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Original Research

MicroRNA-1280 modulates cell growth and invasion of thyroid carcinoma through targeting estrogen receptor α

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Abstract: Thyroid cancer (TC) is one of the most common endocrine malignancies, with a steadily increasing incidence and lethality over the last several decades. ERα is a nuclear hormone receptor that has a key role in different cellular process and participates in the development and progression of thyroid cancer. ERα is the predicted target gene of microRNA-1280 (miR-1280). The present study was designed to delineate the role and underlying mechanism of miR-1280 in regulating thyroid cancer through targeting ERα. In our study, we analyzed the expression level of miR-1280 in thyroid cancer and detected significantly lower miR-1280 levels in TC tissue and cell lines compared with adjacent normal tissue or healthy cell line. We then overexpressed miR-1280 by miRNA mimic transfection and inhibited miR-1280 by miRNA inhibitor transfection. The inhibition of miR-1280 significantly elevated proliferation and invasion ability, whereas overexpression of miR-1280 inhibited cell growth and invasion in TC cells. Additionally, the luciferase reporter assay confirmed a targeting reaction between miR-1280 and ERα. Furthermore, overexpression of miR-1280 inhibited ERα and ERK pathway expression in TC cells, indicating that miR-1280 acts as a tumor suppressor by inhibiting the expression of ERα. Taken together, we demonstrated that overexpressed miR-1280 levels in TC cells may promote cell proliferation and invasion by inhibiting ERα, which might provide a new therapeutic target for thyroid cancer.

Key words: Thyroid cancer, ERα, miR-1280, ERK pathway.

Introduction

Thyroid cancer is one of the most common endocrine malignancies, with a steadily increasing incidence over the last several decades (1). The majority of thyroid cancers are derived from thyroid follicular cells, and classified as well-differentiated (WDTC), poorly differentiated (PDTC) or anaplastic thyroid cancer (2). In addition, WDTCs are divided in to papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) (3). In most cases, well differentiated PTC and FTC have a favorable prognosis (4). However, the minority of thyroid cancer patients will die of their disease due to local cell growth, invasion and distant metastasis (5). Despite advances in diagnostics and the implementation of new therapeutic strategies, the survival rates of thyroid cancer remain dismal. Thus, the development of novel therapeutic strategies by understating the molecular mechanisms involved in the growth and invasion of thyroid cancer cells is urgently needed.

MicroRNA (miRNA) is a type of naturally existing small non-coding RNA with a length of ~24 nucleotides, which regulate the expression of a large number of genes by binding to specific sites in target mRNA, leading to mRNA cleavage/degradation or translational repression (6). It has been reported that ~60% of all protein-coding genes in humans are regulated by miRNAs, which participate in the regulation of various cellular processes including cell proliferation, apoptosis and differentiation (7). Multiple studies have shown that miRNAs are involved in gene expression regulation of various diseases. Dysregulation of miRNA expression has been reported in a number of cancers, and evidences has suggested that some miRNAs can function as oncogenes or tumor suppressor genes (8). Recent research into thyroid cancer has demonstrated that miRNAs play a crucial role in thyroid carcinogenesis (9). Thus, inactivation of oncogenic miRNAs or the restoration of tumor suppressor miRNAs may have great potential for thyroid cancer treatment.

It has been reported that premenopausal women are at the highest risk for PTC and FTC, suggesting that estrogens play an important role in thyroid cancer, and indicating a special role of ER expression in thyroid tumorigenesis (10). It is well known that there are two different isoforms of nuclear estrogen receptors, ERα and ERβ, together mediating estrogens in humans (11). The structural and functional domains of ERα and ERβ are similar, but they differ in tissue distribution and functions (12). It has been demonstrated that ERα is involved in a various of cellular processes including cell proliferation, invasion, apoptosis and differentiation (13). These studies suggested that ERα is a promising molecular target for the prevention and treatment of thyroid cancer.

In this study, using bioinformatics software programs, we predicted miR-1280 to be the target miRNA for ERα, and investigated the potential role of miR-1280 in thyroid cancer. By the overexpression and knockdown of miR-1280, we found that miR-1280 played

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an important role in cell proliferation and invasion in thyroid cancer cells. Furthermore, we confirmed the target reaction between miR-1280 and ERα, thereby suggesting that overexpression of miR-1280 was capable of downregulating ERα expression and inhibited downstream genes expression. Taken together, our results suggest that miR-1280 may provide novel insight into the diagnosis and prognosis of thyroid cancer.

Materials and Methods

Patients

A total of twelve follicular thyroid cancer tissue samples and twelve adjacent non-neoplastic tissue samples were collected from patients and immediately frozen in liquid nitrogen and stored in a freezer at -80˚C. The patients with follicular thyroid cancer were recruited from the First Affiliated Hospital of Zhengzhou University. This study was approved by the ethics Committee of our institution. Written informed consent was provided by all of the participating patients.

RNA extraction and qRT-PCR assay

Total RNA was extracted from the frozen follicular thyroid cancer tissue and adjacent non-neoplastic tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The purity and concentration of the total RNA were measured by using an Ultraviolet Spectrophotometer (Eppendorf, Hamburg, German). cDNA was synthesized using the One Step PrimeScript miRNA cDNA synthesis kit (Takara Biotechnology, Dalian, China). Briefly, 20 μl reactions containing 50 ng of cDNA, 10 μl of 2X SYBR-Green PCR Master Mix, 6.25 U of AMV reverse transcriptase, 10 U of RNase inhibitor and 0.1 mM of primers were subjected to 1 cycle of 95˚C for 10 min, and then 40 cycles of 95˚C for 15 sec, 56˚C for 30 second 72˚C for 45 sec, then 72˚C for 10 min and 4˚C for 5 min. microRNA 1280 specific primers (F: 5'-ATAAGCTTGGTAGCGTGGCCGAG-3'; R: 5'-AT GAATTCTGGTGGCAGCGGTGG-3') were purchased by Sangon (Shanghai, China). U6 primers (F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'; R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'). miR-1280 expression was normalized to U6 RNA. Data were analyzed using the ΔCt method and expressed as the fold change.

Cell culture and transfection

The human follicular thyroid cancer cell line FTC133 and TT were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The normal thyroid follicular epithelial cell line Nthy-ori 3-1 were obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). The FTC133 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA)/ F12 medium. Both TT and Nthy-ori 3-1 cell line were maintained in RPMI-1640 medium. In addition, the three cell lines were equally supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 2 mM L-glutamine, 100U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Rockville, MD, USA), in a humidified chamber with 5% CO_2 at 37˚C. For transfection, the hsa-miR-1280 mimic, hsa-miR-1280 inhibitor, hsa-miR-1280 mimic control or hsa-miR-1280 inhibitor control (Ambion, Austin, Tx, USA) were delivered at a final concentration of 100 nM using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

MTT assay

Cell proliferation was assessed by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded in 96-well plates following miR-1280 transfection for indicated times. Thereafter, the old medium were discarded and fresh medium containing MTT (5 mg/ml MTT in PBS; Sangon, Shanghai, China) were added and incubated for additional 4 h. Dimethyl sulfoxide (Sigma, St. Louis, MO, USA) was used to dissolve the formazan and the absorbance at 490 nm was measured using an ELISA reader (Bio-tek, Winooski, VT, USA).

Cell invasion assay

The invasion ability of cells was analyzed using Transwell cell culture chambers (8 μm pore size; BD Biosciences, San Jose, CA, USA). Briefly, 48 h following transfection, the cells were resuspended with serum-free medium, and 200 μl of the cell suspension was added to the upper chamber. Additionally, medium containing 10% serum was added to the bottom wells of the 24-well chamber. The cells were then removed from the upper part of the filters by scrubbing using a cotton swab following culture for 24 h with 5% CO_2 at 37°C. Afterwards, the membrane was fixed with 4% formaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 15 min. Finally, the number of invading cells was counted at x100 magnification.

Dual-luciferase reporter assay

The fragment from the 3'-UTR of ERα mRNA containing the predicted miR-1280 binding sequences was amplified by PCR using the cDNA of human genomic DNA as a template. The sequence for the mutation within the miR-1280 binding sites was amplified by the point mutation method using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacture's instructions. The primer sequences were ERα WT (F: 5'-CGCGTcctattgttggatattgaatgacagacaatcttatgtagcaaagattatgcctgaaaagggatccA-3'; R: 5'-AGCTTggatccctttttcaggcataatctttgctacataagattgtctgtcattcaatatccaacaataggA-3') and ERα MUT (F: 5'-CGCGTatgaaagtggtacaccttaaagcttttatatgactgtagcagagtatctggtgattgtcaggatccA-3'; R: 5'-AGCTTggatcctgacaatcaccagatactctgctacagtcatataaaagctttaaggtgtaccactttcatA-3'). After subcloning into pGL3 luciferase promoter vector (Promega, Madison, WI, USA), the recombinant plasmids were confirmed by DNA sequencing and co-transfected with hsa-miR-1280 mimic or hsa-miR-1280 mimic control into FTC133 cells and incubated for 48 h. Then, cells were harvested and lysed. The firefly luciferase and Renilla luciferase activity were detected using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA) as per standard protocols and the relative luciferase activity was normalized to Renilla luciferase activity for each transfected well.

Western blot analysis

A total of 25 μg proteins extracted from cells were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were then blocked in 2.5% nonfat milk for 1 h at 37 °C. After washed with Tris-buffered saline with Tween, the membranes were incubated with primary antibodies against ERα, ERK, p-ERK1/2, Cyclin D1 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Then, peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, Hubei, China) diluted in 1:1,000 were added and incubated for 1 h at room temperature. A fluorescent western blotting detection system was used. The band density of each gene was normalized to the corresponding density of β-actin.

Plasmids construction and transfection of ERα overexpression vector

Recombinant ERα vectors were constructed using pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). Briefly, the full-length coding region of ERα was inserted into the Nhe I/Xho I sites of pcDNA3.1 and identified by enzyme digestion and sequencing. The pcDNA3.1- ERα was co-transfected with miR-1280 mimic into FTC133 cells, while the negative control group was transfected with empty pcDNA3.1 plasmid and miR-1280 mimic control.

Statistical analysis

The quantitative data were expressed as mean ± SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Decreased expression of miR-1280 in follicular thyroid cancer tissues and cell lines

The expression level of mature miR-1280 was detected by qRT-PCR. The results showed that the expression of miR-1280 was significantly decreased in human thyroid cancer tissues as compared with the adjacent non-neoplastic tissues (Fig. 1A). In addition, the expression level of miR-1280 was obviously decreased in FTC133 and TT cell line compared with Nthy-ori 3-1 cells (Fig. 1B). These results suggest a critical role of miR-1280 in follicular thyroid cancer.

Inhibition of miR-1280 elevated the cell proliferation and invasion ability of Nthy-ori 3-1

To evaluate the contributions of miR-1280 in normal thyroid follicular epithelial cell, Nthy-ori 3-1 cells were transfected with miR-1280 inhibitor and miR-1280 inhibitor control. As shown in Fig. 2A, miR-1280 expression level was significantly downregulated in Nthy-ori 3-1 cells transfected with miR-1280 inhibitor for 48 h compared with miR-1280 inhibitor control transfection group. Moreover, we found that the inhibition of miR-1280 accelerated cell proliferation of Nthy-ori 3-1 (Fig .2B). The cell invasion ability was analyzed using Transwell cell culture chambers. The results revealed

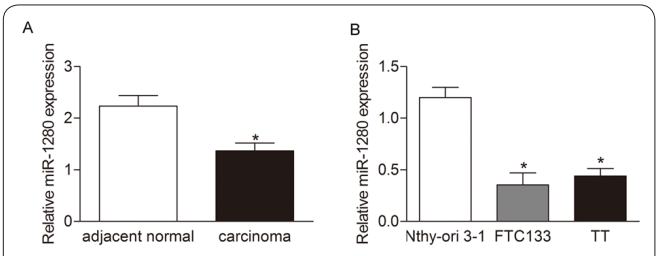

Figure 1. MiR-1280 is significantly decreased in follicular thyroid cancer tissues and cell lines. A. The mRNA expression level of miR-1280 in follicular thyroid cancer and adjacent non-neoplastic tissues was detected by qRT-PCR, miR-1280 expression was normalized to U6 RNA. B. The mRNA expression level of miR-1280 in follicular thyroid cancer cell line FTC133 and TT. Nthy-ori 3-1 cells were used as healthy control cells. Data are represented as means ± SD. *P<0.05, compared with adjacent tissue samples or Nthy-ori 3-1 cells.

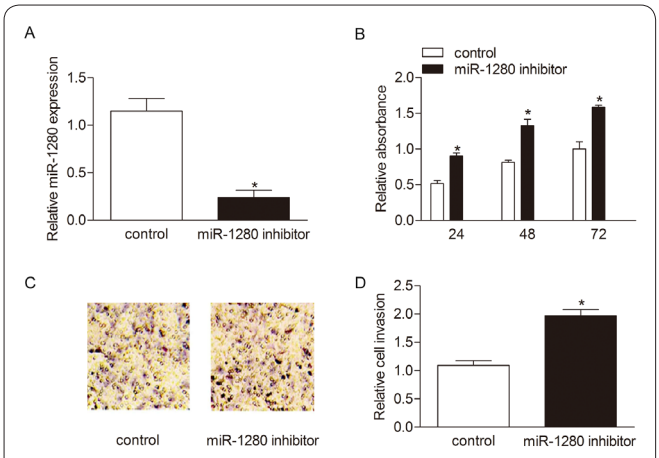

Figure 2. MiR-1280 inhibition elevated cell proliferation and invasion ability of Nthy-ori 3-1. Nthy-ori 3-1 cells were transfected with miR-1280 inhibitor and inhibitor control respectively. A. The mRNA expression level of miR-1280 was detected by qRT-PCR, miR-1280 expression was normalized to U6 RNA. B. Nthy-ori 3-1 transfected with miR-1280 inhibitor and inhibitor control was seeded on 96-well microplates, cultured for 24, 48 and 72 h and stained with MTT. The number of viable cells was determined via the absorbance. C and D. The cell invasion ability of Nthy-ori 3-1 was detected by Transwell cell culture chambers. Data are represented as means ± SD. *P<0.05, compared with control group.

that the invasion ability of Nthy-ori 3-1 was significantly increased in cells transfected with miR-1280 inhibitor compared with those transfected with inhibitor control (Fig. 2C and D).

Overexpression of miR-1280 inhibited the cell proliferation and invasion ability of FTC133

To further explore function of miR-1280 in follicular thyroid cancer cells, FCT133 cells were transfected with miR-1280 mimic and miR-1280 mimic control. The expression of miR-1280 was detected after 48 h of transfection using qRT-PCR. The results showed that miR-1280 expression was significantly increased in FTC133 cells transfected with miR-1280 mimic compared with the mimic control group (Fig. 3A). In addition, the cell growth (Fig. 3B) and cell invasion (Fig. 3C and D) of FTC133 were decreased significantly with miR-138 mimic transfection.

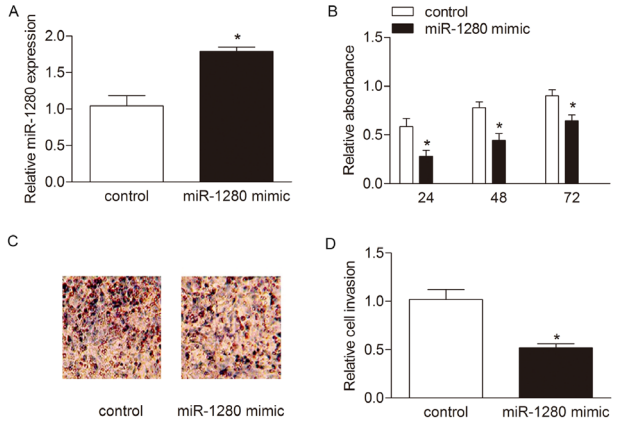

Figure 3. MiR-1280 overexpression inhibited cell proliferation and invasion ability of FTC133. FTC133 cells were transfected with miR-1280 mimic and mimic control respectively. A. The mRNA expression level of miR-1280 was detected by qRT-PCR, miR-1280 expression was normalized to U6 RNA. B. FTC133 transfected with miR-1280 mimic and inhibitor mimic control was seeded on 96-well microplates, cultured for 24, 48 and 72 h and stained with MTT. The number of viable cells was determined via the absorbance. C and D. The cell invasion ability of FTC133 was detected by Transwell cell culture chambers. Data are represented as means ± SD. *P<0.05, compared with control group.

Prediction and identification of target gene of miR-1280

The bioinformatics software programs, miRBase, RegRNA and RNAhybrid were used to predict the target gene of miR-1280. According to the Bioinformatics analysis, the potential binding target sequence of miR-1280 was found in the 3'-UTR of the ERα gene (Fig. 4A). To further delineate whether ERα is a direct target of miR-1280, dual-luciferase reporter assay was performed. Co-transfection of pGL3-ERα-3'-UTR with miR-1280 mimic in FTC133 cells significantly downregulated the relative luciferase activity in comparison with mimic control group, whereas cells that co-transfected of miR-1280 mimic with pGL3-ERα-3'-Mut-3'-UTR containing mutations in the predicted consensus sequences for miR-1280 had no apparent effect on luciferase activity (Fig. 4B). Numerous studies have demonstrated that ERα plays a critical role in the development of thyroid cancer. Western blot results showed that the expression levels of ERα were significantly increased in thyroid cancer cell line FTC133 as compared with normal thyroid follicular epithelial cell line Nthy-ori 3-1 (Fig. 4C and D). To further confirm the target reaction between miR-1280 and ERα in follicular thyroid cancer, we assessed the effect of miR-1280 on ERα expression in FTC133 cells. The results showed that expression level of ERα was significantly decreased by miR-1280 overexpression, whereas knockdown of miR-1280 markedly increase ERα expression level in FTC133 (Fig. 4E and F). We next examined whether miR-1280 can effect ERK pathway. The results showed that the expression of p-ERK1/2 and Cyclin D1 were down-regulated in miR-1280 mimic transfection group, and up-regulated in miR-1280 inhibitor group.

Overexpression of ERα abrogates the effect of miR-1280 on cell proliferation and invasion in FTC133

To verify the contribution of ERα to the biological

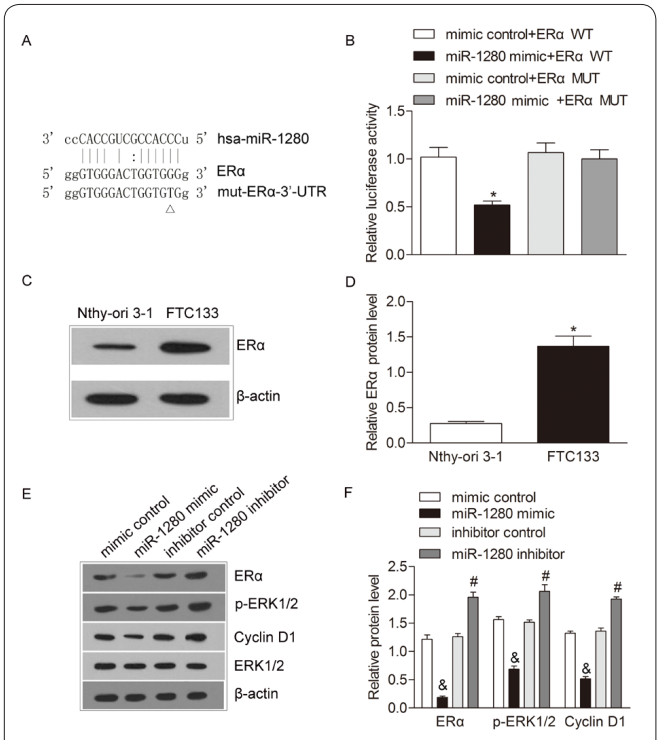

Figure 4. Identification of the targeting reaction between miR-1280 and ERα. The target gene was predicted using bioinformatics software programs and identified by luciferase reporter assay. A. The wild-type (WT) miR-1280 binding sequence and mutant (MUT) miR-1280 binding sequence in the 3'-untranslated region (3'-UTR) of the ERα gene. B. Relative firefly luciferase activity in FTC133 cells co-transfected with miR-1280 mimic or mimic control together with luciferase reporter constructs containing either WT or MUT ERα 3'-UTR. C. The protein expression level of ERα was measured by western blot. D. Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. E. The protein expression level of ERK, p-ERK1/2 and Cyclin D1 were measured by western blot. F. Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. Data are represented as means ± SD. *P<0.05, compared with mimic control+ ERα WT group or Nthy-ori 3-1 group. $^{\&}$P<0.05, compared with mimic control group. $^{\#}$P<0.05, compared with inhibitor control group.

effects of miR-1280 in FTC133, the cells were co-transfected with miR-1280 mimic and ERα overexpression vector harboring no specific miR-1280 binding-specific sequences in the 3'-UTR. The results revealed that the overexpression of ERα (Fig. 5A and B) significantly blocked the inhibition effect of miR-1280 on cell proliferation (Fig.5C) and invasion (Fig.5D and E) in FTC133 cells. In conclusion, these findings suggest that miR-1280 plays an important role in the regulation of the proliferation an invasion ability of FTC133 cells by targeting ERα.

Discussion

As the incidence and the morbidity of thyroid carcinoma increased over the recent decades, development of suitable biomarkers is critical for accurately diagnosing thyroid carcinoma (14). In this study, we describe a previously unknown mechanism for the regulation of thyroid cancer cell proliferation and invasion. We found that the expression levels of miR-1280 were upregulated in thyroid cancer tissues compared with adjacent benign

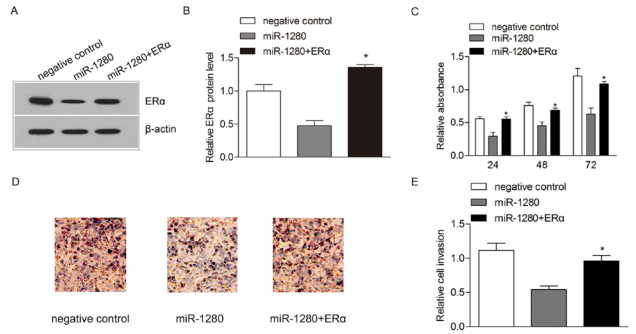

Figure 5. Effect of ERα overexpression on miR-1280-induced enhancement of the proliferation and invasion of FTC133 cells. Negative control group, cells transfected with empty pcDNA3.1 plasmid and miR-1280 mimic control. miR-1280 group, cells transfected with miR-1280 mimic. miR-1280+ ERα group, cells co-transfected with miR-214 mimic and ERα overexpression vectors harboring no miR-1280 binding-specific sequences in the 3'-UTR. A. The expression levels of ERα were detected by western blot analysis. B. Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. C. The proliferation of FTC133 cells was measured by MTT assay. D. The cell invasion ability of FTC133 was detected by Transwell cell culture chambers. Data are represented as means ± SD. *P<0.05, compared with miR-1280 group.

tissues. Moreover, we demonstrated that miR-1280 can regulate the proliferation and invasion of thyroid cancer cells by directly targeting the 3'-UTR of ERα mRNA.

MicroRNAs are known as the endogenous single-stranded non-coding RNAs consisting of approximately 24 nucleotides, which can partially or completely suppress the post-transcriptional expression of target genes and have been demonstrated to suppress or promote different cellular processes, such as cell apoptosis, differentiation, proliferation and invasion (15). Nowadays, amount of studies have demonstrate that microRNAs play critical role in the regulation of various disease including thyroid cancer (16). Though little is known about miR-1280, one study has shown that miR-1280 is expressed in colon and pancreatic cancers according to the expression analysis of 19 colorectal and 17 pancreatic human cancer samples (17). Another study reported that miR-1280 act as a tumor suppressor and has prognostic and diagnostic potential in bladder cancer (18). In our study, we for the first time that miR-1280 was significantly down-regulated in thyroid cancer tissues and cancer cell lines compared to adjacent normal tissues or non-malignant cell line. Moreover, after using mimics and inhibitors of miR-1280 to transfected FTC133 cells, we demonstrated that miR-1280 overexpression inhibited proliferation and invasion ability of FTC133 cell. These results suggested that miR-1280 may functions as a suppressor to regulate the progression of thyroid cancer.

Estrogen is known to be involved in the growth and differentiation of normal mammary gland (19). Recent report suggested that estrogens played an important role in thyroid cancer (10). The biological effect of estrogen in human is mediated by a number of receptors, including ERα. ERα, encoded by the gene ESR1 in humans, is a nuclear hormone receptor that has a key role in different cellular process, including tumorigenesis (20). It has been reported that ERα contributes to tumor growth and affect the cell proliferation in thyroid cancer (21). In the present study, a conservative miR-1280 binding site in the 3'-UTR of ERα was detected using bioinformatics software programs. Moreover, the results of luciferase reporter assay revealed that the overexpression of miR-1280 significantly reduced the luciferase activity when the FTC133 cells transfected with ERα WT, but not in ERα MUT transfection group. These results demonstrated that ERα is a direct target of miR-1280.

As has been previously reported, ERα is important in the cell proliferation process of thyroid cancer cells (22). In our study, we found the expression of ERα was significantly increased in FTC133 compare with normal control cells, which was consistent with the former study. Furthermore, we investigated the potential mechanism of miR-1280 in regulating ERα in thyroid cancer cells. The results showed that overexpression of miR-1280 significantly decreased ERα expression, whereas inhibition of miR-1280 elevated the expression of ERα in FTC133 cells. This result further confirmed the target reaction between miR-1280 and ERα. Therefore, indicated that miR-1280 might be considered a new therapeutic target for patients of thyroid cancer. Studies have been demonstrated that ERα can act through nongenomic mechanism to regulate signal transduction through ERK pathway (23). Moreover, ERα can bind with estrogen response elements in the control regions of genes, such as Cyclin D1, and participate in cell proliferation and invasion (24). Importantly, our study found that miR-1280 overexpression negatively regulated the ERK signaling pathway and Cyclin D1 expression in FTC133. These results implied that the interaction of miR-1280 and ERα inhibited ERα-mediated transactivation activity of Cyclin D1 and ERK pathway, suggest that miR-1280 may play a significant role in ERα regulated cell proliferation and invasion in thyroid cancer.

In order to further confirm the inhibition effect of miR-1280 on cell processing via targeting ERα in thyroid cancer. We constructed ERα overexpression vector and co-transfected with miR-1280 mimic into FTC133 cells. The results showed that overexpression of ERα restored the miR-1280 inhibition effect on proliferation and invasion ability in FTC133 cells. These results suggested that miR-1280 can act as a newly identified miRNA that suppresses the cell proliferation and invasion through directly regulating ERα in thyroid cancer.

In conclusion, our study is the first report to document the tumor suppressor role of miR-1280 in thyroid cancer. MiR-1280 directly targets oncogene ERα, inhibiting the proliferation and invasion of thyroid cancer cells. Our findings provide an important clue to help elucidate the pathogenesis of thyroid cancer and indicate that restoration of tumor suppressor miR-1280 might be useful therapeutically in the treatment of the disease.

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