INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and is the 12th most prevalent malignancy worldwide, with 338,000 newly diagnosed patients in 2012 and approximately 100,000 deaths annually. Clear cell RCC (ccRCC) is pathologically the most common type and accounts for approximately 75% of all cases. Although the prognosis is favorable with surgical resection for nonmetastatic RCC, approximately 20%-30% of RCC patients have metastatic sites at the diagnosis and the 5-year survival rate is less than 20%. In addition, more than 20% of patients develop metastases during postoperative follow-up periods. These clinical issues are caused by a lack of useful biomarkers for early detection of RCC and the inefficiency of therapy for patients with metastatic or treatment-resistant RCC.

Abstract

Analysis of microRNA (miRNA) regulatory networks is useful for exploring novel biomarkers and therapeutic targets in cancer cells. The Cancer Genome Atlas dataset shows that low expression of both strands of pre-miR-101 (miR-101-5p and miR-101-3p) significantly predicted poor prognosis in clear cell renal cell carcinoma (ccRCC). The functional significance of miR-101-5p in cancer cells is poorly understood. Here, we focused on miR-101-5p to investigate the antitumor function and its regulatory networks in ccRCC cells. Ectopic expression of mature miRNAs or siRNAs was investigated in cancer cell lines to characterize cell function, ie, proliferation, apoptosis, migration, and invasion. Genome-wide gene expression and in silico database analyses were undertaken to predict miRNA regulatory networks. Expression of miR-101-5p caused cell cycle arrest and apoptosis in ccRCC cells. Downstream neighbor of son (DONSON) was directly regulated by miR-101-5p, and its aberrant expression was significantly associated with shorter survival in propensity score-matched analysis (P = .0001). Knockdown of DONSON attenuated ccRCC cell aggressiveness. Several replisome genes controlled by DONSON and their expression were closely associated with ccRCC pathogenesis. The antitumor miR-101-5p/DONSON axis and its modulated replisome genes might be a novel diagnostic and therapeutic target for ccRCC.

KEYWORDS

DONSON, microRNA, miR-101-5p, renal cell carcinoma, replisome
MicroRNAs (miRNAs) are classified as noncoding RNAs that are approximately 18-25 bases in size. They are widely found, ranging from plants to humans. MicroRNAs bind to the 3′-UTR of target genes and have many biological functions that are achieved by regulating the expression of protein-coding genes in a sequence-dependent manner. Numerous reports have indicated that miRNAs are closely involved in cell growth, migration, invasion, apoptosis, angiogenesis, and tumor metastasis in various human cancers. Interestingly, a single miRNA can regulate a vast number of protein-coding or noncoding RNAs. Therefore, the analysis of aberrantly expressed miRNAs in human cancers provides us information about cancer-modulating molecular networks.

Previously, we established a miRNA expression signature from autopsy samples of ccRCC patients who relapsed following sunitinib treatment. Based on this signature, we have identified a number of antitumor microRNAs (miR-101-3p, miR-455-duplex, and the miR-29-family) as well as the oncogenes that they control. All of the miRNAs were closely related to ccRCC development. The discovery of oncogenic networks mediated by antitumor miRNAs contributes to the elucidation of the molecular mechanisms mediating the pathogenesis of ccRCC.

Current RNA-sequencing (-seq) technology makes it possible to construct miRNA expression signatures in human cancer. Expressions of several passenger strands of miRNA are significantly up- or down-regulated in cancer tissues from the miRNA signature. Our functional assays showed that several passenger strands of miRNAs (eg, miR-455-5p, miR-144-5p, and miR-145-3p) had antitumor roles, as did the guide strands of miRNAs. In general, the passenger strand of miRNA is degraded and therefore considered to have no function. Our reports differ from the previous concept. Thus, we have discovered a new aspect of miRNA functionality.

Here, we focused on miR-101-5p (the passenger strand) to elucidate the function of miR-101-5p and determine its target oncogenes as useful diagnostic markers in ccRCC. Previous studies have shown that miR-101-3p (the guide strand of the miR-101 duplex) acts as an antitumor miRNA in several cancers by targeting oncogenic genes. In contrast to miR-101-3p, the functional significance of miR-101-5p in cancer cells is poorly understood. Ectopic expression of miR-101-5p attenuated the aggressive phenotype of ccRCC cells. Downstream neighbor of son (DONSON) was directly regulated by miR-101-5p, and its aberrant expression was significantly associated with shorter survival in propensity score-matched analysis. Moreover, several replisome genes controlled by DONSON and their expression were closely associated with ccRCC pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and cell lines

In the present study, 18 clinical ccRCC tissue samples were obtained from patients received nephrectomy at Chiba University Hospital between 2014 and 2015 (Table S1). Also, autopsy specimens were obtained from 5 patients whose disease was resistant to several tyrosine kinase inhibitor (TKI) treatments; samples were obtained from Teikyo University Chiba Medical Center Hospital between 2012 and 2016 (Table S2). We obtained informed consent from all patients and the current research protocol was approved by the Institutional Review Board of Chiba University (acceptance no. 484). Two ccRCC cell lines (786-0 and A498) from ATCC were used in this study. These cell lines were cultured in RPMI-1640 with 10% FBS (HyClone).

2.2 | Transfection of ccRCC cells with miRNAs, siRNAs, and plasmid vectors

MicroRNAs, siRNAs, and vectors were transfected into cancer cells as described in our previous reports using the reagents listed in Table S3.

2.3 | RNA preparation and quantitative RT-PCR

Total RNA including miRNA was isolated using TRizol reagent (Invitrogen) in clinical specimens and ISOGEN reagent (Nippon Gene) in ccRCC cells. TaqMan probes and DONSON primers were used and the reagents are listed in Table S3. Quantitative RT-PCR for miR-101-5p and miR-101-3p was used to validate miRNA expression. To normalize the data for analysis of mRNAs and miRNAs, GUSB and RNU48 were used. The PCR quantification was carried out as previously described.

2.4 | Assays of proliferation, migration, and invasion

Cell proliferation, migration, and invasion were assessed as described previously.

2.5 | Assay of cell cycle

Clear cell RCC cells were transfected with either the transfection reagents alone as a control or miR-101-5p, miR-101-3p, and si-DONSON in 6-well tissue culture plates. Seventy-two hours after transfection, these cells were harvested by trypsinization. Cells were stained with propidium iodide with the CyCletest Plus DNA Reagent Kit (BD Biosciences) and analyzed using the CyAn ADP analyzer (BD Biosciences). The proportion of cells in the G0/G1, S, and G2/M phases were calculated and compared. We undertook each experiment in triplicate.

2.6 | Assay of apoptosis

In order to identify the apoptotic cells, the FITC Annexin V apoptosis detection kit (BD Biosciences) and the BD FACS Celesta flow cytometer (BD Biosciences) were used according to the
FIGURE 1  Expression of miR-101 and its clinical significance in clear cell renal cell carcinoma (ccRCC). A–C, Expression levels of miR-101-5p and miR-101-3p in ccRCC clinical specimens. RNU48 was used as an internal control. Expression levels of miR-101-5p and miR-101-3p were positively correlated by Spearman’s rank test. D, E, Based on The Cancer Genome Atlas database, high grades of RCC were significantly associated with low miR-101 expression levels. F–I, Low expression levels of miR-101 were significantly associated with poor prognosis in RCC patients (disease-free survival and overall survival). RNA-seq, RNA sequencing; RSEM, RNA sequencing by expectation-maximization.
manufacturer’s instructions. We classified these cells as viable cells, dead cells, or early or late apoptotic cells and compared the percentage of apoptotic cells according to each condition. To evaluate apoptosis with western blotting, anti-poly (ADP-ribose) polymerase (PARP) was used. Adriamycin (ADM) was used as a positive control.

### 2.7 Incorporation of miR-101-5p and miR-101-3p into the RNA-induced silencing complex by Ago2 immunoprecipitation

After 72 hours, miRNAs incorporated into the RNA-induced silencing complex (RISC) were isolated using the human AGO2 miRNA isolation kit (Wako Pure Chemical Industries). The method of measuring the amount of miR-101-5p incorporated into RISC was according to a previous study.

### 2.8 Target genes regulated by miR-101-5p and miR-101-3p

Candidate target genes regulated by miR-101-5p or miR-101-3p were identified using in silico and genome-wide gene expression analyses and those obtained from the TargetScan database (http://www.targetscan.org/vert_70/). Upregulated genes in RCC tissues compared with normal renal tissues were identified from public data in the Gene Expression Omnibus (GEO; accession no. GSE36895), from which we narrowed down these genes. Gene expression was analyzed with our oligo microarray data analyses (Human GE 60K; Agilent Technologies) that were deposited into the GEO (on 23 August 2018; http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE118966.

### 2.9 Evaluation of miR-101-5p binding sites by luciferase reporter assay

The 3′-UTR of DONSON and the 3′-UTR lacking the putative miR-101-5p binding site (position 219-225 in 3′-UTR of DONSON) were cloned into the psiCHECK-2 vector. A luciferase reporter assay was undertaken as previously described.

### 2.10 Western blot analysis and immunohistochemistry

Western blotting and immunohistochemistry (IHC) were carried out as described previously. Primary Abs are listed in Table S3.

### RESULTS

### 3.1 Analysis of miR-101-5p and miR-101-3p expression levels in clinical ccRCC tissues and their clinical significance

In the human genome, the chromosomal location of miR-101 is at 1q31.3. The mature sequences of miR-101-5p and miR-101-3p are 5′-CAGUUAUCACAGUGCUAGCU-3′ and 5′-UACAGUACUGUAUAACUGAA-3′, respectively. Both miR-101-5p and miR-101-3p expressions were significantly reduced in ccRCC tissues compared with normal kidney tissue with IHC.
**A** miR-101-5p target genes from TargetScan database (release 7.2)

- 2476 genes
- Downregulated genes (log2 < -1.5) by miR-101-5p transfection from genome-wide expression analysis (GSE18966)
- 124 genes
- Upregulated genes (fold-change > 1.5) in RCC tissues from GEO database (GSE36895)
- 38 genes (Table 1A)
- Gene most related to the prognosis of patients with RCC from TCGA database

**B**

|                | 786-O          | A498          |
|----------------|---------------|---------------|
| miR-101-5p    |               |               |
| Control       | 100 ± 5       | 100 ± 5       |
| miR-101-5p    | 90 ± 5        | 70 ± 5        |

**C**

- DONSON expression
- GAPDH expression

**D**

- Position 219 - 225 of the DONSON 3' UTR

**E**

|                | 786-O       | A498       |
|----------------|-------------|------------|
| miR-101-5p    |             |            |
| Control       | 120 ± 10    | 120 ± 10   |
| miR-101-5p    | 100 ± 10    | 80 ± 10    |

**F**

- DONSON expression (entire cohort)

**G**

- DONSON expression (propensity score-matched cohort)

**H**

Cox proportional hazard model

- DONSON expression (high vs low)
- Age (≥ 60 vs < 60)
- Gender (M vs F)
- Stage (3+4 vs 1+2)
- Grade (3+4 vs 1+2)

**I**

|                | Normal RCC   | Primary RCC |
|----------------|--------------|-------------|
| miR-101-5p    | 0.6 ± 0.2    | 0.4 ± 0.2   |

**J**

- 14 years old, female

**K**

- No. 16
- No. 17
- No. 18

**L**

- No. 1
- No. 2
- No. 3
with those in adjacent noncancerous tissues (P = .027 and P = .0057, respectively; Figure 1A,B). In addition, Spearman's rank analysis showed strong positive correlation between miR-101-5p and miR-101-3p expression levels (R = 0.943, P < .0001; Figure 1C). From a large cohort of TCGA database, low expressions of miR-101-5p and miR-101-3p were significantly associated with high pathological grade (both, P < .0001; Figure 1D,E) and poor clinical outcomes (disease-free survival, P = .0485 and P = .0457; overall survival, P = .0397 and P = .00271, respectively, Figure 1F-I) in ccRCC patients.

### 3.2 | Antitumor functions of miR-101-5p and miR-101-3p in ccRCC cells

Restoration of miR-101-5p and miR-101-3p expression indicated that both miRNAs significantly suppressed cancer cell proliferation (Figure 2A), migration (Figure S1A,B) and invasion potentials (Figure S1C,D). Furthermore, the apoptotic rate was elevated in miR-101-5p and miR-101-3p transfected cells compared to controls (Figure 2B,C). In addition, transfection of miR-101-3p apparently upregulated cleaved PARP expression (Figure 2D). In cell cycle analyses, ectopic expression of these miRNAs confirmed increase in the sub-G1 peak in ccRCC cells (Figure 2E). We analyzed the expression levels of cell cycle-related genes by ectopic expression of miR-101-5p and/or miR-101-3p in ccRCC cells (Table S4). Downregulation of CCNB1, CDK1, CDK2, and CDK4 were detected by microarray data.

Cell migration and invasive abilities were significantly inhibited by ectopic expression of miR-101-5p and miR-101-3p in ccRCC cells. To explain this phenomenon, the expression of epithelial-mesenchymal transition (EMT)-related genes was examined by microarray analyses (Table S4). The mRNA expression levels of CDH2, VIM, ZEB1, TWIST1, SNAI1, and FN1 were reduced by miR-101-5p and/or miR-101-3p transfection into ccRCC cells. Furthermore, we investigated the expression changes of EMT-related proteins (eg, E-cadherin, N-cadherin, SLUG, Vimentin, and TWIST) by ectopic expression of miR-101-5p and miR-101-3p in ccRCC cells (Figure S2). Notably, the expression levels of N-cadherin, SLUG, and Vimentin were suppressed by expressions of miR-101-5p and miR-101-3p in 2 RCC cell lines, 786-O and A498 (Figure S2). Downregulation of TWIST was detected in 786-O cells by miR-101-5p expression. Our present data indicated that expression of these miRNAs regulate the expression of EMT-related proteins and they play critical roles in malignant transformation of ccRCC cells.

In addition, we examined the synergistic effect of the 2 miRNAs on cell proliferation, apoptosis, and the cell cycle. No synergistic effect of antitumor effects in RCC cells by the ectopic expression of the 2 miRNAs, miR-101-5p and miR-101-3p, was observed (Figure S3).

### 3.3 | Incorporation of miR-101-5p into RISC in ccRCC cells

To verify that miR-101-5p (passenger strand) had actual functions in ccRCC cells, it is essential that miRNAs are incorporated into the RISC to control target genes. Immunoprecipitation using anti-Ago2 Abs was carried out after transfection of miR-101-5p into 786-O cells. The amount of miR-101-5p incorporated into the protein was measured by PCR. Levels of miR-101-5p in the immunoprecipitation were much higher than those in mock, miR-control, or miR-101-3p-transfected cells (P < .0001; Figure S4).

### 3.4 | Candidate target genes of miR-101-5p and miR-101-3p

We identified genes that had putative target sites for miR-101 in their 3′-UTR and that showed downregulated expression in ccRCC cells transfected with miR-101 (log2 ratio less than −1.5) and upregulated expression levels (fold-change greater than 1.5) in RCC tissues from the GEO database (Figure 3A). Using this search strategy, 38 and 47 genes were found as candidate target genes for miR-101-5p and miR-101-3p, respectively (Table 1). Among these genes, we focused on DONSON, which is targeted by miR-101-5p and had the strongest relation to the prognosis from TCGA database.

### 3.5 | MicroRNA-101-5p targeted DONSON expression

mRNA and protein levels of DONSON were significantly reduced after transfection of 786-O and A498 cells with miR-101-5p compared to control cells (Figure 3B,C).
TABLE 1  miR-101-duplex regulatory genes in clear cell renal cell carcinoma cells

| Gene symbol | Gene name | Entrez Gene ID | Cytoband | GEO expression fold-change (tumor/normal) | Mock vs miR-101-5p transfection in 786-O cells (log2 ratio) | OS analysis from TCGA database (high vs low expression, \(P\) value) |
|-------------|-----------|---------------|----------|------------------------------------------|------------------------------------------------------------|---------------------------------------------------------------|
| DONSON      | Downstream neighbor of SON | 29980 | hs|21q22.11 | 1.665 | -1.952 | 8.10E-15 |
| EFHD2       | EF-hand domain family, member D2 | 79180 | hs|1p36.21 | 2.000 | -1.933 | 1.16E-05 |
| NAP1L1      | Nucleosome assembly protein 1-like 1 | 4673 | hs|12q21.2 | 1.536 | -1.672 | 1.40E-05 |
| HSPA6       | Heat shock 70 kDa protein 6 (HSP70B') | 3310 | hs|1q23.3 | 2.814 | -1.509 | 0.000116 |
| PBK         | PDZ binding kinase | 55872 | hs|8p21.1 | 2.982 | -2.425 | 0.0018800 |
| DPHSL3      | Dihydropyrimidinase-like 3 | 1809 | hs|5q32 | 2.327 | -1.548 | 0.0038900 |
| EV1A2       | Ecotropic viral integration site 2A | 2123 | hs|17q11.2 | 2.971 | -1.682 | 0.0073700 |
| KIAA1841    | KIAA1841 | 84542 | hs|2p15 | 2.132 | -1.658 | 0.0106000 |
| SNX10       | Sorting nexin 10 | 29887 | hs|7p15.2 | 1.564 | -2.843 | 0.0112000 |
| GINS1       | GINS complex subunit 1 (Psf1 homolog) | 9837 | hs|20q11.21 | 1.532 | -2.077 | 0.0137000 |
| LYSMD2      | LysM, putative peptidoglycan-binding, domain containing 2 | 256586 | hs|15q21.2 | 1.433 | -1.687 | 0.0244000 |
| TBL1XR1     | Transducin (beta)-like 1 X-linked receptor 1 | 79718 | hs|3q26.32 | 1.427 | -1.879 | 0.0396000 |
| KCND2       | Potassium voltage-gated channel, Shal-related subfamily, member 2 | 3751 | hs|7q31.31 | 2.588 | -1.551 | 0.0444000 |
| ITGA5       | Integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | 3678 | hs|12q13.13 | 7.156 | -1.932 | 0.0818000 |
| MEGF6       | Multiple EGF-like-domains 6 | 1953 | hs|1p36.32 | 2.113 | -1.608 | 0.1510000 |
| KDEL2       | KDEL (Lys-Asp-Glu-Leu) containing 2 | 143888 | hs|11q22.3 | 1.710 | -1.651 | 0.1690000 |
| CDI09       | CDI09 molecule | 135228 | hs|6q13 | 1.449 | -1.739 | 0.2230000 |
| MET         | Met proto-oncogene | 4233 | hs|7q31.12 | 2.553 | -2.159 | 0.2240000 |
| HAUS6       | HAUS augmin-like complex, subunit 6 | 54801 | hs|9p22.1 | 1.831 | -2.069 | 0.2980000 |
| FCHSD2      | FCH and double SH3 domains 2 | 9873 | hs|11q13.4 | 1.482 | -1.645 | 0.3190000 |
| QSER1       | Glutamine and serine rich 1 | 79832 | hs|11p13 | 1.565 | -2.025 | 0.3800000 |
| NACAP2      | Non-SMC condensin II complex, subunit G2 | 54892 | hs|7q36.3 | 2.127 | -2.679 | 0.3850000 |
| MEF2C       | Myocyte enhancer factor 2C | 4208 | hs|5q14.3 | 2.693 | -1.988 | 0.4720000 |
| PMP22       | Peripheral myelin protein 22 | 5376 | hs|17p12 | 3.152 | -1.938 | 0.4800000 |
| METAP1D     | Methionyl aminopeptidase type 1D (mitochondrial) | 254042 | hs|2q31.1 | 1.492 | -1.645 | 0.4810000 |
| IL16        | Interleukin 16 | 3603 | hs|15q25.1 | 1.799 | -1.642 | 0.6600000 |
| EGLN3       | Egl-9 family hypoxia-inducible factor 3 | 112399 | hs|14q13.1 | 13.669 | -1.568 | 0.6880000 |
| CKA2        | Cytoskeleton associated protein 2 | 26566 | hs|13q14.3 | 1.454 | -1.803 | 0.8310000 |
| PCSK5       | Proprotein convertase subtilisin/kexin type 5 | 5125 | hs|9q21.13 | 1.490 | -2.535 | 0.0000246 a |
| MXI1        | MAX interactor 1, dimerization protein | 4601 | hs|10q25.2 | 1.987 | -2.013 | 0.0000979 a |
| KCTD20      | Potassium channel tetramerization domain containing 20 | 222658 | hs|6p21.31 | 1.415 | -1.582 | 0.0006140 a |
| LRRCC8C     | Leucine rich repeat containing 8 family, member C | 84230 | hs|1p22.2 | 1.890 | -1.593 | 0.0039200 a |
| RCCTB2      | Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2 | 1102 | hs|13q14.2 | 1.456 | -2.255 | 0.0054900 a |
| EDIL3       | EGF-like repeats and discoidin I-like domains 3 | 10085 | hs|5q14.3 | 2.902 | -2.611 | 0.0085400 a |

(Continues)
### TABLE 1 (Continued)

| Gene symbol | Gene name | Entrez Gene ID | Cytoband | GEO expression fold-change (tumor/normal) | Mock vs miR-101-5p transfection in 786-O cells (log2 ratio) | OS analysis from TCGA database (high vs low expression, P value) |
|--------------|-----------|----------------|----------|------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| TPR          | Translocated promoter region, nuclear basket protein | 7175 | hs|1q31.1 | 1.663 | -1.630 | 0.0124000a |
| NR3C1        | Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) | 2908 | hs|5q31.3 | 2.111 | -1.718 | 0.0178000a |
| STARD13      | StAR-related lipid transfer (START) domain containing 13 | 90627 | hs|13q13.1 | 1.445 | -2.355 | 0.0374000a |
| SETD7        | SET domain containing (lysine methyltransferase) 7 | 80854 | hs|4q31.1 | 2.225 | -2.392 | 0.0459000a |
|              |           |                |          |                                          |                                                  |                                                  |
| (B) miR-101-3p |          |               |          |                                          |                                                  |                                                  |
| JAK3         | Janus kinase 3 | 3718 | hs|19p13.11 | 2.283 | -1.516 | 1.23E-09 |
| MX2          | Myxovirus (influenza virus) resistance 2 (mouse) | 4600 | hs|21q22.3 | 1.508 | -1.830 | 3.81E-08 |
| NAP1L1       | Nucleosome assembly protein 1-like 1 | 4673 | hs|12q21.2 | 1.536 | -1.795 | 1.40E-05 |
| TMEM39B      | Transmembrane protein 39B | 55116 | hs|1p35.1 | 2.416 | -1.037 | 2.64E-05 |
| ANXA2        | Annexin A2 | 302 | hs|15q22.2 | 1.659 | -1.811 | 0.000151 |
| STIL         | SCL/TAL1 interrupting locus | 6491 | hs|1p33 | 2.136 | -1.176 | 0.000255 |
| FBXO32       | F-box protein 32 | 114907 | hs|8q24.13 | 1.485 | -1.079 | 0.000574 |
| LMNB1        | Lamin B1 | 4001 | hs|5q23.2 | 2.245 | -3.395 | 0.002780 |
| AP3S1        | Adaptor-related protein complex 3, sigma 1 subunit | 1176 | hs|5q23.1 | 1.622 | -1.320 | 0.022000 |
| SELPLG       | Selectin P ligand | 6404 | hs|12q41.11 | 2.757 | -1.457 | 0.026600 |
| RASD2        | RASD family, member 2 | 23551 | hs|22q12.3 | 2.707 | -1.957 | 0.080900 |
| NAV1         | Neuron navigator 1 | 89796 | hs|1q21.3 | 1.337 | -1.887 | 0.020350 |
| MADD1        | MADD mitotic arrest deficient-like 1 (yeast) | 4085 | hs|4q27 | 1.954 | -1.418 | 0.092900 |
| TTYH2        | Tweety family member 2 | 94015 | hs|17q25.1 | 1.958 | -1.103 | 0.097200 |
| DDIT4        | DNA-damage-inducible transcript 4 | 54541 | hs|10q22.1 | 3.996 | -1.103 | 0.148000 |
| IKZF3        | IKAROS family zinc finger 3 (Aiolos) | 22806 | hs|1q21.11 | 2.099 | -1.098 | 0.165000 |
| RRM1         | Ribonucleotide reductase M1 | 6240 | hs|1p15.4 | 1.836 | -1.294 | 0.173000 |
| NETO2        | Neuropilin (NRP) and tolloid (TLL)-like 2 | 81831 | hs|16q12.1 | 10.418 | -1.545 | 0.191000 |
| IKZF2        | IKAROS family zinc finger 2 (Helios) | 22807 | hs|2q34 | 1.499 | -2.151 | 0.221000 |
| ZCHC2        | Zinc finger, CCHC domain containing 2 | 54877 | hs|18q21.33 | 2.171 | -1.391 | 0.247000 |
| CEBPA        | CCAAT/enhancer binding protein (C/EBP), alpha | 1050 | hs|19q13.1 | 1.531 | -1.176 | 0.320000 |
| ITGA3        | Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) | 3675 | hs|17q21.33 | 1.486 | -1.224 | 0.325000 |
| CPNE8        | Copine VIII | 144402 | hs|12q12 | 1.513 | -1.306 | 0.352000 |
| STAT1        | Signal transducer and activator of transcription 1, 91kDa | 6772 | hs|2q32.2 | 1.550 | -1.047 | 0.376000 |

(Continues)
The TargetScan Human database showed that there is a binding site for miR-101-5p (positions 219-225) in the DONSON 3′-UTR (Figure 3D). A luciferase reporter assay was carried out using vectors containing these sequences to see if miR-101-5p directly regulates DONSON expression depending on the sequence. Cotransfection of miR-101-5p with vectors

### TABLE 1 (Continued)

| Gene symbol | Gene name                                   | Entrez Gene ID | Cytoband         | GEO expression fold-change (tumor/normal) | Microarray analysis from TCGA database (high vs low expression, P value) |
|-------------|---------------------------------------------|----------------|------------------|------------------------------------------|-------------------------------------------------------------------------|
| STC2        | Stanniocalcin 2                             | 8614           | hs|5q35.1          | 6.507  | -1.152 | 0.440000 |
| NCF2        | Neutrophil cytosolic factor 2               | 4688           | hs|1q25.3          | 3.432  | -1.545 | 0.495000 |
| ZNF532      | Zinc finger protein 532                    | 55205          | hs|18q21.32        | 1.899  | -1.237 | 0.504000 |
| FAM78A      | Family with sequence similarity 78, member A| 286336         | hs|9q34.13         | 4.577  | -1.504 | 0.534000 |
| BAZ2A       | Bromodomain adjacent to zinc finger domain, 2A| 11176          | hs|12q13.3        | 1.700  | -1.433 | 0.739000 |
| MCTP1       | Multiple C2 domains, transmembrane 1        | 79772          | hs|5q15            | 3.092  | -1.720 | 0.749000 |
| RAB27A      | RAB27A, member RAS oncogene family           | 5873           | hs|15q21.3        | 1.452  | -1.602 | 0.766000 |
| CARD8       | Caspase recruitment domain family, member 8 | 22900          | hs|19q13.33       | 1.650  | -1.535 | 0.854000 |
| RPS6KA5     | Ribosomal protein S6 kinase, 90kDa, polypeptide 5| 9252           | hs|14q32.11       | 1.480  | -1.155 | 6.28E-06a |
| PCSK5       | Proprotein convertase subtilisin/kexin type 5| 5125           | hs|9q21.13        | 1.490  | -1.503 | 2.46E-05a |
| ZNF792      | Zinc finger protein 792                    | 126375         | hs|19q13.11       | 1.534  | -1.348 | 0.000156a |
| TGFα        | transforming growth factor, alpha            | 7039           | hs|2p13.3         | 2.497  | -1.122 | 0.000477a |
| NRP1        | Neuropilin 1                                | 8829           | hs|10p11.22       | 1.509  | -1.199 | 0.001140a |
| SPRY1       | Sprouty homolog 1, antagonist of FGF signaling (Drosophila)| 10252          | hs|4q28.1         | 1.622  | -1.125 | 0.003680a |
| CDH5        | Cadherin 5, type 2 (vascular endothelium)   | 1003           | hs|16q21          | 2.616  | -1.448 | 0.009350a |
| ICK         | Intestinal cell (MAK-like) kinase           | 22858          | hs|6p12.2         | 1.619  | -1.514 | 0.011900a |
| CDK19       | Cyclin-dependent kinase 19                  | 23097          | hs|6q21           | 2.174  | -1.108 | 0.016600a |
| NAA15       | N(alpha)-acetyltransferase 15, NatA auxiliary subunit| 80155          | hs|4q31.1         | 1.460  | -2.003 | 0.020100a |
| MLEC        | Malectin                                   | 9761           | hs|12q24.31       | 1.519  | -1.385 | 0.020400a |
| BDP1        | B double prime 1, subunit of RNA polymerase III transcription initiation factor IIB| 55814          | hs|5q13.2         | 1.448  | -1.272 | 0.021800a |
| CBL         | Cbl proto-oncogene, E3 ubiquitin protein ligase| 867            | hs|11q23.3        | 1.800  | -1.027 | 0.051800a |
| EMP1        | Epithelial membrane protein 1               | 2012           | hs|12p13.1        | 1.615  | -1.521 | 0.059700a |
| KDM5A       | Lysine (K)-specific demethylase 5A          | 5927           | hs|12p13.33       | 1.862  | -1.000 | 0.074900a |

GEO, Gene Expression Omnibus; miR, microRNA; OS, overall survival; RCC, renal cell carcinoma; TCGA, The Cancer Genome Atlas.

**Poor prognosis in patients with low gene expression.**

**FIGURE 4** DONSON knockdown assay by siRNA and the effect of cotransfection of DONSON/miR-101-5p. A, Cell proliferation activity after si-DONSON transfection into clear cell renal cell carcinoma ccRCC cells. B, Cell proliferation 72 h after reverse transfection with miR-101-5p and 48 h after forward transfection with the DONSON vector. C, Cell migration 48 h after reverse transfection with miR-101-5p and 24 h after forward transfection with the DONSON vector. D, Cell invasion 48 h after reverse transfection with miR-101-5p and 24 h after forward transfection with the DONSON vector. E-G, Effects of si-DONSON on apoptosis, as assessed by apoptosis assays and western blot analysis of cleaved poly (ADP-ribose) polymerase (PARP), a marker of apoptosis. GAPDH was used as the loading control. Adriamycin (ADM) was used as a positive control. H, Effect of si-DONSON on the cell cycle. Flow cytometric analyses of cell-cycle-phase distributions in control cells and cells transfected with si-DONSON. Bar charts represent the percentages of si-DONSON-transfected cells relative to the control cells in the G0/G1, S, and G2/M phases, respectively. *P < .0001; **P < .005
significantly suppressed luciferase activity compared to control cells ($P = .0012$) (Figure 3E).

3.6 | Clinical significance of DONSON expression in RCC patients

Conventional and propensity score-matched cohort analyses showed that patients with high DONSON expression showed poor prognosis (Figure 3F,G). The clinical background of the patients used for analysis is shown in Table S5. Furthermore, multivariate analysis has shown that gene expression is an independent prognostic factor (Figure 3H).

Combination analyses (miR-101-5p and DONSON) showed that patient group (low expression of miR-101-5p or high expression of DONSON) was a promising prognostic marker of patients with RCC (disease-free survival, $P < .001$; overall survival, $P < .001$) (Figure S5A,B). High expression of DONSON was involved in RCC pathogenesis, eg, tumor stage, metastasis, and grade (Figure S5C-G).

3.7 | Expression of DONSON in sunitinib-naïve and sunitinib-treated specimens

DONSON mRNA expression levels were significantly elevated in primary ccRCC tissues compared with those in adjacent noncancerous tissues ($P = .0051$) (Figure 3I). Furthermore, the expression levels in sunitinib-treated ccRCC tissues were highly expressed compared with those in primary ccRCC tissues ($P < .0001$) (Figure 3I).

In IHC staining, the expression of DONSON was gradually increased in the order of normal tissue, primary (sunitinib-naïve) RCC and sunitinib-treated ccRCC (Figure 3J-L).

3.8 | Knockdown assay and rescue study of DONSON in ccRCC cells

We confirmed that the expression levels of both DONSON mRNA and DONSON protein could be suppressed by si-DONSON transfection of ccRCC cells (Figure S6A,B). Downregulation of DONSON with siRNAs significantly attenuated cell proliferation (Figure 4A), migration and invasive potentials (Figure S6C,D).

In addition, introduction of both DONSON and miR-101-5p significantly restored cell proliferation, migration, and invasive activity, compared to cells transfected with miR-101-5p alone (Figure 4B-D). We confirmed that DONSON and miR-101-5p transfection restored DONSON protein expression (Figure S7).

Furthermore, the proportion of apoptotic cells was elevated in si-DONSON-transfected cells compared to control cells (Figure 4E,F). Transfection of si-DONSON did not apparently upregulate the level of cleaved PARP (Figure 4G). In cell cycle assays, the number of cells in the G$_2$/M phase were significantly elevated in si-DONSON transfected cells than control cells (Figure 4H).

3.9 | DONSON expression analyses combining clinical database and in vitro experiments

We identified differentially expressed genes that had similar expression behaviors to that of DONSON (Figure 5A). The GSEA showed that the top signaling pathway that was enriched in DONSON high RCC patients was the G$_2$/M checkpoint (Figure 5B). Furthermore, using the Kyoto Encyclopedia of Genes and Genomes pathways analysis, we found that the top significantly enriched pathway in 992 genes that had similar expression behavior to that of DONSON was DNA replication (Figure 5C,D). A heatmap visualization of gene expression of DNA replication pathway-related genes is shown in Figure 5E. Most of the genes coexpressed with DONSON were significantly associated with prognosis in ccRCC patients (Figure S8).

3.10 | Downstream genes mediated by DONSON in ccRCC cells

After microarray analysis, we identified 50 genes that were downregulated ($\log_2 < -1.0$) after transfection with si-DONSON (Table S6). DONSON expression was the most downregulated after si-DONSON transfection, indicating that these analyses were reliable and can be analyzed.

4 | DISCUSSION

A remarkable property of miRNA is that a single miRNA species can control a huge number of RNA transcripts under normal and pathologic conditions. Therefore, miRNA-controlled intracellular RNA networks are being investigated in cancer cells. From RNA-seq-based miRNA signatures, some passenger strands of miRNAs, eg, miR-144-5p, miR-455-5p, and miR-532-3p, possess antitumor activity in ccRCC cells and their target genes contributed to its pathogenesis.

Passenger strands of miRNAs are generally not examined. Therefore, characterization of miRNA passenger strands in cancer regulatory networks is important for the development of novel diagnostic approaches.

In this study, we focused on miR-101-5p (the passenger strand of pre-miR-101) and investigated the associated regulatory RNA networks in ccRCC cells. Previous studies of miR-101-3p (the guide strand of pre-miR-101) found that it was often downregulated in a wide range of human cancers and acted as an antitumor miRNA through its targeting of several oncogenes. Our miRNA signature of patients with sunitinib failure showed that miR-101-5p was the most downregulated miRNA in cancer tissues. Moreover, ectopic expression of miR-101-3p significantly blocked the aggressive phenotype. Direct control of enhancer of zeste homolog 2 (EZH2), which functions as an oncogene in various cancers, has been proven in many studies. Our previous study showed that antitumor miR-101-3p directly regulated ubiquitin like with phd and ring finger domains 1 (UHRF1) in ccRCC cells. Aberrant expression of UHRF1 was observed in several cancers and its overexpression facilitated cancer...
FIGURE 5 The Cancer Genome Atlas (TCGA) database analysis of clinical significance and function of DONSON in clear cell renal cell carcinoma. A, Identification of differentially expressed genes in the DONSON high group and the si-DONSON group. B, Gene Set Enrichment Analysis (GSEA) of mRNA expression levels of DONSON high RCC patients. C, Venn diagram showed the overlapped 992 genes among TCGA and RNAi. D, Significantly enriched pathways including 992 genes that showed similar expression behaviors with DONSON using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. E, Heatmap visualization of gene expression of DNA replication pathway-related genes.
cell malignancy. In contrast to those studies of miR-101-3p, investigation of miR-101-5p has rarely been undertaken.

In a few reports, miR-101-5p expression was found to be down-regulated. miR-101-5p was shown to inhibit cell aggressiveness through targeting of C-X-C motif chemokine ligand 6 (CXCL6) in cervical cancer and nonsmall-cell lung carcinoma. To our knowledge, the present study is the first report to show the antitumor functions of miR-101-5p in ccRCC. The elucidation of miR-101-5p-controlled novel oncogenic networks in ccRCC cells is particularly important.

In further analysis of miR-101-5p in ccRCC, we identified candidate target oncogenes using genome-wide expression analysis. A total of 38 oncogenes was found to be regulated by miR-101-5p. Of these oncogenic targets, the high expression of 13 genes was a significant prognostic factor for ccRCC. We focused on DONSON because it was directly regulated by miR-101-5p and it was the best predictor of poor prognosis of patients. Recently, DONSON was found to encode a novel fork protein factor and play an important role in mammalian DNA replication and genome stability. Moreover, its mutation caused microcephalic dwarfism. Previous studies showed that DONSON is a member of replisome complex and protected stalled or damaged replication forks. Also, DONSON interacts with several DNA replication factors and facilitated the activation of the intra-S-phase and G2/M cell cycle checkpoints. Minichromosome maintenance (MCM) proteins are crucial DNA replication genes that interact with DONSON. They are often overexpressed in ccRCC tissues and could be useful prognostic markers in ccRCC patients. Aberrant expression of DONSON was observed in sunitinib-treated ccRCC and silencing DONSON inhibited cell growth and induced apoptosis and cell cycle arrest in G2/M phase. Our GSEA data analysis revealed that the EMT pathway was significantly enriched in the DONSON high expression group. Previous reports showed that EMT-associated genes were overrepresented in TKI-resistant ccRCC tissues compared with pretreatment ccRCC tissues. Tyrosine kinase inhibitor-resistant RCC cells promoted the activities of EMT-related genes, indicating that EMT was involved in the mechanism of resistance to TKI. These findings might explain the aberrant expression of DONSON that was detected in sunitinib-treated ccRCC tissues. Also, our data showed that miR-101-5p regulated the expression of several EMT-related genes, indicating miR-101-5p might have a role in resistance to TKI treatment.

Expression of DONSON was an independent strong prognostic marker (better than tumor stage or pathological grade) and was associated with ccRCC patient survival in a propensity score-matched cohort. Aberrant expression of DONSON has serious effects on the prognosis of patients with ccRCC. However, elucidation of the molecular mechanism for controlling DONSON expression in ccRCC cells is not sufficient. Exploring the causes of overexpression of DONSON in ccRCC cells is an important issue. Recent studies showed that expression of circular RNAs in cancer cells have participated in oncogenesis. Interestingly, overexpression of circ-DONSON (derived from exon 3 to exon 8 of DONSON mRNA) was detected in gastric cancer and its aberrant expression promoted gastric cancer cell aggressiveness through initiated SOX4 expression. The oncogenic roles of the circ-DONSON in ccRCC cells need to be investigated in the future.

We searched genes/pathways in ccRCC cells that were mediated by DONSON. Interestingly, DNA replication, mismatch repair, nucleotide excision repair, and spliceosome pathways were identified as miR-101-5p regulatory pathways. Among these pathways, genes involved in the DNA replication pathway (DNA2, POLE, REFC4, LGI1, POLD4, POLA2, and RNASEH2A) predicted poor prognosis of ccRCC patients. The replisome is a complex molecular machine that comprises the DNA replication apparatus. The replisome unwinds double-stranded DNA into single strands. These findings suggest that aberrantly expressed genes involved in the replisome affected ccRCC pathogenesis.

In conclusion, this is the first research to report that miR-101-5p acted as an antitumor miRNA in ccRCC cells. Several oncogenic targets regulated by miR-101-5p were closely involved with ccRCC pathogenesis. Moreover, we found that DONSON and replisome genes, which we identified from analyses of genes controlled by antitumor miR-101-5p, could be novel prognostic and therapeutic targets in ccRCC.

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CONFLICT OF INTEREST
The authors declare no conflict of interest. NN is an employee of MSD K.K., a subsidiary of Merck & Co., Inc and reports personal fees from MSD K.K. outside this study.

ORCID
Yasutaka Yamada https://orcid.org/0000-0002-0070-1590
Nijiro Nohata https://orcid.org/0000-0002-6816-2984
Takayuki Arai https://orcid.org/0000-0002-3888-9576

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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