Regulation of Oncogenic Targets by the Tumor-Suppressive miR-139 Duplex (miR-139-5p and miR-139-3p) in Renal Cell Carcinoma

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Abstract: We previously found that both the guide and passenger strands of the miR-139 duplex (miR-139-5p and miR-139-3p, respectively) were downregulated in cancer tissues. Analysis of TCGA datasets revealed that low expression of miR-139-5p (p < 0.0001) and miR-139-3p (p < 0.0001) was closely associated with 5-year survival rates of patients with renal cell carcinoma (RCC). Ectopic expression assays showed that miR-139-5p and miR-139-3p acted as tumor-suppressive miRNAs in RCC cells. Here, 19 and 22 genes were identified as putative targets of miR-139-5p and miR-139-3p in RCC cells, respectively. Among these genes, high expression of PLXDC1, TET3, PXN, ARHGEF19, ELK1, DCBLD1, IKBKB, and CSF1 significantly predicted shorter survival in RCC patients according to TCGA analyses (p < 0.05). Importantly, the expression levels of four of these genes, PXN, ARHGEF19, ELK1, and IKBKB, were independent prognostic factors for patient survival (p < 0.05). We focused on PXN (paxillin) and investigated its potential oncogenic role in RCC cells. PXN knockdown significantly inhibited cancer cell migration and invasion, possibly by regulating epithelial–mesenchymal transition. Involvement of the miR-139-3p passenger strand in RCC molecular pathogenesis is a new concept. Analyses of tumor-suppressive-miRNA-mediated molecular networks provide important insights into the molecular pathogenesis of RCC.

Keywords: miR-139-5p; miR-139-3p; renal cell carcinoma (RCC); microRNA; tumor suppressor; paxillin (PXN)

1. Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies, with more than 330,000 cases newly diagnosed in 2018 and more than 100,000 deaths annually [1]. Clear cell RCC is the most common histological subtype of RCC, accounting for approximately 75% of all cases [2]. Approximately 20–30% of RCC patients have metastatic lesions at diagnosis, and thus the 5-year survival rate of these patients is less than 20% [2,3]. In addition, more than 20% of patients develop metastases during the postoperative follow-up period [4]. Elucidation of the molecular mechanisms involved in distant metastasis of RCC will contribute to the development of new diagnostic and therapeutic strategies.
MicroRNAs (miRNAs) are classified as noncoding RNAs approximately 18–25 bases long that are found in a wide range of organisms, from plants to humans [5]. miRNAs bind to the 3′-UTR of their target genes (protein coding and noncoding) in a sequence-dependent manner to regulate their expression. miRNAs are involved in various intracellular processes [6,7]. A unique property of these molecules is that a single miRNA can control a vast number of genes in normal and diseased cells [6,7]. Therefore, aberrant expression of miRNAs disrupts intracellular transcriptional networks, which in turn causes human diseases. In cancer cells, aberrant expression of miRNAs is closely associated with cancer cell progression, metastasis, and drug resistance [8,9].

According to the original theory regarding miRNA biogenesis, the passenger strand of the miRNA duplex is degraded and therefore considered to have no function [6,7]. Our RNA-sequencing-based signatures refute this concept, and our recent studies showed downregulation of both the guide and passenger strands of miRNA duplexes (e.g., miR-30c, miR-99a, miR-101, miR-143, miR-145, and miR-150) in cancer tissues [10–12]. Furthermore, ectopic expression assays revealed that the passenger strand of some miRNAs, similar to the guide strand, functions as tumor-suppressive miRNAs, regulating many oncogenes intracellularly [13–16]. Involvement of miRNA passenger strands in the molecular pathogenesis of human cancers is a new concept, and additional research on passenger strands is needed. A recent study confirmed that both strands of miRNAs are functional and, despite having different seed sequences, cooperate to control molecular pathways across cancer types [17].

In this study, we focused on miR-139-5p (the guide strand) and miR-139-3p (the passenger strand) and investigated their functional significance. We searched for oncogenes that are controlled by miR-139-3p in RCC cells in public databases and identified 22 genes as putative targets of miR-139-3p regulation. Among these genes, high expression of PXN, ELK1, ARHGEF19, DCBLD1, IKBKB, and CSF1 significantly predicted shorter survival in RCC patients according to The Cancer Genome Atlas (TCGA) analyses ($p < 0.05$). We discuss the oncogenic role of PXN in RCC cells.

2. Materials and Methods

2.1. Human RCC Cell Lines

The A498 and 786-O human RCC cell lines were used in this study and were obtained from the Japanese Collection of Research Bioresources Cell Bank. Cell maintenance was performed as we described previously [15].

2.2. RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. For gene expression assays, reverse transcription of the RNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) and TaqMan Gene Expression Assays (Applied Biosystems), according to our previous studies [13–16]. The expression of GAPDH was evaluated as the internal control. We used the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The TaqMan primers and probes used in this study are listed in Table S1.

2.3. Transfection of miRNAs, siRNAs, and Plasmid Vectors into RCC cells

Transfection of miRNAs and siRNAs into RCC cells was performed using Lipofectamine RNAiMAX reagent (Invitrogen) according to our previous studies [13–16]. miRNAs or siRNAs were added to a final concentration of 10 nM. Plasmid vectors were transfected into the cells using Lipofectamine 2000 (Invitrogen). The final concentration was 50 ng/well.
2.4. Functional Assays (Cell Proliferation, Migration, and Invasion Assays) in RCC cells

XTT assay for cell proliferation, wound healing assay for migration, and Matrigel chamber assay for invasion were performed using RCC cells as described previously [13–16]. The reagents used are listed in Table S1.

2.5. Identification of miR-139-5p and miR-139-3p Gene Targets in RCC Cells

The search strategy used to identify miRNA targets is summarized in Figure S1. The expression profiles of miR-139-5p/miR-139-3p-transfected A498 and 786-O cells from the Gene Expression Omnibus (GEO) GSE129043 dataset were used. GSE36895 datasets were also utilized (details below). The TargetScanHuman database (http://www.targetscan.org/vert_72/) was used to predict miRNA-binding sites.

2.6. In silico Analysis of RCC Public Databases

For comparison of gene expression levels between normal and cancer tissues, we utilized the GSE36895 datasets obtained from GEO. GSE36895 contains mRNA microarray data from clear cell RCC tumors, normal kidney tissues, and mouse tumor graft samples obtained using Affymetrix U133 Plus 2.0 whole-genome chips (Affymetrix, Santa Clara, CA, USA). Expression is shown as signal intensities, and for each gene with multiple probes, the mean intensity value was used. In addition, RNA sequencing data from TCGA—Kidney renal clear cell carcinoma (TCGA-KIRC) RNA sequencing datasets were utilized to re-evaluate gene expression levels in normal versus tumor samples.

2.7. Clinicopathological Analysis of RCC

For Kaplan–Meier analyses of overall survival, we downloaded TCGA clinical data (Firehose Legacy) from cBioportal (https://www.cbioportal.org). The patients were divided into two groups by median expression for each gene, according to the data collected from OncoLnc (http://www.oncolnc.org). R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for the analyses.

Multivariate Cox regression analyses were also performed using TCGA-KIRC clinical data and survival data according to the expression level of each gene from OncoLnc to identify factors associated with RCC patient survival. In addition to gene expression, the tumor stage, pathological grade, and age group were evaluated as potential independent prognostic factors. The multivariate analyses were performed using JMP Pro 15.0.0 (SAS Institute Inc., Cary, NC, USA).

We performed gene set enrichment analysis (http://software.broadinstitute.org/gsea/index.jsp) to obtain lists of differentially expressed genes between high and low PXN expression groups in the TCGA-KIRC cohort.

2.8. Plasmid Construction and Dual-Luciferase Reporter Assays

psiCHECK-2 plasmid vectors (Promega, Madison, WI, USA) harboring the wild-type or a deletion sequence of the miR-139-3p-binding site within the 3′-UTR of PXN were prepared. The predicted binding site sequence was obtained from the TargetScanHuman database (release 7.2). Cells were co-transfected with miR-139-3p and the plasmid vectors for 36 h, after which firefly and Renilla luciferase activities in cell lysates were measured consecutively using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activities are expressed as normalized values to firefly luciferase activities. The dual-luciferase reporter assay procedure was described in our previous studies [13–16].

2.9. Western Blotting

Cell lysates were prepared 48 h after transfection with RIPA buffer (Nacalai Tesque, Chukyo-ku, Kyoto, Japan). Then, 20 µg of protein lysates were separated on 4–15% Mini-PROTEAN TGX Precast
Gels (Bio-Rad), and transferred to Immun-Blot PVDF membranes (Bio-Rad). Blocking was performed with Blocking One (Nacalai Tesque) for 30 min. The antibodies used in this study are shown in Table S1.

2.10. Statistical Analyses

For comparisons among multiple groups, Dunnett’s test was applied. The statistical analyses were performed using JMP Pro 15. Significance levels were set to $p < 0.05$ if not otherwise mentioned.

3. Results

3.1. Analysis of miR-139-5p and miR-139-3p Expression Levels in Clinical RCC Tissues and Their Clinical Significance

The expression levels of miR-139-5p and miR-139-3p were evaluated in RNA sequencing data from RCC tissue samples obtained from TCGA. miR-139-5p and miR-139-3p were significantly downregulated in RCC tissues compared with normal tissues ($p < 0.0001$ and $p < 0.0001$, respectively; Figure 1A). Cohort analyses using the TCGA-KIRC datasets revealed that low expression of miR-139-5p and miR-139-3p was associated with poorer survival in patients with RCC ($p < 0.0001$ and $p < 0.0001$, respectively; Figure 1B).

Figure 1. The expression and clinical significance of miR-139-5p and miR-139-3p in renal cell carcinoma (RCC) clinical specimens. (A) Expression of miR-139-5p and miR-139-3p were significantly reduced in The Cancer Genome Atlas—Kidney Renal Clear Cell Carcinoma (TCGA-KIRC) cancer specimens compared with adjacent normal specimens ($p < 0.001$). (B) Spearman’s rank test showed positive correlations between the expression levels of miR-139-5p and miR-139-3p in TCGA-KIRC clinical specimens ($R = 0.8278$, $p < 0.001$). (C) Kaplan–Meier survival curves of patients from TCGA-KIRC cohort. Patients were divided into two groups according to the median expression levels of miR-139-5p or miR-139-3p: high- and low-expression groups. Both miR-139-5p and miR-139-3p expression levels were significantly associated with the 5-year survival rate of RCC patients ($p < 0.0001$).
3.2. Tumor-Suppressive Functions of miR-139-5p and miR-139-3p in RCC Cells

To investigate the tumor-suppressive functions of miR-139-5p and miR-139-3p in RCC cells, we assessed cell proliferation, migration, and invasion after ectopic transfection of miR-139-5p and miR-139-3p into A498 and 786-O cells. Ectopic expression of the two miRNAs did not significantly affect the proliferation of RCC cells (Figure 2A). In contrast, the expression of these miRNAs significantly inhibited the migration and invasive abilities of RCC cells (Figure 2B,C).

Figure 2. Functional assays of cell proliferation, migration, and invasion following ectopic expression of miR-139-5p and miR-139-3p in RCC cell lines A498 and 786-O. N.S.: not significant. (A) Cell proliferation was assessed using XTT assays. Data were collected 72 h after miRNA transfection. (B) Cell migration was assessed using wound healing assays. (C) Cell invasions were determined 48 h after seeding miRNA-transfected cells into chambers using Matrigel invasion assays.
3.3. Identification of Putative Oncogenic Targets Regulated by miR-139-3p and miR-139-5p in RCC Cells

To identify the genes regulated by miR-139-5p and miR-139-3p in RCC cells, we integrated three datasets. First, we obtained RNA microarray data from miR-139-5p- or miR-139-3p-transfected A498 or 786-O cells (GSE129043). Second, we used data from the TargetScanHuman database (release 7.2) to obtain annotated putative targets regulated by each miRNA strand. Third, we extracted genes highly expressed in clinical specimens of RCC from GSE36895.

A schematic of the strategy used to narrow down the gene list is shown in Figure S1. A total of 19 genes regulated by miR-139-5p and 22 by miR-139-3p were finally selected (Table 1A,B).

| Gene Symbol | Gene Name | Entrez Gene ID | miR-139-5p Transfected A498 Cells (log2 FC) | miR-139-5p Transfected 786-O Cells (log2 FC) | GSE36895 | Total Binding Sites | TCGA 5y OS p-Value |
|-------------|-----------|---------------|------------------------------------------|------------------------------------------|------------|----------------------|-------------------|
| PLDCT1      | plexin domain containing 1 | 876869 | -0.615791 | -1.455703 | 1.6528159 | 1 | 0.0098 |
| TET3        | tet methylcytosine dioxygenase 3 | 200424 | -1.074818 | -1.152035 | 0.7350367 | 1 | 0.0264 |
| IRF4        | | 3662 | -2.491284 | -1.563216 | 0.72956836 | 1 | 0.0575 |
| RAR27B      | RAR27B, member RAS oncogene family | 5997 | -0.620716 | -0.979475 | 0.7823266 | 1 | 0.1244 |
| FCHSD2      | FCH and double SH3 domains 2 | 9873 | -1.834753 | -1.364281 | 0.5676638 | 1 | 0.1565 |
| DMD         | dystrophin | 1756 | -0.86734 | -1.1249 | 0.469447 | 1 | 0.3991 |
| APOL6       | apolipoprotein L, 6 | 80380 | -1.363298 | -0.574163 | 0.43652818 | 2 | 0.4733 |
| APIS2       | adapter-related protein complex 1, sigma 2 subunit | 8905 | -0.571341 | -0.63469 | 0.57979524 | 1 | 0.5072 |
| PTTRU       | protein tyrosine phosphatase, receptor type, U | 10076 | -1.430874 | -0.883479 | 0.8481335 | 1 | 0.6152 |
| TRAT1       | T cell receptor associated transmembrane adaptor 1 | 50852 | -1.192173 | -1.781637 | 1.9733818 | 2 | 0.7395 |
| SLCA8A1     | solute carrier family 39 (zinc transporter), member 14 | 23516 | -0.536152 | -0.7059 | 1.040825 | 1 | 0.8125 |
| OTUD14      | OTU deubiquitinate 4 | 54726 | -1.720465 | -1.59549 | 0.21931966 | 1 | 0.9338 |
| CDC47L      | cell division cycle associated 7-like | 55556 | -1.478013 | -0.564377 | 2.1739191 | 1 | 0.969 |
| ZNF678      | zinc finger protein 678 | 339550 | -1.585917 | -0.621852 | 0.25128952 | 2 | 0.0086 |
| FGBBP2      | fibrillogrowth factor binding protein 2 | 83388 | -0.830705 | -1.317887 | 1.5742466 | 1 | 0.0058 |
| APF2B2      | ATPase, Ca++ transporting, plasma membrane 2 | 491 | -1.109898 | -2.668194 | 1.3086246 | 3 | 0.0050 |
| EML1        | echinoerd microtubule associated protein like 1 | 2009 | -0.569747 | -0.549781 | 0.38891175 | 2 | 0.014 |
| PCSK5       | proprotein convertase subtilisin/kexin type 5 | 5125 | -1.751302 | -0.57702 | 0.57546955 | 1 | 0.0006 |
| FAM168A8    | family with sequence similarity 16, member A | 23201 | -1.390649 | -0.959992 | 0.25754136 | 1 | 0.0002 |

* Better prognosis in high-expression group.

Table 1. A. Candidate target genes of miR-139-5p. B. Candidate target genes of miR-139-3p.
3.4. Clinical Significance of miR-139 Target Genes in RCC Pathogenesis

Kaplan–Meier analyses of 5-year overall survival were performed according to high versus low expression of miR-139 target genes. The high expression of two miR-139-5p (PLXDC1 and TET3) and six miR-139-3p target genes (PXN, ARHGEF19, ELK1, CSF1, IKBKB, and DCBLD1) was found to be related to better 5-year overall survival rates of the patients ($p < 0.05$, Table 1A,B, and Figure 3).

![Figure 3](image-url)

**Figure 3.** Clinical significance of miR-139-5p or miR-139-3p target genes in TCGA-KIRC database. High expression of two miR-139-5p target genes (PLXDC1 and TET3) and six miR-139-3p target genes (PXN, ARHGEF19, ELK1, CSF1, IKBKB, and DCBLD1) were significantly associated with poor prognosis in patients with RCC. Kaplan–Meier curves for 5-year overall survival according to the expression of each miR-139-3p target gene are shown.

Furthermore, multivariate analyses identified four genes (PXN, ARHGEF19, ELK1, and IKBKB) as independent prognostic factors for patient survival ($p < 0.05$; Figure 4). We validated the expression levels of these target genes using TCGA-KIRC RNA sequencing dataset. All target genes except ARHGEF19 were upregulated in cancer tissues in this cohort (Figure 5).
Figure 4. Forest plot showing the results of multivariate analyses of eight genes (PLXDC1, TET3, PXN, ARHGEF19, ELK1, CSF1, IKBBB, and DCBLD1). In addition to the gene expression level, the tumor stage, pathological grade, and age group were evaluated as potential independent factors associated with survival. Numbers of cases per each group are shown in Table S2.
For subsequent analyses, we focused on miR-139-3p (the passenger strand) target genes, according to our previous studies emphasizing the novel roles of miRNA passenger strands. Among these target genes, we focused on PXN, which showed the most significant association with RCC patient survival in the multivariate analysis and has been reported previously as an oncogene in other types of cancers [18,19].
3.5. Direct Regulation of PXN by miR-139-3p in RCC Cells

In RCC cells transfected with miR-139-3p, both PXN mRNA and protein levels were significantly downregulated (Figure 6A,B). Western blot images are shown in Figure S2. To validate that miR-139-3p binds directly to PXN to downregulate its expression, we performed dual-luciferase reporter assays. A498 and 786-O cells were co-transfected with plasmid vectors and miR-139-3p. We used two different plasmid vectors: one containing the partial wild-type sequence of the miR-139-3p-binding site predicted by TargetScanHuman database (“wild-type sequence” in Figure 6C) and the other containing this sequence lacking the binding site (“deletion-type sequence” in Figure 6C). Luciferase activity was significantly reduced in cells transfected with the wild-type sequence but not in cells transfected with the deletion sequence (Figure 6D). These results suggest that miR-139-3p directly binds to the 3'-UTR of PXN.

Figure 6. Expression of paxillin (PXN) was regulated directly by miR-139-3p in RCC cells. (A) Expression of PXN mRNA was significantly suppressed in miR-139-3p-transfected A498 and 786-O cells (48 h after transfection). Expression of GAPDH was used as an internal control. (B) Expression of PXN protein was reduced in miR-139-3p-transfected RCC cells (48 h after transfection). GAPDH was used as a loading control. (C) The TargetScanHuman database predicted one putative miR-139-3p-binding site in the 3' UTR of PXN. (D) Dual-luciferase reporter assays showed decreased luminescence activity in RCC cells co-transfected with miR-139-3p together with a vector harboring the “wild-type sequence”. Normalized data were calculated as Renilla/firefly luciferase activity ratios. N.S.: not significant.

3.6. PXN Knockdown Assays in RCC Cells

We assessed the oncogenic functions of PXN in RCC cells by performing knockdown using two small interfering RNAs (siRNAs) targeting PXN. The mRNA and protein levels of PXN were successfully downregulated by either siRNAs in A498 and 786-O cells (Figure 7A,B). Western blot images are shown in Figure S2.
Figure 7. Effects of PXN knockdown on cell proliferation, migration, and invasion in RCC cells. (A, B) Expression of PXN was successfully reduced after siRNA transfection in RCC cells. (C) Cell proliferation was determined by XTT assays. N.S.: not significant. (D) Cell migration was determined by wound healing assays. (E) Cell invasion was determined by Matrigel invasion assays.

Next, functional assays were performed in RCC cells transfected with these siRNAs. Similar to miR-139-3p transfection, transfection of the siRNAs did not suppress cell proliferation (Figure 7C). However, cell migration and invasion were significantly suppressed by siRNA transfection in both cell lines (Figure 7D,E).

3.7. PXN-Mediated Pathways in RCC Cells

We performed gene set enrichment analysis to determine differentially expressed genes between the high and low PXN expression groups of the TCGA-KIRC cohort. In support of the functional assays using siRNAs, the most enriched signaling pathway in the high PXN expression group was “epithelial–mesenchymal transition” (Figure 8 and Table S3). Other significantly enriched pathways (FDR q-value < 0.05) were “hypoxia”, “KRAS signaling”, “myogenesis”, “angiogenesis”, and “apical junction”, supporting the hypothesis that PXN is related to the metastatic ability of cancer cells (Figure 7 and Table S2).
Figure 8. Pathways enriched among the differentially expressed genes in the high PXN expression group according to gene set enrichment analysis. The six significantly enriched pathways (FDR q-value < 0.05) are shown.
4. Discussion

Recent RNA sequencing techniques have accelerated the establishment of miRNA expression signatures. Our recent studies revealed that some passenger strands of miRNAs (e.g., miR-30c-2-3p, miR-99a-3p, miR-101-5p, miR-143-5p, miR-145-3p, and miR-150-3p) act as tumor-suppressive miRNAs by targeting several oncogenes in a wide range of cancers [10–16]. Involvement of the passenger strands of miRNAs in cancer pathogenesis is a new development in cancer research.

A recent in silico study (analysis of molecular profiles in more than 5200 patient samples from 14 different cancers) revealed that both strands of miRNAs are functional across different cancer types. For example, downregulation of both strands of miR-30a and miR-145 was frequently observed in multiple cancers, and their downregulation enhanced aberrant expression of cell cycle-related genes. These malignant events were found to affect the prognosis of cancer patients [17]. Simultaneous analysis of both strands of miRNA duplexes will lead to elucidation of a new molecular mechanism in cancer cells.

In this study, we showed that both strands of the miR-139 duplex acted as tumor-suppressive miRNAs in RCC cells. Accumulating evidence has shown a tumor-suppressive role of miR-139-5p in multiple types of cancers, including RCC [20–22]. Notably, miR-139-5p target genes are involved in cancer activation pathways, e.g., the PI3K/AKT/mTORC1, Wnt/β-catenin, and RTK/RAS/MAPK pathways [23–27]. Therefore, miR-139-5p plays a central role in controlling the malignant transformation of human cancers.

In contrast to miR-139-5p, few studies have evaluated the importance of miR-139-3p in cancer because it is a passenger strand. Recently, it was reported that miR-139-3p directly regulates KDM5B (lysine demethylase 5B), a key regulator of histone 3 lysine 4 demethylation in laryngeal squamous cell carcinoma [28]. Ectopic expression of miR-139-3p suppressed cancer cell malignant behavior by inhibiting the Wnt/β-catenin pathway [28]. In the HeLa cervical cancer cell line, miR-139-3p was reduced upon increasing expression of circular RNA hsa_circ_0031288 [29]. In turn, the decreased expression of miR-139-3p resulted in increased expression of B-cell CLL/lymphoma 6, as a target molecule. These regulatory effects promoted malignant transformation of HeLa cells. In bladder cells, both strands of the miR-139 duplex were downregulated in cancer tissues and exhibited tumor-suppressive effects [30]. Interestingly, matrix metalloprotease 11 (MMP11) was directly regulated by both miR-139 strands, and knockdown of MMP11 attenuated the aggressive phenotype of bladder cancer cells [30]. Our study is the first to show that miR-139-3p (the passenger strand) is involved in RCC pathogenesis, and both strands of the miR-139 duplex are pivotal players in RCC oncogenesis.

We are also interested in the presence of oncogenes controlled by tumor-suppressive miR-139-5p and miR-139-3p in RCC cells. A total of 19 and 22 genes were identified as putative targets of miR-139-5p and miR-139-3p regulation in RCC cells, respectively. Taking advantage of survival data from TCGA datasets, we performed multivariate analysis and found that, among the miR-139 duplex target genes, PXN, ELK1, ARHGEF19, and IKBKB were independent prognostic factors for RCC patient survival. Further genomic analyses of these genes will contribute to elucidating the molecular pathogenesis of RCC.

ELK1 is a well-established transcription factor that is phosphorylated by MAPKs and induces transcription of the c-fos proto-oncogene [31,32]. ARHGEF19 is a RhoGEF that reportedly activates the MAPK pathway and interacts with BRAF in lung cancer [33]. The role of ARHGEF19 in RCC is not well characterized, but the interaction of ARHGEF19 with ELK1 via the MAPK pathway may play a role in RCC development. IKBKB encodes IKK-beta, which phosphorylates the inhibitor of NF-kB, resulting in activation of the NF-kB pathway [34,35]. NF-kB and its downstream inflammatory signaling pathway are closely related to RCC carcinogenesis and aggressiveness [36].

In this study, we focused on PXN (paxillin) because its expression was found to be directly controlled by miR-139-3p in RCC cells and strongly related to RCC molecular pathogenesis. PXN is a focal adhesion scaffold/adaptor protein that contains five LD domains (leucine-aspartate motifs) located at the N-terminus and four cysteine–histidine-rich LIM domains at the C-terminus [37,38]. The LD
domains act as a docking site for focal adhesion-related proteins, e.g., Src (tyrosine-protein kinase), FAK (focal adhesion kinase), PAK (p21-activated kinase), and ILK (integrin-linked kinase) [37,38]. The LIM domains act as binding sites for protein–protein interactions [37,38]. The pathways activated by PXN enhance cancer cell malignant progression and metastasis in a wide range of cancers [39]. Moreover, two key proteins in focal adhesion complexes, PXN and integrin B4, directly bind to each other, and this complex enhanced cisplatin resistance in lung cancer cells [40]. Another study showed that PXN phosphorylation may contribute to cisplatin resistance via the ERK-mediated activation of Bcl-2 transcription in lung cancer [41]. PXN-mediated pathways may be therapeutic targets for attenuating drug-resistance in cancer cells.

In our GSEA analysis, PXN-high-expressed RCC specimen was enriched with epithelial-mesenchymal transition (EMT) signaling pathways. A previous report has shown the suppressive effect of PXN on EMT pathway in cell lines [42]. These findings suggest the speculation that inhibitions of cancer cell migration and invasion by PXN knockdown in our study are due to the regulation of epithelial-mesenchymal transition.

It has been reported that several miRNAs (e.g., miR-132, miR-137, miR-145, miR-212, and miR-218) directly control PXN expression in cancer cells [43–47]. Among these miRNAs, we previously reported the downregulation of miR-145 and miR-218 in RCC cells [48,49]. More recently, DLX6-AS1 (a long noncoding RNA that adsorbs miRNA-199b) promoted epithelial–mesenchymal transition and cisplatin resistance via the miR-199b-5p/PNX axis in breast cancer cells [50]. This is the first report that PXN is directly regulated by tumor-suppressive miR-139-3p in RCC cells. Non-coding RNA-mediated epigenetic regulation of PXN expression will be assessed in the future.

5. Conclusions

Both strands of the miR-139 duplex are closely involved in RCC oncogenesis. This is the first report to reveal that miR-139-3p (the passenger strand) acts as a tumor-suppressive miRNA in RCC. Several genes are controlled by miR-139 and contribute to RCC molecular pathogenesis. Notably, the expression of PXN was directly regulated by miR-139-3p, and its overexpression enhanced RCC malignant transformation. Analyses of tumor-suppressive miRNAs (including the passenger strands) will contribute to the elucidation of new molecular networks in RCC.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9059/8/12/599/s1, Figure S1: Schematic of the strategy used to narrow down the miR-139-5p/miR-139-3p target genes, Figure S2: Western blot images, Table S1: Reagents used in this study, Table S2: The number of cases per group for multivariate analysis, Table S3: Gene set enrichment analysis of genes enriched among the differentially expressed genes in the TCGA-KIRC high PXN expression group.

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