Upregulated miR-155 in Papillary Thyroid Carcinoma Promotes Tumor Growth by Targeting APC and Activating Wnt/\(\beta\)-Catenin Signaling

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Context: MicroRNAs (miRNAs) are strongly implicated in many cancers, including papillary thyroid carcinoma (PTC), which is the most common malignancy in thyroid tissue. Recently, miRNA-155 (miR-155) has been proved to play a substantial role in liposarcoma and breast cancer, but its functions in the context of PTC remain unknown.

Objectives: The objective was to investigate the potential involvement of miR-155 in PTC.

Design: Expression levels of miR-155 were assessed via quantitative real-time PCR in 20 pairs of human PTC and adjacent normal tissues and in 4 human PTC cell lines. Lentiviral miR-155 overexpression models were performed in TPC-1 and CGTH-W3 cells, and the effects on cell growth were evaluated. We have searched for miR-155 targets and identified the hypothesis that miR-155 could promote tumor growth of PTC by targeted regulation of adenomatous polyposis coli (APC) expression and activating the Wnt/\(\beta\)-catenin signaling.

Results: MiR-155 levels were markedly increased in PTC specimens and PTC cell lines. Overexpression of miR-155 dramatically promoted PTC cell viability and colony formation in vitro, whereas miR-155 depletion reduced these parameters. Further studies revealed that APC is a novel miR-155 target, because miR-155 bound directly to its 3’-untranslated region and reduced both the mRNA and protein levels of APC. Similar to the miR-155 over-expression, APC downregulation promoted cell growth, whereas rescued APC expression reversed the promotive effect of miR-155. Furthermore, miR-155 overexpression resulted in activation of \(\beta\)-catenin and induction of several downstream genes including c-Myc, cyclin D1, TCF-1, and LEF-1. Depletion of \(\beta\)-catenin partially prevented miR-155–induced tumor cell viability and colony formation. In xenograft animal experiments, we found overexpressed miR-155 effectively promoted tumor growth of PTC cells.

Conclusions: Our results indicate that miR-155 functions as an oncogene in PTC. By targeting APC, miR-155 efficiently regulates the Wnt/\(\beta\)-catenin signaling. And miR-155 may be a potential therapeutic or diagnostic/prognostic target for treating PTC. (J Clin Endocrinol Metab 98: E1305–E1313, 2013)

Thyroid cancer is the most common malignancy of endocrine system, accounting for 5% to 10% of cancers in women. In the United States, ~1690 deaths were estimated to result from thyroid cancer in 2010 (1). Of the various histologic subtypes, papillary thyroid carcinoma (PTC) accounts for ~80% of cases. Genetically, PTC is characterized by alterations in the RET-RAS-BRAF/MAPK signaling pathway (2, 3). These alterations include

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**Materials and Methods**

**Tissue specimens and cell lines**

Twenty pairs of human PTC and adjacent normal tissues were obtained from Shanghai 10th People’s Hospital (Shanghai, China). The tissues were frozen in liquid nitrogen and stored at −80°C until use. All tumors were reviewed by a single pathologist and classified according to the widely accepted diagnostic histologic criteria (24). Written informed consent for tissue donation (for research purposes) was obtained from the patients, and the protocol was approved by the Institutional Review Board of Shanghai 10th People’s Hospital and Tongji University. Human HEK293T cells, human PTC cell lines TPC-1 (Shanghai Ruiqi Biological Technology Co, Ltd, Shanghai, China), CGTH-W3 (25), K1 (Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China), IHH-4 (26), the human thyroid epithelial cell line Nthy-ori-1 (Shanghai Edinburgh Biological Science and Technology Development Co, Ltd, Shanghai, China), and noncancerous human thyroid epithelial cells (TECs) (27) were cultured in DMEM plus 10% fetal bovine serum (Life Technologies, Inc, Grand Island, New York) at 37°C in a humidified atmosphere containing 5% CO₂. The CGTH-W3 cell line was a generous gift from Dr Jen-Der Lin (Chang Gung Memorial Hospital, Taiwan), The IHH-4 cell line was obtained from Health Science Research Resources Bank (Osaka, Japan) and was established from a 75-year-old man with a PTC, The TECs were isolated from adjacent normal thyroid tissue according to the method described previously in detail (27). Authentication of normal thyrocyte and PTC cells cultured was provided using the short tandem repeat profiling (for the methods and results see in Supplemental Data “Cell Authentication” and Supplemental Figure 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org).

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted using the TRIzol reagent (Life Technologies) according to the manufacturer’s protocol. The RT-PCR primers for miR-155 and U6 were purchased from Ribobio (Guangzhou, China). The PCR primers for APC were 5’-CCA CAG GCA AAT CCT AAG AGA GAA C-3’ and 5’-ATT GTC AAA AGT TTC TGA TAA GGT C-3’. cDNA was synthesized from 1 μg total RNA with a reverse reaction kit (Promega, Madison, Wisconsin). The melting curves were used to confirm the specificity of (PCR) amplification. Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Da-Liang, China). Expression of U6 or β-actin was used as internal control. The relative expression levels of each gene was calculated and normalized using the 2⁻ΔΔCT method relative to β-actin or U6 small nuclear RNA. All of the reactions were run in triplicate.

**Lentivirus production and infection**

The pre-miR-155 sequence was amplified and cloned into the lentivirus-based expression plasmid pCDH-CMV-MCS-EF1coGFP constructs (System Biosciences, Mountain View, California). The primers for pre-miR-155 were 5’-CCA CAG GAA TCC TCT GAG TGC TGA AGG CTT GTA GTC G-3’ (forward) and 5’-CAC GGA TCC AGT CTA AGT TTA TCC AGC AGG G-3’ (reverse). Virus particles were harvested 48 hours after pCDH-CMV-miR-155 transfection with the packaging phas-
mids pRSV/pREV, pCMV/pVSVG, and pMDLG/pRRE into 293T cells using Lipofectamine 2000 reagent (Life Technologies). TPC-1 and CGTH-W3 cells were infected with recombinant lentivirus-transducing units plus 10 \( \mu \text{g/mL} \) polybrene (Sigma, St Louis, Missouri).

### Oligonucleotide transfection

The APC small interfering RNA (siRNA) and \( \beta \)-catenin siRNA were purchased from GeneChem (Shanghai, China). The miR-155 inhibitor anti-miR-155 were obtained from Ambion (Austin, Texas). Target cells were transfected with anti-miR-155, APC siRNA, or \( \beta \)-catenin siRNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. Cells were collected 48 hours after transfection.

### Plasmid construction

The coding sequences of APC were amplified and cloned into pcDNA3.1(+) to generate APC expression vectors. The primers for APC were 5'-GGA TCC GAT GGC TGC AGC ATA TGA TCA GTT GTT-3' (forward) and 5'-GCG GCC GCT TAA ACA GAT GTC ACA AGG TAA GAC CCA GA-3' (reverse). The full-length APC 3'-untranslated region (UTR) was cloned into pGL3 luciferase vector (Ambion). The luc-mut vector, in which the first 5 nucleotides complementary to the miR-155 seed region were mutated by site-directed mutagenesis, was constructed as a mutant control. The primers for APC 3'-UTR were 5'-GGG TAC CCG GAG GCA CTC TTG ATG GTT AGG-3' (forward) and 5'-CCT CGG TAA AGC AAA GCA GGC TGG GTA A-3' (reverse).

### Luciferase reporter assay

TPC-1 and CGTH-W3 cells were seeded in 24-well plates and transiently transfected with appropriate reporter plasmid and miRNA using Lipofectamine 2000. After 48 hours, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase was used for normalization. The experiments were performed independently in triplicate.

### Cell viability analysis

To evaluate cell viability, 2 \( \times \) 10^3 cells were separately plated in 96-well plates. After transfection, cell viability was identified using methylthiazol tetrazolium (MTT) assay. Briefly, 20 \( \mu \text{L} \) of an MTT dye solution (5 mg/mL, Sigma) was added to each well and continued incubation for 4 hours. Then, the supernatant was removed and 150 \( \mu \text{L} \) dimethylsulfoxide was added to stop the reaction. Finally, the OD was determined with a microplate spectrophotometer (ELx800; Bio-TEK, Windham, NH).
ooski, Vermont) at a wavelength of 570 nm, and the viability curve was generated based on absorbance and time.

Colony formation assay
Cells were infected with miR-155-lentivirus to stably overexpress miR-155. After 48 hours of infection, the cells were plated in 6-well plates at 200 per well and grown for 2 weeks. After 2 weeks, the cells were washed twice with PBS, fixed with methanol/acetic acid (3:1, vol/vol), and stained with 0.5% crystal violet. The number of colonies was counted under the microscope.

Western blotting
Proteins from the cells were extracted in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Total cellular protein concentrations were determined using a bichinchoninic acid assay kit (Beyotime). For nuclear extract, cells were washed, lysed, and centrifuged to remove the supernatants. The nuclear fractions were processed using the EpiQuik Nuclear Extraction Kit (Epigentek, Brooklyn, New York) following the manufacturer’s instructions.

The whole-cell extracts and nuclear extracts (20–30 μg) were boiled with equal amounts of loading dye for 10 minutes and separated by 10% PAGE and then transferred onto nitrocellulose membranes (Hybond-ECL; GE HealthCare, Little Chalfont, United Kingdom). Membranes were blocked in PBS with 0.1% Tween 20 containing 5% nonfat milk. The following primary antibodies at the indicated dilutions were used: APC, β-catenin, β-actin, and lamin A (1:1000; Abcam, Cambridge, United Kingdom) and c-Myc, cyclin D1, TCF-1, and LEF-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, California). Primary antibody incubations were kept overnight at 4°C. The membranes were then washed with wash buffer (1× PBS and 0.01% Tween 20) and incubated with a 1:2000 dilution of horse- radish peroxidase-conjugated antirabbit or antimouse secondary antibody. The membranes were washed with wash buffer and developed.

Tumor xenograft animal model
Male athymic nude mice were housed and manipulated according to the protocols approved by the Experimental Animal Center of Tongji University. For each mouse, 5 × 10⁶ miR-155–overexpressing TPC-1 cells were injected sc into the right scapulas in 100 μl serum-free medium. After development of palpable tumor, the tumor volume was monitored every 5 days and assessed by measuring the 2 perpendicular dimensions using a caliper and the formula (a × b²)/2, where a is the larger and b is the smaller dimension of the tumor. At 25 days after inoculation, the mice were killed and tumor weights were assessed. A portion of each tumor was selected for immunohistochemical staining for Ki67, Western blotting for APC and key components of the Wnt/β-catenin pathway, and quantitative real-time PCR (qRT-PCR) analysis for miR-155.

Statistical analysis
Results are presented as mean ± SD from a minimum of 3 replicates. Difference between groups was evaluated by SPSS version 13.0 statistical software with Student’s t test when comparing only 2 groups or assessed by 1-way ANOVA when more than 2 groups were compared. For comparison of paired tissues, a paired Student’s t test was used to determine the statistical significance. The relationship between APC and miR-155 expression was explored by Spearman’s correlation. Differences were considered statistically significant at P < .05.

Results
miR-155 is frequently upregulated in human PTC tissues and cell lines
To verify miR-155 upregulation in PTC, we quantified the expression of miR-155 in 20 fresh-frozen PTC specimens and adjacent normal tissues. The results of qRT-PCR showed that the average expression levels of miR-155 was significantly higher in PTC tissues than adjacent normal tissues (Figure 1A). Consistently, significant upregulation of miR-155 was also observed in 4 hu-
man PTC cell lines (TPC-1, CGTH-W3, K1, and IHH-4) compared with the noncancerous human TECs (Figure 1B). Taken together, these results provide novel evidence for miR-155 upregulation in human PTC tissues and cell lines.

miR-155 promotes PTC cell growth in vitro

To determine whether miR-155 regulates PTC cell growth, we first established miR-155 overexpression models in TPC-1 and CGTH-W3 cells infected with lentiviral vectors carrying the miR-155 gene. Increased expression of miR-155 upon infection in these PTC cell lines was confirmed by qRT-PCR (Figure 2A). We then used these cells to determine their growth curve and colony-formation capacity. As shown in Figure 2, B and C, overexpression of miR-155 significantly increased cell growth and colony formation in TPC-1 and CGTH-W3 cell lines compared with their corresponding controls. In contrast, miR-155 knockdown resulted in decreased cell growth and colony-formation capacity (Figure 2D). Taken together, these results suggest that miR-155 can promote cell growth and colonogenic potential in TPC-1 and CGTH-W3 cells.

miR-155 directly targets APC 3'-UTR

To investigate the molecular mechanisms by which miR-155 promotes PTC cell growth, putative miR-155 targets were predicted using target prediction programs, such as TargetScan and miRanda. Our analysis revealed that APC was a potential target of miR-155. The 3'-UTR of APC mRNA contains a complementary site for the seed region of miR-155 (Figure 3A). To determine whether APC is a direct target of miR-155, the APC 3'-UTR and the mutant containing the miR-155 binding sites were subcloned into a reporter vector downstream of the luciferase gene. Luciferase reporter assays showed that the relative luciferase activity of the reporter that contained wild-type 3'-UTR of APC mRNA was significantly decreased in miR-155–overexpressing cells compared with control cells. However, mutation of the predicted binding site of miR-155 on the APC 3'-UTR rescued the luciferase activity (Figure 3B). Furthermore, the results of qRT-PCR and Western blotting showed that overexpression of miR-155 significantly decreased the levels of APC, whereas miR-155 knockdown prevented miR-155–induced reduction of APC mRNA and protein (Figure 3, C and D). Taken together, these results support the bioinformatics predictions indicating APC 3'-UTR as a direct target of miR-155.

APC is involved in miR-155–induced growth promotion in PTC cells

To investigate whether APC serves as a critical mediator of miR-
miR-155’s role in PTC cells, we performed loss-of-function and gain-of-function studies. First, we knocked down APC using specific RNA interference in TPC-1 cells. As shown in Figure 4A, siRNA against APC significantly promoted cell growth, which is similar to those induced by miR-155. Subsequently, we evaluated whether overexpression of APC could abolish the promotive effect of miR-155. TPC-1 cells stably overexpressing miR-155 were transfected with APC plasmids lacking 3’-UTR. We showed that APC overexpression significantly abolished miR-155–induced cell growth (Figure 4B). These results demonstrate that APC is a functional target of miR-155. To further verify whether miR-155-induced modulation of APC is of clinical relevance, we assessed the expression levels of this target in 20 pairs of clinical PTC (ie, miR-155–overexpressing) samples via Western blotting and qRT-PCR analyses. Compared with paired normal tissues, PTC showed significantly lower APC expression (Figure 4, C and D). Furthermore, we analyzed the correlation between APC level and miR-155 expression in the same patients. As shown in Figure 4E, when the relative expression levels of APC were plotted against that of miR-155 in each patient, a significant inverse correlation was observed (P = .007; R = −0.805).

miR-155 activates the Wnt/β-catenin pathway

Given that APC is implicated in β-catenin phosphorylation/degradation (28), we examined the expression levels of total β-catenin and nuclear β-catenin in PTC cells with miR-155 overexpression. As depicted in Figure 3D, a marked increase in total β-catenin and nuclear β-catenin expression was found. We then sought to determine whether miR-155 could regulate Wnt/β-catenin signaling. TPC-1 and CGTH-W3 cells were transiently transfected with the Wnt signaling reporter TOPFlash or the negative control FOPFlash, along with miR-155 or miR control. We found that TCF/LEF transcriptional activity was significantly increased in miR-155–overexpressing cells, whereas this effect could be inhibited by anti–miR-155 (Figure 5A; the basal activity of TOPFlash/FOPFlash is shown in Supplemental Figure 2), indicating that Wnt/β-catenin signaling is altered by miR-155. The same assay was performed on TECs, and we found further evidence to support our claims (Supplemental Figure 2). To further investigate the functional role of the increased β-catenin induced by miR-155 overexpression, we performed loss-of-function analyses by silencing β-catenin in miR-155–overexpressing TPC-1 cells. We found that silencing of β-catenin prevented miR-155–induced cell viability and colony-formation capacity (Figure 5B). These results further indicate that miR-155 promotes cell growth by activating Wnt/β-catenin signaling. To further investigate the relationship between miR-155 and Wnt/β-catenin signaling in PTC formation, we analyzed the protein levels of β-catenin in the same samples as Figure 4C. As depicted in Figure 5C and Supplemental Figure 3, β-catenin expres-

![Figure 5](https://example.com/figure5)

**Figure 5.** Overexpression of miR-155 activates the Wnt/β-catenin pathway. A, Luciferase activity of TOPFlash/FOPFlash in miR-155–overexpressing cells. ***, P < .01. B, Knockdown of β-catenin partly prevented miR-155–induced cell viability and colony formation in TPC-1 cells. *, P < .05; ***, P < .01. C, Western blot analyses conducted on the sample cohort depicted in Figure 4C identified increased β-catenin expression in PTC. All assays were performed in triplicate. Data are presented as means ± SD.
miR-155 overexpression promotes tumor growth of PTC cells in nude mice

To further investigate the role of miR-155 on promotion of tumor growth in vivo, miR-155–overexpressing or control TPC-1 cells (1 × 10⁶) were injected sc into nude mice (n = 5). At 25 days after implantation, the mice were killed and the tumors were recovered. B, Volume of xenograft tumors. **, P < .01. C, Ki67- and PCNA-stained sections of transplanted tumors. D, Levels of miR-155 in xenograft tumor tissues as determined by qRT-PCR. **, P < .01. E, Western blotting analysis of APC and β-catenin protein in miR-155–overexpressing and control xenograft tumor tissues. F, Expression profile changes of the c-Myc, cyclin D1, TCF-1, and LEF-1 genes at the translational level in TPC-1 and CGTH-W3 cells stably overexpressing miR-155. G, Expression profile changes of the c-Myc, cyclin D1, TCF-1, and LEF-1 genes at the translational level in xenograft tumor tissues derived from miR-155–overexpressing and control TPC-1 cells. Tumors 1, 2, and 3 were obtained from 3 different mice.

Discussion

The upregulation of miR-155 has been observed frequently in PTCs (22, 23). However, to date, the relationship between miR-155 and PTC was not fully understood. In this study, we demonstrated for the first time that miR-155 promotes tumorigenesis in PTC by targeting a novel target, APC, which results in activation of Wnt/β-catenin signaling.
ments conducted in several cancer models determined a role for miR-155 in tumor cell proliferation, migration, and invasion (20, 34). To our knowledge, our study is the first to report a role for miR-155 in PTC.

As is well known, aberrant miRNA expression is closely related to the changes of cell morphology and function. In our study, we found that miR-155 is overexpressed at levels of 20- to 30-fold, but only an ~2-fold increase in growth and colony formation is observed. However, as this study (35) has shown, miRNAs in cancer cells show dynamic changes at levels of 20- to 40-fold, which doesn’t mean that the cell morphology and function also show the same dynamic changes, and only ~1- to 4-fold change in growth and colony formation is observed. Furthermore, this is already significantly changes in cell growth and colony formation increased 1~4-fold. Thus, our results suggest that miR-155 plays an important role in human PTC cell growth.

To date, several targets of miR-155 have been identified, such as SHIP, C/EBPB (33), FOXO3 (34), RHOA, MSH2, MSH6, MLH1, SOCS1 (36), and CK1α (21). On the basis of bioinformatics analysis, we further predicted several additional miR-155 targets, including APC, GSK-3β and CK1α. Our present experimental results confirmed that APC is a functional target of miR-155 in PTC cells. There are several lines of evidence to support this. First, miR-155 overexpression significantly downregulated APC by directly targeting the 3′-UTR of APC mRNA, confirmed using luciferase-reporter-gene assays. This effect was largely eliminated when the sites in APC 3′-UTR targeted by miR-155 were mutated. Moreover, both mRNA and protein of APC were significantly decreased in miR-155-overexpressing cells. We further found that knockdown of APC promoted cell growth similar to the phenotypes induced by miR-155 restoration, whereas APC overexpression could abolish the growth-promotive effect of miR-155. These results strongly suggested that miR-155-induced cell growth was partly mediated by repressing APC expression. It is worth noting that GSK-3β and CK1α are another 2 potential targets predicted by TargetScan. However, our results showed that overexpression of miR-155 did not affect GSK3β and CK1α expression in TPC-1 and CGTH-W3 cells (data not shown). Moreover, the reporter assay also showed that miR-155 did not repress the luciferase expression of pGL3-GSK3β 3′-UTR and pGL3-CK1α 3′-UTR (data not shown).

Our results further demonstrate that miR-155-mediated downregulation of APC leads to activation of Wnt/β-catenin signaling in PTC cells. First, miR-155 overexpression caused β-catenin accumulation and nuclear translocation. Second, miR-155 overexpression increased TCF/LEF transcriptional activity, and this effect was blocked by anti-miR inhibition of miR-155. The importance of β-catenin in miR-155–induced tumor cell growth is also supported by the observation that siRNA depletion of β-catenin prevented miR-155–induced PTC viability and colony-formation capacity. Consistent with these findings, miR-155 overexpression was found to enhance the expression of several β-catenin downstream genes in PTC cells.

Upregulation of β-catenin occurs in a variety of cancers, namely colorectal, breast, and ovarian cancers (37, 38). Mutations that activate β-catenin occur late in thyroid tumor progression, being detected in undifferentiated (anaplastic) thyroid carcinomas (39). Increased free β-catenin pools have been observed in thyroid carcinomas secondary to reduced E-cadherin expression (40). However, the exact mechanism for β-catenin activation in PTC has not been completely defined. Our findings in this study demonstrate a key role of miR-155 for β-catenin activation in human PTC cells. This finding is noteworthy, given that miR-155 is commonly upregulated in human PTC and that β-catenin is a key molecule implicated in tumorigenesis in PTC. Thus, miR-155 overexpression is a new mechanism that activates β-catenin in human PTC, and this pathway may play an important role in tumorigenesis of PTC.

This study identified miR-155 as an oncomiR in human PTC by targeting APC, consequently resulting in activation of Wnt/β-catenin signaling and driving PTC cell growth. Our findings suggest that miR-155 may be a potential target for future prevention and treatment of human PTC.

Acknowledgments

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