Impact of novel oncogenic pathways regulated by antitumor miR-451a in renal cell carcinoma

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Recent analyses of our microRNA (miRNA) expression signatures obtained from several types of cancer have provided novel information on their molecular pathology. In renal cell carcinoma (RCC), expression of microRNA-451a (miR-451a) was significantly downregulated in patient specimens and low expression of miR-451a was significantly associated with poor prognosis of RCC patients (P = .00305) based on data in The Cancer Genome Atlas. The aims of the present study were to investigate the antitumor roles of miR-451a and to identify novel oncogenic networks it regulated in RCC cells. Ectopic expression of miR-451a significantly inhibited cancer cell migration and invasion by RCC cell lines, suggesting that miR-451a had antitumor roles. To identify oncogenes regulated by miR-451a in RCC cells, we analyzed genome-wide gene expression data and examined information in in silico databases. A total of 16 oncogenes were found to be possible targets of miR-451a regulation. Interestingly, high expression of 9 genes (PMM2, CRELD2, CLEC2D, SPC25, BST2, EVL, TBX15, DPYSL3, and NAMPT) was significantly associated with poor prognosis. In this study, we focused on phosphomannomutase 2 (PMM2), which was the most strongly associated with prognosis. Overexpression of PMM2 was detected in clinical specimens and Spearman’s rank test indicated a negative correlation between the expression levels of miR-451a and PMM2 (P = .0409). Knockdown of PMM2 in RCC cells inhibited cancer cell migration and invasion, indicating overexpression of PMM2 could promote malignancy. Analytic strategies based on antitumor miRNAs is an effective tool for identification of novel pathways of cancer.

KEYWORDS
antitumor, microRNA, miR-451a, PMM2, renal cell carcinoma

1 INTRODUCTION

Renal cell carcinoma (RCC) is the most common form of kidney cancer and is diagnosed in more than 350,000 patients worldwide, making it the seventh most common site for tumors.1 Although patients with stage I RCC had a 5-years survival rate above 90%, those with advanced RCC had a 5-years survival rate of only 23%.2 In fact, 25%-30% of patients have metastasis at the time of diagnosis.3 Furthermore, distant metastasis and recurrence are found even when surgical resection is carried out for localized RCC, cases that are associated with poor prognosis. Recently, molecular targeted therapy and immunotherapy have been used for patients with metastatic or
recurrent RCC. However, the therapeutic benefits are limited, and RCC is generally insensitive to radiation and chemotherapy. Thus, development of novel therapeutic strategies is needed. We believe that novel genomic approaches are required to elucidate the underlying molecular mechanism of metastatic RCC.

MicroRNA (miRNA) is a single-stranded low molecular RNA of 19-22 bases that possesses important functions. For example, miRNA regulates the expression of target genes by inhibiting translation and/or accelerates the degradation of functional RNAs (protein coding/non-protein coding genes). In human cells, a single miRNA can regulate many different protein-coding or non-coding mRNAs and a single mRNA can be regulated by several different miRNAs. Thus, aberrant expression of miRNA could disrupt regulated RNA networks in cancer cells. Therefore, it is important to elucidate the aberrant expression of miRNAs in each type of cancer to better understand the molecular mechanism of cancer pathogenesis.

We previously identified antitumor miRNAs that regulated novel oncogenic pathways based on miRNA expression signatures. Downregulation of miR-451a was detected by our studies of miRNA signatures and several types of cancers. Moreover, a large cohort analysis using The Cancer Genome Atlas (TCGA) database showed that low expression of miR-451a was associated with poor survival of patients with RCC. The antitumor role of miR-451a in RCC has been reported; however, the molecular pathways regulated by miR-451a have not been fully elucidated. MicroRNA biogenesis is unique, in that a single miRNA can target a vast number of RNAs in cells. Continuous analyses of antitumor miRNA-regulated molecular pathways are essential for understanding RCC pathogenesis. Here, we aimed to investigate novel oncogenic pathways regulated by antitumor miR-451a in RCC cells and involving in RCC pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Patients, cancer tissue collection, cell lines, and cell culture

We postoperatively collected cancerous and normal tissues from 15 RCC patients at Chiba University Hospital (Chiba, Japan) between 2012 and 2015. Clinicopathological characteristics of the 15 patients are listed in Table 1. Tumor stages were determined by the General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma based on the AJCC-UICC TNM classification. All patients gave signed, informed consent for the use of the tissues for research purposes.

We used 2 human RCC cell lines (786-O and A498) obtained from ATCC (Manassas, VA, USA) as previously described. These cell lines were maintained in RPMI-1640 with 10% FBS (HyClone, Logan, UT, USA).

2.2 | Transfections with mature miRNA, siRNA, or plasmid vectors

We used the following mature miRNA species in these experiments: mature miRNA and pre-miR miRNA precursors (has-miR-451a; P/N: AM17100; Applied Biosystems, Foster City, CA, USA). The following siRNAs were used: Stealth Select RNAi siRNA, si-PMM2 (HSS108164 and HSS108165; Invitrogen, Carlsbad, CA, USA), and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). PMM2 plasmid vectors were designed and provided by OriGene (cat. no. RC203472; Rockville, MD, USA). MicroRNAs and siRNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax transfection reagent (Invitrogen), as previously described. Plasmid vectors were incubated with Opti-MEM and

| TABLE 1 | Clinical features of 15 patients with clear cell renal cell carcinoma |
|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Patient no. | Age, years | Gender | Grade | pT | INF | v | ly | eg or ig | fc | im | rc | rp | s |
| 1 | 71 | F | G2 | T1a | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 2 | 74 | M | G1 > G2 | T1a | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 3 | 59 | M | G3 > G2 | T1b | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 4 | 52 | M | G2 > G3 > G1 | T1a | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 5 | 64 | M | G2 > G3 | T1b | a | 0 | 0 | eg | 1 | 1 | 0 | 0 | 0 |
| 6 | 67 | M | G2 > G3 > G1 | T3a | b | 1 | 0 | ig | 0 | 1 | 1 | 0 | 0 |
| 7 | 67 | M | G2 > G3 > G1 | T3a | b | 1 | 0 | ig | 1 | 0 | 0 | 0 | 0 |
| 8 | 59 | M | G3 > G2 | T3a | b | 1 | 0 | ig | 0 | 0 | 0 | 0 | 0 |
| 9 | 73 | M | G1 > G3 | T2a | a | 0 | 1 | eg | 1 | 0 | 0 | 0 | 0 |
| 10 | 77 | M | G1 > G2 | T1b | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 11 | 77 | M | G2 > G1 | T3a | a | 1 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 12 | 51 | M | G2 > G1 | T1 | a | 0 | 0 | eg | 0 | 0 | 0 | 0 | 0 |
| 13 | 78 | M | G2 > G1 > G3 | T1b | b | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 14 | 57 | M | G1 > G2 | T1a | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 15 | 54 | M | G2 > G1 | T3a | a | 0 | 0 | eg | 0 | 0 | 1 | 0 | 0 |

eg, expansive growth; F, female; fc, capsular formation; ig, infiltrative growth; im, intrarenal metastasis; INF, infiltration; ly, lymph node; M, male; rc, renal capsule invasion; rp, pelvis invasion; s, sinus invasion; v, vein.
Lipofectamine 3000 reagent (Invitrogen) by forward transfection following the manufacturer’s protocol.

2.3 | Quantitative real-time RT-PCR

Total RNA was extracted from human tissues and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol, as we described previously. The procedure for PCR quantification has been outlined. Expression levels of miR-451a (assay ID:0001141; Applied Biosystems) were analyzed by TaqMan quantitative real-time RT-PCR (qRT-PCR) (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to the expression of RNU48 (assay ID:001006; Applied Biosystems). TaqMan probes and primers for PMM2 (P/N: Hs00756707_m1; Applied Biosystems), GAPDH (internal control; P/N: Hs02758991_m1; Applied Biosystems), and GUSB (internal control; P/N: Hs00939627_ml; Applied Biosystems) were assay-on-demand gene expression products. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.4 | Cell proliferation, migration, and invasion assays

Cell proliferation, migration, and invasion assays have been described.
2.5 | Identification of putative target genes regulated by miR-451a in RCC cells

To identify miR-451a target genes, we used in silico analyses and genome-wide gene expression analyses, as outlined previously.20-25 We used the TargetScanHuman 7.0 (August 2015 release) (http://www.targetscan.org/), TCGA, and OncoLnc datasets (http://www.oncolnc.org/) to select and narrow down putative miRNA target genes.27-29 An oligo microarray (Human Ge 60K; Agilent Technologies) was used for gene expression analysis. We deposited the microarray data into the Gene Expression Omnibus (GEO) database.

2.6 | Western blot analysis

Cells were collected 48 hours after transfection, and lysates were prepared. Immunoblotting was undertaken with rabbit anti-PMM2 antibodies (1:500 dilution, SAB2702078; Sigma-Aldrich, St. Louis, MO, USA). Anti-GAPDH antibodies (1:10 000, ab8245; Abcam) were used as an internal loading control. The procedures have been described.20-25

2.7 | Plasmid construction and dual luciferase reporter assays

The partial wild-type sequence of the PMM2 3’-UTR or that with deletion of the miR-451a putative target site was inserted between the SgfI-Pmel restriction sites in the 3’-UTR of the hRLuc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedures were covered in earlier reports.20-25

2.8 | Immunohistochemistry

We used a tissue microarray that was incubated overnight at 4°C with anti-PMM2 antibodies (1:100 dilution, SAB2702078; Sigma-Aldrich). The sliced slides were treated with biotinylated goat antibodies (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The procedures were described earlier.20-25

2.9 | Regulation of targets downstream of PMM2 in RCC

We analyzed PMM2-regulated pathways in RCC cells. We analyzed gene expression using si-PMM2-transfected 786-O cells. Microarray data were used for expression profiling of si-PMM2 transfectants. The microarray data were deposited into the GEO database (accession no. GSE107008).

2.10 | The Cancer Genome Atlas database analysis of RCC

To assess the clinical significance of miRNAs and their targeted genes, we used the RNA sequencing database in TCGA. The definition of high and low expression divided half of the clinical data population in the order of expression. Gene expression and clinical data were obtained from cBioportal and OncoLnc (data downloaded November 1, 2017).27-29

2.11 | Statistical analysis

Relationships between 2 groups and the numerical values obtained by qRT-PCR were analyzed by Mann-Whitney U-tests and paired t tests. Spearman’s rank test was used to analyze the correlation between the expression levels of miR-451a and PMM2. Relationships among more than 3 variables and numerical values were analyzed with Bonferroni-adjusted Mann-Whitney U-tests. Survival analysis was carried out using the Kaplan-Meier method, log-rank tests, and multivariable Cox hazard regression analyses with JMP software (version 13; SAS Institute, Cary, NC, USA). Other statistical analyses were carried out using Expert StatView (version 5; SAS Institute).

3 | RESULTS

3.1 | Expression levels of miR-451a in RCC clinical specimens and cell lines

Expression levels of miR-451a were significantly downregulated in RCC tissues compared with those in non-cancerous tissues (P = .0166; Figure 1A). Furthermore, expression levels of miR-451a in 786-O and A498 cells were markedly downregulated (Figure 1A).
| Gene symbol | Gene name                          | Conserved sites count | Poorly conserved sites count | GEO expression data fold-change (tumor/normal) | A498 miR-451a transfection (Log2 ratio) | 786-O miR-451a transfection (Log2 ratio) | Average A498/786-O miR-451a transfection (Log2 ratio) | Cytoband | TCGA data OS (P-value) |
|-------------|-----------------------------------|-----------------------|-----------------------------|-----------------------------------------------|----------------------------------------|-----------------------------------------|-----------------------------------------------|----------|------------------------|
| PMM2        | Phosphomannomutase 2              | 1                     | 0                           | 1.580                                         | -1.617                                 | -1.020                                  | -1.200                                         | hs16p13.2| .00000218              |
| CRELD2      | Cysteine-rich with EGF-like domains 2 | 0                     | 1                           | 1.656                                         | -1.724                                 | -2.256                                  | -1.990                                         | hs22q13.33| .000000831             |
| CLEC2D      | C-type lectin domain family 2, member D | 0                     | 1                           | 2.557                                         | -1.014                                 | -1.455                                  | -1.235                                         |           | .00009140              |
| SPC25       | SPC25, NDC80 kinetochore complex component | 0                     | 1                           | 3.213                                         | -2.497                                 | -2.321                                  | -2.409                                         |           | .00009800              |
| BST2        | Bone marrow stromal cell antigen 2 | 0                     | 1                           | 2.061                                         | -0.668                                 | -1.723                                  | -1.196                                         | hs19p13.11| .00015300              |
| EVL         | Enah/Vasp-like                     | 0                     | 1                           | 1.896                                         | -1.197                                 | -1.510                                  | -1.354                                         | hs14q32.2| .00125000              |
| TBX15       | T-box 15                           | 0                     | 1                           | 4.118                                         | -1.224                                 | -1.618                                  | -1.421                                         | hs1p12    | .00193000              |
| DPYSL3      | Dihydropyrimidinase-like 3        | 0                     | 1                           | 2.326                                         | -1.325                                 | -1.344                                  | -1.335                                         | hs5q32    | .00389000              |
| NAMPT       | Nicotinamide phosphoribosyltransferase | 0                     | 1                           | 2.174                                         | -2.369                                 | -0.534                                  | -1.452                                         | hs7q22.3  | .01380000              |
| MEGF6       | Multiple EGF-like-domains 6        | 0                     | 1                           | 2.112                                         | -0.917                                 | -1.181                                  | -1.364                                         | hs1p36.32| .15100000              |
| CRIP2       | Cysteine-rich protein 2            | 0                     | 1                           | 1.590                                         | -1.899                                 | -1.593                                  | -1.746                                         | hs14q32.33| .18800000              |
| CAV1        | Caveolin 1, caveola protein, 22kda | 0                     | 1                           | 6.729                                         | -1.196                                 | -2.144                                  | -1.670                                         | hs7q31.2  | .20100000              |
| PSMB8       | Proteasome (prosome, macropain) subunit, beta type, 8 | 1                     | 0                           | 2.682                                         | -1.502                                 | -1.906                                  | -1.704                                         | hs6p21.32| .25500000              |
| CDH11       | Cadherin 11, type 2, OB-cadherin (osteoblast) | 0                     | 1                           | 1.847                                         | -0.791                                 | -1.788                                  | -1.290                                         | hs16q21   | .42600000              |
| EGLN3       | Egl-9 family hypoxia-inducible factor 3 | 0                     | 1                           | 13.668                                        | -0.740                                 | -1.940                                  | -1.340                                         | hs14q13.1| .68800000              |
| SLC39A14    | Solute carrier family 39 (zinc transporter), member 14 | 0                     | 1                           | 2.057                                         | -1.391                                 | -1.386                                  | -1.389                                         | hs8p21.3  | .83600000              |

GEO, Gene Expression Omnibus; OS, overall survival; TCGA, The Cancer Genome Atlas.
In the human genome, miR-451a has formed a miRNA cluster with other miRNAs (miR-144-5p, miR-144-3p, miR-451b, and miR-4732) on human chromosome 17q11.2 region (Figure S1). We also checked expression of these miRNAs in RCC clinical specimens. Expression levels of miR-144-5p and miR-144-3p were significantly downregulated in RCC tissues. On the contrary, it was revealed that the expression levels of miR-451b and miR-4732 were extremely low in normal kidney and RCC tissues (Figure S1).

To investigate the molecular mechanisms of silencing of miR-451a in RCC cells, 786-O cells were treated with the demethylating agent 5-aza-2′-deoxycytidine. Expression of miR-451a was not dramatically elevated by 5-aza-2′-deoxycytidine treatment (Figure S1). A large cohort analysis (n = 506) using data from TCGA database showed that low expression of miR-451a was associated with poor survival of patients with RCC (P = .00305; Figure 1B).

### 3.2 Effects of ectopic expression of miR-451a in RCC cell lines

To investigate the antitumor functions of miR-451a in RCC cells, we applied to gain-of-function analyses in this study. Ectopic expression of miR-451a in RCC cell lines (786-O and A498) did not affect cell proliferation (Figure 1C). Cell migration and invasion activities were significantly inhibited in miR-451a transfectant cells compared with those in mock or miRNA-control transfectant cells (Figure 1D,E).

### 3.3 Screening of candidate targets by miR-451a regulation in RCC cells

We undertook in silico and gene expression analyses to identify genes targeted by miR-451a. The strategy for selection of miR-451a target genes is shown in Figure 2. Using the TargetScanHuman 7.0 database, we identified 541 genes that had putative target sites for miR-451a in their 3′-UTRs. Among these genes, we identified 64 that showed increased expression levels in RCC tissues (fold-change >1.5) using the database (GEO database accession number: GSE36895).

Next, we identified 16 genes that were downregulated after transfection of 786-O and A498 cells with miR-451a (average log2 ratio < −1.0; Table 2). Next, using the OncoLnc database, we investigated whether the expression levels of the 16 candidate genes affected the prognosis of RCC patients. Kaplan-Meier survival curves revealed that high expression levels of 9 of the genes were...
associated with poor prognosis in patients with RCC (Figure 3). Finally, we focused on PMM2 because it showed the most evident difference in OncoLnc prognostic analysis ($P = .000000218$; Table 2, Figure 3) and there are few reports on cancer studies.

### 3.4 PMM2 directly regulated by miR-451a in RCC cells

Expression levels of PMM2/PMM2 were reduced by miR-451a transfection at mRNA and protein levels (Figure 4A,B).

Furthermore, we carried out luciferase reporter assays to elucidate whether PMM2 mRNA had a functional target site for miR-451a. The TargetScan database predicted that miR-451a was bound at position 1127-1133 in the 3′-UTR of PMM2 (Figure 4C). We used vectors encoding a partial wild-type sequence of the 3′-UTR of PMM2 mRNA, including the predicted miR-451a target site, or a vector lacking the miR-451a target site. Luminescence intensity was significantly reduced by co-transfection with miR-451a and the vector carrying the wild-type 3′-UTR of PMM2. In contrast, luminescence intensity was not reduced when the target site of miR-451a was deleted from the vectors (Figure 4D).

### 3.5 Effects of PMM2 knockdown in RCC cell lines

To investigate the functional significance of PMM2, we carried out loss-of-function studies using transfection of si-PMM2 into 786-O and A498 cells. First, we evaluated the knockdown efficiency of si-PMM2 transfection, using 2 types of si-PMM2 (si-PMM2_1 and si-PMM2_2). Using qRT-PCR and Western blot analyses, we confirmed that the expression levels of PMM2 mRNA and protein were significantly reduced (Figure 5A,B). Furthermore, functional assays showed that si-PMM2 transfection significantly inhibited cell proliferation, migration, and invasion in comparison with mock or siRNA-control transfected cells (Figure 5C-E).

### 3.6 Expression of PMM2/PMM2 in RCC clinical specimens and cell lines

We used qRT-PCR to investigate the mRNA expression levels of PMM2 in 15 pairs of RCC tissues, adjacent noncancerous tissues and RCC cell lines. Expression of PMM2 was significantly upregulated in RCC tissues compared with that in normal tissues ($P = .0026$; Figure 6A) and also markedly upregulated in RCC cell lines.

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**FIGURE 4** Regulation of PMM2 expression by miR-451a in renal cell carcinoma cells. A, Expression levels of PMM2 mRNA 48 hours after transfection of 10 nmol/L miR-451a into cell lines. GUSB was used as an internal control. *$P < .0001$*. B, Protein expression of phosphomannomutase 2 (PMM2) 72 hours after transfection with miR-451a. GAPDH was used as a loading control. C, miR-451a binding sites in the 3′-UTR of PMM2 mRNA. D, Dual luciferase reporter assays using vectors encoding putative miR-451a target sites (positions 1127-1133) in the PMM2 3′-UTR for both wild-type and deleted regions. Normalized data were calculated as the ratio of Renilla/firefly luciferase activities. *$P < .005$*
Furthermore, Spearman’s rank test revealed a negative correlation between the expression levels of miR-451a and PMM2 \( (P = 0.0409, R = -0.38; \text{Figure 6B}) \).

Moreover, we carried out immunohistochemistry to analyze PMM2 protein expression in an RCC tissue microarray (cat. no. KD806; US Biomax, Rockville, MD, USA). Patient characteristics for samples used in the tissue microarray are as described in http://www.biomax.us/tissue-arrays/Kidney/KD806. The PMM2 protein was strongly expressed in several cancer lesions (Figure 6C).

### 3.7 Effects of co-transfection of PMM2/miR-451a in 786-O cells

We undertook PMM2 rescue studies in 786-O cells to elucidate whether the molecular pathway of PMM2/miR-451a was significant for the progression of RCC. Figure 7A shows the results of Western blot analysis of PMM2 protein expression. Functional assays showed that the migration and invasive abilities of RCC cells were significantly recovered by PMM2 and miR-451a transfection compared with cells transfected with miR-451a alone (Figure 7C,D). These results suggested that PMM2 had an important role in the aggressiveness of RCC.

### 3.8 Downstream genes affected by silencing of PMM2 in 786-O cells

We carried out genome-wide gene expression analyses by transfecting si-PMM2 into 786-O cells to elucidate which genes were modulated by PMM2 knockdown. We focused on genes that were significantly downregulated by transfection of both si-PMM2 \(_1\) and si-PMM2 \(_2\) (average \(\log_2 \text{[si-PMM2/mock]} < -1.0\)). Genes significantly downregulated by silencing of PMM2 are listed in Table 3. Among these genes, high expression levels of CD7, CCNE2, ASB2, RCN3, HSF1, PAQR4, CD37, SOX11, XAF1, and DEPDC1 were associated with poor prognosis in RCC patients based on TCGA database (Figure 8).
Clinical significance of PMM2 in RCC pathogenesis using TCGA database

To investigate the clinical significance of PMM2 in RCC pathogenesis, we asked whether the PMM2 expression level affected disease-free survival (DFS) in RCC. We found that high expression levels of PMM2 were significantly associated with low DFS in RCC patients (P < .0001; Figure 9A). Furthermore, we analyzed the relationships among PMM2 expression levels and tumor T stage, lymph node metastasis, disease stage, and histologic grade in RCC. PMM2 expression levels were significantly higher in the more advanced tumor stages and histologic grades (Figure 9B-E).

Additionally, we undertook univariable and multivariable Cox hazard regression analyses to investigate the clinical significance of PMM2 expression along with other clinical factors in the overall survival of RCC patients. Multivariate analysis showed that high PMM2 expression and tumor stage were independent predictive factors for overall survival (hazard ratio = 1.26, P = .0487 and hazard ratio = 0.76, P = .0353, respectively; Table 4). These results suggested that PMM2 may be closely associated with tumor progression and malignancy in RCC.

4 | DISCUSSION

The aberrant expression of miRNAs disrupts regulated RNA networks in various type of cancer cells and therefore contributes substantially to the pathogenesis of human cancer. Therefore, understanding miRNA signatures is essential to clarifying their roles in the pathology of human cancer cells. We previously characterized several miRNAs that had significant antitumor functions in RCC cells. For example, miR-101 was significantly reduced in RCC tissues and the restoration of miR-101 inhibited RCC aggressiveness through targeting the UHRF1 gene. More recently, we showed that miR-10a-5p was downregulated in primary and tyrosine-kinase inhibitor-treated RCC specimens and directly regulated the SKA1 gene. SKA1 was overexpressed and knockdown of SKA1 inhibited migration and invasion of RCC cells.

Our present data showed that expression of miR-451a was significantly reduced in primary RCC. Furthermore, ectopic expression of miR-451a resulted in inhibition of cancer cell migratory and invasive abilities in RCC, indicating that miR-451a functions as a tumor suppressor in RCC. Previous reports showed that expression of miR-451 was downregulated in several types of human cancers. In bladder cancer, miR-451 expression was significantly reduced in cancer tissues compared with adjacent tissues and that restoration of miR-451 reduced cancer cell migration and invasive abilities. Furthermore, low expression of miR-451 was associated with advanced tumor stage and high pathological grade in bladder cancer. Our previous data showed that miR-451a acted as an antitumor miRNA through targeting ESDN/DCBLD2 in head and neck squamous cell carcinoma.

In this study, we identified 16 putative oncogenic targets by miR-451a regulation in RCC cells. Interestingly, among these targets,
expression of 9 genes (PMM2, CRELD2, CLEC2D, SPC25, BST2, EVL, TBX15, DPYSL3, and NAMPT) were significantly associated with poor prognosis of the patients with RCC by TCGA database analyses. These targets might be potential therapeutic targets for RCC and the search for RNA networks controlled by antitumor miR-451a and its targets are important for elucidation of the pathogenesis of RCC.

In the present study, we identified PMM2 as an oncogenic gene in RCC cells. Moreover, overexpressed PMM2 was involved in RCC pathogenesis. PMM2 codes for an enzyme that converts mannose-6-phosphate to mannose-1-phosphate and participates in a metabolic pathway in glycan synthesis.33-35 Protein glycosylation is an important contributor to cancer progression, including cell growth, tumor-induced immunomodulation, and eventual metastasis.36 Previous reports indicated that cancer cells, including RCC, are characterized by an aberrant increase in protein N-glycosylation.37,38 In clear cell RCC, upregulation of protein glycosylation in cancer cells may be useful in diagnosis and determining disease prognosis.39 Furthermore, N-glycosylation is involved in cell adhesion and is associated with reduced expression of E-cadherin, which modulates the metastatic potential of cancer cells.37,40 We hypothesize that this pathway might contribute to cancer pathogenesis such that upregulation of PMM2 might enhance cancer cell aggressiveness by increased N-glycosylation. In support of this hypothesis, TCGA database showed that high expression of PMM2 was associated with poor prognosis even in other types of cancer, for example, bladder cancer, breast cancer, head and neck squamous cell carcinoma, glioma, and melanoma (Figure S2).

Interestingly, a previous report showed that miR-451 was controlled by glucose levels and regulated cancer aggressiveness through the AMP-activated protein kinase pathway and mTOR activation in glioblastoma.15 Thus, miR-451 regulated pathways may be involved in glucose-related metabolic pathways and regulate cancer aggressiveness. Further research into miR-451/PMM2-modulated pathways in cancer will be necessary. To investigate PMM2-mediated pathways in RCC, we undertook genome-wide gene expression analyses using PMM2 knockdown cells. We identified 27 genes that were regulated by PMM2 in RCC. Among them, the expression of 10 genes was elevated (CD7, CCNE2, ASB2, RCN3,
| Gene symbol | Gene name                                | GEO expression data fold-change (tumor/normal) | Log2 (si-PMM2_1/mock) | Log2 (si-PMM2_2/mock) | Average Log2 (si-PMM2/mock) | Cytoband | TCGA data OS (P-value) |
|-------------|------------------------------------------|-----------------------------------------------|-----------------------|-----------------------|----------------------------|----------|------------------------|
| GNLY        | Granulysin                               | 5.987                                         | −2.465                | −2.548                | −2.506                     | hs:2p11.2| .09360000              |
| TTF2        | Transcription termination factor, RNA polymerase II | 1.312                                         | −3.407                | −1.139                | −2.273                     | hs:1p13.1| .27800000              |
| CD7         | CD7 molecule                             | 1.981                                         | −1.935                | −2.522                | −2.228                     | hs:17q25.3| .0001220               |
| CCNE2       | Cyclin E2                                | 2.430                                         | −3.101                | −1.251                | −2.176                     | hs:8q22.1| .00664000              |
| NLGN1       | Neuroligin 1                             | 2.422                                         | −1.377                | −2.500                | −1.938                     | hs:3q26.31| .03910000*             |
| TMEM184B    | Transmembrane protein 184B               | 1.525                                         | −1.128                | −2.639                | −1.883                     | hs:22q13.1| .96300000              |
| ASB2        | Ankyrin repeat and SOCS box containing 2 | 1.804                                         | −2.534                | −1.194                | −1.864                     | hs:14q32.12| .01680000              |
| ASPH        | Aspartate β-hydroxylase                  | 1.727                                         | −1.414                | −2.244                | −1.829                     | hs:8q12.3 | .22000000              |
| MAML3       | Mastermind-like 3 (Drosophila)           | 1.364                                         | −1.644                | −1.923                | −1.784                     | hs:4q31.1 | .00010700*             |
| RCN3        | Reticulocalbin 3, EF-hand calcium binding domain | 1.314                                         | −2.325                | −1.103                | −1.714                     | hs:19q13.33| .0000636              |
| CAMK1       | Calcium/calmodulin-dependent protein kinase I | 1.324                                         | −1.566                | −1.471                | −1.519                     | hs:3p25.3 | .54600000              |
| P2RX7       | Purinergic receptor P2X, ligand gated ion channel, 7 | 8.047                                         | −1.746                | −1.134                | −1.440                     | hs:12q24.31| .95700000              |
| KDM1B       | Lysine (K)-specific demethylase 1B        | 1.342                                         | −1.291                | −1.544                | −1.417                     | hs:6p22.3 | .00939000*             |
| OAS3        | 2′-5′-oligoadenylate synthetase 3, 100kda | 2.919                                         | −1.490                | −1.287                | −1.388                     | hs:12q24.13| .97300000              |
| HSF1        | Heat shock transcription factor 1        | 1.324                                         | −1.685                | −1.083                | −1.384                     | hs:8q24.3 | .00126000              |
| PMM2        | Phosphomannomutase 2                     | 1.580                                         | −1.304                | −1.376                | −1.340                     | hs:16p13.2| .000000218             |

(Continues)
| Gene symbol | Gene name                                                                 | GEO expression data fold-change | Log2 (si-PMM2_1/mock) | Log2 (si-PMM2_2/mock) | Average Log2 (si-PMM2/mock) | Cytoband | TCGA data OS (P-value) |
|-------------|---------------------------------------------------------------------------|---------------------------------|------------------------|------------------------|----------------------------|----------|------------------------|
| CDKN2B      | Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)                 | 3.355                           | -1.237                 | -1.434                 | -1.335                     | hs9p21.3 | .00542000*             |
| PAQR4       | Progestin and adipok receptor family member IV                            | 5.134                           | -1.567                 | -1.087                 | -1.327                     | hs16p13.3 | .00152000              |
| CD37        | CD37 molecule                                                             | 6.272                           | -1.154                 | -1.420                 | -1.287                     | hs19q13.33 | .01880000             |
| SOX11       | SRY (sex determining region Y)-box 11                                     | 5.969                           | -1.334                 | -1.236                 | -1.285                     | hs2p25.2  | .04050000              |
| XAF1        | XIAP associated factor 1                                                  | 2.349                           | -1.044                 | -1.489                 | -1.266                     | hs17p13.1 | .00001720             |
| DEPDC1      | DEP domain containing 1                                                   | 2.606                           | -1.253                 | -1.197                 | -1.225                     | hs1p31.2  | .00011100             |
| SAMHD1      | SAM domain and HD domain 1                                                | 2.764                           | -1.225                 | -1.163                 | -1.194                     | hs20q11.23 | .16600000             |
| P2RY1       | Purinergic receptor p2y, g-protein coupled, 1                             | 1.427                           | -1.167                 | -1.142                 | -1.155                     | hs3q25.2  | .00051400*            |
| LOC100422737| Uncharacterized loc100422737                                              | 1.322                           | -1.093                 | -1.199                 | -1.146                     | hs6q21    | No data                |
| DOCK9       | Dedicator of cytokinesis 9                                                | 1.614                           | -1.158                 | -1.087                 | -1.123                     | hs13q32.3 | .00001100*            |
| SLC39A10    | Solute carrier family 39 (zinc transporter), member 10                    | 1.331                           | -1.074                 | -1.137                 | -1.105                     | hs2q32.3  | .01820000*            |
| RNF125      | Ring finger protein 125, e3 ubiquitin protein ligase                       | 1.526                           | -1.060                 | -1.060                 | -1.060                     | hs18q12.1 | .03820000*            |

GEO, Gene Expression Omnibus; OS, overall survival; TCGA, The Cancer Genome Atlas. 
* poor prognosis with low expression.
**FIGURE 8** The Cancer Genome Atlas database analysis of PMM2-mediated downstream genes in renal cell carcinoma. Kaplan-Meier curves of 10 genes whose high expression levels were associated with poor prognosis in renal cell carcinoma.

**FIGURE 9** Kaplan-Meier curves for disease-free survival based on PMM2 expression in patients with renal cell carcinoma, and expression levels of PMM2 according to T stage, N stage, tumor stage, and histologic grade. All patients’ data were obtained from The Cancer Genome Atlas database. A, Kaplan-Meier survival curves for disease-free survival based on PMM2 expression in patients with renal cell carcinoma. B-E, Relationships between expression levels of PMM2 and T stage, N stage, disease stage, and histologic grade. *P < .05, **P < .005, ***P < .0001
abilities. In clinical analysis, high expression of silencing significantly inhibited cancer cell migration and invasive phomannomutase 2 was strongly expressed in RCC cells and its PMM2 significantly associated with shorter DFS and lower survival rates. In short, acted as a tumor suppressor through the targeting of downregulated in clinical RCC cells. Moreover, PMM2-mediated pathways may improve our understanding of RCC aggressiveness.

In conclusion, our data revealed that expression of miR-451a was significantly downregulated in clinical RCC cells. Moreover, miR-451a acted as a tumor suppressor through the targeting of PMM2. Phosphomannomutase 2 was strongly expressed in RCC cells and its silencing significantly inhibited cancer cell migration and invasive abilities. In clinical analysis, high expression of PMM2 was significantly associated with shorter DFS and lower survival rates. In short, PMM2-regulated genes are deeply involved in RCC pathogenesis. Elucidation of the pathways mediated by the miR-451a/PMM2 axis should improve our understanding of oncogenic mechanisms and lead to new treatment strategies in RCC.

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### CONFLICT OF INTEREST

The authors have no conflict of interest.

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