MicroRNA-26a/b directly regulate La-related protein 1 and inhibit cancer cell invasion in prostate cancer

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Abstract. Our past studies of microRNA (miRNA) expression signatures of cancers including prostate cancer (PCa) revealed that microRNA-26a and microRNA-26b (miR-26a and miR-26b) were significantly downregulated in cancer tissues. In the present study, we found that restoration of miR-26a or miR-26b significantly inhibited PCa cell invasion. Gene expression data and in silico analysis showed that the gene encoding La-related protein 1 (LARP1) was a putative candidate of miR-26a and miR-26b regulation. Moreover, luciferase reporter assays revealed that LARP1 was a direct target of both miR-26a and miR-26b. Overexpression of LARP1 was observed in PCa clinical specimens and knockdown of LARP1 inhibited cancer cell migration. Therefore, LARP1 acted as an oncogene in PCa cells. Furthermore, ‘ribosome’, ‘RNA transport’ and ‘mTOR signaling pathway’ were identified as LARP1-regulated pathways. Our present data suggested that loss of tumor-suppressive miR-26a and miR-26b enhanced cancer cell invasion in PCa through direct regulation of oncogenic LARP1. Elucidation of the molecular networks regulated by tumor-suppressive miRNAs will provide insights into the molecular mechanisms of PCa oncogenesis and metastasis.

Introduction

Prostate cancer (PCa) is the most common cause of cancer in men, accounting for about one-quarter of all cases in adult males, and the second leading cause of cancer-related deaths among men in developed countries (1). Multiple treatment options are available for localized PCa with a 5-year survival rate of almost 100%. In contrast, advanced PCa is difficult to cure. Metastatic PCa is initially treated by androgen-deprivation therapy (ADT); however, it gradually becomes resistant to first-line ADT and progresses to castration-resistant PCa (CRPC) (2,3). Therefore, it is important to develop a deeper understanding of the molecular mechanisms underlying PCa metastasis through the use of novel approaches.

The discovery of non-coding RNAs (ncRNAs) in the human genome was an important conceptual breakthrough in the study of cancer (4). Further improvements in our understanding of ncRNAs are necessary to enhance our understanding of the mechanisms of cancer initiation, development and metastasis. In that regard, microRNAs (miRNAs) are small endogenous ncRNA molecules (19-22 bases in length) that regulate protein-coding gene expression by repressing mRNA translation or cleaving RNA transcripts in a sequence-specific manner (5). A substantial amount of evidence suggests that miRNAs are aberrantly expressed in many human cancers and play significant roles in human oncogenesis and metastasis (6-9).

Analyses of miRNA expression signatures in PCa have revealed that several miRNAs that normally acted as tumor-suppressors were downregulated in cancer tissues (10-15). It is believed that normal regulatory mechanisms can be disrupted by the aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. Therefore, identification of aberrantly expressed miRNAs is an important first step toward elucidating the details of miRNA-mediated oncogenic pathways. Recent study of several miRNA signatures of PCa showed that the expression of miR-26a and miR-26b was frequently reduced in cancer tissues compared with normal prostate tissues (16), suggesting that miR-26a and miR-26b act as tumor suppressors in PCa.

The aim of the present study was to investigate the functional significance of miR-26a and miR-26b. This investigation included identifying their molecular targets and the downstream genes modulated in PCa cells. Our data demonstrated that restoration of mature miR-26a and miR-26b inhibited cancer cell invasion. Moreover, gene expression data and in silico database analysis showed that the gene coding for La-related protein 1 (LARP1), an RNA-binding protein, was a direct regulatory target of both miR-26a and miR-26b. Silencing of LARP1 significantly inhibited cancer cell inva-
Table I. The patient characteristics.

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**Materials and methods**

**Clinical prostate specimens and cell culture.** Clinical prostate specimens were obtained from patients with PCa who underwent radical prostatectomy at Chiba University Hospital from 2009 to 2013. Seventeen paired samples of PCa and corresponding normal tissues from prostatectomy specimens were used for the present study. Those samples that were considered normal were free of cancer cells as determined by pathological examination. The background of the patients and pathological characteristics are summarized in Table I. The protocol was approved by the Institutional Review Board of Chiba University. All patients provided written informed consent for tissue donation for research purposes before tissue collection.

We used the human PCa cell lines PC3 and PC3M, obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC3 originated from a PCa patient with bone metastasis. PC3M was obtained upon injection of PC3 cells into nude mice and was derived from a liver metastasis following intrasplenic injection of PC3. PC3 and PC3M cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

**RNA isolation.** Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

**Quantitative real-time RT-PCR.** The procedure for PCR quantification was conducted as previously described (10,12,15). The expression of miR-26a (assay ID: 000405; Applied Biosystems, Foster City, CA, USA) and miR-26b (assay ID: 000407; Applied Biosystems) was analyzed by TaqMan quantitative real-time PCR and normalized to the expression of RNU48 (assay ID: 001006; Applied Biosystems). TaqMan probes and primers for LARP1 (P/N: Hs00391726_m1; Applied Biosystems), GUSB (the internal control; P/N: Hs00939627_m1; Applied Biosystems) and GAPDH (the internal control; P/N: Hs02758991_g1; Applied Biosystems) were assay-on-demand gene expression products.

**Transfection with mature miRNA and small interfering RNA (siRNA).** The following mature miRNA species were used in the present study: Ambion Pre-miR miRNA precursor for hsa-miR-26a-5p (product ID: PM10249; Applied Biosystems) and Ambion Pre-miR miRNA precursor for hsa-miR-26b-5p (product ID: PM12899; Applied Biosystems). The following siRNAs were used: Stealth Select RNAi siRNAs; si-LARP1 (cat no. HSS118648, HSS118649; Invitrogen) and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). RNAs were incubated with OPTI-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen) as previously described (10,12,15).

**Cell proliferation and invasion assays.** Cells were transfected with 10 nm miRNA or siRNA by reverse transfection and plated in 96-well plates at 3x10³ cells/well. After 72 h, cell proliferation was determined with the XTT assay using a Cell Proliferation kit II (Roche Applied Sciences, Tokyo, Japan), as previously reported (10,12,15).

A cell invasion assay was carried out using modified Boyden chambers containing Transwell-precoated Matrigel membrane filter inserts with 8-µm pores in 24-well tissue culture plates, with cells plated at 1x10⁵ cells/well (BD Biosciences) as previously reported (10,12,15). All experiments were performed in triplicate.

**Genome-wide gene expression and in silico analysis for the identification of genes regulated by miR-26a and miR-26b.** To identify miR-26a and miR-26b target genes, we used a combination of in silico and genome-wide gene expression analyses. First, we screened genes using TargetScan Release 6.2 (http://www.targetscan.org/). Next, to identify downregulated genes in clinical PCa specimens, we analyzed publicly available gene expression data set in the GEO database (accession number: GSE29079). We merged these data sets and selected putative miR-26a and miR-26b target genes in the present study.
Identification of downstream pathways and genes regulated by LARP1. To identify molecular pathways regulated by LARP1 gene expression in cancer cells, we performed gene expression analysis using si-LARP1-transfected PC3 cells. An oligomicroarray (human 60 k v; Agilent Technologies) was used for gene expression studies. Gene expression data were categorized according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways using the GeneCodis program (http://genecodis.dacya.ucm.es). The strategy behind this analysis procedure has been described (12,14,15,19).

Western blotting. Cells were harvested 72 h after transfection, and lysates were prepared. Cell lysates (20 µg protein) were separated on Mini-PROTEAN TGX gels (Bio-Rad Laboratories, hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with rabbit anti-LARP1 antibodies (1:200, sc-102006; santa Cruz Biotechnology, Inc., Dallas, TX, USA). Anti-GAPDH antibodies (1:1,000, ab8245; Abcam) were used as an internal loading control. Membranes were washed and incubated with anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies (7074; Cell signaling Technology, Danvers, MA, USA). Complexes were visualized with Clarity western ECL substrate (Bio-Rad Laboratories).

Plasmid construction and dual-luciferase reporter assays. Partial wild-type (WT) sequences of the LARP1 3'-untranslated region (UTR) or those with deleted miR-26a/26b target sites (positions 2527-2533, 3337-3344 and 3494-3500 of the LARP1 3'-UTR) were inserted between the XhoI-Pmel restriction sites in the 3'-UTR of the hRluc gene in the psiChECk-2 vector (C8021; Promega, Madison, WI, USA). The synthesized DNA was cloned into the psiCHECK-2 vector. PC3 cells were transfected with 50 ng of the prepared vector and 10 nM miR-26a and miR-26b using Lipofectamine 2000 (Invitrogen). The activities of firefly and Renilla luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the ratio of Renilla/firefly luciferase activities as previously described (10,12,15).

Immunohistochemistry. A total of 17 radical prostatectomy specimens were used (Table I). Tissue specimens were immunostained with the UltraVision Detection system (Thermo Fisher Scientific, Fremont, CA, USA) following the manufacturer's protocol. Primary rabbit polyclonal antibodies against LARP1 (SC-102006; Santa Cruz Biotechnology) were diluted 1:50. The slides were treated with biotinylated goat antibodies.

Statistical analysis. The relationships between 2 groups and the numerical values obtained by qRT-PCR were analyzed using the Mann-Whitney U test. The relationships among more than 3 variables and numerical values were analyzed using the Bonferroni-adjusted Mann-Whitney U test. All analyses were performed using Expert StatView (version 5; SAS Institute Inc., Cary, NC, USA).

Results

Expression levels of miR-26a/26b in PCa specimens and cell lines. First, we evaluated the expression of miR-26a and miR-26b in 17 radical prostatectomy specimens (Table I; nos. 1-17). Patients had a median PSA level of 8.88 ng/ml (range, 4.48-29.93 ng/ml) and 35.3% of patients were classified as cT3a or cT3b according to the TNM classification. The expression levels of miR-26a and miR-26b were significantly lower in cancer tissues than in non-cancerous tissues (P=0.0084 and P=0.0103, respectively; Fig. 1A and B). Additionally, PC3 and PC3M cells also exhibited low expression of miR-26a and miR-26b compared to normal prostate tissues (Fig. 1A and B).

Spearman's rank test showed positive correlations between the expression of miR-26a and miR-26b (R=0.812 and P<0.0001; Fig. 1C).

Effects of miR-26a/26b restoration on the proliferative and invasive properties of PC3 and PC3M cells. To investigate the functional effects of miR-26a and miR-26b, we performed gain-of-function studies using miRNA transfection of PC3 and PC3M cells. XTT assays demonstrated that cell proliferation was not inhibited in miR-26a/26b transfectants in comparison with mock- or miR-control transfectod PC3 cells (Fig. 2A).
however, restoration of miR-26a and miR-26b did inhibit PC3M cell proliferation (Fig. 2A). In cell invasion assays, miR-26a and miR-26b transfection significantly inhibited cell invasion compared with mock- or miR-control transfectants in both PC3 and PC3M cells (Fig. 2B).

Identification of candidate target genes of miR-26a/26b in PCa cells. To identify target genes of miR-26a and miR-26b, we performed in silico analysis and gene expression analysis. First, the TargetScan program showed that 2,589 genes had putative target sites for miR-26a and miR-26b in their 3’-UTR regions. To gain further insight into which genes were affected by tumor-suppressive miR-26a and miR-26b in PCa, the genes were analyzed with available gene expression data from GEO (accession no. GSE29079), and we selected genes that were upregulated (log2 ratio >1.0). Ten candidate genes were identified as targets of miR-26a and miR-26b (Table II). Of these, we focused on the LARP1 gene for further analyses because it has two putatively conserved target sites and one poorly conserved target site. Moreover, the functional significance of LARP1 in PCa cells had not been determined.

LARP1 was directly regulated by miR-26a and miR-26b. Next, we performed qRT-PCR and western blotting to confirm that restoration of miR-26a and miR-26b resulted in downregulation of LARP1 in PC3 and PC3M cells. The mRNA and protein expression levels of LARP1 were significantly repressed in miR-26a and miR-26b transfectants in comparison with mock or miR-control transfectants (P<0.0005; Fig. 3A and B).

We then performed luciferase reporter assays in PC3 cells to determine whether LARP1 mRNA was directly regulated by miR-26a and miR-26b. The TargetScan database predicted that three putative miR-26a/26b-binding sites existed in the 3’-UTR of LARP1 (positions 2527-2533, 3337-3344 and 3494-3500; Fig. 3C). We used vectors encoding either the partial WT sequence of the 3’- uTR of LARP1 mRNA, including the predicted miR-26a/26b target sites, or deletion vectors that lacked the miR-26a/26b target sites. We found that the luminescence intensities were significantly reduced.
by transfection with miR-26a/26b and vectors carrying the WT 3'-UTR of LARP1 (positions 2527-2533 and 3337-3344), whereas transfection with deletion vectors blocked the decrease in luminescence (P<0.0001; Fig. 3C). These data suggested that miR-26a/26b bound directly to specific sites in the 3'-UTR of LARP1 mRNA.

Effects of silencing LARP1 on cell proliferation and invasion in PCa cell lines. To investigate the functional role of LARP1, we performed loss-of-function studies using si-LARP1 transfectants. First, we evaluated the knockdown efficiency of si-LARP1 transfection in PC3 and PC3M cells. qRT-PCR and western blotting indicated that si-LARP1 transfection effectively downregulated LARP1 expression in PC3 and PC3M cells (P<0.0001; Fig. 4A and B).

In functional assays, cell proliferation was inhibited by transfection with si-LARP1 in comparison with mock- or si-control-transfected PC3 and PC3M cells (Fig. 4C). Similarly, Matrigel invasion assays demonstrated that cell invasion was significantly inhibited in si-LARP1 transfectants in comparison with mock- or si-control-transfected PC3 and PC3M cells (P<0.0001; Fig. 4D).

Expression of LARP1 protein in PCa clinical specimens. We validated strong LARP1 expression in radical prostatectomy specimens by immunohistochemical staining. LARP1 was
strongly detected in several PCa specimens, whereas no or low expression was observed in non-cancerous lesions (Fig. 5).

**Genes downstream of LARP1 in PC3 cells.** To further investigate the roles of LARP1 in PC3 cells, we performed genome-wide gene expression analysis using si-LARP1. We categorized 358 genes significantly downregulated by si-LARP1-1 and si-LARP1-2 compared with mock transfected (log$_2$ FC < -0.5) by KEGG pathway analysis. As Table III shows, 17 pathways were significantly downregulated by si-LARP1, the most prominent of which was the ribosome pathway (P=7.07E-35).

**Discussion**

A growing body of evidence has shown that miRNAs are involved in several biological processes. Importantly, they are closely associated with human oncogenesis and metastasis (17). In normal cells, miRNA closely regulates RNA molecular networks. In contrast, aberrantly expressed miRNAs can disrupt the otherwise tightly regulated relationship between miRNA and mRNA, leading to growth and metastasis of cancer cells. Therefore, identification of aberrantly expressed miRNAs in cancer cells is the first step in elucidating abnormal molecular signaling networks contributing to oncogenesis.

Based on the miRNA expression signature of PCa, we have identified downregulated miRNAs and proved their tumor-suppressive functions in PCa cells. They include miR-1/133a, miR-143/145, miR-23b/27b/24-1, the miR-29 family and miR-218 (10-15).

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 PCa and other types of cancers (16,18,19). 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 two differ in miR-26b (miRBase release 21; http://www.mirbase.org/). The seed sequences of these miRNAs are identical, suggesting that miR-26-family regulated genes are identical in human cells. Silencing of protein-coding RNAs and miRNAs results from aberrant DNA methylation and epigenetic modification (20). Aberrant DNA hypermethylation by overexpression of DNMT3b causes the silencing of miR-26a and miR-26b in breast cancer cell lines (21). Expression of miR-26a was increased by treatment with 5-aza-2-deoxycytidine in a prostate cancer cell line (22). Notably, MYC protein directly binds to promoter regions of
Table III. Significantly enriched KEGG pathways and involved genes modulated by si-LARP1 in PC3 cells.

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Figure 5. Immunohistochemical staining of LARP1 in prostate clinical specimens. Differences in LARP1 expression were observed in cancer lesions and adjacent normal prostate tissues in the same fields. (A) Patient number 6 and (B) patient number 16. Overexpression of LARP1 was observed in cancer lesions. In contrast, negative staining of LARP1 was seen in normal prostate glands and stromal tissues. (Upper panel, original magnification, x100; lower panel, original magnification, x200).
Interestingly, restoration of targeted cells (23). A recent study showed that Ezh2 expression and suppressed proliferation of PCa. We focused on LARP1, which has an oncogenic function in PCa cells. Overexpression of LARP1 enhanced cancer cell migration and invasion in oral squamous cell carcinoma through direct regulation of TMEM184B. Moreover, silencing of TMEM184B inhibited cancer cell migration and invasion and regulated actin cytoskeleton pathway-related genes.

In prostate cancer, several studies reported that miR-26a and miR-26b were downregulated in PCa clinical specimens and that they functioned as tumor suppressors that targeted several oncogenic genes. Enhancer of zeste homolog 2 (EZH2) is a histone-lysine N-methyltransferase enzyme and component of the polycomb repressive complex 2 (27,28). Overexpression of EZH2 is observed in several cancers, including PCa (29-31). Interestingly, restoration of miR-26a and miR-26b reduced EZH2 expression and suppressed proliferation of PCa cells (23). A recent study showed that miR-26a directly targeted LIN28B and ZCCHC11 (32). Moreover, the present study demonstrated that ZCCHC11 was overexpressed in human cancers and that ZCCHC11 promoted cancer cell growth and metastasis (32). It is well known that LIN28B, an RNA-binding protein, has suppressive roles in let-7 family biogenesis. Moreover, the LIN28/let-7 regulatory circuit widely influences development and human cancers (33-36). Previous studies showed that downregulation of the let-7 family was frequently observed in PCa tissues and they acted as tumor-suppressive miRNAs in PCa cells (37,38). Therefore, it appears that restoration of miR-26a enhanced let-7 biogenesis through its targeting of LIN28B as a let-7 suppressor, and consequently, inhibited cancer cell proliferation and metastasis (32).

Identification of miRNA regulatory networks in cancer cells might contribute to the elucidation of novel molecular mechanisms of human oncogenesis and metastasis. In the present study, we performed a combination of genome-wide gene expression analysis and in silico analysis to identify miR-26a and miR-26b targets in PCa cells. Recent miRNA studies in our laboratory have utilized this strategy to successfully identify novel molecular targets and pathways regulated by tumor-suppressive miRNAs in several cancers, including PCa (12-15). A total of 10 putative candidate genes regulated by miR-26a/b are documented in this study. Among them, we focused on LARP1, an RNA-binding protein, because the functional roles of the gene in the development of PCa have not been elucidated. Our data confirmed overexpression of LARP1 in clinical specimens of PCa and silencing LARP1 inhibited PCa cell migration. These data strongly suggested that LARP1 has an oncogenic function in PCa cells. Overexpression of LARP1 was reported in other types of cancers, including hepatocellular carcinoma, cervical cancer and non-small cell lung cancer, and expression of the gene has correlated with clinical outcomes (39,40).

Recent studies showed that LARP1 plays a critical role in the stabilization and translation of 5′-terminal oligopyrimidine tract (TOP) mRNAs, such as ribosomal proteins and elongation factors by interacting with their 5′- and 3′-untranslated regions (41-43). Moreover, recent studies indicated that LARP1 is phosphorylated by mTOR and is a key regulator of mTORC1 signaling (40,44). We analyzed genes downstream of LARP1 by using si-LARP1 transfecants. The data showed that several genes were involved in ‘ribosome’, ‘RNA transport’ and ‘mTOR signaling pathways’. These findings suggest that LARP1 is deeply involved in cancer progression and development through stabilization and regulation of TOP mRNAs in mTOR pathways.

In conclusion, downregulation of miR-26a and miR-26b was validated in PCa clinical specimens and these miRNAs were shown to function as tumor suppressors in PCa. To the best of our knowledge, this is the first report demonstrating that tumor-suppressive miR-26a/b directly targeted LARP1 as an RNA-binding protein in PCa cells. Moreover, LARP1 was upregulated in PCa clinical specimens and contributed to cancer cell invasion, indicating that it functioned as an oncogene. The identification of novel molecular pathways and targets regulated by miR-26a/b may lead to a better understanding of PCa oncogenesis and metastasis.

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