MicroRNA-146b-3p Promotes Cell Metastasis by Directly Targeting *NF2* in Human Papillary Thyroid Cancer

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Background: MiR-146b has been reported to be overexpressed in papillary thyroid cancer (PTC) tissues and associated with aggressive PTC. MiR-146b is regarded as a relevant diagnostic marker for this type of cancer. MiR-146b-5p has been confirmed to increase cell proliferation by repressing SMAD4. However, detailed functional analysis of another mature form of miR-146b, miR-146b-3p, has not been carried out. This study aimed to identify the differential expression of miR-146b-5p and miR-146b-3p in more aggressive PTC associated with lymph node metastasis, and further elucidate the contribution and mechanism of miR-146b-3p in the process of PTC metastasis.

Methods: Expression of miR-146b-5p and miR-146b-3p was assessed in formalin-fixed paraffin-embedded tissue samples from PTC patients, and the relationship with lymph node metastasis was analyzed. A variety of PTC cells, including BHP10-3, BHP10-3SC_{mice}, and K1 cells, were cultured and treated with miR-146b-5p or miR-146b-3p mimics/inhibitors. The cell migration and invasion abilities were characterized by the real-time cell analyzer assay and Transwell™ assay. PTC xenograft models were used to examine the effect of miR-146b-3p on PTC metastatic ability *in vivo*. Direct downstream targets of miR-146b-3p were analyzed by luciferase reporter assay and Western blotting. The mechanism by which miR-146b-3p affects cell metastasis was further characterized by co-transfection with merlin, the protein product of the *NF2* gene.

Results: MiR-146b-5p and miR-146b-3p expression was significantly higher in thyroid cancer tissues and cell lines than in normal thyroid tissue and cells. Moreover, expression of miR-146b-5p and miR-146b-3p was further increased in thyroid metastatic nodes than in thyroid cancer. After overexpression of miR-146b-5p or miR-146b-3p in BHP10-3 or K1 cells, PTC migration and invasion were increased. Notably, miR-146b-3p increased cell migration and invasion more obviously than did miR-146b-5p. Overexpression of miR-146b-3p also significantly promoted PTC tumor metastasis *in vivo*. Luciferase reporter assay results revealed that *NF2* is a downstream target of miR-146b-3p in PTC cells, as miR-146b-3p bound directly to the 3' untranslated region of *NF2*, thus reducing protein levels of NF2. Overexpression of merlin reversed the enhanced aggressive effects of miR-146b-3p.

Conclusions: Overexpression of miR-146b-5p and miR-146b-3p is associated with PTC metastasis. MiR-146b-3p enhances cell invasion and metastasis more obviously than miR-146b-5p through the suppression of the *NF2* gene. These findings suggest a potential diagnostic and therapeutic value of these miRNAs in PTC metastasis.

Keywords: PTC, miR-146b-5p, miR-146b-3p, metastasis, NF2

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Introduction

PAPILLARY THYROID CANCER (PTC) is the most commonly diagnosed thyroid cancer, accounting for $\geq 80\%$ of thyroid malignancies (1). Although PTC is usually indolent and curable with surgical thyroidectomy, a subset of patients who are not diagnosed in a timely manner will have aggressive disease characterized by local recurrence and/or distant metastasis. Therefore, the identification of patients with an aggressive phenotype is important for determining the need for adjuvant therapy and long-term follow-up monitoring.

MicroRNAs (miRNAs) are small, noncoding RNAs that negatively regulate gene expression through binding to the 3' untranslated region (UTR) of target messenger RNAs (mRNAs) and by blocking the translation or degradation of the mRNAs (2). MiRNAs have been reported to be able to post-transcriptionally regulate up to a third of human genes, suggesting that miRNAs may have important roles in physiological and pathological processes, including human carcinogenesis (3). MiRNA expression is tissue specific, and dysregulation of miRNA expression has been described in a variety of tumors, including lung, liver, breast, and colorectal cancer (4,5). In thyroid cancer, the miRNA expression pattern depends on cellular origin and degree of tumor differentiation. Numerous studies have demonstrated an increased aberrant miRNA expression, including miR-146b, miR-221, and miR-222, in PTCs compared to normal thyroid tissues (6–11). Recently, further studies have reported that miRNAs such as miR-146b (12–15) are strongly associated with more aggressive PTC.

In humans, miR-146b-5p and miR-146b-3p are products of the same *MIR146B* gene that is located on chromosome 10 at position q24.32. A number of studies have examined the role of miR-146b-5p in cancer, including lung, breast, glioma, and pancreatic cancer. MiR-146b-5p was proven to decrease the migration and invasiveness of cancer cells by targeting transcripts of the *MMP16* and *EGFR* genes and negatively regulating the nuclear factor- κ B (NF- κ B) pathway (16–18). MiRNAs behave in a dual mode in cancer as either oncogenes or tumor suppressors, depending on tissue type and specific targets (19). Unlike in other cancers, miR-146b-5p has a confirmed oncogenic role regulating TGF- β signal transduction by repressing SMAD4 in PTC (20) and promoting metastasis by targeting ZNRF3 (21). Furthermore, miR-146b-5p has been shown to be sequentially upregulated in different stages of PTC (22,23). While miR-146b-5p is relatively well characterized, such functional studies of miR-146b-3p have rarely been performed. In a human non-small cell lung cancer study, miR-146b-5p and miR-146b-3p had opposite prognostic values (24), which indicated that although they are encoded by the same gene, miR-146b-3p might have a different role from miR-146b-5p and other important functions in human carcinogenesis. However, the possible roles and mechanisms of miR-146b-3p in human PTC are still not well established.

This study aimed to identify differential expression of miR-146b-5p and miR-146b-3p in more aggressive forms of PTC, such as those with lymph node metastasis (LNM), and it further elucidated the contribution and mechanism of miR-146b-3p in the process of PTC metastasis. The study confirms that both miR-146b-5p and miR-146b-3p are overexpressed in PTC with LNM. MiR-146b-3p enhanced cell migration and invasion more obviously than did miR-146b-5p. Moreover,

the *NF2* gene was identified as a direct and functional target of miR-146b-3p in PTC.

Methods

Tissue samples

A total of 72 formalin-fixed paraffin-embedded (FFPE) tissues samples from 24 patients were obtained at the Department of Pathology in Shandong Provincial Hospital affiliated to Shandong University. All tissues samples were pathologically confirmed as PTC tissues, matched adjacent non-neoplastic thyroid tissues, normal lymph node tissues, or LNM tissues (Fig. 1A). The histological variants of most of tissue samples (23/24) were classical variant PTC (CV-PTC), and only one case was follicular variant PTC (FV-PTC). Detailed patient information is shown in Table 1. The research was approved by the Ethics Committee of Shandong Provincial Hospital.

Cell culture

Three human PTC cell lines were cultured. BHP10-3 and BHP10-3SC_{micc} cells were kindly provided by Dr. Gary Clayman (The University of Texas M.D. Anderson Cancer Center, Houston, TX), and K1 cells were kindly provided by Dr. Xingsong Tian (Department of Breast and Thyroid Surgery, Shandong Provincial Hospital affiliated to Shandong University, Shandong, China). For imaging analysis *in vivo*, high tumorigenic BHP10-3SC_{micc} cells were stably labeled for bioluminescence and red fluorescent protein (named BHP10-3SC_{micc}luc; JiuyuJintai Biotechnology Co., Beijing, China). Cells were cultured in RPMI 1640 medium (BHP10-3 and BHP10-3SC_{micc}; HyClone) or Dulbecco's modified Eagle's medium (DMEM; K1; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (SAFC Bioscience, St. Louis, MO). The procedure to obtain primary normal human thyrocytes (PHT) has been carefully standardized (25,26). Briefly, normal tissue specimens were digested in 2 mg/mL collagenase I (Sigma-Aldrich, St. Louis, MO) and 0.125% trypsin for 1 h. After thyrocytes were filtered and washed, they were seeded and cultured in DMEM/F12 medium (HyClone) containing 10% newborn calf serum (Gibco) and bovine thyrotropin (TSH; 2 mIU/mL; Sigma-Aldrich).

MicroRNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

MicroRNA was extracted from FFPE human tissues and human thyroid cell lines using a miRNeasy FFPE kit (BioTeke Corporation, Beijing, China) and TRIzol reagent (Invitrogen, Carlsbad, CA) separately. MiR-146b-5p and miR-146b-3p were reverse transcribed and quantified with quantitative reverse transcription polymerase chain reaction (qRT-PCR) using an All-in-OneTM miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD). The miRNA primers were purchased from GeneCopoeia. RUN6B was used as an endogenous control.

Plasmid construction, transfection with microRNA mimics/inhibitor, and expression constructs

Recombinant plasmids with 3' UTRs of potential target genes (*NF2* and *MTSS1*) based on miRbase and RNA22

FIG. 1. MiR-146b-5p and miR-146b-3p expression is significantly increased in PTC tissues and further enhanced in MLN. (A) The histological changes of different thyroid tissues and LN tissues stained with H&E (magnification $\times 100$). (B) Relative expression levels of mature miR-146b-5p (left panels) and miR-146b-3p (right panels) in 72 samples, including PTC, matched normal thyroid tissue, MLN, and LN, were determined by qRT-PCR and normalized against an endogenous control RUN6B. Data were analyzed using a $\Delta\Delta Ct$ approach and expressed as miR-146b/RUN6B ratio ($-2^{\Delta\Delta Ct[\text{miR-146b-RUN6B}]}$). (C) Fold change of expression of mature miR-146b-5p (left panels) and miR-146b-3p (right panels) in 14 pairs of MLN and their corresponding primary PTC. Data were analyzed using $\log_2$ fold change ($\Delta\Delta Ct [\text{MLN/each corresponding PTC}]$). Significant upregulation of miR-146b in paired samples was defined as $\log_2$ fold change >1 , which means twofold higher. (D) Relative expression levels of miR-146b-5p and miR-146b-3p in PTC patients with different aggressive abilities (PTC with LNM [PTC-M] vs. PTC without LNM [PTC-NM]) by qRT-PCR and normalized against an endogenous control RUN6B. NT, normal thyroid tissues; PTC, papillary thyroid cancer; MLN, metastatic lymph nodes; LN, lymph node; H&E, hematoxylin and eosin; qRT-PCR, quantitative reverse transcription polymerase chain reaction. # $p < 0.05$; ## $p < 0.005$. Color images available online at www.liebertpub.com/thy

information were constructed. The oligonucleotides were ligated downstream of the *Renilla* luciferase open reading frame in the pMIR-REPORTTM miRNA expression reporter vector (Ambion, Waltham, MA) using *SacI* and *HindIII* restriction sites. The primers and corresponding point mutation sequences used for plasmid inserts are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/thy). Expression vector for merlin (pcDNA3 merlin) was purchased from Addgene (Cambridge, MA; Addgene 11623). All transfections were carried out using Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

For BHP10-3 and K1 cells, microRNA mimics and negative control (miR-NC; RiboBio Co., Guangzhou, China) were used at a final concentration of 50 nM. For BHP10-3SC_{mice} cells, microRNA inhibitor and negative control (anti-NC; RiboBio Co.) were transfected at a final concentration of 100 nM. The pcDNA3 merlin and respective empty-vector control (pcDNA3) were transfected at 4 μg per 60 mm culture dish.

Dual luciferase activity assay

For luciferase reporter activity assays, cells were seeded in 24-well plates and co-transfected with either miR-146b-3p

TABLE 1. INITIAL CLINICOPATHOLOGIC CHARACTERISTICS OF PTC PATIENTS

Clinicopathologic features	PTC with LNM	PTC without LNM	p-Value
Number	14	10	
Mean age, years (range)	36 (21–57)	50 (34–68)	0.014
Men, n (%)	3 (21.4%)	3 (30%)	0.65
Pathological stage ^a			0.62
I/II	9	9	
III/IV	5	1	
LN, n (range)	21 (4–47)	15 (7–32)	0.22
Metastatic LN, n (range)	10 (1–39)	0	0.004
Mean tumor size, cm (range)	2.3 (0.8–5.5)	3.1 (1.8–5)	0.11

^aUsing the American Joint Committee on Cancer (AJCC) Staging for Thyroid Cancer (seventh edition, 2010).

PTC, papillary thyroid carcinoma; LNM, lymph node metastasis; LN, lymph node.

mimics or inhibitor together with 0.5 µg/well of the constructed pMIR-REPORTTM miRNA target expression vectors. Forty-eight hours after transfection, luciferase activities were measured using the dual luciferase reporter assay system kit (Promega, Madison, WI) according to the manufacturer's instructions using a LB942 luminescence reader (Berthold Technologies, Bad Wildbad, Germany). Values were double normalized to firefly luciferase activity and to cells transfected with empty pMIR-REPORTTM control vectors.

Real-time cell analyzer migration and invasion assays

Real-time cell analyzer (RTCA) migration and invasion assay measures the effect of any treatment in a real-time setting (27). For RTCA migration or invasion experiments, transfections were performed as described above. Cells were then starved in serum-free medium for 12 h and seeded in RTCA CIM-16 plate (xCELLigence Roche, Penzberg, Germany) in serum-free medium. Full growth medium was used as a chemoattractant in the lower chamber. For invasion assays, the CIM-16 plates were initially coated with Matrigel (BD Biosciences, Bedford, MA) diluted in serum-free medium at a ratio of 1:40. Real-time measurements were performed at 96 h for invasion assays or 24 h for migration assays using the RTCA device (xCELLigence Roche).

TranswellTM migration and invasion assays

For TranswellTM migration and invasion experiments, cells were starved in serum-free medium for 12 h after transfection and seeded on 24-well TranswellTM plates with a polycarbonate membrane and an 8 µm pore size (Corning, Inc., Corning, NY) in serum-free medium. Full growth medium was used as a chemoattractant in the lower chamber. For invasion assays, the 24-well TranswellTM plates were initially coated with Matrigel diluted in serum-free medium at a ratio of 1:30. After 24 or 48 h, respectively, for the migration or invasion assays, cells remaining in the upper chamber or on the upper membrane were carefully removed. Cells adhering to the lower membrane were stained with hematoxylin and

imaged using a Zeiss A2 microscope (Carl Zeiss, Oberkochen, Germany). The number of cells was counted using ImageJ.

Animal studies

The animal experimental protocol was approved by the Animal Ethics Committee of Shandong Provincial Hospital. Fifteen male BALB/c nude mice (four weeks old) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and acclimated to the specific pathogen-free standard housing conditions for one week. For the establishment of the PTC xenograft model, BHP10-3SCmluc cells were suspended in sterile phosphate-buffered saline (PBS) at a concentration of 2×10^7 /mL with a volume of 250 µL and inoculated intramuscularly into the left leg of nude mice. As shown in Figure 5A, after two weeks, mice were imaged for bioluminescence *in vivo* to exclude any that were not successfully xenografted. The successfully xenografted nude mice were randomly divided into two groups: hsa-miR-146b-3p mimics (miR-146b-3p agomir) or agomir Ncontrol (NC agomir; RiboBio Co.) were directly injected into the implanted tumor at the dose of 2.5 nmol in 100 µL PBS per mouse twice per week for a total of eight times. Tumor and metastasis bioluminescent imaging *in vivo* was analyzed weekly. Animals were sacrificed at the end of the sixth week after xenografting, and ectopic implanted tumors, metastasis tumor tissues, or lymph nodes were collected for further fluorescent imaging *ex vivo*, histopathology, and Western blotting analysis.

Bioluminescent imaging in vivo and fluorescent imaging ex vivo

To monitor the primary tumor growth and metastasis development, the LB983 NightOWL imaging system (Berthold Technologies) was used for animal imaging and analysis. For *in vivo* bioluminescent imaging (Luciferin), mice were anesthetized with 10 mg/mL pentobarbital in sterile water and injected intraperitoneally with 10 µL/g D-luciferin (15 mg/mL in DPBS; Promega) of body weight. Imaging was started after 10 minutes of injection. For metastasis assays, the primary tumor was covered because its bioluminescence signal was too strong. For *ex vivo* fluorescent imaging (red fluorescent protein [RFP]), mice lung and lymph nodes were excised, placed in the LB983 chamber, and imaged using red fluorescence.

Hematoxylin and eosin staining

The tumor tissues, lung, and lymph nodes were fixed in 4% paraformaldehyde, embedded in paraffin, serially cut into sections 4 µm thick, and then stained with hematoxylin and eosin (H&E) to evaluate the histopathology following standard procedures. Images were acquired with a Zeiss A2 microscope (Carl Zeiss).

Protein extraction and Western blotting analysis

Total proteins were extracted from cells or tissue using radio immunoprecipitation assay lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and determined using a BCA Protein Assay Kit (Shenenergy Biocolor Bioscience & Technology Company, Shanghai, China). Total cell protein

(40 µg) was electrotransferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA), and after incubation in 5% bovine serum albumin for one hour, the blots were probed with primary polyclonal antibody against merlin (21686-1-AP, Proteintech, Rosemont, IL) at a 1:1000 dilution overnight at 4°C. GAPDH (60004-1-Ig; Proteintech) was used on the same membranes as an internal control. Detection was performed with the FluorChem Q (ProteinSimple, San Jose, CA).

Statistical analysis

All statistical analyses were performed using PASW Statistics for Windows v18.0 (SPSS, Inc., Chicago, IL), and data are expressed as the mean ± standard deviation. Differences between two groups were compared using an unpaired Student's *t*-test or Mann-Whitney's *U*-test. Analysis of variance was used to compare the means of multiple groups. All of the calculated *p*-values are two-sided. Differences were regarded as statistically significant at *p* < 0.05.

Results

MiRNA-146b expression is significantly increased in PTC tissues and further enhanced in metastatic lymph nodes

Both miR-146b-5p and miR-146b-3p are products of the same *MIR146B* gene. Studies have reported that miR-146b is overexpressed in PTC tissues and showed an association with aggressive PTC. However, these studies all focused on miR-146b-5p, while the functional role of miR-146b-3p in PTC metastasis remains elusive. This study sought to confirm that both miR-146b-5p and miR-146b-3p expression was higher in PTC tissues than in normal thyroid tissue and further enhanced in thyroid metastatic lymph nodes. The expression levels of mature miR-146b-5p and miR-146b-3p in clinical samples (72 samples) were examined by qRT-PCR and normalized against an endogenous control (RUN6B RNA). In 14 patients with LNM (51 samples), it was found that median miR-146b-5p and miR-146b-3p expression levels were 42- and 24-fold higher,

respectively, in PTC than in the non-tumorous thyroid tissues (median expression = 7.59/0.18, 12.48/0.51). Intriguingly, median miR-146b-5p and miR-146b-3p expression levels in metastatic lymph nodes were further increased (median expression = 20.94 and 42.58, respectively), while the expression levels in normal lymph nodes were very low (median expression = 0.9 and 1.31, respectively). Overall, miR-146b was significantly upregulated in metastatic lymph nodes (*p* < 0.05; Fig. 1B). As shown in Figure 1C, when comparing paired metastatic lymph nodes with their corresponding primary PTC tissues, in seven (50%) patients, the ratio of miR-146b-5p and miR-146b-3p was >1.0, indicating that PTC cells with high miR-146b expression might prefer to metastasize to lymph nodes. Further, the different expression levels of miR-146b-5p and miR-146b-3p in PTC with or without LNM were analyzed. As shown in Figure 1D, median miR-146b-5p and miR-146b-3p expression levels were significantly higher in PTC with LNM than in PTC without metastasis (median miR-146b-5p expression = 7.59 and 2.22, and median miR-146b-3p expression = 12.48 and 3.48, respectively), indicating that expression levels of miR-146b positively correlate with PTC LNM.

Furthermore, the relationship between miR-146b-5p or miR-146b-3p and clinicopathologic features of PTC patients was analyzed. As Table 2 shows, the expression levels of miR-146b-3p and miR-146b-5p were significantly positively associated with LNM (*p* = 0.035 and 0.016, respectively). There were no significant differences in the expression levels of miR-146b-3p or miR-146b-5p based on other clinicopathologic parameters, including age at the time of diagnosis, multicentricity, and tumor staging. In addition, 7/24 PTC patients were followed for 8–10 years, and none of them had a recurrence (data not shown).

Expression of miRNA-146b is significantly increased in PTC cell lines and positively correlated with cell metastasis ability

To analyze further the role of the expression of both miR-146b-5p and miR-146b-3p on tumor metastasis, PHT and

TABLE 2. CORRELATION ANALYSIS BETWEEN miR-146b-3P OR miR-146b-5P AND CLINICOPATHOLOGIC FEATURES

Clinicopathologic features	n	miR-146b-3p	p	miR-146b-5p	p
Age at the time of diagnosis, years					
<45	13	2.97 (0.70–47.58)	0.543	2.10 (0.70–29.85)	0.582
≥45	11	4.81 (1.09–22.17)		2.91 (0.90–12.50)	
Sex					
Female	18	4.61 (0.70–47.58)	1.000	2.43 (0.70–29.85)	0.689
Male	6	4.94 (1.60–10.36)		2.67 (0.73–8.33)	
Tumor size (cm)					
<2	13	9.78 (2.55–47.58)	0.046	6.54 (0.90–29.85)	0.052
≥2	11	2.97 (0.66–10.36)		1.95 (0.70–8.33)	
Tumor staging (AJCC)					
I/II	18	3.01 (0.66–47.58)	0.083	2.05 (0.70–29.85)	0.096
III/IV	6	9.99 (4.42–22.17)		6.62 (1.95–12.50)	
Number of lesions					
Single	16	3.34 (0.66–22.17)	0.126	2.05 (0.70–12.5)	0.066
Multiple	8	10.02 (1.09–47.58)		5.14 (1.11–29.84)	
LNM					
No	10	2.95 (1.09–10.36)	0.035	1.61 (0.73–8.33)	0.016
Yes	14	9.99 (0.66–47.58)		5.07 (0.70–29.85)	

different PTC cell lines (K1, BHP10-3, and BHP10-3SC_{mice}) were cultured, and cell migration and invasion abilities were analyzed. The expression of miR-146b was further assessed by qRT-PCR. As shown in Figure 2A and B, BHP10-3SC_{mice} cells had the most aggressive abilities among the three PTC cell lines. Although derived from BHP10-3 cells, BHP10-3SC_{mice} cells are extremely tumorigenic and invasive (28). Corresponding to these results, both miR-146b-5p and miR-146b-3p expression was significantly higher in PTC cell lines than in the normal human thyrocytes, and miR-146b expression was further increased in BHP10-3SC_{mice} cells compared to BHP10-3 and K1 cells (Fig. 2C). Notably, although miR-146b-3p expression was lower than miR-146b-5p expression in the PHT and different PTC cell lines, the level of miR-146b, especially miR-146b-3p, was positively correlated with the migration and invasion abilities.

MiR-146b-3p enhances the migratory and invasive abilities more obviously than miR-146b-5p in BHP10-3 cells, and inhibiting miR-146b-3p expression can reduce the metastatic ability of BHP10-3SC_{mice} cells

To verify the role of miR-146b-5p and miR-146b-3p in the metastasis of PTC, the migration and invasion capacities of BHP10-3 cells and K1 cells transfected with miR-146b-5p or miR-146b-3p mimics were assessed in a real-time assay using RTCA. Overexpression of miR-146b was confirmed by real-time PCR (Fig. 3A and Supplementary Fig. S1A). As indicated in Figure 3B and C, the real-time cell migratory and invasive

rates of BHP10-3 cells infected with miR-146b-5p or miR-146b-3p mimics were higher than the rates in negative controls (infected with miR-NC). Furthermore, the effects of miR-146b on PTC cells were confirmed in endpoint Transwell™ assays. As shown in Figure 3D and E and Supplementary Figure S1B and C, in accordance with the RTCA assay results, increased expression of miR-146b-3p or -5p significantly enhanced the migration and invasion abilities of BHP10-3 cells and K1 cells when compared to control treatment. Notably, compared to miR-146b-5p mimics, miR-146b-3p mimics promoted migration and invasion more obviously ($p < 0.005$).

MiR-146b-3p or miR-146b-5p inhibitors were further used to confirm the effect of miRNA inhibitors on the PTC migratory and invasive abilities using the Transwell™ assay in BHP10-3SC_{mice} cells, which highly express miR-146b-3p and miR-146b-5p. As shown in Figure 3F, the miR-146b inhibitor obviously decreased the expression of miR-146b in BHP10-3SC_{mice} cells; both cell migration and invasion decreased significantly after transfection with the miR-146b inhibitor (Fig. 3G and H). Consistent with the cell transfection results with microRNA mimics, inhibition of miR-146b-3p expression reduced PTC migration and invasion more significantly than did inhibition of miR-146b-5p.

NF2 is a direct downstream target of miR-146b-3p

MicroRNAs usually exert their function by suppressing the expression of target mRNAs. Therefore, next the study searched for the target genes of miR-146b-3p in PTC. According to

FIG. 2. Expression of miRNA-146b is significantly increased in PTC cell lines and positively correlated with the ability of cell invasion. (A) Transwell™ assay was performed to determine the migration or invasive ability of BHP10-3, BHP10-3SC mouse and K1 cells. Representative images showed migrated or invasive cells in the lower chamber stained with hematoxylin. (B) Cell count of Transwell™ assay for above three cells was statistically analyzed. (C) Fold changes of miR-146b-5p and -3p expressions in PTC cells with different invasive abilities (BHP10-3 and BHP10-3SC_{mice}) were calculated relative to miR-146b-5p expression in PHT by qRT-PCR and normalized against an endogenous control RUN6B. PHT, primary normal human thyrocytes. ^{##} $p < 0.005$ vs. BHP10-3SC_{mice}; ^{**} $p < 0.005$ vs. PHT. Color images available online at www.liebertpub.com/thy

FIG. 3. MiR-146b-3p enhances the migratory and invasive abilities more obviously than miR-146b-5p in BHP10-3 cells. Inhibiting miR-146b-3p expression can reduce the ability of metastasis in BHP10-3SC mouse cells. (A) Expression of miR-146b-5p or -3p was confirmed by real-time PCR after BHP10-3 cells were transfected with miR146b-5p mimics or -3p mimics. (B and C) BHP10-3 cells were transiently transfected with miR-146b-3p mimics (50 nM), miR-146b-5p mimics (50 nM), or negative control (miR-NC; 50 nM). Real-time measurements were performed in 24 h for the migration assay or 96 h for the invasion assay using the real-time cell analyzer system. (D) Transwell™ assay was performed to determine the migration or invasive ability of BHP10-3 cells after being transfected with miR-146b-3p mimics, miR-146b-5p mimics, or miR-NC for 24 or 48 h. Representative images showed migrated or invasive BHP10-3 cells in the lower chamber stained with hematoxylin. (E) Cell count of Transwell™ assay for the above three treatments was statistically analyzed. (F) Expression of miR-146b-5p or -3p was confirmed by real-time PCR after BHP10-3SC mouse cells were transfected with miR146b-5p inhibitor or -3p inhibitor. (G) Transwell™ assay was performed to determine the migration or invasive ability of BHP10-3SC mouse cells after transfection with miR-146b-3p inhibitor, miR-146b-5p inhibitor, or anti-NC for 24 or 48 h. Representative images show migrated or invasive BHP10-3SC mouse cells in the lower chamber stained with hematoxylin. (H) Cell count of Transwell™ assay for the above two treatments was statistically analyzed. # $p < 0.05$, ## $p < 0.005$ vs. miR-NC or anti-NC. Color images available online at www.liebertpub.com/thy

the prediction of miRanda (microrna.org and miRbase), RNA22 (<http://cbcsrv.watson.ibm.com/rna22.html>), and PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), it was found that *NF2* and *MTSS1* contained two predicted miR-146b-3p binding sites in their 3' UTRs (Fig. 4A).

NF2 is located on human chromosome 22q12. As a tumor suppressor gene, mutations in the *NF2* gene or merlin (the tumor suppressor protein encoded by the *NF2* gene) inactivation have been detected in multiple human cancer types, including mesotheliomas, thyroid, breast, and prostate cancer (29–32). *MTSS1*, metastasis suppressor 1, which is located on

FIG. 4. NF2 is a direct downstream target for miR-146b-3p. (A) Diagram of miR-146b-3p putative seed sequences in the 3' UTR of candidate mRNA. Based on bioinformation software, *NF2* and *MTSS1* were found to contain two possible candidate miR-146b-3p binding sites on 3' UTR separately. (B and C) Relative luciferase activity analysis in BHP10-3 cells transiently co-transfected with 3' UTR of wild-type or mutant recombinant *NF2* or *MTSS1* luciferase plasmids with miR-NC or miR-146b-3p mimics. The two wild-type recombinant plasmids of *NF2* (NF2-P1 and NF2-P2) and two wild-type recombinant plasmids of *MTSS1* (MTSS1-P1 and MTSS1-P2) were co-transfected into cells (B). The wild type of two NF2 3' UTR and mutant of these NF2 3' UTR were transfected into cells (C). (D) Merlin protein expression was detected by Western blot analysis after transfection of miR-146b-3p mimics in BHP10-3 cells. (E) Relative luciferase activity analysis in BHP10-3SC mouse cells transiently co-transfected with 3' UTR of wild-type or mutant recombinant *NF2* luciferase plasmids with anti-NC or miR-146b-3p inhibitor. (F) Merlin protein expression was detected by Western blot analysis after miR-146b-3p inhibition in BHP10-3SC mouse cells. Renilla luciferase vector was used as an internal control. The relative luciferase activities of group transfected with miR-NC or anti-NC were set as 1. UTR, untranslated region. # $p < 0.05$; ## $p < 0.005$ vs. miR-NC or anti-NC. Color images available online at www.liebertpub.com/thy

human chromosome 8q24, was identified as a potential metastasis suppressor gene in bladder cancer, prostate cancer, and breast cancer (33–37) and a powerful indicator of early stage HCC development (38). To verify whether *NF2* and *MTSS1* are the direct targets of miR-146b-3p, the study constructed vectors containing wild-type or mutant 3' UTRs of *NF2* or *MTSS1* into a luciferase receptor vector and co-transfected them with miR-146b-3p mimics or mimic controls into BHP10-3 and K1 cell lines. The transfection efficiency was normalized by co-transfection with a Renilla reporter vector. Luciferase reporter assays showed that miR-146b-3p obviously inhibits the relative luciferase activities of two wild-type *NF2* 3' UTRs by approximately 36% and 43% relative to those in the mimic controls, while the luciferase activities of the *MTSS1* 3' UTRs were not decreased in PTC cells (Fig. 4B and Supplementary Fig. S2A). Moreover, with two mutated *NF2* 3' UTR plasmids, there was no significant difference in the relative luciferase activity between control and miR-146b-3p mimic transfections (Fig. 4C and Supplementary Fig. S2B). In addition, the regulation of miR-146b-3p on the 3' UTR of *NF2* was further confirmed by transfection with miR-146b-3p inhibitor or control into BHP10-3SC_{mice} cells. As Figure 4E shows, inhibition of miR-146b-3p obviously increased the relative luciferase activities of the two wild-type *NF2* 3' UTRs, and mutated *NF2* 3' UTR plasmids exhibited no significant change. These results suggest that miR-146b-3p could directly bind to the 3' UTR of *NF2* but not that of *MTSS1*.

Western blot analysis was performed to observe any changes in merlin protein expression with miR-146b-3p mimics or inhibitor transfection. As shown in Figure 4D and F and Supplementary Fig. S2C, endogenous merlin protein expression was significantly inhibited or enhanced after miR-146b-3p mimic or inhibitor transfection. Taken together, these observations indicate that *NF2* is a direct downstream target of miR-146b-3p in PTC cells.

miR-146b-3p promotes tumor metastasis in vivo

To characterize further the effect of miR-146b-3p on PTC metastatic ability *in vivo*, BHP10-3SC_{mice} cells with high tumorigenicity were chosen, and they were engineered with stably labeled dual reporters of bioluminescence (luciferase) and RFP, named BHP10-3SCmluc. Next, a PTC xenograft model was established via intramuscular injection with BHP10-3SCmluc cells. Further mice were intratumorally injected with miR-146b-3p agomir and imaged.

All mice were successfully xenografted. As shown in Figure 5B, with the prolongation of feeding time, the body weight of the mice decreased gradually. Although mice in the miR-146b-3p agomir-treated group had lower body weights than those in the control group (NC agomir-treated), the difference was not statistically significant. Tumor metastasis was detected by *in vivo* bioluminescence imaging weekly. On the fifth week after xenografting, it was observed that the mice with miR-146b-3p agomir treatment had a lung metastasis (data not shown). At the end of the sixth week, as shown in Figure 5C, not only was tumor metastasis detected by *in vivo* bioluminescence imaging, but also pulmonary and LNM was observed by *ex vivo* fluorescence imaging. At the terminal detection, the tumor size of the treatment group with miR-146b-3p agomir treatment was slightly larger than that

of the control group, but there was no significant difference (Fig. 5D). At the same time, compared to total metastasis in the control mice, the total metastasis occurrence number was much higher in the miR-146b-3p agomir-treated mice (1/10 vs. 3/5 separately; Fig. 5E). Histopathology with H&E staining was carried out to confirm the metastasis (Fig. 5F). To clarify the cellular mechanisms underlying miR-146b-3p-mediated tumor metastasis, resected tissues from those treated xenograft tumors were analyzed to verify miR-146b-3p and merlin expression. Consistent with the above results, at the endpoint of the experiment, miR-146b-3p agomir treatment significantly increased the expression of miR-146b-3p (Fig. 5G) and decreased the expression of merlin (Fig. 5H) *in vivo*. All these data indicate that high expression of miR-146b-3p could promote PTC tumor metastasis *in vivo*.

Reintroduction of NF2 abrogated the miR-146b-3p-induced metastasis enhancement

To confirm further that *NF2* is the functional target gene of miR-146b-3p and that overexpression of merlin can attenuate the effect of miR-146b-3p on the metastatic abilities in BHP10-3 cells and K1 cells, miR-146b-3p mimics and pcDNA3 merlin were co-transfected into BHP10-3 cells and K1 cells using transfection with miR-146b-3p mimics, mimic controls, or pcDNA3 merlin as controls. As shown in Figure 6A and B and Supplementary Fig. S3A and B, the expression of merlin recovered after pcDNA3 merlin transfection. Interestingly, Transwell™ assays showed that the migration or invasive ability of BHP10-3 cells and K1 cells decreased after co-transfection with miR-146b-3p mimics and pcDNA3 merlin (Fig. 6C and D and Supplementary Fig. S3C and D). RTCA assay showed that compared to the migratory and invasive abilities of cells after miR-146b-3p mimic transfection, the migratory and invasive abilities of BHP10-3 cells decreased significantly in BHP10-3 cells co-transfected with miR-146b-3p mimics and pcDNA3 merlin (Fig. 6E and F). The results further revealed that reintroduction of merlin expression significantly abrogated the metastatic potential induced by miR-146b-3p in BHP10-3 cells, suggesting that *NF2* is the functional target gene for miR-146b-3p in BHP10-3 cells.

Discussion

As the most common type of thyroid cancer, the incidence of PTC has increased rapidly in recent years (1). A substantial proportion of PTC patients, especially those with lymph node and distant metastasis, are prone to relapse and can have a poor prognosis. Identification of the molecular pathogenesis of PTC metastasis is important to identify effective diagnostic and therapeutic strategies for these patients. This study found that miR-146b-3p, as an ignored mature expression product of miR-146b, is significantly overexpressed in PTC metastasis lymph nodes and is positively correlated with migration and invasion abilities. In addition, the results demonstrate that the tumor suppressor gene *NF2* is the target of miR-146b-3p. These findings might contribute to the early diagnosis and prevention of PTC metastasis.

MicroRNA expression is markedly tissue specific. Aberrant expression of tissue-specific miRNA is associated with various types of cancers arising in the lung, pancreas, breast, and colon (15). MiR-146b has been identified as an oncogene in PTC based on its increased expression in PTC tissue

FIG. 5. MiR-146b-3p promotes tumor metastasis *in vivo*. (A) Schematic of male BALB/c PTC intramuscular xenograft nude mice model. (B) Body weight of miR-146b-3p agomir or NC agomir (control) treated BHP10-3SCmluc inoculated nude mice ($n=5, 10$). (C) For spontaneous tumor metastasis images were taken from three representative miR-146b-3p agomir or NC agomir (control) treated model separately. Bioluminescence images were obtained from the chest side of the mice *in vivo* (upper), and fluorescence images were obtained from the lungs and lymph nodes of sacrificed mice *ex vivo* (lower) at the end time point. (D) The size and morphology of the miR-146b-3p agomir or NC agomir treated BHP10-3SCmluc xenografts in nude mice. (E) Ratio of metastasis of miR-146b-3p agomir or NC agomir (control) treated BHP10-3SCmluc inoculated nude mice. (F) Histopathological characteristics of a representative intramuscular xenograft tumor, pulmonary and lymph node metastases of sacrificed mouse stained with H&E (magnification $\times 100$). The expression of miR-146b-3p (G) and protein levels of merlin (H) were determined by qRT-PCR and Western blot assay separately. The tissues were derived from xenograft mouse tumors treated with miR-146b-3p agomir or NC agomir. $**p < 0.001$. Color images available online at www.liebertpub.com/thy

(14,39), but few studies have analyzed the expression of miR-146b in PTC with LNM. In particular, the relationship between miR-146b-5p and -3p and aggressive clinical pathological features of PTC remains unclear. Metastasis, which is the spread of cancer from its site of origin and subsequent colonization of distant organs, includes a multistage process involving cancer cell motility and transit in the blood or lymph. Only a subset of tumor cells with more aggressive characteristics can overcome these diverse challenges and produce colonization (40). Spreading of cancer cells by PTC occurs principally via the lymphatic system, resulting in re-

gional LNM (41). Some publications have reported that miRNA-146b expression is increased in PTC or PTC with LNM in contrast to the expression in benign tissues or PTC without LNM by analyzing primary tumor samples of PTC patients with different degrees of aggressiveness (42). However, further analysis of the different expression levels of miR-146b between primary and metastatic lymph nodes from the same PTC patients, which can better reflect the characteristics of cell clusters tending to invade, has not been performed. On the other hand, miR-146b-5p, as the popular mature product of miR-146b, has been reported to be the

FIG. 6. Reintroduction of NF2 abrogates miR-146b-3p induced metastasis enhancement of BHP10-3 cells. BHP10-3 cells were co-transfected with miR-146b-3p mimics and pcDNA3 merlin or pcDNA3 (empty vector control), miR-NC (mimics control), or pcDNA3 merlin was respectively transfected into cells as control. (A) The protein level of endogenous merlin was detected by Western blot analysis after transfection. (B) The densitometric histogram of protein bands and results were expressed as ratio of corresponding protein to GAPDH. (C) Transwell™ assay was performed to determine the migration or invasive ability of BHP10-3 cells after being transfected for 24 or 48 h. Representative images show migrated or invasive BHP10-3 cells in the lower chamber stained with hematoxylin. (D) Cell count of Transwell™ assay for different treatment was statistically analyzed. (E and F) The migratory and invasive capacities of BHP10-3 cells were performed by RTCA assay after being transfected. # $p < 0.05$; ## $p < 0.005$ vs. miR-NC. Color images available online at www.liebertpub.com/thy

major effective mature type, while the expression and function of miR-146b-3p has remained elusive.

This work focused on miR-146b expression in PTC with LNM and detected levels of miR-146b-5p and miR-146b-3p in patients with PTC of different degrees of aggressiveness. Similar to the reports in the literature, upregulation of miR-146b-5p was confirmed in PTC with LNM compared to that in PTC without LNM. As expected, a similar phenomenon

was detected for miR-146b-3p expression. Furthermore, the two mature types of miR-146b were analyzed among tissues pathologically confirmed as primary PTC and LNM, matched to adjacent non-neoplastic thyroid tissues and normal lymph node tissues from the same patient as controls. The results indicate that miR-146b-5p and miR-146b-3p expression is higher in PTC tissue compared to that in adjacent non-neoplastic thyroid tissue. Intriguingly, expression

of miR-146b-5p and miR-146b-3p was further increased in thyroid metastatic lymph nodes compared to that in primary PTC, which indicates that the cell clusters that reached the lymph nodes expressed higher miR-146b-5p and miR-146b-3p levels, with stronger metastatic abilities. Further, the relationship between the expression of miR-146b-3p or -5p and clinicopathologic features of patients with PTC was analyzed. The expression levels of miR-146b-3p and miR-146b-5p were significantly positively associated with LNM. Because of the incomplete information for the other 17 PTC patients, follow-up was available only for a small portion (7/24) of the PTC patients, and none of these patients had a recurrence. There were no significant differences in the expression levels of miR-146b-3p or miR-146b-5p with other clinicopathologic parameters, including age at the time of diagnosis, multicentricity, and tumor staging. The results are partially consistent with a study by Han *et al.* (43) that demonstrated that miR-146b-3p, miR-146b-5p, and miR-222 are predictive of PTC associated with central LNM. These results suggest that upregulation of miR-146b-3p, consistent with miR-146b-5p, is positively correlated with PTC LNM. MiR-146b-3p, similar to miR-146b-5p, can be a predictor of thyroid tumorigenesis and metastatic ability in PTC.

To verify further the biological bases of the association between miR-146b-5p and -3p expression with PTC metastasis, PHT and PTC cell lines (BHP10-3 cells derived from *RET/PTC1* rearrangement and K1 cells derived from *BRAF*^{V600E} mutation) (44) were used to analyze the expression of miR-146b-5p and miR-146b-3p. BHP10-3SC_{mice} cells, as a colony of BHP10-3 with the same *RET/PTC1* rearrangement background, proved extremely tumorigenic and invasive (28). It was interesting to observe that the expression of both miR-146b-5p and -3p increased in PTC cells, especially in BHP10-3SC_{mice} cells, which was similar to the results in different samples of PTC patients. In addition, although the amount of miR-146b-3p was much lower than that of miR-146b-5p in PHT and different PTC cell lines, it was discovered that miR-146b-3p was more positively correlated with the invasion ability of PTC cells than miR-146b-5p.

MiR-146b-5p is a well characterized miRNA and is involved in many cancers, including lung and breast cancer and glioblastomas (16–18). MiR-146b-5p enhances cell proliferation by repressing SMAD4 and disrupting TGF- β signal transduction in thyroid cancer (20). Recently, studies suggested that miR-146b-5p enhanced the migratory and invasive abilities in normal rat thyroid cell lines (PCCI3) and different tumor follicular cells (TPC-1 and BCPAP) (45). In these cell line studies, miR-146b-5p overexpression was achieved using expression of the PC-CMV-146b plasmid, which is also the precursor for miR-146b-3p. The effect on promoting cell migratory and invasive abilities may be the common effect of miR-146b-5p and -3p, rather than the individual action of miR-146b-5p. Deng *et al.* (21) presented that miR-146b-5p directly enhanced Wnt/ β -catenin signaling through the downregulation of its target *ZNRF3*, thus promoting metastasis in PTC. However, the effect of miR-146b-3p was ignored.

In recent years, researchers have realized that although arising from the same precursor, unlike miR-146b-5p, miR-146b-3p might play a different and important role in cancer. Research on A549 lung cancer cells has shown that miR-146b-5p and miR-146b-3p have negative effects on the

malignant phenotype of lung cancer cells (24). Garcilaso *et al.* (46) found that miR-146b-3p targets two genes, *PAX8* and *SLC5A5*, key markers for differentiated thyroid phenotype, might modulate thyroid cell differentiation and iodide uptake for improved treatment of advanced thyroid cancer. In this research, miR-146b-5p and miR-146b-3p mimics or inhibitors were used to overexpress or inhibit different mature products of miR-146b separately. The effect of miR-146b-5p and miR-146b-3p overexpression on the metastatic nature of the BHP10-3 cells was determined using the RTCA migration and invasion assays. Unlike the Transwell™ migration and invasion assay as an endpoint assay, xCELLigence RTCA technology provided real-time data to be a highly accurate platform to monitor living cell behavior (47) and evaluate the migratory and invasive behaviors of BHP10-3 cells altered with miR-146b overexpression. Notably, miR-146b-3p enhances the migratory and invasive abilities more obviously than miR-146b-5p in the RTCA assay. Conventional Transwell™ migration and invasion assay correlate very well with this finding. These results indicate that instead of miR-146b-5p, miR-146b-3p plays a crucial role in promoting PTC cell metastasis and is specifically involved in PTC cell migration and invasion.

To illustrate how miRNA-146b-3p promotes the invasion and migration of PTC, the study screened for potential target genes of miR-146b-3p by bioinformatics software and identified *NF2* and *MTSS1*. Subsequent studies using luciferase reporter assays revealed that miR-146b-3p binds directly to the 3' UTR of *NF2* but not *MTSS1*. Germline and somatic mutations in the *NF2* gene have been detected in hereditary and sporadic tumors. In the familial syndrome, a germline mutations is found, together with an acquired somatic mutation (Knudson two-hit mechanism) (48). *NF2* was originally reported in the neurofibromatosis type 2 cancer syndrome, which is characterized by a highly specific subset of central and peripheral nervous system tumors (29). Studies have revealed that as a tumor suppressor gene, *NF2* plays a broad role in restraining cell proliferation by regulating several important signaling pathways, such as the mTOR, PI3K/AKT, and hippo signaling pathways (29,49–51), and it translocates to the nucleus to inhibit CRL4^{DCAF1} E3 ubiquitin ligase (52). *NF2* was one of the most frequent allelic losses (75%) in medullary thyroid carcinomas (53). Recently, Fagin *et al.* demonstrated that *NF2*/merlin inactivation improves mutant *RAS* signaling by promoting YAP/TEAD-driven oncogenic transcription, resulting in development of poorly differentiated thyroid cancers (54). Furthermore, the ever-increasing breadth of mechanistic insights revealed that *NF2* plays a crucial role in tumor metastasis. McClatchey *et al.* (55) observed that *NF2* heterozygous mice develop multiple malignant tumor types with a high frequency of metastasis to distant sites. However, whether *NF2* contributes to PTC metastasis remains unclear.

This study first elucidated that the enhanced effects of miR-146b-3p on PTC cell migration and invasion abilities were attenuated by reintroduction of merlin expression, which suggests that *NF2*/merlin is involved in the regulation of PTC cell metastasis. Notably, although some studies (56–58) reported that merlin could interact with adherens junctions, which are firmly linked to tumor invasion, and suppress tumor metastasis by attenuating the FOXM1-mediated Wnt/ β -catenin signaling, the expression of β -catenin was

unchanged when miR-146b-3p or merlin was overexpressed (data not shown), suggesting that NF2/merlin inhibited PTC metastasis in a Wnt/ β -catenin-independent manner. Additional studies regarding the potential mechanisms will be done in the future.

The study also verified the role of miR-146b-3p on PTC metastatic ability *in vivo*. Due to the limitation of relatively low tumorigenicity and aggressiveness of PTC, there is no highly aggressive PTC animal model that can be used to evaluate the potential treatment effect of miR-146b-3p inhibition. An *in vivo* nude mouse xenograft model with high PTC tumorigenicity cells was used, and the miR-146b-3p agomir was subsequently injected into the tumor *in situ*. Although the tumor volumes in nude mice were not markedly changed, the ratio of lymph node and distant metastasis was significantly increased in high miR-146b-3p expression cancers compared to the control PTC. Additionally, the level of merlin notably decreased with the increased miR-146b-3p expression. These results further indicate that high expression of miR-146b-3p inhibit expression of merlin and promote PTC tumor metastasis *in vivo*.

In summary, the present results demonstrate that miR-146b-3p, as one mature product of miR-146b, is notably upregulated in PTC with LNM and metastatic lymph nodes and plays a more important role than miR-146b-5p in human PTC cell migration and invasion by directly targeting NF2. These findings are important for the early diagnosis of PTC patients with the tendency to develop metastases and aggressive phenotypes. Moreover, these findings may provide new gene therapy targets for PTC metastasis in the future.

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Author Disclosure Statement

The authors declare that they have no competing interest.

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