MiR-146b-5p Promotes Metastasis and Induces Epithelial-Mesenchymal Transition in Thyroid Cancer by Targeting ZNRF3

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Key Words
MiR-146b-5p • Metastasis • Epithelial-mesenchymal transition • Thyroid cancer • ZNRF3

Abstract
Background/Aims: Micro-RNA (miR)-146b-5p is overexpressed in papillary thyroid carcinoma (PTC) and associated with extrathyroidal invasion and advanced tumor stage. In the present study, we showed that miR-146b-5p is upregulated in PTC with lymph node metastasis. Methods: A computational search and luciferase assay identified zinc RING finger 3 (ZNRF3), a negative regulator of Wnt/β-catenin signaling, as a direct target of miR-146b-5p in PTC. Results: MiR-146b-5p promoted migration and invasiveness and induced epithelial-mesenchymal transition (EMT) of PTC cells, whereas ZNRF3 overexpression reversed this effect. MiR-146b-5p increased the cell surface levels of the Wnt receptors Frizzled-6 and LRP6 and enhanced Wnt/β-catenin signaling by downregulating ZNRF3, whereas an inhibitor of Wnt/β-catenin suppressed the effect of miR-146b-5p on migration, invasiveness and EMT of PTC cells. Conclusion: These results indicate that miR-146b-5p induces EMT and may promote PTC metastasis through the regulation of Wnt/β-catenin signaling, and suggest novel potential therapeutic targets for the treatment of PTC.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor, and its incidence has been steadily increasing in the last decades [1]. Several factors are implicated in the development of PTC including genetic alterations, growth factors and radiation, and the prognosis of patients with PTC is associated with age, tumor size, and histological parameters such as lymph node invasion and distant metastasis [2]. The histological variants of PTC, which are related to tumor aggressiveness and therefore to prognosis, include conventional PTC, papillary thyroid microcarcinoma, follicular variant and tall cell variant [3].

MicroRNAs (miRNAs) are small (22-nucleotide) noncoding single stranded RNAs that regulate gene expression by binding to the 3’ untranslated region (3’ UTR) of their target mRNAs, modulating mRNA stability and/or translation [4]. The dysregulation or aberrant expression of miRNAs has been implicated in the pathogenesis of human cancer and several miRNAs have been shown to be dysregulated in thyroid tumors including PTC [5]. MiRNAs upregulated in PTC include miR-187, -221, -146b, -155, -122a, -31, -205 and -224 [6, 7] and a number of miRNAs are downregulated in PTC and contribute to the development and progression of the disease [3]. MiR-146b was shown to be significantly overexpressed in PTCs with extrathyroidal invasion and associated with high-risk PTC with BRAF mutation [5]. Furthermore, miR-146b expression is an independent risk factor for poor prognosis in PTC together with advanced tumor stage and cervical lymph node metastasis [8]. Overexpression of miR-146b-5p was shown to significantly increase cell proliferation through the modulation of TGF-β signaling in thyroid cancer [9]. However, the role of miR-146b-5p in extrathyroidal invasion remains unclear.

Epithelial-mesenchymal transition (EMT) is a crucial step in the process of migration of carcinoma tumors from the primary site into surrounding tissues [10]. Multiple signaling pathways are involved in triggering EMT in normal and transformed cells such as the TGF-β, Wnt-β-catenin and Notch pathways [11]. These signaling pathways activate several EMT-related transcription factors, such as Snail (Snai1), Slug (Snai2) and EF1/ZEB1, which directly or indirectly inhibit E-cadherin production [12].

In the present study, we investigated the effect of miR-146b-5p on thyroid carcinogenesis and extrathyroidal invasion to examine the underlying mechanisms. We identified zinc RING finger 3 (ZNRF3) as a direct target of miR-146b-5p and showed that miR-146b-5p promotes cell migration and invasion and increases the cell surface levels of Wnt receptors through the suppression of ZNRF3. Our results indicate that miR-146b-5p promotes EMT in PTC through a mechanism involving the regulation of Wnt/β-catenin signaling and suggest novel potential therapeutic targets for the treatment of PTC.

Materials and Methods

Tissue samples and papillary carcinoma cell lines

Tumor specimens and tumor-adjacent tissues were obtained from 60 PTC patients (Table 1) who underwent thyroidectomy in Shanghai 6th People’s Hospital between January 2006 and January 2009. Thirty patients were confirmed to have lymph node metastasis at the time of the operation. The study was approved by the Research Ethics Committee of Shanghai Jiao Tong University and informed consent was obtained from all patients.

Two human papillary thyroid carcinoma cell lines, namely TPC-1 (Shanghai Ruqi Biological Technology Co., LTD) and K1 (Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China), and human thyroid follicular epithelial cells (Nthy-ori 3-1) (JENNIO Biological Technology, Guangzhou, China) were cultured in DMEM (Gibco) containing 10% FBS (Life Technologies). Authentication of K1 and TPC-1 cells was performed previously [13].

Real-time PCR

Total RNA was extracted from surgical specimens or cell lines using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For miRNA expression analysis, 10 ng of total
RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) and RT primers provided with the miR-146b-5p Taqman miRNA Assay (Applied Biosystems) according to the manufacturer’s instructions. MiR-146b-5p expression was detected from the cDNA product, using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan miRNA sequence-specific probes (Applied Biosystems). U6 small nuclear 2 (RNU6B) was used for normalization.

For mRNA expression analysis, 3 μg of total RNA was converted into cDNA using M-MLV Reverse Transcriptase (Invitrogen). PCR products were amplified using SYBR Green Universal PCR Master Mix (Applied Biosystems) and the following primers: forward 5′-CATCGTCAACAAGCAGAAAGTG-3′ and reverse 5′-GGAGACCACGACGAAGAAAG-3′ for ZNRF3. β-actin was used as an endogenous control for mRNA normalization. Amplification was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems). Relative quantification of MiRNA and mRNA for each sample was performed using the comparative Ct method (2-△△Ct) [14].

**Luciferase reporter assays**

Total cDNA from TPC-1 and K1 cells was used to amplify the 3′UTRs of ZNRF3 by PCR using the forward primer 5′-ACTAGTGTATAGCAGCACATTTCATTT-3′ and the reverse primer 5′-AAG CTT CAG GGT CTT GTG TTA GTC-3′. Site-directed mutagenesis of miR-146b-5p binding sites was carried out using a QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). Then, the segments were cloned downstream of the luciferase reporter gene in the pMIR-REPORT plasmid (Ambion, Austin, USA) to generate pMir-Report-ZNRF3-wt plasmid and pMir-Report-ZNRF3-mut plasmid.

For reporter assays, TPC-1 and K1 cells were transfected with the plasmids pMir-Report-ZNRF3-wt or -mut, in combination with 10 nM miR-146b-5p mimic, mimic NC, 10 nM anti-miR-146b-5p or anti-NC (Ambion) using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were washed and lysed. Luciferase activity was assessed using DualGlo Luciferase Assay System (Promega) and normalized by the control vector containing Renilla luciferase.

**Western blotting**

Cells were trypsinized and lysed in the presence of protease inhibitors. Total protein lysates (30 μg) were separated by 10% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were incubated with the primary antibodies anti-ZNRF3, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-lipoprotein receptor-related protein (LRP)6,
anti-β-catenin (all from Santa Cruz, CA, USA), and anti-Frizzled (FZD)1, anti-FZD2, anti-FZD6, and anti-Slug (all from Abcam, Cambridge, MA, USA). The anti-β-actin antibody (Santa Cruz) was used to normalize the protein input. After washing, membranes were incubated with secondary antibodies for 1 h at room temperature and visualized using an Enhanced Chemiluminescence kit (GE HealthCare) according to the manufacturer’s instructions.

**In vitro migration and invasion assay**

The migratory and invasive capacities of TPC and K1 cells were assessed using the BioCoat Invasion Chamber system (BD Biosciences, San Jose, CA, USA). After treatment, cells were diluted in serum-free DMEM and plated on the top of Matrigel invasion chambers (8-μm pore size; BD Biosciences) with Matrigel Basement Membrane Matrix (for invasion test) or with BSA (for migration test). A volume of 2.5mL of 10% FBS-DMEM was added to the lower compartment. The migration assay was performed for 24 h at 37°C in a 5% CO₂ incubator. After incubation, nonmigrating cells were removed by scrubbing, and migrating cells present on the lower surface of the membrane were stained with Hematoxylin and Eosin (Sigma-Aldrich). Cells from 10 fields were counted at 400×. The data are expressed as mean±SD of triplicate filters.

**Flow cytometric analysis**

Cells were collected using trypsin-free cell dissociation buffer (Invitrogen) and resuspended in FACS buffer (PBS with 1% BSA and 0.02% sodium azide). After blocking with mouse serum, cells were incubated with anti-FZD6 or anti-LRP6 antibody (R&D system) for 1 h at 4°C, followed by incubation with conjugated secondary antibodies. After washing in FACS buffer, cells were subjected to multichannel analysis using a FACScan flow cytometer (BD) and analyzed using Cell-Quest 3.3 software.

**Lentivirus construction and infection**

ZNRF3 cDNA was cloned by PCR using the forward primer 5’-AGC TAC TAG TAT GAG GCC GCG CTC GGG CGG CCC-3’ and reverse primer 5’-AGC TAA GCT TTC AGG CTC CCG GGC TGC TGC TGT CTG-3’. For lentivirus construction, the ZNRF3 cDNA clone was inserted into pHBLV-CMVIE-IRES-Puro lentiviral vectors (Hanbio, Shanghai, China). The recombinant lentivirus with the ZNRF3 gene coding sequence was produced by co-transfection of 293T cells with the plasmids PSPAX2 and PMD2G using LipoFiter™ (Hanbio, Shanghai, China). Lentivirus-containing supernatant was harvested 48 h after transfection and filtered through 0.22-μm cellulose acetate filters (Millipore, USA). Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at 50,000 × g).

To establish stable ZNRF3-overexpressing cell lines, TPC-1 cells were transduced with lentiviral vector at an MOI of approximately 10 in the presence of 5 μg/ml polybrene. After 24 h, the culture medium was removed and fresh medium was added. At 72 hours after transduction, puromycin (5 mg/ml) was added to medium for stable cell line selection. The empty lentivirus vector-puromycin was used as the negative control. After antibiotic selection for 3 weeks, cells stably overexpressing the ZNRF3 gene were obtained. To establish stable ZNRF3-knockdown cell lines, K1 cells were transduced with ZNRF3 shRNA Lentiviral vector (OriGene, Rockville, MD, USA) and selected following the manufacturer’s instructions. After harvesting cells, the expression level of ZNRF3 was determined by western blotting and real-time-PCR.

**Statistical analysis**

Data were obtained from three independent replicates and presented as the mean±SD. The statistical significance of the difference was analyzed by one-way analysis of variance (more than two groups) or Student’s t-test (two groups only). Differences were considered significant at p<0.05.

**Results**

**MiR-146b-5p is upregulated and ZNRF3 is downregulated in papillary thyroid carcinoma with lymph node metastasis**

The expression of miR-146b-5p was examined in 60 tumor-adjacent tissues, and primary PTC tumors without (n=30) and with (n=30) lymph node metastasis (LNM) by real time PCR. The results showed that miR-146b-5p is significantly upregulated in tumors with LNM.
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compared to those without (p<0.05) (Fig. 1A). Based on a computational search identifying ZNRF3 as a potential target of miR-146b-5p, we examined its levels of expression in the same PTC tissue samples by real time PCR and found significantly lower levels of ZNRF3 mRNA in tumors with LNM than in those without (p<0.05) (Fig. 1B). A scatter diagram confirmed that miR-146b-5p expression and ZNRF3 expression are negatively correlated (Fig. 1C).

**Fig. 1.** miR-146b-5p is upregulated and ZNRF3 is downregulated in papillary thyroid carcinoma with lymph node metastasis. A and B, miR-146b-5p (A) and ZNRF3 (B) expression in 30 primary tumors with lymph node metastasis (LNM), 30 without lymph node metastasis (PT) and 60 tumor-adjacent normal tissues (control) was analyzed by real-time PCR. (C) A scatter diagram shows that miR-146b-5p expression and ZNRF3 expression are negatively correlated. * p<0.05, compared with the control; #p<0.05, compared with PT.

**ZNRF3 is a target of miR-146b-5p in PTC**

MiR146b-5p was expressed at higher levels in TPC-1 cells and K1 cells than in control human thyroid follicular epithelial cells (Nthy-ori 3-1), and miR146b-5p expression was significantly higher in K1 cells than in TPC-1 cells (Fig. 2A). Binding sites for miR-146b-5p in the 3′-UTR of ZNRF3 were identified by bioinformatics analysis (Fig. 2B). To verify that ZNRF3 is a direct target of miR-146b-5p, ZNRF3 wild type (wt) or mutant (mt) 3′-UTR was subcloned into a luciferase reporter vector and transfected with or without mimic NC, miR-146b-5p mimics, anti-NC or anti-miR-146b into the PTC cell lines TPC-1 and K1. Luciferase reporter assays showed that miR-146b-5p significantly inhibited the luciferase activity of the wild-type ZNRF3 3′-UTR by approximately 50% in TPC-1 cells and 30% in K1 cells relative to the control, whereas cotransfection with anti-miR-146b significantly increased luciferase activity (p<0.05) (Fig. 2C and 2D). Transfection of the mutant ZNRF3 3′-UTR with the same constructs had no effect on luciferase activity, confirming that ZNRF3 is a direct target of miR-146b-5p. To further examine whether miR-146b-5p modulates the expression of ZNRF3
Fig. 2. ZNRF3 is a target of miR-146b-5p in PTC. A, Real-time PCR analysis showed that miR-146b-5p was expressed at higher levels in TPC-1 cells and K1 cells than in control human thyroid follicular epithelial cells (Nthy-ori 3-1). B, The predicted binding site (ZNRF3 3'UTR-wt) and the mutated binding site (ZNRF3 3'UTR-mut) of miR-146b-5p on ZNRF3. C-D, A luciferase reporter plasmid carrying ZNRF3 3'UTR-wt or ZNRF3 3'UTR-mut was transfected into TPC-1 cells (C) or K1 cells (D) alone, co-transfected with mimic NC or miR-146b-5p mimic or with anti-NC or anti-miR-146b and luciferase activity was measured. E-H, TPC-1 cells or K1 cells were transfected or not with mimic NC, miR-146b-5p mimic, anti-NC or anti-miR-146b, and miR-146b-5p expression was analyzed in TPC-1 cells (E) or K1 cells (F) by real-time PCR. ZNRF3 protein expression was analyzed in TPC-1 cells (G) or K1 cells (H) by western blotting. *p<0.05, compared with control; **p<0.01, compared with control; #p<0.05, compared with PT.
of ZNRF3 were analyzed by real-time PCR and western blotting. Transfection of the miR-146b-5p mimics significantly increased the mRNA level of miR-146b-5p and significantly downregulated the protein expression of ZNRF3, whereas anti-miR-146b had the opposite effects in both cell lines (Fig. 2F–H).

**MiR-146b-5p promotes migration and invasiveness of PTC cells by downregulating ZNRF3**

The relation between miR-146b-5p and its target ZNRF3 and its role in the behavior of PTC cells were analyzed by real-time PCR and western blotting. Transfection of the miR-146b-5p mimics significantly increased the mRNA level of miR-146b-5p and significantly downregulated the protein expression of ZNRF3, whereas anti-miR-146b had the opposite effects in both cell lines (Fig. 2F–H).
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Cellular Physiology and Biochemistry

MiR-146b-5p induces EMT of PTC cells by downregulating ZNRF3

TPC-1 cells were transfected with or without miR-146b-5p mimics and LV-ZNRF3 or co-transfected with miR-146b-5p mimic and LV-ZNRF3, and the expression of EMT markers was analyzed by western blotting. The results showed that miR-146b significantly promoted whereas ZNRF3 silencing significantly inhibited cell migration and invasion of PTC cells by downregulating the expression of ZNRF3.

Fig. 4. miR-146b-5p induces EMT of PTC cells through the suppression of ZNRF3. A, TPC-1 cells were transfected or not with mimic NC, miR-146b-5p mimic, LV-NC, LV-ZNRF3 or co-transfected with miR-146b-5p mimic and LV-ZNRF3, and the expression of EMT markers was analyzed by western blotting. B, K1 cells were transfected or not with miR-146b-5p shRNA NC, miR-146b-5p shRNA, ZNRF3 shRNA NC, ZNRF3 shRNA or co-transfected with miR-146b-5p shRNA and ZNRF3 shRNA, and the expression of EMT markers was analyzed by western blotting.

Fig. 5. miR-146b-5p increases cell surface levels of FZD6 and LRP6 through the suppression of ZNRF3. TPC-1 cells were transfected or not with miR-146b-5p mimic, LV-ZNRF3 or co-transfected with miR-146b-5p mimic and LV-ZNRF3. A, The protein levels of FZD1, FZD2, FZD6 and LRP6 were analyzed by western blotting. B, The cell surface levels of FZD6 and LRP6 were determined by flow cytometry assay.

and ZNRF3 protein expression was analyzed by western blotting. Transfection with miR-146b-5p shRNA significantly upregulated the expression of ZNRF3, whereas co-transfection of miR-146b-5p shRNA and ZNRF3 shRNA reversed this effect (Fig. 3B). Cell migration and invasion assays showed that overexpression of miR-146b significantly promoted whereas ZNRF3 overexpression significantly inhibited cell migration and invasion in TPC-1 (Fig. 3 C and E) and co-expression of ZNRF3 reversed the effect of miR-146b-5p. Silencing of miR-146b significantly inhibited cell migration, and co-expression of ZNRF3 shRNA reversed the effect of miR-146b-5p silencing in K1 cells (p<0.05) (Fig. 3D). Silencing of miR-146b significantly inhibited whereas ZNRF3 silencing significantly promoted cell invasion, and co-expression of ZNRF3 shRNA reversed the effect of miR-146b-5p silencing in K1 cells (p<0.05) (Fig. 3F). These results indicated that miR-146b-5p promotes the migration and invasion of PTC cells by downregulating the expression of ZNRF3.

MiR-146b-5p induces EMT of PTC cells by downregulating ZNRF3
expression of the epithelial marker E-cadherin and upregulated the expression of the mesenchymal markers N-cadherin and vimentin and this effect was suppressed by co-transfection with ZNRF3 in TPC-1 cells (Fig. 4A), whereas miR-146b silencing upregulated the expression of E-cadherin and downregulated the expression of N-cadherin and vimentin, and this effect was suppressed by co-transfection with ZNRF3 shRNA in K1 cells (Fig. 4B). These results indicated that miR-146b promotes EMT in PTC cells by downregulating the expression of ZNRF3.

MiR-146b-5p promotes invasiveness and induces EMT of PTC cells by modulating Wnt/β-catenin signaling

ZNRF3 inhibits Wnt signaling by promoting Wnt receptor ubiquitination and degradation [15, 16]. Wnt binds to receptors of the FZD family, disrupting the function of a destruction complex that normally ubiquitinates and targets β-catenin for degradation. The FZD gene family in mammals comprises at least 10 members [17] and FZD1, FZD2, and FZD6 are expressed in thyroid cells [18]. We therefore examined the expression of FZD receptors and low-density LPR6, a co-receptor involved in the interaction between the Wnt protein and the FZD receptors leading to the accumulation of β-catenin, in TPC-1 cells transfected with or without miR-146b-5p mimics, LV-ZNRF3 or co-transfected with miR-146b-5p mimics and LV-ZNRF3. The Wnt inhibitor IWR-1-endo (300 nM) was used to treat TPC-1 cells or miR-146b-5p transfected TPC-1 cells. The protein levels of β-catenin, slug and EMT markers were analyzed by western blotting. B-C, TPC-1 cells were transfected or not with miR-146b-5p mimic or treated with or without IWR-1-endo. Migration (B) and invasiveness (C) were detected. *p<0.05, compared with control; #p<0.05, compared with miR-146b.

To confirm the involvement of the Wnt/β-catenin pathway in the miR-146b-5p mediated promotion of EMT in PTC, the protein expression of β-catenin, markers of EMT and the E-cadherin transcriptional repressor Slug were analyzed by western blotting in
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Discussion

The overexpression and oncogenic role of miR-146b-5p in PTC have been reported previously [5, 8, 9]. However, to date, the relationship between miR-146b-5p and PTC invasiveness has remained unclear. In the present study, we show that miR-146b-5p plays a role in the invasiveness of PTC through the regulation of the Wnt signaling pathway. We identified ZNRF3, a negative regulator of Wnt/β-catenin signaling, as a direct target of miR-146b-5p and showed that miR-146b-5p promotes invasiveness and EMT in PTC by downregulating ZNRF3, resulting in enhanced Wnt signaling and the promotion of tumor cell migration and invasion.

The Wnt/β-catenin pathway plays a crucial role in the development of thyroid cancer [19] and a Wnt/GSK3β/β-Trcp1 axis regulating Slug (Snail2) activity and thus EMT was recently identified in breast cancer [20]. The EMT process involves the dissolution of intercellular adhesions and the acquisition of a more motile mesenchymal phenotype, a crucial mechanism by which carcinoma cells enhance their invasive capacity [21]. In the present study, we found that miR-146b-5p promoted PTC cell migration and invasion and downregulated the epithelial marker E-cadherin, whereas it upregulated the expression of the mesenchymal markers vimentin and N-cadherin, suggesting that miR-146b-5p induces alterations in thyroid cancer cells resembling those of the EMT process. Furthermore, miR-146b-5p upregulated the expression of Slug and this effect was reversed by a Wnt antagonist, confirming the role of miR-146b-5p in promoting EMT through a process involving the regulation of Wnt signaling.

TGF-beta signaling has been shown as a potent driver of EMT through the activation of Smad2/3, which form complexes with Smad4, resulting in their translocation to the nucleus and the regulation of the transcription of target genes leading to the induction of EMT [22]. Furthermore, crosstalk between TGF-β/Smad signaling and Wnt signaling has been reported in the context of EMT. However, a previous study found that miR-146b-5p represses Smad4 and disrupts TGF-β signal transduction in PTC [9]. The present results showing a direct effect of miR-146b-5p on enhancing Wnt/β-catenin signaling through the downregulation of its target ZNRF3 provide a potential mechanism by which miR-146b-5p induces EMT and thus promotes metastasis in PTC.

ZNRF3 is a unique transmembrane E3 ubiquitin ligase that interacts with the FZD and LRP 5/6 complexes, leading to the ubiquitination and degradation of the heterodimeric receptors, thereby suppressing Wnt-driven signal transduction [15]. We found that miR-146b-5p increases the cell surface levels of FZD6 and LRP6 through the suppression of ZNRF3, resulting in enhanced Wnt/β-catenin signaling. These findings reveal a novel mechanism of miR-146b-5p mediated induction of EMT and suggest that ZNRF3 plays a tumor suppressor role in PTC. A similar role for ZNRF3 was demonstrated in human gastric adenocarcinoma in a recent study that showed that ZNRF3 inhibits gastric cancer cell growth and promotes apoptosis through the modulation of the Wnt/β-catenin signaling pathway [23]. Mutations
in ZNRF3 and its homolog RING finger 43 (RNF43) have been linked to pancreatic ductal adenocarcinoma and mucinous ovarian tumors [24, 25] confirming the role of these ubiquitin ligases as tumor suppressors.

In the present study, we identified a regulatory mechanism linking miR-146b-5p and its target ZNRF3 to EMT through the modulation of Wnt/β-catenin signaling. We found that miR-146b-5p is upregulated in PTC with LNM and identified ZNRF3 as its direct target. We demonstrated that miR-146b-5p promotes EMT in PTC by suppressing ZNRF3, leading to the activation of Wnt/β-catenin signaling. Our results provide insight into the role of miR-146b-5p in thyroid carcinogenesis and metastasis and suggest possible therapeutic targets for the treatment of metastatic PTC.

**Disclosure Statement**

We have no conflict of interest to report.

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