microRNA-141 inhibits thyroid cancer cell growth and metastasis by targeting insulin receptor substrate 2

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Abstract: microRNA-141 (miR-141), a member of the miR-200 family, and has been reported to involve in tumor initiation and development in many types of cancers. However, the function and underlying molecular mechanism of miR-141 in thyroid cancer remains unclear. Therefore, the aim of this study is to identify its expression, function, and molecular mechanism in thyroid cancer. In this study, we found that miR-141 expression levels were downregulated in human thyroid cancer specimens compared to the adjacent normal tissues, and its expression were strongly correlated with clinical stages and lymph node metastases. Function assays showed that overexpression of miR-141 inhibited cell proliferation, induced cell apoptosis, and decreased migration, invasion in thyroid cancer cells, as well as tumor growth in nude mice. Moreover, insulin receptor substrate 2 (IRS2), a known oncogene, was confirmed as a direct target of miR-141, and IRS2 expression levels were upregulated in thyroid cancer, and its expression were inversely correlated with miR-141 expression levels in human thyroid cancer specimens. Forced expression of IRS2 reversed the inhibition effect induced by miR-141 overexpression in thyroid cancer cells. Taken together, our study provides the first evidence that miR-141 suppressed thyroid cancer cell growth and metastasis through inhibition of IRS2. Thus, miR-141 might serve as a promising therapeutic strategy for thyroid cancer treatment.

Keywords: Thyroid cancer, proliferation, invasion, IRS2

Introduction

Thyroid cancer is the most common endocrine malignancy, and one of the most rapidly growing in many countries [1]. Thyroid cancer originate from parafollicular cells (medullary) and follicular cells (non-medullary), which account for over 95% of all thyroid cancer cases and are categorized into four histological types, including follicular thyroid cancer (FTC), papillary thyroid cancer (PTC), anaplastic thyroid cancer (ATC), and Hürthle cell carcinoma (HCC) [1, 2]. Despite the advances of diagnostic and therapeutic approaches have greatly improved long-term survival of thyroid cancer, a significant proportion of patients with locoregional recurrence or distant metastases within 10 years [3]. Hence, better understanding of the molecular mechanisms underlying carcinogenesis and progression in thyroid cancer would contribute to improve diagnosis, therapy and prevention.

MicroRNAs (miRNAs) are a class of endogenous, single-stranded, short (18-24 nucleotides in length), highly conserved noncoding RNAs that regulate gene expression at the posttranscriptional level by binding to the 3'-untranslated region (UTR) of target mRNAs, resulting in mRNA cleavage or translation inhibition [4]. It has been showed that miRNAs can involved in a serials of biological processes, such as the cell proliferation, cell cycle, apoptosis, migration, invasion, and differentiation [5, 6]. In recent years, miRNAs have been recognized as critical regulators in development and progression of cancer, and function as oncogenes or tumor suppressor genes in a variety of tumors including thyroid cancer [7-9].

MicroRNA-141 (miR-141), a member of the miR-200 family, has been reported to play a crucial role in the pathogenesis of various malignant tumors, such as gastric cancer [10], colorectal
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

cancer [11], breast cancer [12], hepatocellular carcinoma [13], bladder cancer [14], non-small cell lung cancer [15] and renal cell carcinoma [16]. However, the role and molecular mechanism of miR-141 in thyroid cancer has not been determined. Therefore, in this study, we investigate the potential role and molecular mechanism of miR-141 in thyroid cancer.

Materials and methods

Patients and tissue samples

Human thyroid cancer specimens (30 pairs) and adjacent normal tissues were obtained from patients who underwent surgical resection in the Department of Thyroid Surgery, First Hospital of Jilin University (Changchun, China). All tissue samples were snap-frozen in liquid nitrogen immediately after surgery and stored at -80°C until use. All samples were histologically classified by clinical pathologist. All patients provided written informed consent for the use of their tissues. The experiment protocols have been approved by the ethics committees of Jilin University (Changchun, China).

Cell culture and transfection

The human thyroid cell line, TPC-1 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in 1640 medium (Gibco, USA) supplemented with 10% fetal calf serum (FBS, Gibco BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO2 incubator. miR-141 mimic (miR-141), and corresponding miRNA negative control (miR-NC) were brought from Qiagen (Frederick, MD, USA). Overexpression IRS2 plasmid and blank vector were purchased from RiboBio (Guangzhou, China). These molecular productions were transfected into TPC-1 cells using lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions.

RNA extraction and quantitative RT-PCR

Total RNAs were isolated from harvested cells or human tissues using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. To measure expression levels of miR-141, cDNA was synthesized using miScript reverse transcription kit (Qiagen). Then quantitative PCR assay was performed using the TaqMan miRNA assay kits (Applied Biosystems, Foster City, CA, USA) through the specific primers of miR-141 and U6 (Applied Biosystems) under an ABI7900 real-time PCR system (Applied Biosystems). U6 was used as internal control. To determine the mRNA levels of IRS2, total RNAs were reversely transcribed by oligodT primer using RT Reagent Kit (Takara, Dalian, China). Then quantitative PCR assay was performed using the Real-time PCR Mixture Reagent (Takara) through the specific primers of IRS2 and as β-actin previously described [17] under an ABI7900 real-time PCR system. Housekeeping gene β-actin was used as internal control. Relative gene expression was calculated by the 2-ΔΔCt method.

Cell proliferation assays

Cells proliferation was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, the cells were seeded into 96-well plates (5 × 103 cells/well). After transfection, CCK-8 (10 μl, Dojindo) was added to each well at indicated time points (24 h, 48 h, 72 h) and incubated at 37°C for additionally 4 h. The absorbance at 450 nm was measured under a microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA).

Cell cycle and apoptosis analysis

Transfected cells were harvested by trypsin and washed with phosphate-buffered saline (PBS) at 48 h posttransfection. For cell cycle analysis, the cells were fixed with 75% ethanol at 4°C overnight, then fixed cells were washed with PBS, treated with RNase A (50 μg/mL) in PBS at 37°C for 20 min, and then mixed with propidiumiodide (PI, 50 μg/mL, Sigma, USA) for 30 min in the dark at room temperature. The stained cells were determined with fluorescence-activated cell sorting (FACS) by flow cytometry (FACSCalibur, Becton-Dickinson, Bedford, MA, USA). For cell apoptosis assay, the apoptosis assay was performed using an Annexin-V-FLUOS Staining kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions 48 h after transfection. The apoptosis ratio was analyzed with FACS using the Cell Quest software (Becton-Dickinson).

Cell migration and invasion assays

To examine the migration ability of cells in vitro, a wound-healing assay was performed. In briefly, transfected cells were seeded in 3.5-cm plates and grown to a density of 70 to 80%. Afterwards, artificial wound were created by 200 μl pipette tips. Wound healing was observed and photographed at different time.
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

points (0 and 24 h) using light microscope (Olympus, Japan). The migrating distance was measured from five different areas for each wound.

Cell invasion was determined using Transwell chamber with a pore size of 8 μm, and the inserts were coated with 20 μl Matrigel (1:3 dilution, BD Bioscience, San Jose, CA, USA). The 2 × 10^4 transfected cells were added to upper transwell chambers in serum-free medium. RPMI1640 medium containing 10% FBS was added into the lower chamber as the chemoattractant. After incubation for 48 h in a humidified atmosphere of 5% CO₂ at 37°C, noninvading cells were removed from the top well with a cotton swab, while the bottom cells were fixed in 90% alcohol and stained with 0.1% crystal violet for 5 min, then photographed under a microscope (Olympus). The number of invaded cells was counted at five randomly selected fields.

**Dual-luciferase reporter assay**

3'-untranslated region (3'-UTR) regions of IRS2 containing predicted miR-141 seed-matching sites and corresponding mutant sites were amplified by PCR using human cDNA template, and inserted into inserted downstream of the firefly luciferase gene in a pGL3-promoter vector (Ambion, Austin, TX, USA). These constructs were validated by DNA sequencing.

For dual-luciferase assay, TPC-1 cells were seeded in a 24-well plate and co-transfected with the wild type or mutant IRS2 reporter plasmid, pRL-TK plasmid, and miR-141 or miR-NC. At 48 h after transfection, luciferase activities in the cells were determined using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

**Western blot analysis**

Total proteins from cells or tissues were obtained using cell Pierce lysis buffer (Rockford, IL, USA). Protein concentrations were quantified by using the bicinchoninic acid protein assay kit (Beyotime). Equivalent quantities (30 μg) of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Santa Cruz Biotechnology, Inc, USA). After blocking with 5% nonfat dry milk, the membranes were incubated at 4°C overnight with primary antibodies against IRS2 (1:1000, Cell Signaling Technology, Boston, MA, USA) and β-actin (1:3000, Cell Signaling Technology). Then, the membranes were washed and incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (HRP, Santa Cruz) at a 1:5000 dilution at room temperature for 2 h. Proteins were visualized with chemiluminescence detection (Signagen, Rockville, MD, USA).

**In vivo tumorgenesis assay**

Female nude mice (BALB/c-null, 6-week-old) were purchased from Jilin Laboratory Animal Center (Changchun, China), and bred in special pathogen-free (SPF) condition. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines of Jilin University (Changchun, China).

TPC-1 cells (2 × 10^6) stable expression miR-141 or miR-NC were suspended in 100 μl of serum-free RPMI 1640 medium, and injected subcutaneously into each side of the posterior flank of nude mouse (n=10), respectively. Tumor sizes were measured every five days from the 15th day of injection. Tumor volumes were calculated using vernier caliper according to the formula: volume = 1/2 × (Length × Width²). Mice were sacrificed 35 days after injection, and tumor tissues were striped and weighted. Tumor tissues were snap-frozen in liquid nitrogen immediately and stored at -80°C for detection IRS2 expression.

**Statistical analysis**

The data were expressed as the mean ± SD (standard deviation) from at least three independent experiments. All data are analyzed by Statistical SPSS Version 19.0 (IBM, Chicago, USA). Group differences were compared using two-tailed Student’s T-test or one-way ANOVA. The correlations between miR-141 expression levels and IRS2 levels in human thyroid cancer tissues were analyzed using Spearman's rank test. The differences were considered to be statistically significant at P < 0.05.

**Results**

miR-141 expression was downregulated in thyroid cancer tissues

To determine the expression levels of miR-141 in human thyroid cancer specimens, quantita-
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

Figure 1. miR-141 expression was downregulated in thyroid cancer tissues. A. Relative miR-141 expression levels were analyzed by quantitative RT-PCR (qRT-PCR) in 30 pairs of thyroid cancer specimens and adjacent normal tissues. U6 RNA levels were used as an internal control. B. Relative expression levels of miR-141 in different TNM stages of cancer tissues. C. Relative expression levels of miR-141 in thyroid cancer tissues with or without lymph node metastasis. **P<0.01.

Figure 2. miR-141 inhibits cell proliferation, induces cell apoptosis in thyroid cancer cells. (A) Relative expression levels of miR-141 were determined in thyroid cancer cells transfected with miR-141 mimic or miR-NC by qRT-PCR. (B-D) Cell proliferation (B), cycle distribution (C) and apoptosis (D) were determined in thyroid cancer cells transfected with miR-141 mimic or miR-NC. **P<0.01.

tive RT-PCR (qRT-PCR) analysis was performed in 30 pairs of thyroid tumor specimens and matched adjacent normal tissues. As shown in Figure 1A, miR-141 expression levels in thyroid tumor tissues were significantly lower than those in adjacent normal tissues (Figure 1A). In addition, we found that miR-141 expression levels in tumor tissues were correlated with the clinical stages and lymph node metastasis of patients with thyroid cancer. The expression
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

levels of miR-141 in patients with advance TNM stage (III-IV) and with lymph node metastases were significantly downregulated compared with patients with TNM stage (I and II) and without lymph node metastases (Figure 1B and 1C), respectively. Taken together, low expression levels of miR-141 in thyroid tumor tissues were closely related with advanced clinical stages and metastases, indicating that miR-141 involved in thyroid cancer procession.

**MiR-141 inhibits cell proliferation, induces cell apoptosis in thyroid cancer cells**

To examine the role of miR-141 human thyroid cancer growth, TPC-1 cells were transfected with miR-141 mimic or miR-NC. qRT-PCR analysis demonstrated miR-141 was highly expressed in cells transfected with miR-141 mimic compared to cells transfected with miR-NC (Figure 2A). CCK8 assay showed that overexpression of miR-141 in TPC-1 cells significantly inhibited cell proliferation (Figure 2B). As proliferation directly associated with cell cycle distribution, the effect of miR-141 on cell cycle progression was evaluated in TPC-1 cells. As expected, the percentage of G0/G1 phase cells increased, and the percentage of S phase cells decreased in TPC-1 cells transfected with miR-141 mimic compared to cells transfected with miR-NC (Figure 2C). In addition, cell apoptosis was investigated in TPC-1 cells transfected with miR-141 mimic or miR-NC. As showed in Figure 2D, overexpression of miR-141 could increase cell apoptosis ratio. These results suggested that miR-141 inhibited thyroid cancer growth by regulating cell cycle at G0/G1 stage and promoting cell apoptosis.

**MiR-141 inhibits cell migration and invasion in thyroid cancer cells**

Our above results demonstrated that miR-141 down-regulation was associated with lymph node metastasis in patients with thyroid cancer, therefore, to investigate whether miR-141
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

Figure 4. IRS2 is a direct target of miR-141. (A) The complementary pairings of miR-141 with IRS2 wild-type (Wt) and mutant (Mut) 3' UTR reporter constructs are shown. (B) The luciferase activity was determined in TPC-1 cells at 48 hour after co-transfection with the miR-141 mimic or miR-NC and IRS2 wild-type (Wt) and mutant (Mut) 3' UTR reporter plasmid. (C, D) The IRS2 expression on mRNA level (C) and protein level (D) were detected in TPC-1 cells transfected with miR-141 mimic or miR-NC by qRT-PCR and Western blot assays, respectively. β-actin was used to an internal control. (E) Spearman's correlation analysis was used to determine the correlations between the expression levels of IRS2 and miR-141 in human thyroid cancer specimens. **P<0.01.

Effect on metastasis in vitro, migration and invasion assays were performed in TPC-1 cells transfected with miR-141 mimic or miR-NC by wound healing and transwell assay, respective-
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

We found that overexpression of miR-141 significantly inhibited cell migration (Figure 3A) and invasion (Figure 3B) in TPC-1 cells. IRS2 is a direct target of miR-141

To investigate mechanism of miR-141 in inhibiting human thyroid cancer procession, TargetScan search program was used to predict targets of miR-141. IRS was one of the putative targets of miR-141 (Figure 4A). To explore whether miR-141 targets IRS2 by binding to its 3'-UTR region, TPC-1 cells were co-transfected with the wild type (Wt) or mutant (Mut) IRS2 luciferase reporter plasmid and miR-141 or miR-NC. After 48 h transfection, the luciferase activities in these cells were determined. We found that luciferase activities were significantly decreased in the cells transfected with the wild type IRS2 reporter plasmid, but not in the cells with the mutant reporter plasmid (Figure 4B). In addition, we also forced expression of miR-141 could decrease IRS2 expression on mRNA level (Figure 4C) and protein level (Figure 4D). These results suggest that miR-141 directly targets IRS2 by binding its seed region of the 3'-UTR region in human thyroid cancer cells.

Furthermore, we measured levels of IRS2 in human thyroid cancer specimens and adjacent normal tissues. The results of qRT-PCR showed that the mRNA expression levels of IRS2 were significantly higher in tumor tissues than those in the normal tissues (Figure 4E). In addition, we also determine the correlation between IRS2 mRNA levels and miR-141 levels in the same human CRC specimens using Spearman’s rank correlation analysis. We found that the IRS2 mRNA level and miR-141 expression were inversely correlated in human thyroid cancer specimens (Figure 4F, Spearman’s correlation $r=-0.643$, $P<0.001$).

Restoration of IRS2 rescues the effects of miR-141 in thyroid cancer cells

To investigate the functional relevance of IRS2 targeting by miR-141 in thyroid cancer, we assessed whether IRS2 overexpression reverses miR-141 effect on cell proliferation, apoptosis, migration and invasion. TPC-1 cells were co-transfected with miR-141 mimic or miR-NC and overexpression IRS2 plasmid or blank vector. We found that IRS2 expression both on mRNA level (Figure 5A) and protein level (Figure 5B) was restored in miR-141 combination with
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

IRS2 overexpression plasmid compared to miR-141 combination with vector group. In addition, we also found that forced expression of IRS2 also partially abrogated effect of miR-141 on cell proliferation, apoptosis, migration and invasion (Figure 5C-F), suggesting that miR-141 suppresses human thyroid cancer cell proliferation, migration and invasion, and induces cell apoptosis by inhibiting its target IRS2.

miR-141 suppresses thyroid cancer tumorigenicity in vivo by targeting IRS2

To examine miR-141 biofunction in suppression of thyroid cancer cell tumorigenicity in vivo, TPC-1 cells stable expression miR-141 or miR-NC was respectively injected into nude mice, and tumor sizes were started to be measured after 15 days of injection. Compared to miR-NC group, tumor growth was lower in miR-141 group from Day 20 to Day 35 (Figure 6A). Mice were sacrificed 35 days after injection, and tumor tissues were stripped and weighted. It was found that miR-141 group showed smaller size and lower tumor weight than that of miR-NC group (Figure 6B and 6C). Furthermore, we also determined IRS2 expression of tumor tissue. We found that IRS2 expression was significantly decreased in miR-141 group compared to miR-NC group (Figure 6D). These data indicated that miR-141 could suppress thyroid cancer tumorigenicity in vivo by repressing IRS2.

Discussion

Recently, a large number of miRNAs contributing to cell proliferation, migration and invasion in thyroid cancer have been identified [7-9]. For example, miR-137 inhibited proliferation, colony formation ability, and invasion, with suppressed expression of cyclin E, MMP2, p-ERK, and p-AKT in thyroid cancer cells by targeting
miR-141 inhibits thyroid cancer cell growth by targeting IRS2

epidermal growth factor receptor (EGFR) [18]. miR-539 suppressed migration and invasion in human thyroid cancer cells by targeting CARMA1 [19]. miR-34a promoted proliferation and suppress apoptosis in papillary thyroid carcinoma cells via PI3K/Akt/Bad pathway by regulating growth arrest specific1 (GAS1) GAS1 expression [20]. miR-126 inhibited thyroid cancer cell proliferation, colony formations, migration and invasion, promoted cell apoptosis and cell cycle arrest at G1 stage in vitro, as well as inhibited tumor growth by targeting LRP6 regulating Wnt/β-catenin signaling pathway [21]. Data from the current study provide evidence that miR-141 expression was downregulated in thyroid cancer, and that restoration of miR-141 expression inhibited proliferation, migration and invasion, promoted cell apoptosis in thyroid cancer cells, and suppresses tumor growth in nude mice model, which provides a new insight into the mechanism of thyroid cancer progression.

miR-141 has been shown to be decreased, and function as a tumor suppressor miRNA in many types of cancer, such as gastric cancer [22], breast hepatocellular carcinoma [23, 24], renal cell carcinoma [16], ovarian cancer [25] and esophageal cancer [26] by targeting a number of oncogene, such as EphA2, E2F3, ZEB2, Tiam1, KEAP1 and SOX17 [16, 22-26]. However, some researches reveal that miR-141 expression is up-regulated, and function as oncogene in bladder cancer [14], non-small cell lung cancer [15, 27], and prostate cancer [28]. These inconsistent findings indicate that dysregulation of miR-141 in various cancers may be dependent on details tumor type and the cellular microenvironment. However, the detail biological function and underlying molecular mechanism of miR-141 in thyroid cancer remains largely unclear. Here, we found that miR-141 expression was downregulated in thyroid cancer tissues, and that miR-141 inhibited thyroid cancer tumor growth in vitro and in vivo by targeting IRS2. These findings indicate that miR-141 might act as a tumor suppressor in thyroid cancer.

Insulin receptor substrate 2 (IRS2), located in the 13q34 region, belongs to the insulin receptor substrate (IRS) family of proteins that interact with -SH2 domain containing proteins mainly PI3K during insulin action [29, 30]. It has been showed that IRS2 expression was upregulated in many types of cancer including thyroid cancer [31]. In addition, IRS2 has been reported to contribute to tumorigenesis through promoting cancer cell proliferation and inhibited cancer cell apoptosis, suggesting that IRS2 function as oncogene [32, 33]. A number of miRNAs have been shown to be able to regulate IRS2 expression, such as miR-146 [34], miR-30a [35], miR-145 [36] and miR-135a [37]. In the present study, IRS2 was confirmed as a direct target of miR-141 by luciferase activity assay, qRT-PCR and Western blot. IRS2 expression levels were upregulated in thyroid cancer, and its expression were inversely correlated with miR-141 expression levels in human thyroid cancer specimens. Forced expression of IRS2 reversed the inhibition effect mediated by miR-141 overexpression in thyroid cancer cells. These results suggested that miR-141 exerted suppressor role in thyroid cancer by targeting IRS2.

In conclusion, this is the first study to demonstrate that the expression of miR-141 was downregulated in thyroid cancer, and its expression was significantly associated with TNM stage and lymph node metastasis. Moreover, we also found that miR-141 inhibits thyroid cancer cell proliferation, migration and invasion, induced cell apoptosis, as well as suppress tumor growth in vivo via directly targeting IRS2. These results provide new insights into the mechanism of thyroid cancer progression, and suggest that miR-141 may potentially serve as an anti-tumor agent in the treatment of thyroid cancer.

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Disclosure of conflict of interest

None.

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References

miR-141 inhibits thyroid cancer cell growth by targeting IRS2

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