



MiR-1284 suppresses the proliferation and migration of thyroid cancer

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ARTICLE INFO

Original paper

Article history:

Received: July 16, 2023

Accepted: August 19, 2023

Published: August 31, 2023

Keywords:

Thyroid cancer, MiR-1284, exosomes, proliferation

ABSTRACT

To elucidate the role of microRNA-1284 (miR-1284) in the onset of thyroid cancer (TC) and its underlying mechanism. Differential expressions of miR-1284 in TC and thyroid tissues were detected. Regulatory effects of miR-1284 on proliferative, migratory, apoptotic potentials and cell cycle progression were assessed. In addition, miR-1284 levels in TC tissues and peripheral blood of TC patients were determined as well. Through collecting culture medium and exosomes of PTC cells, changes in miR-1284 levels were examined. MiR-1284 was downregulated in TC than normal thyroid tissues. Overexpression of miR-1284 attenuated proliferative and migratory potentials, but induced apoptosis in TPC-1 and FTC-133 cells. Moreover, overexpression of miR-1284 upregulated E-cadherin and downregulated N-cadherin in papillary TC (PTC) cells. MiR-1284 was downregulated in TC tissues, while its level in the peripheral blood of TC patients was upregulated. Besides, miR-1284 was upregulated in the culture medium and exosomes of PTC cells, which was reversed by Brefeldin A treatment. Overexpression of miR-1284 suppresses proliferative and migratory potentials and induces apoptosis in TC. Upregulated miR-1284 in the peripheral blood of TC patients may be derived from exosomes secreted by PTC cells.

Doi: <http://dx.doi.org/10.14715/cmb/2023.69.8.38>

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Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, and its incidence has gradually increased over the past decades (1, 2). There are four histological subtypes of TC originating from follicular cells, including papillary TC (PTC), follicular TC (FTC), poorly differentiated TC (PDTC) and anaplastic TC (ATC). Among them, PTC accounts for over 80% of all thyroid malignancies (3-5). Generally speaking, the 10-year survival of PTC is higher than 90%. However, approximately 5% of PTC patients are accompanied by invasive tumors through local infiltration, capsule invasion, lymphatic metastasis and distant metastasis. It is reported that 10-15% of PTC patients develop recurrence, leading to a decline in overall survival (4,6).

MicroRNAs (miRNAs) are endogenous, single-strand, non-coding RNAs that were initially discovered in *Caenorhabditis elegans* (7). They are extensively involved in cell phenotype regulations (8-11). Recent evidences have shown multiple differentially expressed miRNAs in human cancers (12,13). Serving as oncogenes or tumor suppressors, miRNAs are involved in cancer development (14). Owing to the specific biological and functional characteristics, miRNAs have become novel diagnostic and therapeutic targets in cancers (15).

Previous studies have shown the potential role of miR-1284 in many types of cancers. By targeting EIF4A1, miR-1284 alleviates the development of gastric cancer (16). MiR-1284 enhances cisplatin sensitivity in cervical cancer by downregulating HMGB1 (17). In breast cancer, miR-1284 inhibits tumor cell growth and invasiveness by

targeting ZIC2 (18). In this research, we aimed to uncover the role of miR-1284 in the development of TC, which contributes to health management in TC patients.

Materials and Methods

Sample collection

Tissue samples were collected from our hospital. All samples were pathologically diagnosed. Biopsy samples analyzed by thyroid fine needle aspiration (FNA) were obtained according to clinical protocols using the Bethesda System for Reporting Thyroid Cytopathology (Cibas&Ali 2009). This study got approval from the Ethics Committee of Huadong Hospital Affiliated with Fudan University and was conducted after the informed consent of each subject. This study was conducted in accordance with the Declaration of Helsinki.

RNA and exosome extraction

Tissue RNAs were extracted using RNAiso Plus reagent, and RNAs in culture medium were obtained using miRNeasy kit (Qiagen, Hilden, Germany). Exosomes from the culture medium and serum samples were isolated using Total Exosomes Isolation Kit (Life Technologies, Gaithersburg, MD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Reverse transcription of RNAs was performed by the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and complementary deoxyribose nucleic acid (cDNA) was sent for qRT-PCR. The relative level of the target was calculated

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using the 2- $\Delta\Delta C_t$ method. Primer sequences were as follows. E-cadherin: F: 5'-TGGAGGAATTCTTGCTTTGC-3'; R: 5'-CGTACATGTCAGCCAGCTTC-3'; N-cadherin: F: 5'-CCTCCAGAGTTTACTGCCATGAC-3'; R: 5'-GTAGGATCTCCGCCACTGATTC-3'.

Cell culture and transfection

PTC cell lines (TPC1 and FTC133) were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) in a 5% CO₂ incubator at 37°C. One day prior to transfection, 4-5×10⁴ cells per well were inoculated in 6-well plates and transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA).

Isolation of primary thyroid cells

Fresh thyroid tissues were repeatedly washed in D-Hanks for the removal of blood and impurities. Tissues were lysed in 250 U/mL type II collagenase with 0.25% trypsin at 37°C water bath for 90 min. The mixture was filtrated using a 200-mesh sieve and washed in DMEM for 2-3 times. The cell survival rate calculated by Trypan Blue was higher than 90%. After cell culture in DMEM containing 10% FBS, 80 U/mL penicillin, 0.08 mg/ml streptomycin, 2.975 g/L HEPES and 10 mU/L TSH for 24 hours, nonadherent cells were washed and those adherent ones were primary thyroid follicular epithelial cells. Medium was replaced every 2-3 days and cell passage was conducted every 5-7 days.

Cell counting kit-8 (CCK-8) assay

Cells were inoculated into 96-well plates with 2×10³ cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells in a 96-well plate (4×10³ cells/well) were incubated with EdU solution (Sigma-Aldrich, St. Louis, MO, USA) in the dark, and 30 min later, they were dyed with Hoechst 33342 for another 30 min. EdU-positive ratio was calculated by the number of EdU-positive cells to that of Hoechst 33342-labeled nuclei.

Cell cycle determination

2×10⁶ cells were collected and centrifuged at 1000 rpm for 5 min. The precipitant was incubated in pre-cold 75% ethanol at 4°C overnight. On the other day, cells were washed and incubated in 1% bovine serum albumin (BSA), following treatment with 100 μL of Propidium Iodide (PI) and 100 μL of RNase. After 37°C in dark incubation for 30 min, the cell cycle was determined.

Cell apoptosis determination

The cell suspension was prepared at 1×10⁶ cells/ml. 10 μL of Annexin-V and 380 μL of loading buffer were added in 100 μL of suspension. Following 15-min incubation in the dark, cell apoptosis was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Transwell assay

100 μL of suspension (1×10⁶ cells/mL) were inoculated

in the upper Transwell chamber (Millipore, Billerica, MA, USA) and inserted in a 24-well plate with 500 μL of medium containing 10% FBS in the bottom. After 48 h incubation, bottom cells were reacted with 15 min methanol, and 20 min crystal violet and captured using a microscope. Migratory cells were counted in 10 random fields per sample (magnification 200×).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as mean±SD (standard deviation). Differences between the two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using a One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). *P*<0.05 considered the difference was statistically significant.

Results

MiR-1284 was downregulated in TC

Thyroid, PTC and PDTC/ATC tissues were collected. Compared with normal thyroid tissues, miR-1284 was downregulated in PTC and PDTC/ATC tissues (Figure 1A). Overexpression of miR-1284 in TPC-1 and FTC-133 cells markedly reduced viability (Figure 1B). Similarly, the EdU-positive rate decreased in PTC cells overexpressing miR-1284 (Figure 1C).

Overexpression of miR-1284 stimulated apoptosis in TC

MiR-1284 level was markedly higher in primary thyroid cells than in PTC cell lines (TPC-1 and FTC-133) (Figure 2A). Furthermore, overexpression of miR-1284 arrested the cell cycle in the G2/M phase and reduced the cell ratio in the S phase (Figure 2B). We detected regulatory effects of miR-1284 on expression levels of cell cycle genes. As qRT-PCR data showed, p21 and p27 were upre-

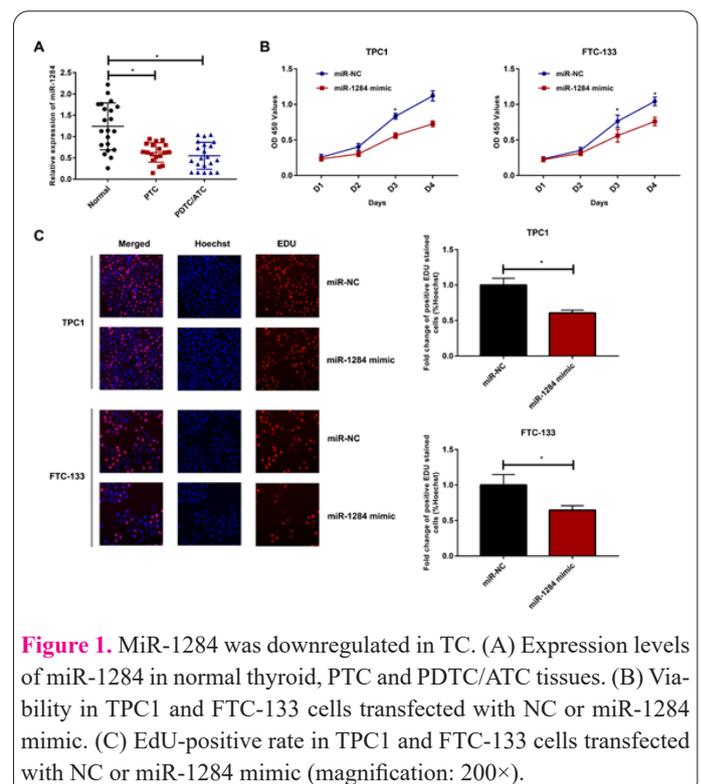


Figure 1. MiR-1284 was downregulated in TC. (A) Expression levels of miR-1284 in normal thyroid, PTC and PDTC/ATC tissues. (B) Viability in TPC1 and FTC-133 cells transfected with NC or miR-1284 mimic. (C) EdU-positive rate in TPC1 and FTC-133 cells transfected with NC or miR-1284 mimic (magnification: 200×).

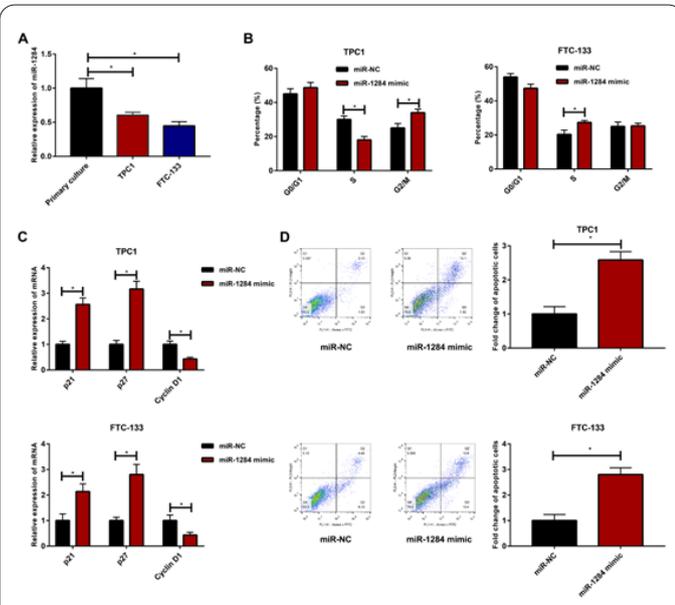


Figure 2. Overexpression of miR-1284 stimulated apoptosis in TC. (A) Expression level of miR-1284 in primary thyroid cells and TC cell lines (TPC1 and FTC-133). (B) Cell cycle distribution in TPC1 and FTC-133 cells transfected with NC or miR-1284 mimic. (C) Expression levels of p21, p27 and Cyclin D1 in TPC1 and FTC-133 cells transfected with NC or miR-1284 mimic. (D) Apoptosis in TPC1 and FTC-133 cells transfected with NC or miR-1284 mimic.

gulated, while cyclin D1 was downregulated in PTC cells overexpressing miR-1284 (Figure 2C). Flow cytometry data revealed that overexpression of miR-1284 remarkably enhanced the apoptotic rate in PTC cells (Figure 2D).

Overexpression of miR-1284 suppressed migratory potential in TC

Transwell assay demonstrated that overexpression of miR-1284 attenuated migratory potential in PTC cells (Figure 3A, 3B). In addition, changes in epithelial marker expressions were detected. Upregulated E-cadherin and downregulated N-cadherin were observed in PTC cells overexpressing miR-1284 (Figure 3C, 3D).

MiR-1284 was upregulated in the peripheral blood of TC patients

Because miR-1284 was downregulated in TC tissues, we next explored whether miR-1284 exerted diagnostic potential in thyroid FNA. Compared with benign TC tissues, miR-1284 abundance in PTC and FTC tissues was markedly lower (Figure 4A). The peripheral blood level of miR-1284 was higher in PTC patients than in both healthy subjects and patients with benign TC (Figure 4B). Subsequently, culture medium and exosomes were collected from cultured PTC cells. A higher level of miR-1284 was observed in the culture medium of PTC cells than in isolated exosomes (Figure 4C, 4D). It is suggested that upregulated serum level of miR-1284 in PTC patients may be derived from exosomes isolated from TC. The application of Brefeldin A can block BIG2 activation and secretion of exosomes from tumor cells. Here, Brefeldin A treatment markedly reduced miR-1284 level in exosomes, further confirming our speculation (Figure 4E, 4F).

Discussion

PTC is the most common subtype of TC. The incidence

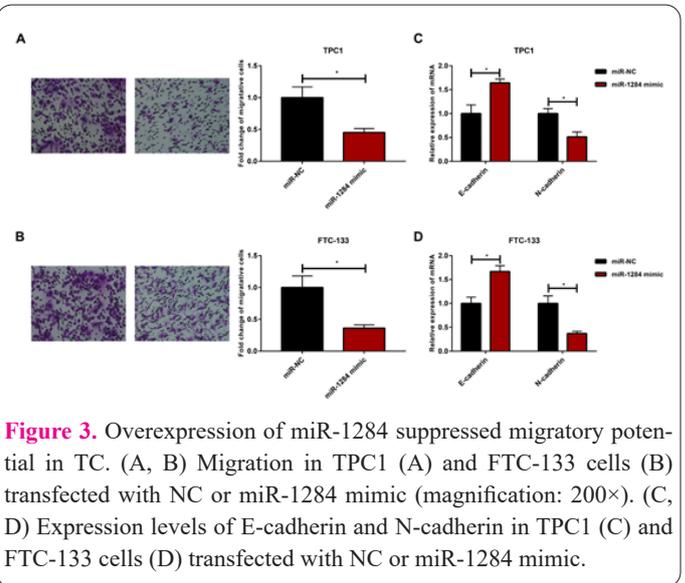


Figure 3. Overexpression of miR-1284 suppressed migratory potential in TC. (A, B) Migration in TPC1 (A) and FTC-133 cells (B) transfected with NC or miR-1284 mimic (magnification: 200×). (C, D) Expression levels of E-cadherin and N-cadherin in TPC1 (C) and FTC-133 cells (D) transfected with NC or miR-1284 mimic.

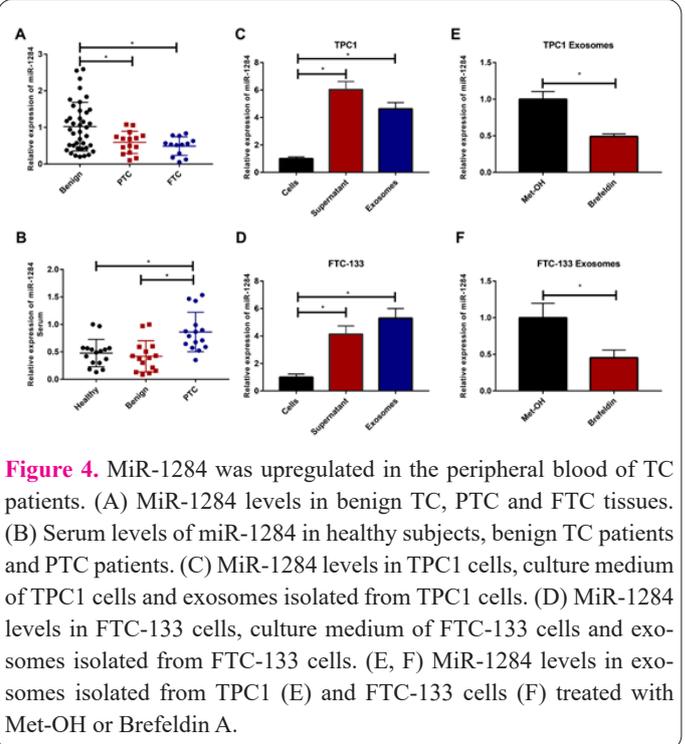


Figure 4. MiR-1284 was upregulated in the peripheral blood of TC patients. (A) MiR-1284 levels in benign TC, PTC and FTC tissues. (B) Serum levels of miR-1284 in healthy subjects, benign TC patients and PTC patients. (C) MiR-1284 levels in TPC1 cells, culture medium of TPC1 cells and exosomes isolated from TPC1 cells. (D) MiR-1284 levels in FTC-133 cells, culture medium of FTC-133 cells and exosomes isolated from FTC-133 cells. (E, F) MiR-1284 levels in exosomes isolated from TPC1 (E) and FTC-133 cells (F) treated with Met-OH or Brefeldin A.

of PTC has increased globally (1,2). It is well known that PTC is an inert tumor with low mortality and a good prognosis. However, lymphatic metastasis of PTC greatly limits the therapeutic efficacy.

MiRNAs exert vital functions in tumor development. They are detectable in many types of body fluids. Owing to the feature of differential expression, miRNAs are promising candidates in tumor diagnosis, treatment and prognosis (19,20). Non-invasive detection of miRNA levels provides a novel approach to tumor biology. Li et al. (21) pointed out that miR-25-3p and miR-451a are highly expressed in the serum of PTC patients, which are markedly reduced after surgical resection of PTC. It is suggested that miRNA levels in tumor tissues display guiding significance in the therapeutic management of tumors. In this paper, miR-1284 was downregulated in PTC tissues, while it was elevated in the peripheral blood of PTC patients. Overexpression of miR-1284 inhibited proliferative and migratory potentials of PTC and induced apoptosis.

Exosomes are considered to be messengers of intercellular communication. They are bilayer-membrane vesicles

with a diameter of 20-200 nm, which are produced by the fusion of living cell polyvesicles with cell membranes. Exosomes are widely derived from fibroblasts, mast cells, stem cells, and tumor cells. They are also present in various body fluids, such as plasma, saliva, milk, cerebrospinal fluid, and urine (22-25). A previous study showed that exosomes in the culture medium of TPC-1 cells carry miR-146 and miR-222 (26). Our findings uncovered that PTC cells transfer miR-1284 from TPC mass to peripheral blood by secreting exosomes.

Overexpression of miR-1284 suppresses proliferative and migratory potentials and induces apoptosis in TC. Upregulated miR-1284 in the peripheral blood of TC patients may be derived from exosomes secreted by PTC cells.

Conflict of Interest

The authors declared no conflict of interest.

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