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ORIGINAL ARTICLE

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miR-33a inhibits cell growth in renal cancer by downregulation of MDM4 expression

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Abstract

Background: *MicroRNA-33a* (*miR-33a*) plays the role of the tumor suppressor gene by regulating the expression level of downstream genes. However, the effects of *miR-33a* in renal cell cancer (RCC) remain unknown. Our study was designed to investigate the expression level and potential function of *miR-33a* in RCC.

Methods: RT-qPCR was applied to measure the levels of *miR-33a* in RCC tissues and cell lines. Western blotting and luciferase reporter assay were used to detect the relationship between *miR-33a* and Mouse double minute 4 (*MDM4)* in RCC cells. CCK-8 and flow cytometry were applied to detected cell viability and cell cycle. Animal models and TUNEL assay were applied to detect the effect of *miR-33a* on the growth of RCC and cell apoptosis.

Results: We found that the levels of *miR-33a* were significantly decreased in RCC tissues and cell lines. Moreover, the low expression of *miR-33a* in RCC patients indicated a shorter overall survival (OS). Notably, *MDM4* as a direct target of *miR-33a* in RCC, the expression level of *MDM4* was significantly increased in RCC cells group than the control group. Furthermore, *miR-33a* overexpression significantly inhibited RCC cells growth than the control group, while the inhibitory effects of *miR-33a* were reversed upon the overexpression of *MDM4*. Luciferase reporter assays showed that there was a direct interaction between *miR-33a* and 3′ UTR of *MDM4* mRNA. In vivo, tumor volumes and weight were significantly decreased in the transfected *miR-33a* mimics group than the control group.

Conclusion: Taken together, our study indicates that *miR-33a* inhibits RCC cell growth by targeting *MDM4*.

KEYWORDS

cell proliferation, *MDM4*, *MiR-33a*, renal cell cancer

1 | INTRODUCTION

Renal cell cancer (RCC) remains one of the most common urogenital tumors in men and women (Chen et al., 2016; Siegel, Miller, Miller, & Jemal, 2017), and it accounts for more than 3% of adult malignancies (Chen et al., 2016). Although the curative methods are surgical tumor or radical resection for renal cell carcinoma, more than 30% patients with localized RCC develop metastasis and recurrence after surgical resection (Akhavan et al., 2015; Li et., 2016;

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Pantuck, Zisman, & Belldegrun, 2001). Therefore, better tumor biomarkers and therapeutic targets for diagnosis and treatment of RCC are necessary.

MicroRNAs (miRNAs) are comprised of 22 nucleotides and abundant family of evolutionarily conserved small, non-coding, and endogenous RNAs, which are closely associated with processes of cell biology (Bartel, 2009; Du et al., 2015; Li, Wu, et al., 2016; Wei, Wang, Wang, Ye, & Chen, 2016). Recently, a large number of studies found that abnormal miRNAs expression is closely related to tumor pathogenesis and they are promising therapeutic targets or biomarkers for cancers (Bartel, 2009; Dallavalle et al., 2016; Du et al., 2015; Henrique & Jeronimo, 2017; Li, Wu, et al., 2016; Wei et al., 2016).

miR-33a has been defined as a tumor suppressor miRNA in a variety of cancers including prostate cancer, gallbladder cancer, melanoma, osteosarcoma, and breast cancer through directly targeting downstream genes (Huang et al., 2018; Kang, Li, Li, Zhao, He, & Shi, 2018; Karatas et al., 2017; Li et al., 2018; Zhang et al., 2015, 2016; Zhou et al., 2015). However, the clinical significance and the effect of *miR-33a* in RCC remain poorly understood.

The *P53* (OMIM 191,170), a tumor suppressor, plays a pivotal role in a variety of physiological processes (AJ, 1997; Vogelstein, 2000). Inactivation of *P53* function is critical to tumorigenesis, progression, and metastasis. Mouse double minute 4(*MDM4*) protein (OMIM 602,704) can inhibit *P53* transcriptional activity directly as *MDM4* contains a *P53* binding domain in a variety of malignancies (Gansmo et al., 2015; Li & Lozano, 2013; Marine & Jochemsen, 2016). The activity of *P53* is reduced by *MDM4* overexpression, which contributes to tumorigenesis (Gansmo et al., 2015; Jeffreena Miranda et al., 2017). Furthermore, *MDM4* is overexpressed in prostate cancer, gastric cancer, hematologic malignancies, lung cancer, etc (Bao, Song, Xu, Qu, & Xue, 2016; Cao, Xu, & Li, 2015; Gansmo et al., 2015; Marine & Jochemsen, 2016; Miranda et al., 2017; Xiong et al., 2016; Xu et al., 2016). Those studies demonstrated that *MDM4* might be closely involved in tumorigenesis. However, the role of *MDM4* in RCC remains unclear. Moreover, *miR-33a* as a suppressor gene downregulating the expression of *MDM4* in RCC has not yet been reported. Therefore, we explored the clinical significance of *miR-33a* and its roles in the pathogenesis of RCC, and implored it is as a promising biomarker and therapeutic target for RCC.

2 | MATERIALS AND METHODS

2.1 | Human tissue samples

The study protocol was approved by the Local Ethics Committees of Guizhou Provincial People's Hospital, and all the patients were approved and signed the written informed consent. The samples of patient-matched RCC and control (adjacent normal renal tissues) were obtained from patients who underwent laparoscopic or open nephrectomy at Guizhou Provincial People's Hospital between 2007 and 2011. Thirty cases of RCC tissues were identified as renal clear cell cancer by histopathological analysis. Those patients with a history of any other types of tumors or received chemo- or radiotherapy before surgery were excluded. All tissues were stored at −80℃ after obtaining.

2.2 | Cell culture

Normal primary renal tubular HK-2 cell lines and RCC cell lines (Caki-1, ACHN and 786-O) were purchased from China Center For Type Culture Collection (Wuhan, China). HK-2 cells were cultured in RPMI 1640 medium (Gibco, USA) and RCC cells were cultured in DMEM medium (Gibco, USA) with 10% fetal calf serum (FCS, Gibco) in an atmosphere of 5% CO_2 at 37ºC in cell humidified incubator.

2.3 | Cell transfection

miR-33a inhibitor, mimics, and negative control of *miR-33a* (inhibitor- and mimics-NC) were purchased from RiboBio (Guangzhou, China). *MDM4* siRNAs and nontargeting siRNA were purchased from GenePharma (Shanghai, China). In brief, 50nM of *miR-33a* mimics and 100nM of *miR-33a* inhibitor were transfected into the 786-O, ACHN, and Caki-1 RCC cells by Lipofectamine 3000 (Invitrogen, China) following the manufacturer's protocol in 6-well cell plate. Hundred nanomolar of MDM4 siRNA was used to knockdown of endogenous MDM4 following the manufacturer's protocol.

2.4 | Real-time quantitative RT-PCR (qPCR)

TRIzol reagents (Invitrogen, USA) were used to extract the total RNA following the manufacturer's protocol. cDNAs were compounded by the PrimeScript RT reagent kit (Takara). Next, qPCRs were conducted using 7,500 Real-Time PCR System (Applied Biosystems) with SYBR Green PCR Master mix (Applied Biosystems, USA) for mRNA analysis, GAPDH was employed to normalize the expression of *MDM4*. qPCRs were conducted using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, USA) for miRNA analysis, U6 was employed to normalize the expression of *miR-33a*. The primer sequences are shown below:

miR-33a (F: 5′-GGTGCATTGTAGTTGCATTGC-3′, R: 5′-GTGCAGGGTCCG AGGTATTC-3′); U6 (5′-GCGCGT CGTGAAGCGTTC-3′,R:5′-GTGCAGGGTCCGAGGT-3′); *MDM4* (F: 5′-CTCAGTGTCAACATCTGACAG-3′, R: 5′-CATATGCTG CTCCTGCTGATC-3′); GAPDH

(F:5′-ATGGGGAAGGTGAAGGTCG-3′, R: 5′-GGGGTCA TT GATGGCAACAATA-3′).

2.5 | Western blotting

RIPA buffer was used to lysing RCC cells to extract total protein. The Bradford assay (Bio-Rad, USA) was applied to measure the protein concentration of each samples. Next, 10% SDS-PAGE gel was used to separate the protein, and electrotransferred to ECL nitrocellulose membranes, and BSA with 0.1% Tris-buffered saline-Tween 20 (TBST) was used to block the membranes for 2 hr. TBST was used to wash the membranes of each samples and incubated overnight with *MDM4* primary antibody (1:2000 dilution) and GAPDH primary antibody (1:3,000 dilution). Subsequently incubated with matched secondary antibodies (1:3,000 dilution) for 2 hr at room temperate after the membranes were washed with TBST. Finally, ECL western blot analysis substrate was used to quantify the results.

2.6 | Luciferase reporter assay

The bioinformatics algorithms from Targetscan and miRwalk were used. Wild type 3′-UTR of *MDM4* and mutant controls were constructed and inserted into the psiCheck2 Luciferase vector (Promega, USA). Next, the *MDM4*-mutant or *MDM4*-wild type and *miR-33a* mimics were co-transduced into cells by lipofectamine 3,000. After 48 hr, luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega Corporation Madison, USA) based on the manufacturer's protocol.

2.7 | CCK-8 assay

Caki-1 and 786-O cells were cultured in 96-well cell plates with 1×10^5 cells/well density for 24 hr after transfection. CCK-8 assays were used to measure the effects of *miR-33a* and *MDM4* on RCC cells growth based on the manufacturer's protocol. The results were performed from three independent experiments with triplicate.

2.8 | Flow cytometry

Cells were harvested and fixed by 70% alcohol, and then stored for 2 hr at 4℃. Then the cells were washed by PBS and according to the instructions exposed to PI (50mg/ml, BD Pharmingen, San Jose, CA) 1 hr and avoid light. Last we quantified the distribution of the cells through FCM (ModFit software).

2.9 | Experimental mouse model

Six-week old nude mice (BALB/c) were inoculated subcutaneously with 5×10^6 Caki-1 cells transfected with or without miR- mimic or miR- inhibitor. The tumors were measured once a week with microcalipers and the tumors were collected and weighed at the end of the experiment, and then tumors were fixed in 4% of paraformaldehyde for further analyses. The tumor volume was calculated through the following formula: Tumor volumes (mm^3) = 1/2 × (length × width^2).

2.10 | Terminal deoxynucleotidyltransferase dUTP nick end labeling assay

According to the protocol of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, cell apoptosis was detected in tumor tissues and staining was performed. Apoptosis was evaluated by counting the positive cells as well as the total number of cells at five random fields.

2.11 | Statistical analysis

The software of Statistical Package for the Social Sciences Version 16 was used to statistical analysis. All data were calculated as means values ± *SD* (standard deviations). The Student *t*-test was utilized to analyze the results between treated and control groups. The value of $P < 0.05$ was considered to have statistical significance.

3 | RESULTS

3.1 | *miR-33a* is decreased in RCC clinical tissues and cells

In this study, the level of *miR-33a* was detected by RT-qPCR in 30 pairs of RCC specimens and adjacent normal renal tissues (Figure 1a). We found that the level of *miR-33a* was obviously decreased in the RCC tissues group than the adjacent normal tissue group. Moreover, compared with HK-2 cell line, the level of *miR-33a* was also significantly reduced in the RCC cells (Caki-1, ACHN, and 786-O) (Figure 1b).

The relationship between patient prognosis and the level of *miR-33a* was analyzed. Patients with RCC were divided into low (n = 15) and high (n = 15) *miR-33a* expression groups following the median value of expression levels of *miR-33a* among RCC specimens, Kaplan–Meier curve was plotted for overall survival (OS). Statistical analysis showed that RCC patients with higher expression level of *miR-33a* had significantly longer OS than patients with lower expression level of *miR-33a* ($p = 0.0178$) (Figure 1c).

3.2 | The effect of *miR-33a* on cell growth in RCC cell

When transfected *miR-33a* inhibitor and mimics into Caki-1 and 786-O cells, the results showed high-transfection

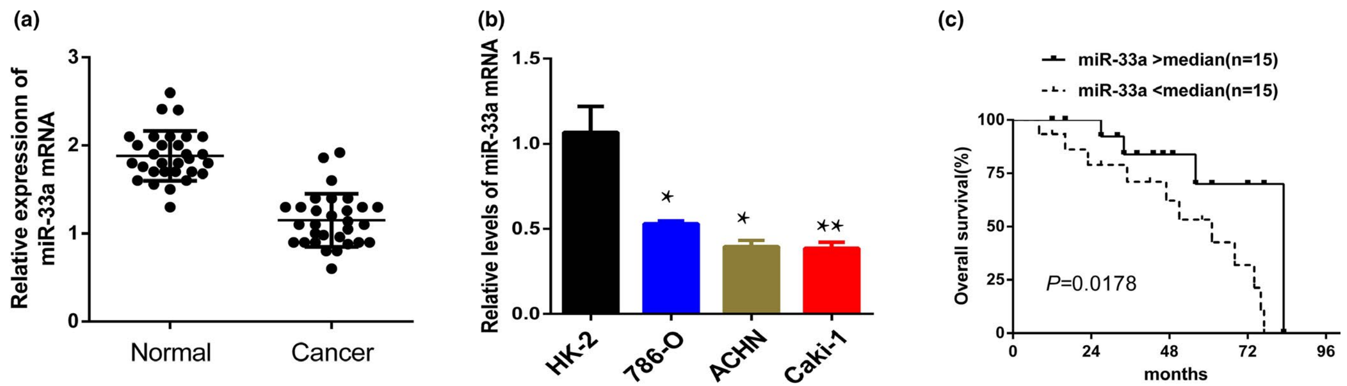

FIGURE 1 Expression of *miR-33a* in renal cell cancer (RCC) tissues and cell lines. (A, B) RT-qPCR analysis of *miR-33a* expression in RCC tissues and cell lines. *$p < 0.05$, **$p < 0.01$ versus adjacent normal renal tissues or HK-2 cells; (C) Kaplan–Meier analysis of overall survival in 30 RCC patients with low median ($n = 15$) and high median ($n = 15$) expression levels of *miR-33a*. **$p < 0.01$ versus HK-2 cell

efficiency (Figure 2a). The RCC cell viability was measured by CCK-8 assay, and the results indicated that *miR-33a* mimics significantly inhibited the proliferation rate of RCC cells (Caki-1 and 786-O) compared with the control group (Figure 2b; $p < 0.05$).

And then, FCM results showed that the *miR-33a* mimics significantly increased the percentages of cells in the G0/G1 phase, which showed that the overexpression of *miR-33a* could suppress RCC cells proliferation while *miR-33a* inhibitor could promote RCC cell proliferation (Figure 2c and d).

3.3 | *MDM4* is a direct target of *miR-33a* in RCC cells

Bioinformatic algorithms were employed for target prediction, including miRsystem, miRanda, miRBase, and TargetScan which predicted the potential targets of *miR-33a.* The results revealed that *MDM4* was the potential direct target of *miR-33a* (Figure 3a). Dual-Luciferase reporter system indicated that *miR-33a* mimics notably reduced the luciferase activity of *MDM4*-wt, while *miR-33a* failed to repress the expression of luciferase containing a mutant binding site (Figure 3b and c). The above results indicated that *miR-33a* negatively regulated the expression level of *MDM4* via directly targeting its 3′ UTR regions. Moreover, *miR-33a* overexpression resulted in decreased expression levels of *MDM4* protein, while *miR-33a* knockdown increased the expression of *MDM4* protein in Caki-1 cells (Figure 3d and e).

siRNA-targeting *MDM4* was compounded and transfected into RCC Caki-1 cells. In our study, the results indicated that siRNA-*MDM4* transfection downregulated the protein expression level of *MDM4* (Figure 3f). Moreover, *miR-33a* inhibitor transfection increased *MDM4* protein expression, whereas *MDM4* siRNA transfection partially reversed the effect of *miR-33a* inhibitor on the expression of *MDM4* protein (Figure 3f).

3.4 | The effect of *MDM4* on cell growth in RCC

RT-qPCR results indicated that the expression level of *MDM4* was significantly upregulated in RCC cells (Caki-1, ACHN, and 786-O) compared to that in HK-2 cells (Figure 4a). Besides, western blot analysis also further corroborated the observation in RT-qPCR results (Figure 4b and c). The relationship between OS and the level of *MDM4* was analyzed. Patients with RCC were divided into low ($n = 15$) and high ($n = 15$) *MDM4* expression groups following the median value of expression levels of *MDM4* among RCC specimens, Kaplan–Meier curve was plotted for OS. Statistical analysis showed that patients with a higher expression level of *MDM4* had significantly shorter OS than patients with lower expression level of *MDM4* ($p = 0.0372$) (Figure 4d).

CCK-8 assay showed that *miR-33a* inhibitor significantly promotes the proliferation rate of RCC cells (Caki-1) compared with the control group (Figure 4e; $p < 0.05$), whereas si-*MDM4* transfection can partially reverse this effect in Caki-1 cells (Figure 4e).Western blot detected the status of *p53* after the transfection of *miR-33a* mimics and -inhibitor, and the results showed that *miR-33a* mimics transfection upregulated the expression of *p53* and *miR-33a* inhibitor inhibited the expression of *p53* in Caki-1 cells (Figure 4f).

3.5 | mi-33a inhibits renal cancer growth in vivo

In vivo, nude mice were subcutaneously injected with RCC Caki-1 cells or cells transfected with *miR-33a* mimics or inhibitor to form RCC. We found that tumor volumes in the *miR-33a* inhibitor group were significantly larger than the control group (Figure 5a), and *miR-33a* mimics could partially inhibit the growth of RCC (Figure 4a). Similarly, *miR-33a* mimics or inhibitor had the similar effect on tumor weight

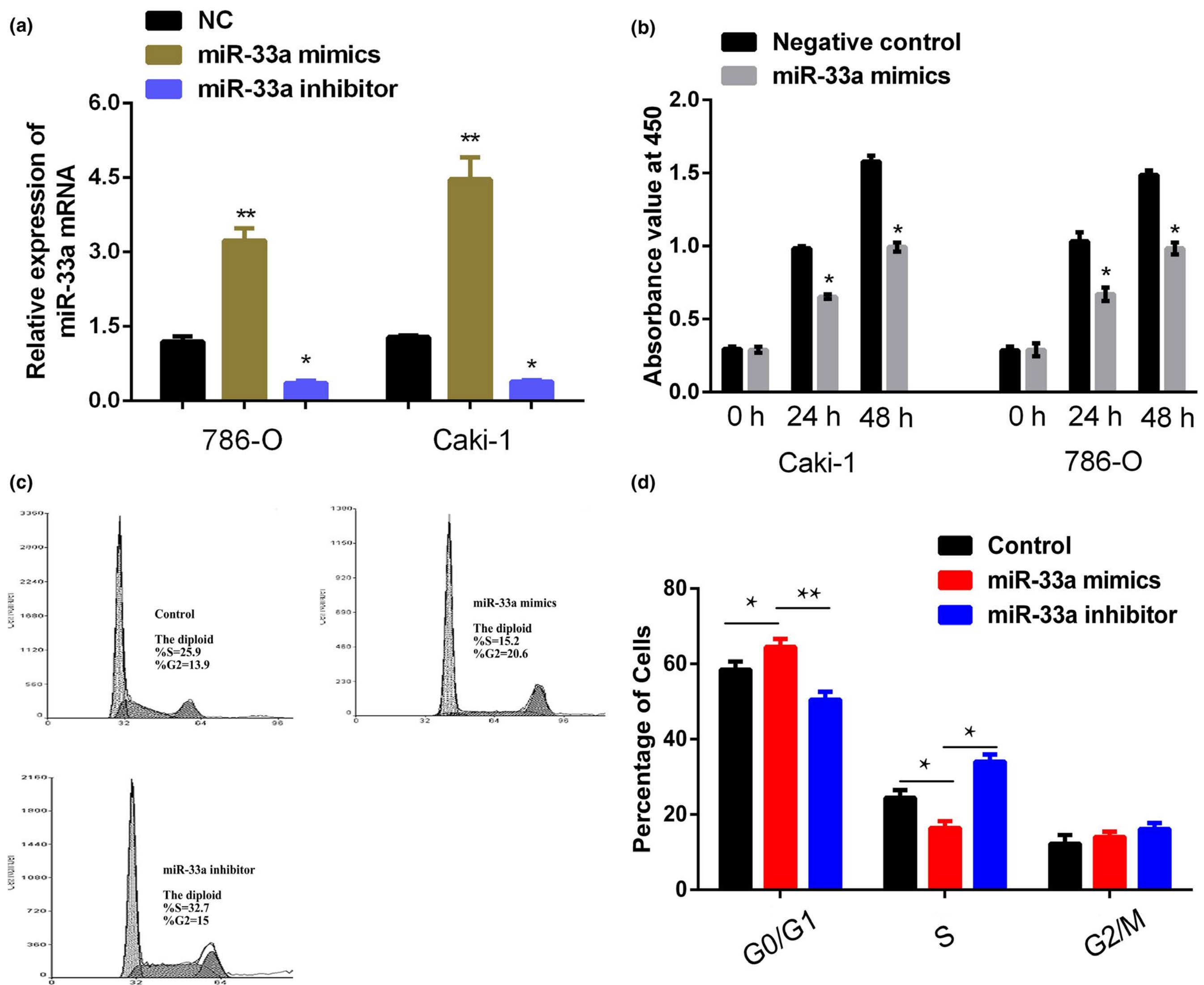

FIGURE 2 Inhibitory effects of *miR-33a* on cell proliferation and cell cycle in renal cell cancer (RCC) cell lines. (a) Expression of *miR-33a* in Caki-1 and 786-O cells after transfection with *miR-33a* mimics and *miR-33a* inhibitor or NC. NC represents negative control of miRNA. Results were expressed as of three independent experiments, with at least three replicates in each independent experiment, $^{**}p < 0.01$ versus NC. (b) CCK-8 assays indicated that the effects of *miR-33a* on the growth of RCC cell lines. Results were expressed as of three independent experiments, with at least three replicates in each independent experiment, $^{*}p < 0.05$ versus NC. (c, d) The results of flow cytometry showed that upregulation of *miR-33a* significantly increased the percentages of cells in the G0/G1 phase, which showed that the upregulation of *miR-33a* could suppress RCC cells proliferation. $^{*}p < 0.05$ versus Control

(Figure 5b and c). Furthermore, we found that cells apoptosis induced by *miR-33a* mimics were more than the control group (Figure 5d). In summary, the above results showed that *miR-33a* mimic could inhibit RCC growth in vivo.

4 | DISCUSSION

It is well known that miRNAs play a pivotal role in all kinds of processes of cell biology (Bartel, 2009; Ratert et al., 2013; Trujillo, Yue, Yue, Tang, O'Gorman, & Chen, 2010). Many researchers have found that *miR-33a* plays the role of anticancer in various human cancers, and *miR-33a* high expression could impede the cancer cell proliferation, invasion, migration, and regulate the chemoresistance in vivo (Chang et al., 2017; Du et al., 2017; Huang et al., 2018; Kang et al., 2018; Karatas et al., 2017; Li et al., 2018; Tian, Wei, Wei, Tian, Qiu, & Xu, 2016). In our study, we found that the level of *miR-33a* was remarkably reduced in RCC samples and cells than the adjacent normal renal tissues and HK-2 cells, respectively. Similarly, our study also showed that *miR-33a* overexpression impeded the cell growth of RCC, while *miR-33a* lower expression had an opposite effect. All the above results demonstrate that

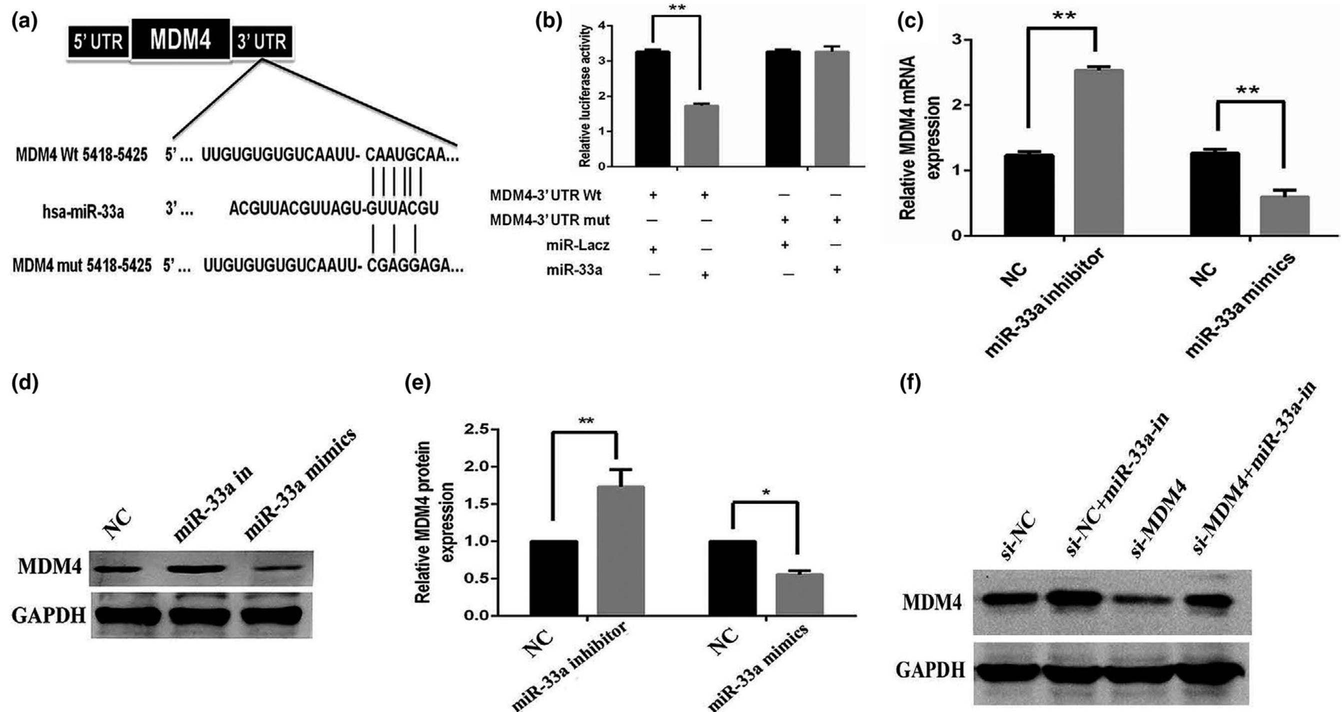

FIGURE 3 *miR-33a* directly targets Mouse double minute 4 (MDM4). (a) The predicted *miR-33a* binding site within *MDM4* 3′ UTR and *miR-33a* mutated version by site mutagenesis; (b) Caki-1 cells were transfected with reporter constructs containing either wild type (WT) *MDM4*, or *MDM*4 3′ UTR with mutation (MUT), along with *miR-33a* mimics, or negative control, respectively. Relative luciferase activity was measured. (c) qPCR analysis of *MDM4* expression in renal cell cancer (RCC) Caki-1 cells after overexpression or knockdown of *miR-33a*. (d, e) Western blotting analysis of *MDM4* protein expression in RCC Caki-1 cells after overexpression or knockdown of *miR-33a*. (f) Western blot analysis revealed that transfection of *MDM4* siRNA into Caki-1 cells resulted in decreased *MDM4* expression compared to the cells transfected with scrambled siRNA. These effects of siRNA were attenuated by anti-*miR-33a* inhibitor transfection. NC represents normal control, *$p < 0.05$, **$p < 0.01$ versus NC

miR-33a downregulation might be involved in the etiology and pathogenesis of RCC.

MDM4 can inhibit the function of *P53* during a number of types of cancer for *MDM4* shares an N-terminal *p53*-binding domain with *MDM2* (OMIM 164,785) (Gansmo et al., 2015; Xu et al., 2016). The overexpression of *MDM4* in a variety of tumors may be related to inhibition of the function of *P53* and result in tumorigenesis, tumor progression, and metastasis (Bao et al., 2016; Cao et al., 2015; Marine & Jochemsen, 2016). Many studies reported the overexpression of *MDM4* in prostate cancer, gastric cancer, hematologic malignancies, etc (Gansmo et al., 2015; Marine & Jochemsen, 2016; Miranda et al., 2017; Wang et al., 2016). All those reports indicate that *MDM4* may be closely involved in tumorigenesis, tumor progression, and metastasis.

Dysregulation of miRNA was closely associated with kinds of cancers, and the microRNA's function was related to negatively regulate target gene expression for translational suppression or mRNA degradation (Bartel, 2009; Li et al., 2014; Sandbothe et al., 2017). Recently, many studies demonstrated that *miR-33a* is widely involved in tumorigenesis by directly regulating its targeted genes. Zhang et al demonstrated that *miR-33a* suppresses gallbladder cancer progression by targeted Twist (Zhang et al., 2016). Zhou et al demonstrated that *miR-33a* could inhibit cell proliferation, invasion and metastasis in melanoma by binding *HIF-1α* (OMIM 606,615) (Zhou et al., 2015). Kang et al indicated that *miR-33a* inhibits the growth of lung cancer cells by negatively regulating the expression of *CAND1* (OMIM 607,727; Kang et al., 2018). In addition, the experts found that *miR-33a*, as a negative regulator, inhibit cells growth, invasion, and metastasis in breast cancer (Zhang et al., 2015). In our investigation, Targetscan predicted that *MDM4* was the direct target of *miR-33a*, and then our results demonstrated that the *MDM4* 3′UTR was the direct target of *miR-33a* by luciferase assays. CCK-8 assays demonstrated that *miR-33a* mimics could impede RCC cell growth. Moreover, RT-qPCR and western blotting suggested that *miR-33a* overexpression remarkably reduced the expression level of *MDM4*. However, the expression of *MDM4* could partially rescue by *miR-33a* inhibitor, suggesting that *MDM4* is a direct target of *miR-33a* in RCC.

In conclusion, we demonstrated that *miR-33a* directly targeted the expression of *MDM4* in RCC cells. On the other hand, our study provided evidence that *miR-33a* overexpression could inhibit cells growth in RCC. All above results indicated

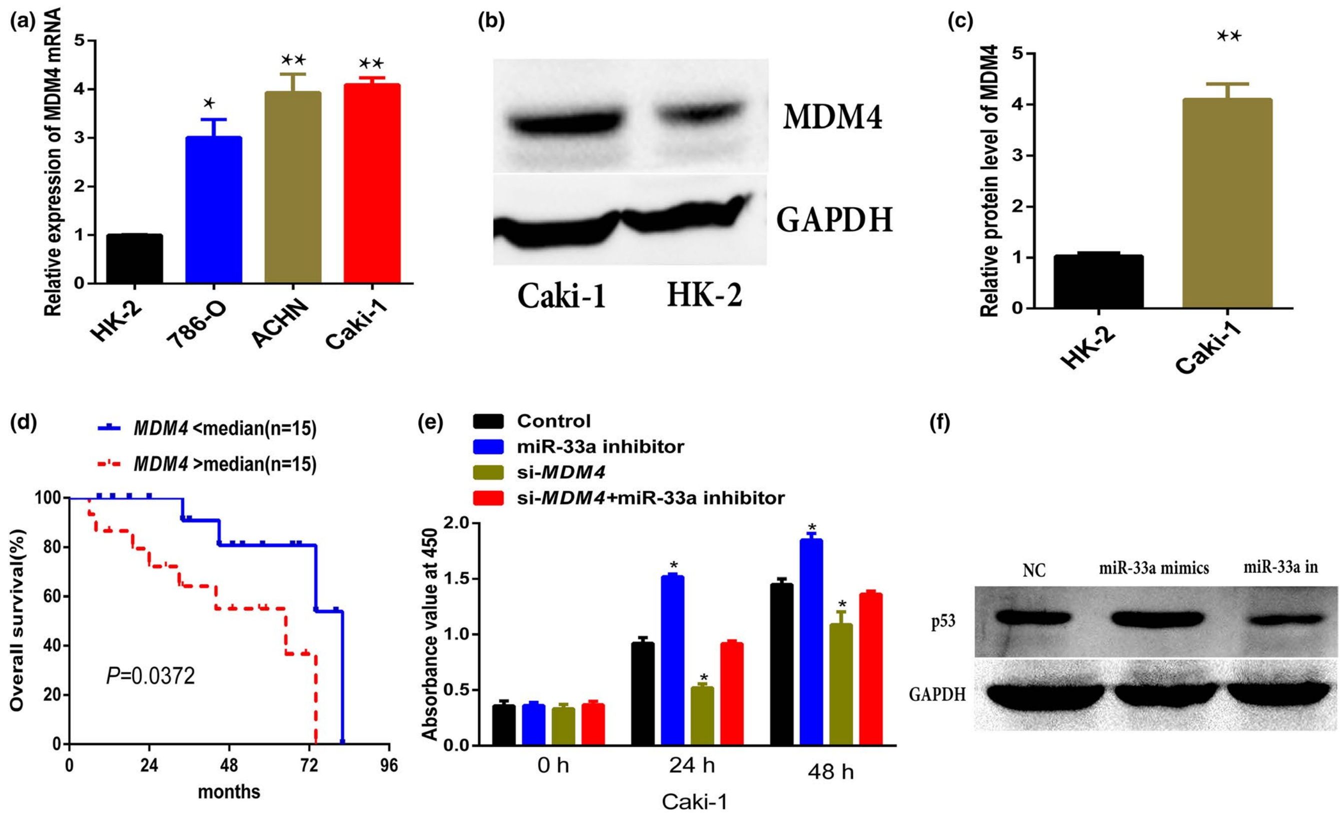

FIGURE 4 The effect of mouse double minute 4 (*MDM4*) on cell growth in renal cell cancer (RCC). (a, b, c) RT-qPCR and western blot analysis of *MDM4* expression in RCC cells. (d) Kaplan–Meier analysis of overall survival in 30 RCC patients with low median (n = 15) and high median (n = 15) expression levels of *MDM4*. (e) CCK-8 assays indicated that the effects of *MDM4* on growth of RCC cell lines. Results were expressed as $\pm\bar{x}s$ of three independent experiments, with at least three replicates in each independent experiment, *$p < 0.05$ versus NC. (f) Western blot analysis of *p53* expression after *miR-33a* mimics and -inhibitor transfection in RCC cells

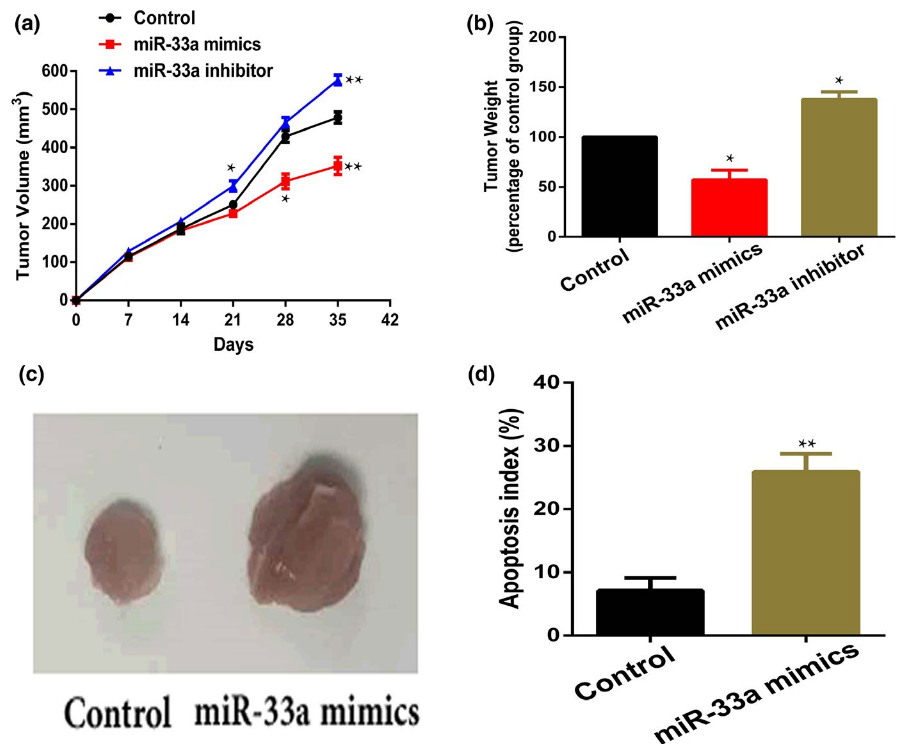

FIGURE 5 *miR-33a* mimics inhibited renal cell cancer tumor growth in vivo. (a, b) *miR-33a* mimics could suppress the tumor growth and reduced tumor weight compared with the control group, *$p < 0.05$, **$p < 0.01$ versus Control. (c) Images of tumor tissues from different groups on day 35. (d) TUNEL assay was performed to detect cell apoptosis in tumor tissues, **$p < 0.01$ versus Control

that *miR-33a* inhibits cell growth by downregulating *MDM4* in RCC. Taken together, *miR-33a* may be served as a potential target for the patients with RCC.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

JKH wrote the manuscript and operated the experiments; SF involved in study supervision and edited the manuscript; BY and LGH performed the molecular experiments; ZP and ZJG performed the animal experiments. All the authors reviewed the manuscript.

ETHICAL APPROVAL

The ethics committee approved the study and all patients were informed about the study and a signed written consent was obtained.

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