Mir-451 Correlates with Prognosis of Renal Cell Carcinoma Patients and Inhibits Cellular Proliferation of Renal Cell Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background:
Renal cell carcinoma (RCC) is a common cancer, accounting for about 2–3% of all adult cancers. A novel diagnostic biomarker and therapeutic target is urgently needed. However, the function of miR-451 in RCC remains unknown. Here, we explored the role of miR-451 in RCC.

Material/Methods:
MiR-451 levels in RCC tissues and cells were tested by qRT-PCR. Cells were transfected miR-451 mimics or miR-451 ASO by Lipofectamine. The cellular proliferation was tested by MTT analysis. The apoptosis rate was revealed by FACS. Bioinformatics algorithms from TargetScanHuman were used to predict the target genes of miR-451. The PSMB8 protein level was tested by Western blot. The interaction between miR-451 and PSMB8 was confirmed by dual luciferase assays.

Results:
MiR-451 level of RCC tissues was lower than in normal tissues, which was correlated to the patients' survival rate. Low levels of miR-451 in RCC cells promoted the growth of cells and inhibited cells apoptosis and vice versa. The targeted gene of miR-451 is PSMB8.

Conclusions:
MiR-451 acts as an anti-oncogene in RCC. Our data offer a new therapeutic target for further research.

MeSH Keywords:
Apoptosis • Carcinoma, Renal Cell • Cell Proliferation • MicroRNAs

Full-text PDF:
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Background

Renal cell carcinoma (RCC) accounts for 2–3% of all malignant tumor in adults, whose incidence ranks seventh in men and ninth in women [1]. The most common renal cancer histology is clear cell RCC (ccRCC), which accounts for 70–80% of all RCC, characterized by loss of 3p in >90% of cases [2,3]. Early-stage ccRCC is potentially curable by surgery, although approximately 6% of patients newly diagnosed with RCC present with metastases [4]. The prognosis for metastatic ccRCC remains poor, with a 5-year survival rate <10%, although molecular-targeted drugs are currently administered for the treatment of metastatic ccRCC. This calls for urgent research into therapeutic approaches for RCC.

MicroRNAs (miRNAs) are a group of small non-coding RNAs that negatively regulate gene expression through inhibiting translation of messenger RNA (mRNA) or targeting mRNAs for degradation [5]. Currently, more than 1000 miRNAs have been identified in human cells, and these regulate an estimated 30% of all gene expression [6]. Aberrant miRNA expression is involved in tumorigenesis of several cancers, including ccRCC [7].

The role of miR-451 in bladder cancer was initially reported in 2014 [8] in a study reporting a low level of miR-451 in bladder cancer tissues, and in which miR-451 was shown to be a tumor-suppressing factor via epithelial mesenchymal transition. Another study indicated that miR-451 showed a strong correlation with progression-free survival of RCC patients [9]. Unfortunately, the exact role of miR-451 in RCC and the underlying mechanism remain unclear.

In this study, the role of miR-451 in RCC was the main focus. The effect of miR-451 on cellular proliferation and cell apoptosis and the potential targeted genes of miR-451 were researched. Our hope was to provide a description of miR-451 in RCC and a potential therapeutic target for further investigation.

Material and Methods

Patients and cancer tissues

We postoperatively acquired 51 RCC tissues and the corresponding peripheral normal tissues in 2011 from the Third People's Hospital, Chengdu (Chengdu, China). All patients gave signed, informed consent for the use of their tissues for scientific research. Ethics approval for the study was obtained from the Ethics Committee/Institutional Review Board of Sichuan University (Chengdu, China). Diagnoses were made according to the pathological evidence by the senior pathologists of the Third People's Hospital, using the specimens in accordance with the classification criteria of the WHO. RCC tissues were

Supplementary Table 1. Characteristics of renal cell carcinoma patients.

| Variables	Values, mean ±SD
Gender	
Male	24
Female	27
Age at diagnosis	59.3±14.5
Tumor stage	
T1–T2	26
T3–T4	15
Cell type	
Clear cell	51
Chromophobe	0
Overall follow-up (days)	
Mean	1421
Median	1572
Range	342–2500

acquired prior to radiotherapy and chemotherapy, and were instantly stored at –80°C for subsequent analysis. All the RCC patients were followed for 36 months and a set of complete clinical data was recorded properly. The clinical information is provided in Supplementary Table 1.

Cell culture

Human renal caner cell lines ACHN, Caki-1, 786-O, A498, and HEK293T cells were purchased from the Cell Bank of Sichuan University (Chengdu, China). HEK293T was maintained in RPMI1640 with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA). ACHN, Caki-1, 786-O, and A498 were cultured in DMEM medium with 10% fetal bovine serum (Gibco BRL, Grand Island, NY).

MTT assay

Cells were seeded at a density of 5×10^3 cell per well in 96-well plates. After incubation for 48 h, 20 μl of 3,(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg/ml in PBS) was added to each well for 3 h at 37°C. Subsequently, a culture medium with MTT was removed and a 200 ll DMSO were added (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd, Shanghai, China). Absorbance was read at 490 nm using a Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). The data are expressed as percentage of growth relative to that of cells in the untreated group [10,11].
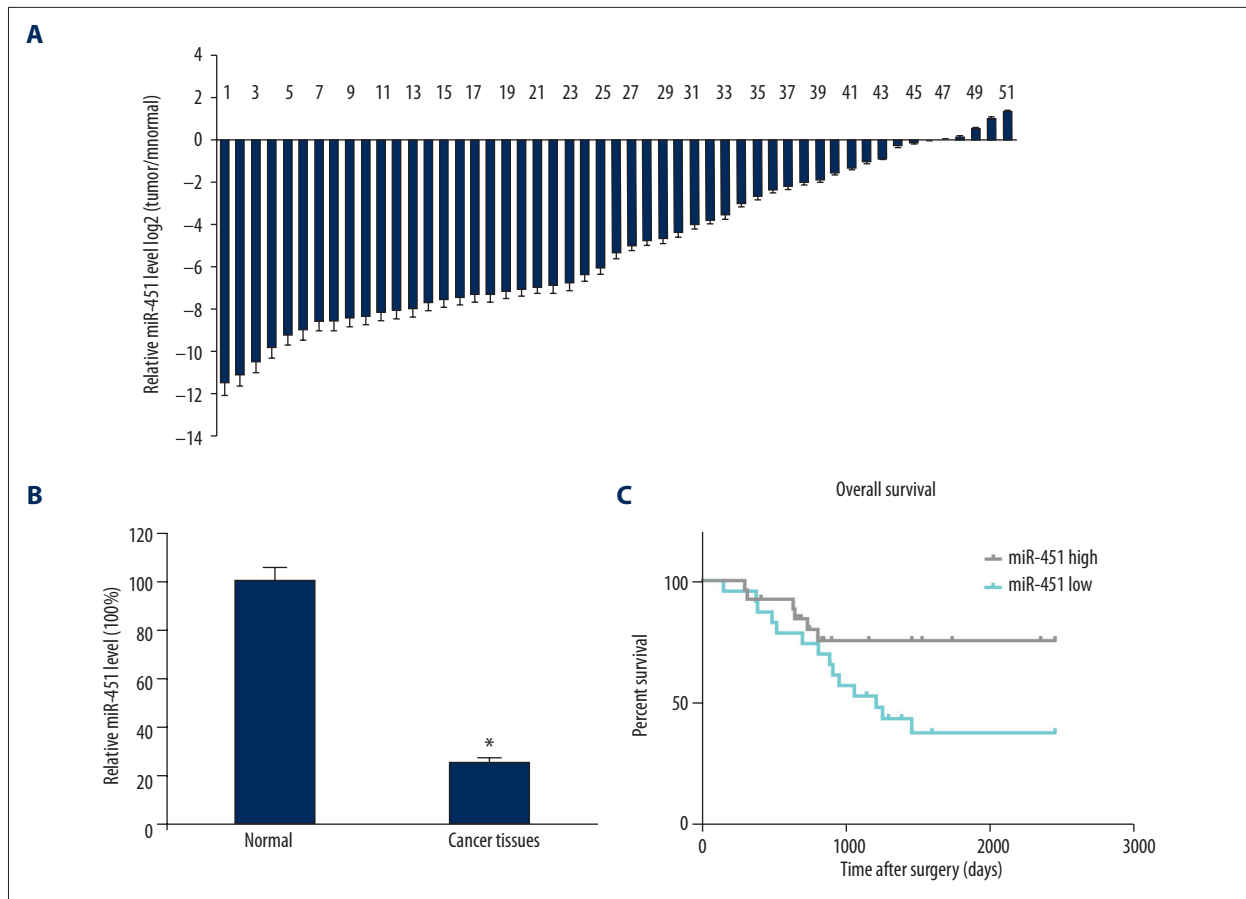
Figure 1. Low miR-451 level in RCC tissues was correlated with low survival rate of RCC patients. We collected 51 pairs of RCC tissues and the corresponding tumor-adjacent normal tissues from the Third People's Hospital. The miR-451 levels were examined by qRT-PCR (**A**). The mean expression of miR-451 in RCC tissues and tumor-adjacent normal tissues were compared. The mean level of miR-451 in tumor-adjacent normal tissues was arbitrarily defined as 100% (**B**). Kaplan-Meier plot of overall survival in RCC patients after the operation according to the expression of miR-451. The median value of all 51 cases was chosen as the cutoff point for separating miR-451 high-expression tumors ($n=25$) from miR-451 low-expression tumors ($n=25$) (**C**). Data are presented as mean ±s.d. of 3 separate experiments. * P<0.05.

MiRNAs quantification

RNA was extracted by Trizol reagent (Invitrogen, CA) as standard method [12]. TaqMan miRNA assays (Applied Bio- systems, CA) were used for semiquantitative determination of the expression of miRNAs according to the manufacturer's instructions. Briefly, 10 ng of total RNA was reverse transcribed using miRNA-specific stem-loop RT primers, MultiScribe reverse transcriptase, RT buffer, dNTPs, and RNase inhibitor (Applied Biosystems) in the GeneAmp 9700 PCR system (Applied Biosystems) under the following conditions: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Real-time PCR was performed on the resulting complementary DNA (cDNA) using miRNA-specific TaqMan primers and TaqMan Universal PCR Master Mix in a 7500 real-time PCR system (Applied Biosystems) with the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min [13]. The relative expression was normalized to the expression of U6 (Applied Biosystems). Relative fold changes of gene expression were calculated by the $^{\triangle\triangle}$CT method [14] and the values are expressed as $2^{-\triangle\triangle CT}$.

MiRNA transfection

MiR-451 mimics and miR-451 antisense oligonucleotides (miR-451 ASO) and the miRNA mimics negative control were purchased from Ambion (Ambion Life Technologies, Austin, TX). ACHN, Caki-1, 786-O, and A498 cells were seeded into 24-well plates and then transfected with miR-451 mimics or miR-451 ASO by Lipofectamine (Invitrogen, Shanghai, China), according to the manufacturer's instructions [15].
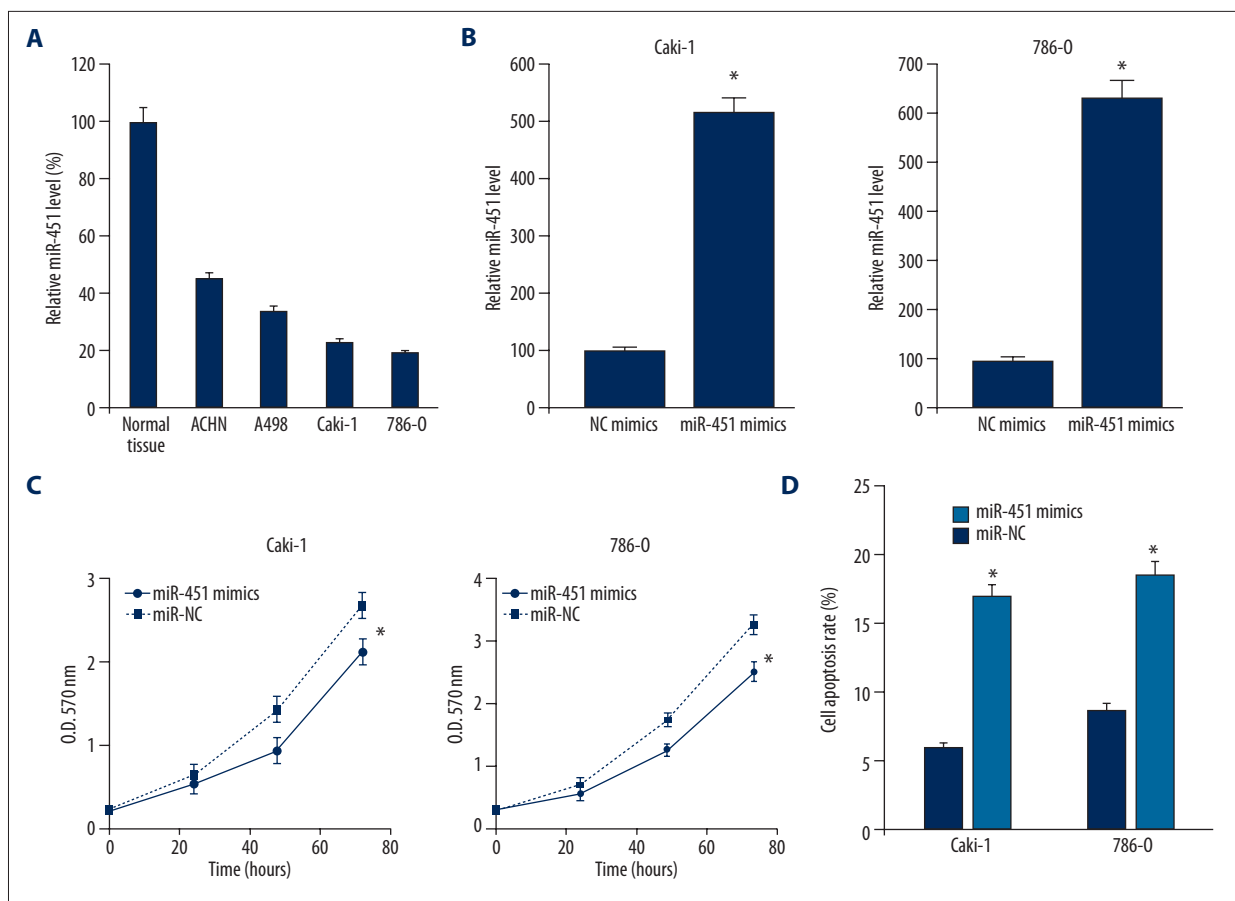
Figure 2. MiR-451 mimics transfection inhibited the cellular proliferation of Caki-1 and 786-O cells. The miR-451 levels in normal renal tissues, ACHN, A498, Caki-1, and 786-O cells were assayed by qRT-PCR. The miR-451 level in normal renal tissues was arbitrarily defined as 100% (**A**). At 48 h after miR-451 mimics transfection, the miR-451 levels in Caki-1 and 786-O cells were assayed by qRT-PCR. The miR-451 levels of cells transfected with miRNA-NC were arbitrarily defined as 100% (**B**). Following the miR-451 mimics transfection, the cellular proliferation of Caki-1 and 786-O cells was assayed by MTT analysis (**C**). At 48 h after transfection of miR-451 mimics, cell apoptosis was examined by FACS (**D**). Data are presented as mean±s.d. of 3 separate experiments. * P<0.05.

Apoptosis assay for flow cytometry

Cells were harvested and washed with PBS 3 times. The Fc receptor was blocked by 3% fetal bovine albumin. Cells were stained with FITC-labeled annexin-V antibody for 20 min. Before analyzing by FACS, PI was added.

Western blot

The proteins of cell were separated by electrophoresis and then were transfer to PVDF filter membranes. The PVDF membranes were then incubated with blocking buffer (TBST, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 50% skimmed milk) for 1 h at room temperature, followed by overnight treatment with the primary antibody at 4°C, and then with an HRP-conjugated secondary antibody. The reactive bands were detected by ECL after washing. Antibodies used in this experiment

were rabbit polyclonal anti-PSMB8 (sc-134503) and anti-β-actin from Santa Cruz Antibodies (Santa Cruz, CA).

MicroRNAs targets prediction

The online software TargetScanHuman [16–19] was used to predict the potential targeted genes of miR-451.

PSMB8 3'UTR reporter analysis

Luciferase assays were performed according to the manufacturer's protocol. We seeded 293T cells into 96-well plates at a density of 6×10⁴ per well. The mutations in the miR-451 seed regions of the PSMB8 3'UTR were generated using the QuikChange Multi site-directed mutagenesis kit (Promega, Fitchburg, WI). RL reporter plasmids (3.6 fmol) and pGL3-control (500 ng for normalization) (Promega, Fitchburg, WI) were
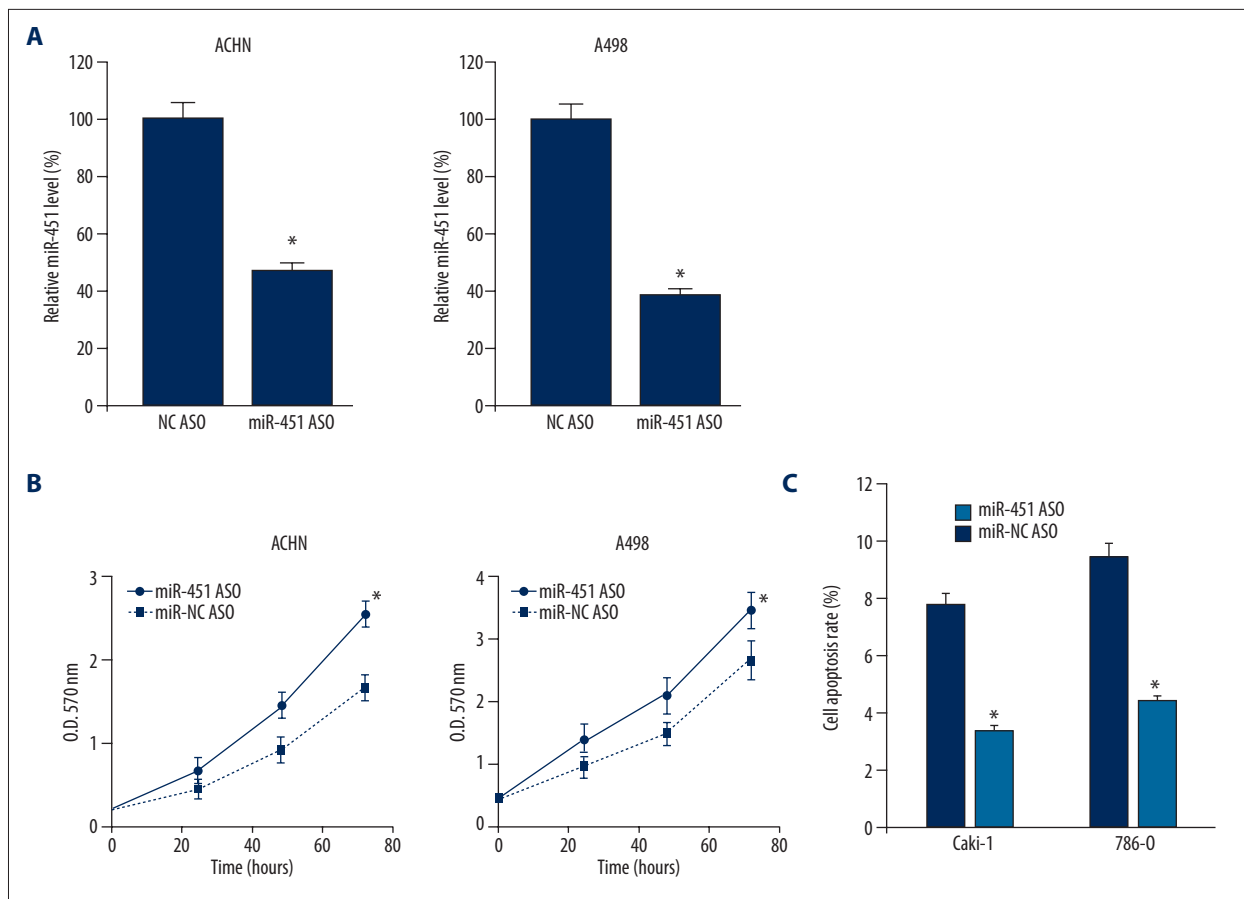
Figure 3. MiR-451 ASO transfection inhibited the cellular proliferation of ACHN and A498 cells. At 48 h after transfection of miR-451 ASO into ACHN and A498 cells, the miR-451 levels in the both cells were examined by qRT-PCR. The miR-451 levels of cells transfected with miRNA-NC were arbitrarily defined as 100% (**A**). Following the miR-451 ASO transfection, the cellular proliferation of ACHN and A498 cells was assayed by MTT analysis (**B**). At 48 h after miR-451 mimics transfection, cell apoptosis was measured by Annexin V-FITC/PI double-staining FACS analysis (**C**). Data are presented as mean ±s.d. of 3 separate experiments. * P<0.05.

transfected with Lipofectamine 2000 (Invitrogen, Canada) into cells. Cells were collected after 48 h for assay using the Dual Luciferase reporter assay system (Promega, Fitchburg, WI) [20,21].
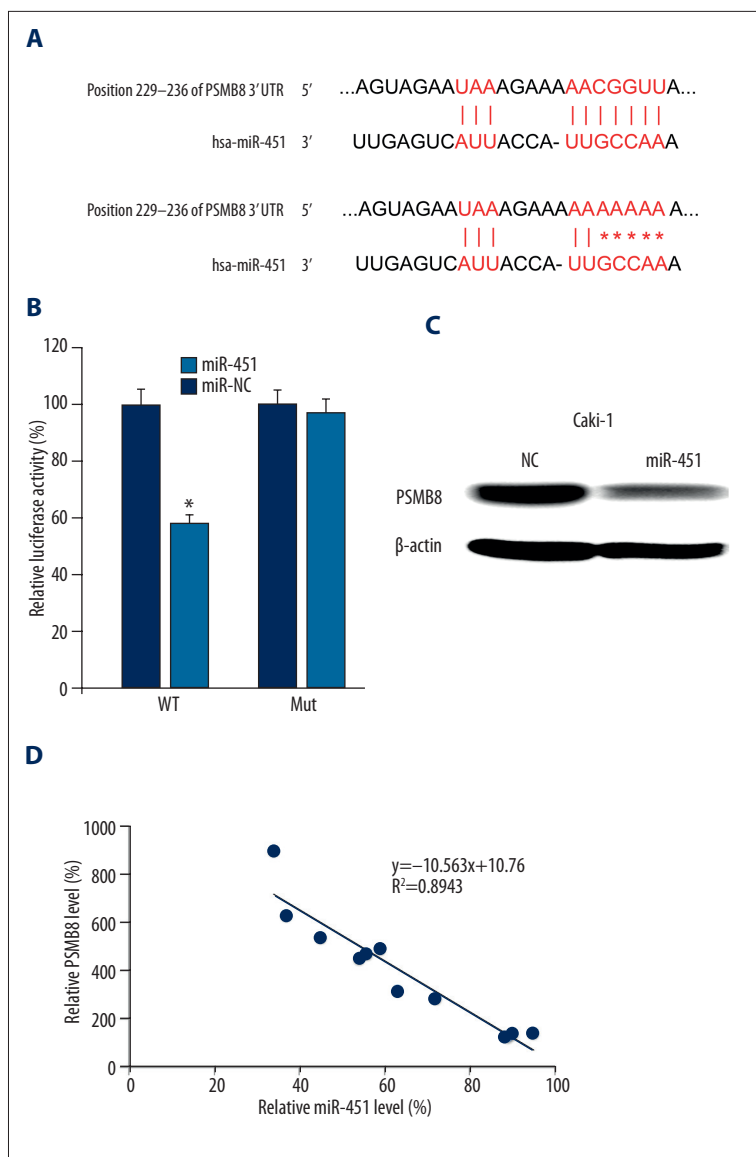
Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Chicago, IL). Overall survival of patients was estimated by the Kaplan-Meier method. The two-tailed Student's t test was used to analyze the difference between 2 groups and ANOVA was used to analyze the difference between 3 groups. The differences in expression of miR-451 between RCC tissues and corresponding normal tissues were tested by the Wilcoxon matched-pairs signed rank test. Correlation analysis was performed by two-tailed Person's correlation coefficient analysis. Differences were considered statistically significant when the p value was less than 0.05.

Results

Down-regulation of miR-451 was correlated with lower survival rate of RCC patients

To study the role of miR-451 in RCC, we initially tested the miR-451 expression in RCC tissues by qRT-PCR. The relative miR-451 level in RCC tissue and the corresponding tumor-adjacent normal tissues were compared. The research showed that in all 51 pairs, there were only 5 pairs with higher levels of miR-451 in RCC tissues (Figure 1A). Significantly, the miR-451 mean level of all RCC was lower than mean level in normal tissues (Figure 1B). By using Kaplan-Meyer analysis, we showed that the low expression of miR-451 was correlated with lower patient survival rate (Figure 1C).

A

Position 229–236 of PSMB8 3′UTR   5′   …AGUAGAAUAAAGAAAAACGGUUA…

                                                         | | | | | | | | | |

hsa-miR-451   3′   UUGAGUCAUUACCA- UUGCCAAA

Position 229–236 of PSMB8 3′UTR   5′   …AGUAGAAUAAAGAAAAAAAAAA A…

                                                         | | | | | * * * * *

hsa-miR-451   3′   UUGAGUCAUUACCA- UUGCCAAA

B

C

Caki-1

D

$y=-10.563x+10.76$
$R^2=0.8943$

Figure 4. PSMB8 was one of the targeted genes of miR-451. The putative targeted genes of miR-451 were analyzed by use of TargetScanHuman software, and the position of PSMB8 and mutated sequence were shown (**A**). The RL reporter plasmids (RL-PSMB8 and its mutated version) and miR-451 or miR-NC were co-transfected into Caki-1 cells, and a firefly luciferase control reporter was used for normalization. At 48 h following transfection, the luciferase activities were tested. Then the ratio of RL activity of firefly luciferase activity in the miR-451-treated group was calculated, with the luciferase activity of miR-NC group defined as 100% (**B**). At 48 h following miR-451 mimics transfection, Caki-1 cells were collected for PSMB8 protein assay by Western blot (**C**). *PSMB8* mRNA and miR-451 levels in 10 RCC tissues were examined by qRT-PCR. Then the correlation between *PSMB8* mRNA and miR-451 was analyzed by two-tailed Person's correlation coefficient analysis (**D**). All data are presented as the mean ±s.d. of 3 separate experiments. * $P<0.05$.

Up-regulation of miR-451 inhibited the growth of RCC cells and induced apoptosis of cells

Next, we examined the miR-451 levels in RCC cell lines by qRT-PCR, and we found that, compared with normal renal tissues, the miR-451 level was decreased in RCC cells (Figure 2A). Because miR-451 levels in Caki-1 and 786-O cells appeared to be lower than in other RCC cells, the Caki-1 and 786-O cells were transfected with miR-451 mimics. At 48 h using after transfection, the miR-451 levels in Caki-1 and 786-O cells were assayed by qRT-PCR. We found that miR-451 levels in Caki-1 and 786-O cells were overexpressed by miR-451 mimics transfection (Figure 2B). Subsequently, cell growth was examined by MTT analysis, showing that miR-451 mimics transfection suppressed the growth of Caki-1 and 786-O cells (Figure 2C). Next, the apoptosis rate of these cells were tested by FACS apoptosis

analysis, and data showed that up-regulation of miR-451 promoted cell apoptosis in Caki-1 and 786-O cells (Figure 2D).

Inhibition of miR-451 promoted the proliferation of RCC cells and inhibited cell apoptosis

ACHN and A498 cells showed higher levels of miR-451 than other cells; therefore, we down-regulated the miR-451 levels in ACHN and A498 cells by transfecting them with miR-451 ASO. The miR-451 levels were assayed by qRT-PCR (Figure 3A). Then the cells growth was assayed by MTT analysis and data showed that miR-451 ASO transfection favored the growth of ACHN and A498 cells (Figure 3B). Next, 48 h following the miR-451 ASO transfection, we examined the cell apoptosis by FACS analysis. These data showed that down-regulation of miR-451 decreased the cell apoptosis rate (Figure 3C).

The PSMB8 gene was targeted by miR-451

To research the targeted genes of miR-451, bioinformatics methods were used. We found that the *PSMB8* gene is one of the targeted genes of miR-451. The binding site and the mutated version are depicted in Figure 4A. The intact 3'UTR of *PSMB8* and its mutated version were cloned into luciferase reporter plasmids that were then used for co-transfection with miR-451 into 293T cells. We discovered that the transcripts carrying the PSMB8 3'UTR exhibited a significant reduction in luciferase activity in the presence of miR-451. In contrast, the control had no significant effect on the luciferase activity (P>0.05) (Figure 4B). At 48 h after miR-451 mimics transfection, the PSMB8 protein level in Caki-1 cells were assayed by Western blot, and we found that overexpression of miR-451 inhibited the PSMB8 protein level in Caki-1 cells (Figure 4C). Finally, the *PSMB8* mRNA levels and miR-451 levels in 9 RCC samples were assayed by qRT-PCR, and data showed that there is a negative correlation between *PSMB8* mRNA levels and miR-451 levels (Figure 4D).

Discussion

Here, we researched the function of miR-451 in RCC. We discovered that the RCC tissues showed a lower level of miR-451, and the low expression of miR-451 was correlated with lower patient survival rate. Low level of miR-451 in RCC cells may promote the growth of cells and vice versa. The targeted gene of miR-451 is *PSMB8*.

To the best of our best knowledge, this is the first report of miR-451 in RCC. MiR-451 has been investigated in a previous study [22] reporting that up-regulation of miR-451 suppressed the proliferation, invasion, and metastasis of A549 lung cancer cells. The inflammation-related gene *PSMB8* is a target for miR-451. Our data showed a similar role of miR-451 in RCC.

Interestingly, *PSMB8* has been proven to be involved in inflammatory disease. Research has shown that *PSMB8* influenced the intensity of NF-κB-mediated inflammation in inflammatory bowel disease [23,24] and caused auto-inflammatory disorder [25]. *PSMB8* knockout mice developed significantly attenuated colitis and had limited secretion of proinflammatory cytokines and chemokines [26]. Inhibition of PSMB8 can block cytokine production and attenuates progression of experimental arthritis [27].

Previous studies suggest that inflammation plays important roles in RCC [28], including a prognostic role [29]. Our data suggest that inflammation influences the development of cancer via PSMB8, which is regulated by miR-451.

MiR-451 also plays roles in inflammation-related diseases. For example, in rheumatoid arthritis, miR-451 suppresses neutrophil chemotaxis via p38 MAPK pathways [30]. Thus, we hypothesized that miR-451 may play a role in RCC via a pathway of inflammation.

Conclusions

Our data revealed the role of miR-451 in RCC. We found that the down-regulation of miR-451 was correlated with lower survival rate of RCC patients, and that overexpression of miR-451 inhibits the proliferation of RCC cells and vice versa. The targeted gene of miR-451 is PSMB8. We hope our study provides a new therapeutic target in RCC.

Disclosure statement

The authors have declared that no competing interests exist.

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