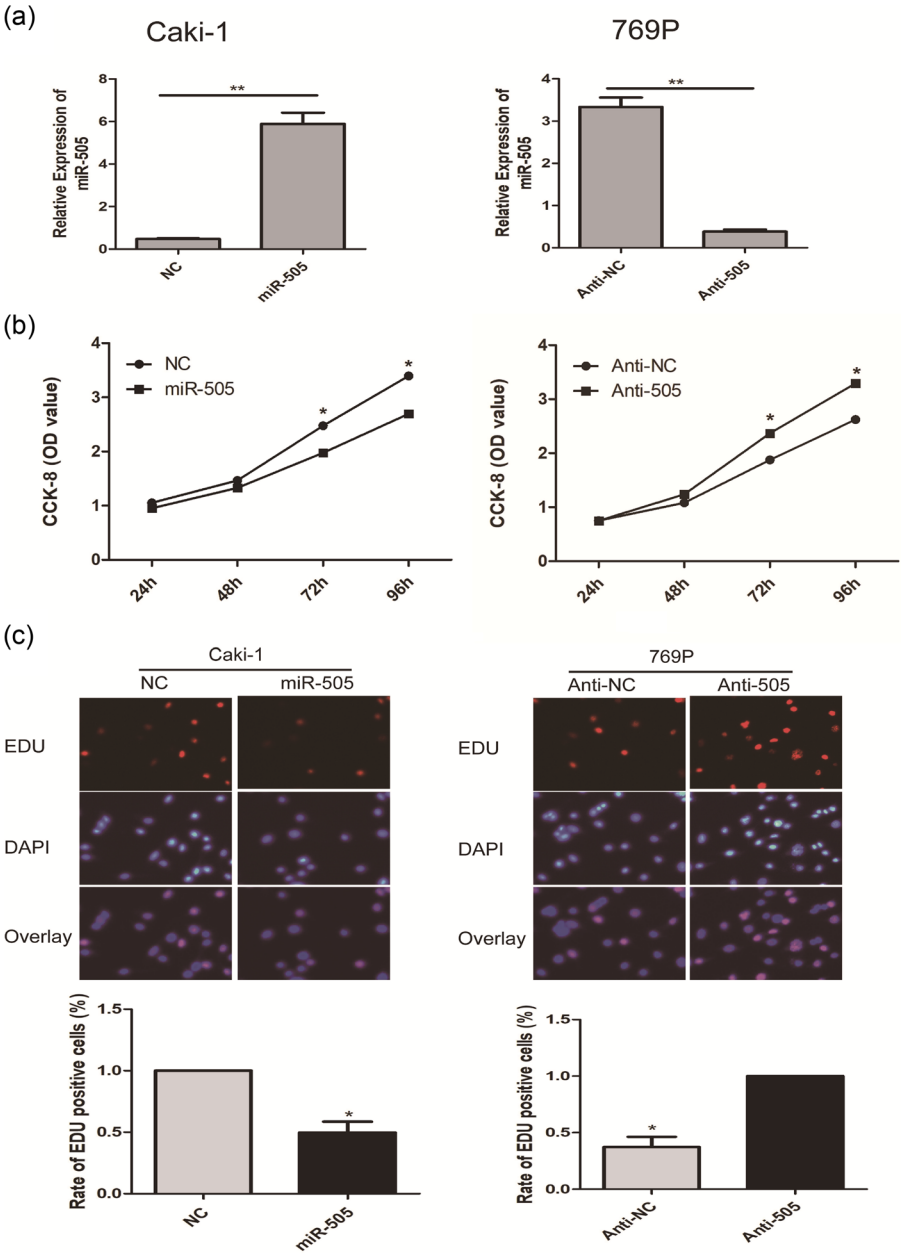

FIGURE 2 miR-505 suppresses cell proliferation in RCC. (a,b) The results of miR-505 expression in cell lines transfected with miR-505 mimics and miR-505-inhibitor lentivirus respectively were validated in Caki-1 and 769P, respectively, by using qRT-PCR. (c) CCK-8 assays for Caki-1 and 769P were determined after transfected miR-505 mimics and miR-505-inhibitor respectively compared with the control. (c) EDU proliferation assay for the effect of miR-505 expression alteration on cell motility. Data are represented as mean ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$. CCK-8: cell counting kit-8; DAPI: 4′,6-diamidino-2-phenylindole; EDU: 5-ethynyl-2′-deoxyuridine; miR: microRNA; NC: negative control; qRT-PCR: quantitative real-time polymerase chain reaction; RCC: renal cell carcinoma [Color figure can be viewed at wileyonlinelibrary.com]

expression level of HMGB1 decreased in miR-505 downexpression 769P cell cotransfected with si-HMGB1 (Figure 4a,b). Moreover, the overexpression of HMGB1 significantly increased cell proliferation, which was rescued by overexpression of transfected in Caki-1 cell (Figure 4c,e). Whereas HMGB1 markers changes reversely in response to cotransfection with miR-505 mimics and pcDNA3.1-HMGB1 (Figure 4d,f). Taken together, these findings suggested that miR-505 suppressed proliferation by directly targeting HMGB1 in RCC cells.

3.6 | miR-505 inhibits RCC proliferation in vivo

To further evaluate the effect of miR-505 on the proliferation of RCC cell lines in vivo, we established a xenograft model by utilizing Caki-1 overexpression miR-505 and 769P knock-down miR-505, subcutaneously into the flank of each mouse. The nude was monitored by the IVIS Lumina II system. As shown in Figure 5a, miR-505 overexpressed mice presented less metastatic nodes than the control group, while miR-505-inhibited mice resulted in obvious increased nodes compared with the control group. The detailed information of the nude has been listed in Figure 5b. The HMGB1 and PCNA expression levels were significantly decreased in high miR-505 expression of RCC samples. In summary, these results indicated that miR-505 inhibited tumor proliferation of renal cell carcinoma in vivo.

4 | DISCUSSION

RCC is one of the most deadly malignant tumor in the world, and its 5-year survival rate is <20% owing to diagnosis at an advanced stage (Capitanio et al., 2018). Despite the advances in surgery, chemotherapy

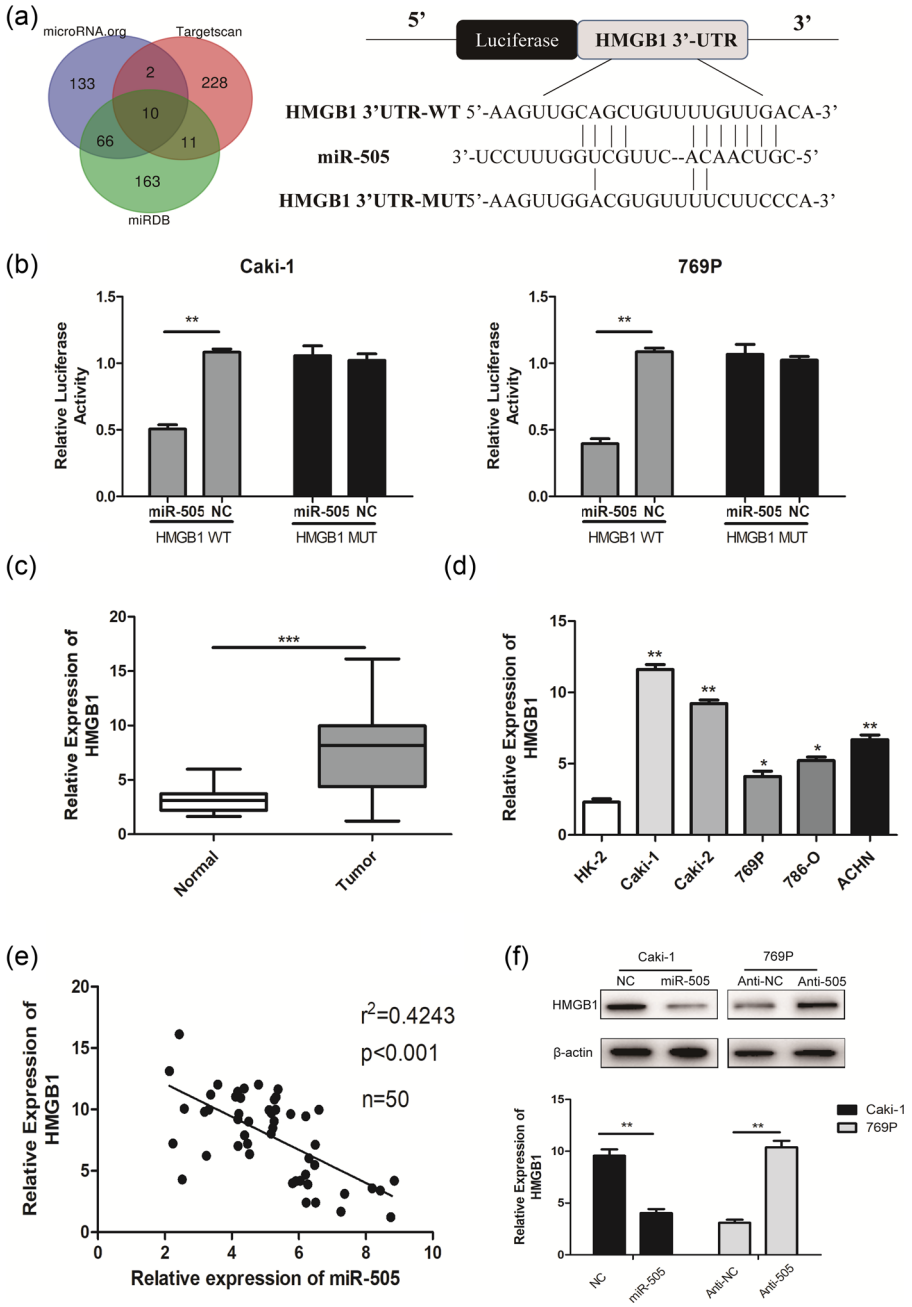

FIGURE 3 miR-505 regulates HMGB1 expression by directly binding its 3′-UTR. (a) The candidate gene targets were predicted by intersecting outputs from three distinct prediction algorithms (TargetScan, miRDB, and microRNA.org). (b) Luciferase reporter assay was conducted to verify that miR-505 directly bound to the 3′-UTR region of HMGB1. Luciferase activity was analyzed in cells cotransfected with miR-505-mimics or negative control with pGL3-HMGB1-WT or pGL3-HMGB1-MUT. (c) The mRNA expression level of HMGB1 relative to GAPDH in human RCC tissues and corresponding adjacent tissues was detected by using qRT-PCR. (d) Expression levels of HMGB1 in 5 renal cancer cell lines (Caki-1,Caki-2, ACHN, 769P, 786-O) and normal human renal tubular epithelial cell (HK-2). (e) A negative correlation was observed between miR-505 and HMGB1 in RCC samples. (f) HMGB1 protein expression and mRNA expression levels in transfected 769P and Caki-1 cells were analyzed by western blot analysis and qRT-PCR. β-Actin served as a control. Data are represented as mean ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$. HMGB1: high mobility group box 1; miR: microRNA; mRNA: messenger RNA; MUT: mutant; NC: negative control; qRT-PCR: quantitative real-time polymerase chain reaction; RCC: renal cell carcinoma; 3′-UTR: 3′-untranslated region; WT: wild-type [Color figure can be viewed at wileyonlinelibrary.com]

and radiotherapy, RCC patients are still suffering from the side effects of chemotherapy and considerably high recurrence rate (Buti et al., 2013; De Meerleer et al., 2014; Diamond et al., 2015; Xing & He, 2016). Therefore, it is urgent to find novel molecules or new therapeutic targets that could suppress the proliferation of RCC (Ciccarese et al., 2015).

In recent years, miRNAs have been demonstrated to play pivotal roles in tumorigenesis and proliferation of various cancers including RCC

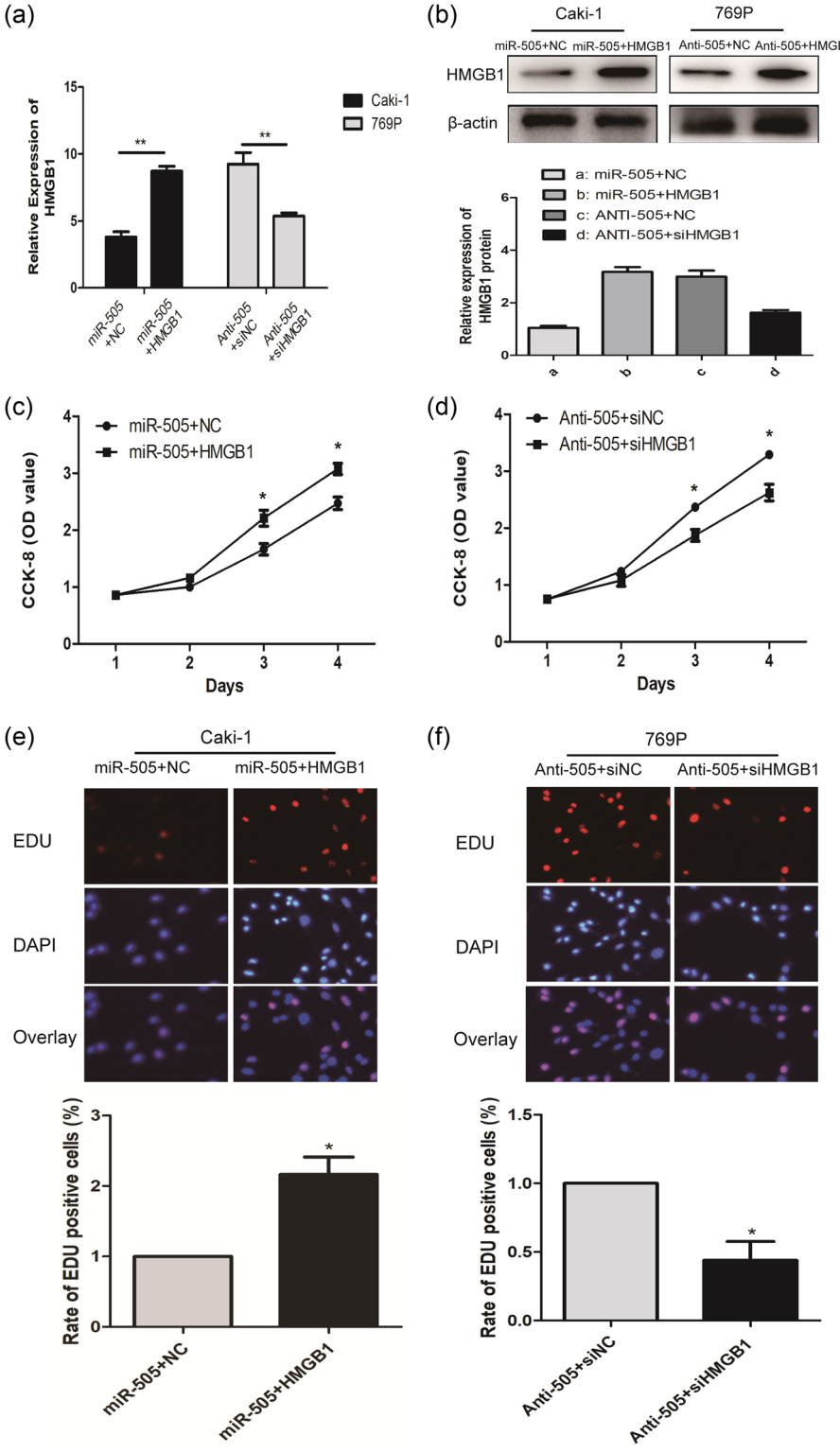

FIGURE 4 miR-505 suppresses proliferation in RCC cells by targeting HMGB1. (a) The expression of HMGB1 was verified by qRT-PCR in cotransfected cell lines. (b) Western blot was used to verify the expression of HMGB1 in cotransfected cell lines. (c,d) The roles of miR-505 and HMGB1 in the regulation of RCC cell proliferation were examined by CCK-8 assay in Caki-1 and 769P, respectively. (e,f) The roles of miR-505 and HMGB1 in the regulation of RCC cell proliferation were examined by EDU proliferation assay in Caki-1 and 769P, respectively. The rescue experiment for miR-505 overexpression was performed by ectopic expression of HMGB1 without its 3′-UTR in Caki-1 cells. Similar rescue experiment for miR-505 silencing was performed by downregulation of HMGB1 in 769P cells. Data are represented as mean ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$. CCK-8: cell counting kit-8; DAPI: 4′,6-diamidino-2-phenylindole; EDU: 5-ethynyl-2′-deoxyuridine; HMGB1: high mobility group box 1; miR: microRNA; NC: negative control; qRT-PCR: quantitative real-time polymerase chain reaction; RCC: renal cell carcinoma; 3′-UTR: 3′-untranslated region [Color figure can be viewed at wileyonlinelibrary.com]

(Morais et al., 2017; Ran et al., 2017). In the present years, several studies have explored the role of miR-505 in the development of various cancers (Y. Chen et al., 2018; Liu et al., 2018; C. Zhang et al., 2018). The results of these studies have suggested that the overexpression of miR-505 suppressed the proliferation of many different types of tumors (Y. Chen et al., 2018; Liu et al., 2018; C. Zhang et al., 2018). For example, Liu et al. (2018) reported that miR-505 acted as a tumor suppressor in the proliferation, migration, and invasion of osteosarcoma cells. Meanwhile, another study by Ma et al. (2017) revealed that miR-505 could predict prognosis and suppress the malignant progression in cervical carcinoma by targeting FZD4. However, the functions of miR-505 in RCC have not previously been reported. Herein, a series of experiments both in vitro and in vivo were to investigate the role of miR-505 in RCC progression and found that miR-505 was downregulated in RCC cell lines and tissue

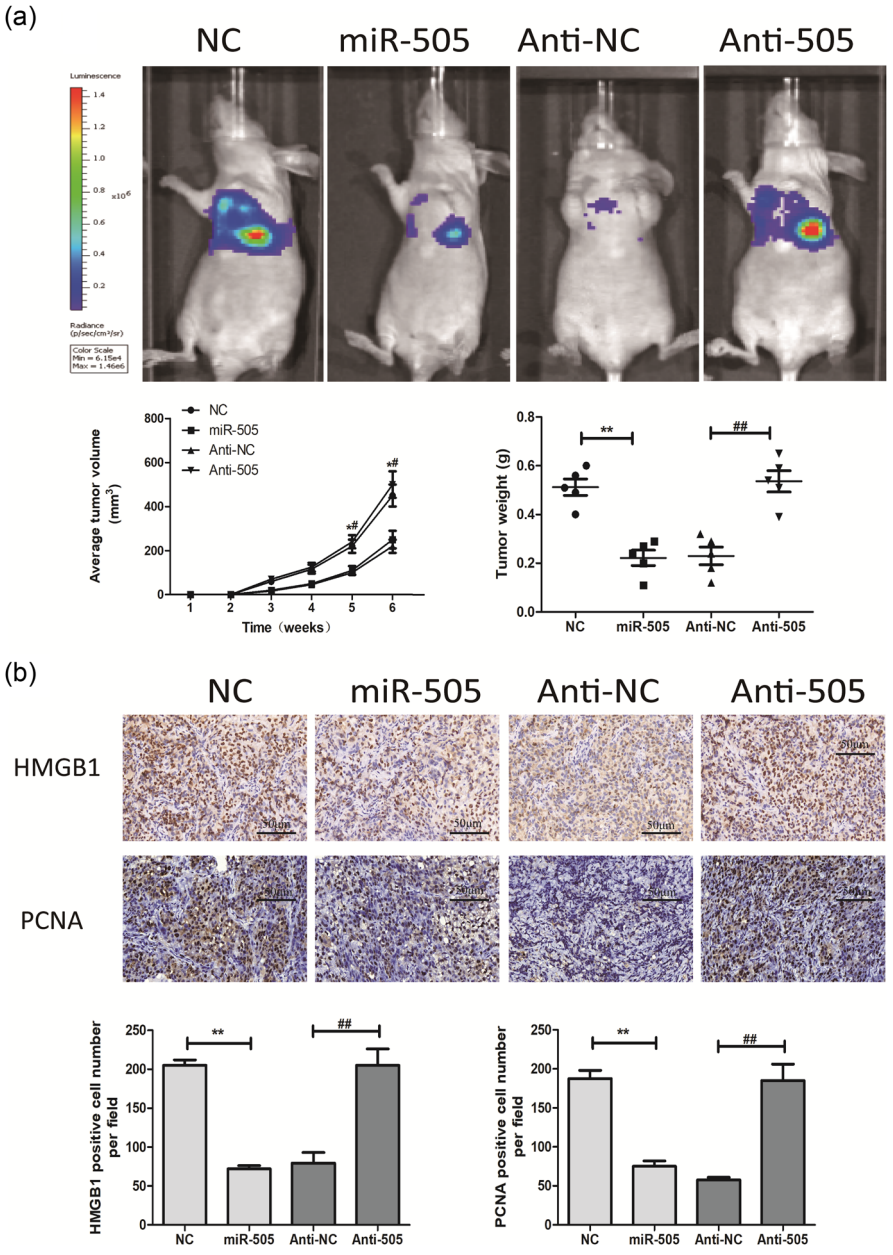

FIGURE 5 miR-505 inhibits RCC metastasis in vivo. (a) The tumor formation was detected by IVIS Lumina II system. (b,c) Immunohistochemical staining against HMGB1 and PCNA assay was utilized to determine the alteration in the samples collected from nude mice of renal tissues. Data are represented as mean ± *SD*. **significant difference vs. NC ($P < 0.01$); ##significant difference vs. Anti-NC ($P < 0.01$). HMGB1: high mobility group box 1; miR: microRNA; NC: negative control; PCNA: proliferating cell nuclear antigen; RCC: renal cell carcinoma [Color figure can be viewed at wileyonlinelibrary.com]

specimens. This result also confirmed that miR-505 expression might be related to proliferation ability. The overexpression of miR-505 in RCC inhibited tumor cell proliferation in vitro. Furthermore, the in vivo animal experiments demonstrated that the overexpression of miR-505 could inhibit the tumor cell proliferation in RCC.

To the best of our knowledge, miRNAs are involved in the progression of cancers by directly regulating the expression of specific target genes (Morais et al., 2017; Yan et al., 2018). HMGB1 was identified as a direct downstream target gene of miR-505 using bioinformatics method and it was further confirmed by the luciferase reporter assay. HMGB1 was overexpressed in RCC, and it also exhibited significantly elevated expression in renal cancer tissues that was closely related to the clinical prognosis of RCC patients (Wu & Yang, 2018; Yan, Ying, & Cai, 2018). In addition, previous studies have already showed that knock-down of HMGB1 in RCC cell lines in vitro led to reduced growth in the tumor microenvironment, which provided a novel theoretical basis for preventing RCC using HMGB1 as the target (J. Li et al., 2017; Wu et al., 2018). Subsequently, miR-505 could rescue the stimulation effect of HMGB1 on the proliferation of RCC cells. Based on the above results, we demonstrated that HMGB1 was a direct downstream target of miR-505.

To proliferation, tumor cells would undergo a pivotal physiological process. Increasing evidence has suggested that miRNAs, as one part of other factors, might be responsible for the main and sole driver for the onset and progression of RCC (Morais et al., 2017; Ran et al., 2017). IHC showed the correlation between the expressions of miR-505 as well as both HMGB1 and PCNA. The in vitro experiment demonstrated that overexpression of miR-505 led to the decreased expression of HMGB1 by western blot analysis. Depending on the rescue assay, the expression level of HMGB1 was altered reversely in response to cotransfection with miR-505 mimics and pcDNA3.1-HMGB1 in RCC cells. Therefore, we speculated that the overexpression of miR-505 could inhibit proliferation via suppression of HMGB1 in RCC.

5 | CONCLUSION

In summary, the current study demonstrated miR-505 was considered as a tumor suppressor by negatively regulating HMGB1 to suppress the cell proliferation in RCC, thereby serving as a novel therapeutic target for the clinical treatment of RCC.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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