Regulation of the collagen cross-linking enzymes LOXL2 and PLOD2 by tumor-suppressive microRNA-26a/b in renal cell carcinoma

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Abstract. Our recent studies of microRNA (miRNA) expression signatures in human cancers revealed that microRNA-26a (miRNA-26a) and microRNA-26b (miRNA-26b) were significantly reduced in cancer tissues. To date, few reports have provided functional analyses of miR-26a or miR-26b in renal cell carcinoma (RCC). The aim of the present study was to investigate the functional significance of miR-26a and miR-26b in RCC and to identify novel miR-26a/b-mediated cancer pathways and target genes involved in RCC oncogenesis and metastasis. Downregulation of miR-26a or miR-26b was confirmed in RCC clinical specimens. Restoration of miR-26a or miR-26b in RCC cell lines (786-O and A498) revealed that these miRNAs significantly inhibited cancer cell migration and invasion. Our in silico analysis and luciferase reporter assays showed that lysyl oxidase-like 2 (LOXL2) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) were directly regulated by these miRNAs. Moreover, downregulating the PLOD2 gene significantly inhibited cell migration and invasion in RCC cells. Thus, our data showed that two genes promoting metastasis, LOXL2 and PLOD2, were epigenetically regulated by tumor-suppressive microRNAs, miR-26a and miR-26b, providing important insights into the molecular mechanisms of RCC metastasis.

Introduction

Renal cell carcinoma (RCC) is a disease in which cells in the kidney tubules undergo oncogenic transformation. RCC has multiple subtypes and may occur in hereditary (2-3% of RCC) or sporadic forms (1,2). RCC is the third most common urological cancer and accounts for 3% of all adult neoplasias. Clear cell RCC (cRCC) is the most common subtype of sporadic RCC (~80%) (1). The standard curative treatment for localized diseases remains surgical excision with total nephrectomy. In contrast, at diagnosis, ~30% of RCCs have already metastasized. The 5-year survival rate in patients with advanced stage RCC is poor (5-10%) due to recurrence or distant metastasis (3,4). Recent molecularly targeted therapy has improved the survival rate of patients with advanced RCC (5,6). However, almost all patients eventually relapse or show distant metastasis due to acquired resistance to molecularly targeted therapy. Identifying molecular pathways responsible for RCC metastasis could provide novel approaches for the development of therapies that block the RCC metastatic pathways.

The discovery of microRNA (miRNA) in the human genome provided new directions in cancer research. The miRNAs are endogenous small RNA molecules (19-22 bases long) that regulate protein coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (7,8). Numerous studies have shown that miRNAs are aberrantly expressed in many human cancers, and they have significant roles in the initiation, development and metastasis of those cancers (9-11). Moreover, normal regulatory mechanisms can be disrupted by the aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. Therefore, identifying aberrantly expressed miRNAs is an important first step toward elucidating miRNA-mediated oncogenic pathways.

Using miRNA expression signatures, we have identified molecular pathways in RCC that are mediated by aberrantly expressed miRNAs (12-15). For example, downregulation of tumor-suppressive miR-218 promoted cancer cell migration and invasion through dysregulation of the focal adhesion pathway. In this regard, caveolin-2 has an oncogenic function in RCC cells (13). The epithelial-mesenchymal transition (EMT)-related miR-200 family (miR-200a/b/c, miR-141 and miR-429) is significantly downregulated in RCC where they act as tumor suppressors that target the focal adhesion and ErbB signaling pathways (14). The miR-143/145 cluster was frequently reduced in RCC tissues; restoration of these miRNAs significantly inhibited RCC cell proliferation and invasion through targeting of hexokinase-2 (16). More recently, expression of the miR-23b/27b cluster was significantly decreased in ccRCC.

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Table I. Characteristics of ccRCC clinical specimens.

| No. | Pathology | Grade | pT  | INF | v | ly | eg or ig | fc | im | rc | rp | s  |
|-----|-----------|-------|-----|-----|---|----|---------|----|----|----|----|----|
| 1   | Clear cell | G2    | T1a | a   | 0 | 0 | eg      | 1  | 0  | 0  | 0  | 0  |
| 2   | Clear cell | G1>G2 | T1a | a   | 0 | 0 | eg      | 1  | 0  | 0  | 0  | 0  |
| 3   | Clear cell | G3>G2 | T1b | a   | 0 | 0 | eg      | 1  | 0  | 0  | 0  | 0  |
| 4   | Clear cell | G2>G3 | G1 | T1a | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 5   | Clear cell | G2>G3 | 1 | T1b | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 6   | Clear cell | G2>G3 | 3 | T3a | a | 1 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 7   | Clear cell | G2>G3 | G1 | T3a | b | 1 | 0 | ig | 0 | 1 | 1 | 0 | 0 |
| 8   | Clear cell | G2>G3 | G1 | T3a | b | 1 | 0 | ig | 0 | 0 | 0 | 0 | 0 |
| 10  | Clear cell | G1>G2 | 1 | T1b | a | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 11  | Clear cell | G2>G1 G3 | 3 | T3a | b | 1 | 0 | ig | 0 | 0 | 0 | 0 | 0 |
| 12  | Clear cell | G2    | 2 | T1a | a | 0 | 0 | eg | 0 | 0 | 0 | 0 | 0 |
| 13  | Clear cell | G2>G1 G3 | T1b | b | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 14  | Clear cell | G2>G1 | G1 | T1a | b | 0 | 0 | eg | 1 | 0 | 0 | 0 | 0 |
| 15  | Clear cell | G2    | 2 | T1b | a | 0 | 0 | eg | 0 | 0 | 0 | 0 | 0 |

INF, infiltration; v, vein; ly, lymph node; eg, expansive growth; ig, infiltrative growth; fc, capsular formation; im, intrarenal metastasis; rc, renal capsule invasion; rp, pelvis invasion; s, sinus invasion.

tissues and associated with pathological grade and stage of the disease (17).

Our miRNA expression signatures of human cancers revealed that miR-26a and miR-26b were frequently downregulated in various types of cancer tissues (10,18,19), suggesting that these miRNAs act as tumor suppressors targeting several oncogenic pathways. Database searches revealed that there were few reports of functional analyses of miR-26a or miR-26b in RCC. The aim of the present study was to investigate the functional significance of miR-26a and miR-26b and to identify molecular targets and pathways contributing to metastasis in RCC cells by miR-26a or miR-26b regulation. We expect that this analysis will provide important insights into the potential molecular mechanisms of RCC oncogenesis and metastasis and will facilitate the development of therapeutic strategies for the treatment of the disease.

Materials and methods

RCC clinical specimens and cell culture. A total of 15 pairs of ccRCC specimens and corresponding non-cancerous specimens were collected from patients who had undergone radical nephrectomy at Chiba University Hospital (Chiba, Japan) from 2012 to 2015. These specimens were staged according to the General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma based on the American Joint Committee on Cancer (AJCC)-UICC TNM classification. The clinicopathological characteristics of the patients are summarized in Table I. Before tissue collection, written informed consent of tissue donation for research purposes was obtained from all the patients.

We used two human RCC cell lines (786-O and A498) obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) as previously described (12-14).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The procedure for PCR quantification was previously described. TaqMan probes and primers for LOXL2 (P/N: Hs00158757_ml; Applied Biosystems, Foster City, CA, USA), PLOD2 (P/N: Hs01118190_ml; Applied Biosystems) and GUSB (the internal control; P/N: Hs00939627_ml; Applied Biosystems) were assay-on-demand gene expression products. The expression levels of miR-26a (assay ID: 000405; Applied Biosystems) and miR-26b (assay ID: 000407; Applied Biosystems) were analyzed by TaqMan quantitative real-time RT-PCR (TaqMan MicroRNA assay; Applied Biosystems) and normalized to the expression of RNU48 as previously described (12,20,21).

Transfection with mature miRNAs and siRNAs. The following mature miRNAs were used: Ambion Pre-miR miRNA precursor for hsa-miR-26a-5p (product ID: PM10249; Applied Biosystems) and for hsa-miR-26b-5p (product ID: PM12899; Applied Biosystems). The following siRNAs were used: Stealth Select RNAi si-RNA, si-PLOD2 (cat nos. HSS108124 and HSS182371; Invitrogen) and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax transfection reagent (Invitrogen) as previously described (12,20,21).

Cell proliferation, migration and invasion assays. 786-O and A498 cells were transfected with 10 nM miRNAs or si-RNAs by reverse transfection. Cell proliferation, migration and invasion assays were performed as previously described (12,20,21).

Western blotting. Cells were harvested 72 h after transfection, and lysates were prepared. Protein lysates (20 µg) were separated on Mini-PROTEAN TGX gels (Bio-Rad Laboratories,
Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with rabbit anti-LOXL2 antibodies (1:1000; ab96233; Abcam, Cambridge, UK) and rabbit anti-PLOD2 antibodies (1:300; 21214-1-AP; Proteintech Group, Inc., Chicago, IL, USA). Anti-GAPDH antibodies (1:1,000; ab8245; Abcam) were used as an internal loading control. The membranes were washed and incubated with anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies (#7074; Cell Signaling Technology). Complexes were visualized with Clarity Western Substrate (Bio-Rad Laboratories).

Screening of miR-26a and miR-26b target genes using in silico analysis and gene expression data. To identify miR-26a/b target genes, we used in silico analysis and genome-wide gene expression analysis. First, we screened genes using TargetScan release 6.2 (http://www.targetscan.org/). Next, to identify upregulated genes in ccRCC clinical specimens, we analyzed publicly available gene expression profiles in the GEO database (accession nos. GSE22541 and GSE36895). Our strategies for miRNA target screening were previously described (12,20,21).

Plasmid construction and dual-luciferase reporter assay. Partial wild-type sequences of the LOXL2 and PLOD2 3′-untranslated region (UTR) or those with deleted miR-26a/b binding sites were inserted between the Xhol-PmeI restriction sites in the 3′-UTR of the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure for the dual-luciferase reporter assay was previously described (12,20,21).

Statistical analysis. The relationships between the two groups and the numerical values obtained by real-time RT-PCR were analyzed using the Mann-Whitney U-test. The relationships among the three variables and numerical values were analyzed using the Bonferroni-adjusted Mann-Whitney U test. Spearman's rank test was used to evaluate the correlations between the expression of (miR-26a and LOXL2), (miR-26a and PLOD2), (miR-26b and LOXL2) and (miR-26b and PLOD2). All analyses were performed using Expert StatView (version 5; SAS Institute, Inc., Cary, NC, USA).

Results

Expression levels of miR-26a and miR-26b in ccRCC clinical specimens and cell lines. The expression levels of miR-26a and miR-26b were significantly lower in ccRCC specimens than in corresponding non-cancerous specimens (P=0.0171 and P=0.0136, respectively; Fig. 1A and B). In 786-O and A498 cells, the expression levels of miR-26a or miR-26b were lower than in non-cancerous specimens.
Effects of miR-26a and miR-26b restoration on cell proliferation, migration and invasion activities in ccRCC cells. To investigate the functional effects of miR-26a or miR-26b, we performed gain-of-function studies using mature miRNA transfection of 786-O and A498 cells. The XTT assays demonstrated that cell proliferation was not inhibited in miR-26a or miR-26b transfectants in comparison with the mock or miR-control transfectants (Fig. 1C). In contrast, the migration assays demonstrated that cell migration activity was significantly inhibited in miR-26a or miR-26b transfectant cells in comparison with the mock or miR-control transfectants (Fig. 1D). The Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited in miR-26a or miR-26b transfectant cells in comparison with the mock or miR-control transfectants (Fig. 1E).

Identification of candidate target genes of miR-26a and miR-26b in ccRCC cells. To identify target genes of miR-26a and miR-26b (the seed sequences of the two miRNAs are identical), we used in silico analysis and genome-wide gene expression data. First, we searched the TargetScan database (release 6.2: http://www.targetscan.org/) and identified 3,419 genes that had putative target sites for miR-26a and miR-26b in their 3'-UTRs. Next, we pared down the list of putative candidate genes based on upregulated genes determined by the gene expression data set of RCC clinical specimens in the GEO (Gene Expression Omnibus) database (accession numbers: GSE36895, GSE22541). The flow chart outlining our strategy for identification of candidate target genes of miR-26a and miR-26b is shown in Fig. 2.

From this selection, 39 candidate genes were identified as targets of miR-26a and miR-26b (Table II). Among these candidate genes, we focused on LOXL2 and PLOD2 genes because these genes have two conserved target sites for miR-26a and miR-26b in their 3'-UTRs, and function as collagen cross-linking enzymes associated with extracellular matrix (ECM) stiffness. Recent studies showed that aberrantly expressed ECM contributes to cancer cell metastasis (22,23). Therefore, these two genes were chosen for further analysis.

Direct regulation of LOXL2 and PLOD2 by miR-26a and miR-26b in ccRCC cells. We first performed qRT-PCR and Western blotting to investigate whether expression of the LOXL2 gene and protein were reduced by restoration of miR-26a or miR-26b in 786-O and A498 cells. We found that the mRNA and protein expression levels of LOXL2/LOXL2 were significantly repressed in miR-26a or miR-26b transfectant cells in comparison with mock or miR-control transfectants (Fig. 3A and B).
**Table II. Putative candidate target genes regulated by miR-26a and miR-26b in RCC cells.**

| Entrez gene ID | Symbol | Gene name | Location | No. of conserved target sites | No. of poorly conserved target sites | GEO average fold-change |
|----------------|--------|-----------|----------|-------------------------------|-------------------------------------|-------------------------|
| 5352           | PLOD2  | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 | 3q24     | 2                             | 0                                   | 2.2220507               |
| 4017           | LOXL2  | Lysyl oxidase-like 2                              | 8p21.3   | 2                             | 0                                   | 2.7719142               |
| 2146           | EZH2   | Enhancer of zeste homolog 2 (*Drosophila*)        | 7q35-q36 | 1                             | 0                                   | 2.0032272               |
| 3625           | INHHB  | Inhibin, β B                                      | 2cen-q13 | 1                             | 0                                   | 3.7558112               |
| 3678           | ITGA5  | Integrin, α 5 (fibronectin receptor, α polypeptide) | 12q11-q13 | 1                             | 0                                   | 2.8391342               |
| 23023          | TMCC1  | Transmembrane and coiled-coil domain family 1     | 3q22.1   | 1                             | 1                                   | 2.226072                |
| 1404           | HAPLN1 | Hyaluronan and proteoglycan link protein 1         | 5q14.3   | 1                             | 1                                   | 2.7813237               |
| 7903           | ST8SIA4| ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase | 5q21     | 1                             | 0                                   | 3.1741676               |
| 1846           | DUSP4  | Dual specificity phosphatase 4                    | 8p12-p11 | 1                             | 0                                   | 2.1518986               |
| 6890           | TAP1   | Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) | 6p21.3   | 0                             | 1                                   | 2.0403051               |
| 7272           | TTK    | TTK protein kinase                                 | 6q14.1   | 0                             | 1                                   | 2.3837836               |
| 170384         | FUT11  | Fucosyltransferase 11 (α (1,3) fucosyltransferase) | 10q22.2  | 0                             | 1                                   | 2.0443428               |
| 22974          | TPX2   | TPX2, microtubule-associated, homolog              | 20q11.2  | 0                             | 1                                   | 2.662108                |
| 2210           | FCGR1B | Fc fragment of IgG, high affinity Ib, receptor (CD64) | 1p11.2   | 0                             | 1                                   | 2.294377                |
| 4747           | NEFL   | Neurofilament, light polypeptide                   | 8p21     | 0                             | 1                                   | 2.1319628               |
| 5836           | PYGL   | Phosphorylase, glycogen, liver                    | 14q21-q22 | 0                             | 1                                   | 2.0643747               |
| 1234           | CCR5   | Chemokine (C-C motif) receptor 5                   | 3p21.31  | 0                             | 1                                   | 3.3846455               |
| 55165          | CEP55  | Centrosomal protein 55 kDa                        | 10q23.33 | 0                             | 1                                   | 2.0711598               |
| 10288          | LILRB2 | Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2 | 19q13.4  | 0                             | 1                                   | 2.454539                |
| 1356           | CP     | Ceruloplasmin (ferroxidase)                        | 3q23-q25 | 0                             | 1                                   | 3.9467278               |
| 3910           | LAMA4  | Laminin, α 4                                      | 6q21     | 0                             | 1                                   | 2.2182174               |
| 163404         | LPPR5  | Lipid phosphate phosphatase-related protein type 5 | 1p21.3   | 0                             | 1                                   | 2.450066                |
| 5027           | P2RX7  | Purinergic receptor P2X, ligand-gated ion channel, 7 | 12q24    | 0                             | 3                                   | 3.0084689               |
| 330            | BIRC3  | Baculoviral IAP repeat containing 3                | 11q22    | 0                             | 1                                   | 2.2927191               |
| 6507           | SLC1A3 | Solute carrier family 1                           | 5p13     | 0                             | 1                                   | 2.1052346               |
| 2335           | FNI    | Fibronectin 1                                      | 2q34     | 0                             | 1                                   | 2.4469628               |
| 8701           | DNAH11 | Dynein, axonal, heavy chain 11                     | 7p21     | 0                             | 1                                   | 2.2785249               |
| 79850          | FAM57A | Family with sequence similarity 57, member A       | 17p13.3  | 0                             | 1                                   | 2.2900116               |
| 1462           | VCAN   | Versican                                          | 5q14.3   | 0                             | 1                                   | 2.524361                |
| 128346         | C1orf162| Chromosome 1 open reading frame 162                | 1p13.2   | 0                             | 1                                   | 2.2255776               |
| 4015           | LOX    | Lysyl oxidase                                     | 5q23.2   | 0                             | 1                                   | 3.3194032               |
| 115761         | ARL11  | ADP-ribosylation factor-like 11                    | 13q14.2  | 0                             | 1                                   | 2.4013827               |
| 286336         | FAM78A | Family with sequence similarity 78, member A       | 9q34     | 0                             | 1                                   | 2.1942985               |
| 6664           | SOX11  | SRY (sex determining region Y)-box 11             | 2p25     | 0                             | 1                                   | 2.577679                |
| 9770           | RASSF2 | Ras association (RalGDS/AF-6) domain family member 2 | 20p13    | 0                             | 1                                   | 2.619857                |
| 57823          | SLAMF7 | SLAM family member 7                              | 1q23.1-q24.1 | 0                             | 1                                   | 2.063896                |
| 58475          | MS4A7  | Membrane-spanning 4-domains, subfamily A, member 7 | 11q12    | 0                             | 1                                   | 2.0315962               |
| 79742          | CXorf36| Chromosome X open reading frame 36                 | Xp11.3   | 0                             | 1                                   | 2.3148956               |
| 146857         | SLFN13 | Schlafen family member 14                         | 17q12    | 0                             | 1                                   | 2.6972997               |
Figure 4. Expression of the gene encoding PLOD2 is suppressed by transfection of RCC cell lines 786-O and A498 with miR-26a or miR-26b. (A) PLOD2 mRNA expression was evaluated by quantitative RT-PCR 72 h after transfection with miR-26a or miR-26b (10 nM). GUSB was used as an internal control. *P<0.01. (B) PLOD2 protein expression was evaluated by western blotting 72 h after transfection with miR-26a or miR-26b (10 nM). GAPDH was used as a loading control. (C) miR-26a and miR-26b binding site in the 3'-UTR of PLOD2 mRNA. (D) Luciferase reporter assays in A498 cells using a vector encoding a putative miR-26a and miR-26b target sites at position 905-912 and 1188-1194 of the PLOD2 3'-UTR. Renilla luciferase values were normalized to firefly luciferase values. *P<0.0001.

Figure 5. Effects on RCC cell proliferation, migration and invasion after silencing of PLOD2 mRNA and protein expression with si-PLOD2 transfection. (A) PLOD2 mRNA expression levels were evaluated by quantitative RT-PCR 72 h after transfection with si-PLOD2 (10 nM). GUSB was used as an internal control. *P<0.0001. (B) PLOD2 protein expression levels were evaluated by western blotting 72 h after transfection with si-PLOD2 (10 nM). GAPDH was used as a loading control. (C) Cell proliferation was determined by XTT assays. (D) Cell migration activity was determined by wound-healing assays. (E) Cell invasion activity was determined by Matrigel invasion assays. *P<0.0001.
Next, to investigate whether LOXL2 mRNA had target sites for miR-26a or miR-26b, we performed luciferase reporter assays in 786-O cells. We used vectors encoding either the partial wild-type sequence of the 3'-UTR of LOXL2, including the predicted miR-26a/b target sites, or deletion vectors lacking the miR-26a/b target sites. We found that the luminescence intensities were significantly reduced by transfection with miR-26a or miR-26b and vectors carrying the wild-type 3'-UTR of LOXL2, whereas transfection with deletion vectors blocked the decrease in luminescence. These data suggested that miR-26a or miR-26b bound directly to specific sites in the 3'-UTR of LOXL2 (Fig. 3C and D).

We also found that the mRNA and protein expression levels of PLOD2 were significantly repressed in miR-26a or miR-26b transfectant cells in comparison with mock or negative control transfectants (Fig. 4A and B). We also observed that the luminescence intensities were significantly reduced by transfection with miR-26a or miR-26b and vectors carrying the wild-type 3'-UTR of PLOD2, whereas transfection with deletion vectors blocked the decrease in luminescence. These data suggested that miR-26a or miR-26b bound directly to specific sites in the 3'-UTR of PLOD2 (Fig. 4C and D).

Expression of LOXL2 and PLOD2 in ccRCC clinical specimens. A total of 15 pairs of ccRCC specimens and corresponding non-cancerous specimens were used for expression studies of LOXL2 and PLOD2 using RT-PCR. We showed that LOXL2 and PLOD2 were significantly upregulated in cancer tissues compared with normal tissues (P=0.0001 and P<0.0001, respectively; Fig. 6A and B). Furthermore, Spearman's rank test showed a negative correlation between the expression of miR-26a/PLOD2 and miR-26b/PLOD2 (Fig. 6E and F).

Discussion
A growing body of evidence has shown that aberrantly expressed miRNAs can disrupt tightly regulated RNA networks in cancer cells and promote human oncogenesis and metastasis (7,9,24-26). Recently, our studies identified a
variety of novel RCC molecular pathways regulated by tumor-suppressive miRNAs (12-15). In the present study, we focused on miR-26a and miR-26b because the expression levels of these miRNAs were reduced in the miRNA signatures of various types of cancers (10,18,19,27). Moreover, the functional roles of these miRNAs in RCC cells are not clear. Our present data showed that miR-26a and miR-26b act as tumor suppressors that modulate cancer cell migration and invasion in RCC cells. Our previous studies of oral cancer and prostate cancer demonstrated the tumor-suppressive roles of these miRNAs (19,20), and those findings support the present results obtained with RCC cells. Downregulation and tumor-suppressive roles of miR-26a or miR-26b have been reported in several types of cancer, such as bladder, breast, hepatocellular carcinoma and oral cancer (19,28-30).

In the human genome, the miR-26 family consists of three subtypes of miRNAs: miR-26a-1, miR-26a-2 and miR-26b. The mature sequences of miR-26a-1 and miR-26a-2 are identical, whereas the two nucleotides differ from that of miR-26b (miRBase release 21; http://www.mirbase.org/). The molecular mechanisms responsible for silencing the expression of the miR-26 family are still unclear. A recent study indicated that MYC oncogene directly bound to the promoter regions of miR-26a-1, miR-26a-2 and miR-26b and negatively regulated expression of these miRNAs in prostate cancer cells (31). Overexpression of MYC was observed in RCC clinical specimens (15,32), suggesting MYC might be a mediator for expression control of tumor-suppressive miRNAs in cancer cells.

A single miRNA may regulate multiple protein-coding genes; indeed, bioinformatics studies have shown that miRNAs regulate 30-60% of the protein-coding genes in the human genome (7,33). Reduced expression of tumor-suppressive miRNAs may cause overexpression of oncogenic genes in cancer cells. To better understand RCC oncogenesis and metastasis, we identified miR-26a and miR-26b target genes using in silico analysis. Recent miRNA studies in our laboratory have utilized this strategy to identify novel molecular targets and pathways regulated by tumor-suppressive miRNAs in several cancers, including RCC (12,20).

A total of 39 putative target genes of miR-26a and miR-26b were identified in the present study. Among these genes, we focused on LOXL2 and PLOD2 because they function as collagen cross-linking enzymes. Numerous studies have shown that aberrant expression of collagen cross-linking enzymes promotes extracellular matrix (ECM) stiffening, resulting in enhanced cancer cell migration and invasion (22,34-39). Overexpression of ECM components has been observed in several cancers (21,23,40). Recently, a number of studies indicated that several miRNAs regulated ECM component genes, and aberrantly expressed miRNAs have contributed to cancer cell progression by dysregulation of cell adhesion, polarity and ECM remodeling (21,23). Our past studies found that the tumor-suppressive miR-29-family (miR-29a, miR-29b and miR-29c) and miR-218 directly regulated laminins (LAMC2 and LAMB3) and integrins (ITGA6 and ITGB3), such that restoration of these miRNAs inhibited cancer cell migration and invasion (21,41,42).

Once collagen is secreted, collagen cross-linking occurs on lysine and hydroxylysine residues by the lysyl oxidase (LOX) family of enzymes (22,43). More recently, we showed that the miR-29s-family directly targeted LOXL2 in RCC and lung cancers (12). Overexpression of LOXL2 was observed in RCC clinical specimens and silencing of LOXL2 inhibited cancer cell migration and invasion in ccRCC cell lines (12). Other research groups found that increased expression of LOXL2 is correlated with disease progression, including RCC (34,44). The function of the LOX-family is covalent crosslinking of collagen and/or elastin in the ECM (35,36). Aberrant expression of LOX-family proteins has been reported in several diseases, including cancers (34-39). Interestingly, LOXL2 is a direct transcriptional target of HIF-1. Moreover, nuclear LOXL2 interacts with transcription factor SNAIL1 and represses E-cadherin as well as induces EMT (45,46). In this study, we demonstrated direct regulation of LOXL2 by miR-26a and miR-26b in RCC cells as observed with the miR-29s-family. These findings showed that tumor-suppressive miR-26a/b-LOXL2 is the pivotal pathway contributing to cancer cell migration and invasion in RCC.

In this study, we also focused on the PLOD2 (procollagen-lysine 2-oxoglutarate-dioxygenase) gene as a target of miR-26a and miR-26b and demonstrated the direct regulation of these miRNAs by luciferase reporter assays. PLOD2 encodes an enzyme that mediates collagen lysine hydroxylation. Collagen cross-linking that are derived from hydroxylated lysine residues have increased stability compared with non-hydroxylated lysine residues (22,47). Overexpression of PLOD2 in ccRCC clinical specimens and promoting migration and invasion in cancer cells were observed in the present study. In breast cancer, Kaplan-Meier curves of disease-specific survival stratified by PLOD2 expression revealed that high PLOD2 expression was significantly associated with decreased disease-specific survival (48). Moreover, PLOD2 expression promoted tumor stiffness and was required for metastasis to lymph nodes and lungs (22,48).

In conclusion, miR-26a and miR-26b were significantly downregulated in ccRCC clinical specimens and appeared to function as tumor suppressors through regulation of collagen cross-linking enzymes, LOXL2 and PLOD2, both of which function as oncogenes in this disease. The identification of novel molecular targets and pathways regulated by the tumor-suppressive miR-26a and miR-26b may lead to a better understanding of ccRCC and the development of new therapeutic strategies to treat this disease.

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