miR-584 Suppresses Invasion and Cell Migration of Thyroid Carcinoma by Regulating the Target Oncogene ROCK1

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Introduction
Thyroid carcinoma (TC) remains the most frequent endocrine system tumor and is the kind of malignancy with the fastest growing prevalence rate in the recent 20 years. Papillary TC (PTC) is the class of tumor with the highest morbidity, comprising over 80\% of the TCs [1]. The tumor cells in patients with clinically advanced PTC often invade the throat, the trachea, the epiglottis, the esophagus, cervical vessels, and the surrounding structures; they even metastasize to the bone and can cause lung, brain, and systemic metastases. With the tumor being hard to cut out completely, the high risk, high concurrency and insensitivity to radiation chemotherapy make patients with advanced PTC feel quite hopeless. So, studies on the invasion and migration pathways of TC in order to find more efficient molecular targets have become a research hotspot in recent years’ investigations in the field of gene therapy.

MicroRNAs (miRNAs) are small noncoding RNAs of 19–22 nucleotides that inhibit protein expression by binding to the 3’-untranslated region (3’-UTR) of a specific target mRNA to block its translation [2]. They also play crucial roles in cell proliferation and differentiation, apoptosis signaling, and the formation and progression of tumors [3]. miRNA analysis was reported as a potential diagnostic tool for PTC [4], and the miRNA signature was suggested to be indicative of the degree of aggressiveness of PTC [5]. But little is known on the role of miRNAs in suppressing invasion and migration of TC, let alone their functions and mechanisms of action. It has been reported that the expression of miR-584 can be up-regulated in breast cancer [1], renal clear-cell carcinoma [6], colon cancer [7], rectal cancer [8], myeloid leukemia [9], and malignant mesothelioma [10], to suppress the invasion and migration of tumors by targeting \textit{PHACTR1} [1], \textit{Rock-1} [6], \textit{NGX6} [7], etc. Uncovering the target gene of miR-584 to control TC invasion and migration is of central importance in the diagnosis, treatment, and prognosis of TC, and for blocking tumor invasion and migration.

Keywords
Thyroid carcinoma · miR-584 · ROCK-1 · Invasion · Cell migration

Summary

Background: Uncovering the target gene of miR-584 to control thyroid carcinoma (TC) invasion and migration is of central importance in the diagnosis, treatment, and prognosis of TC. To validate whether miR-584 has a tumor-suppressive role in thyroid cancer cells by targeting \textit{ROCK1}, a series of experiments were conducted to figure out the mechanism of action of miR-584.

Material and Methods: Migration analyses and cell proliferation assays were performed using miR-584-transfected cells. The expression levels of miR-584 in TC were detected by using real-time polymerase chain reaction (PCR). Western blot analyses were conducted to find out the relationship between the tumor suppressor miR-584 and the target oncogene \textit{ROCK1} protein expression levels. Wound healing experiments were used to examine the relationships between miR-584 and the migration of thyroid cancer K1 cells and the effects of \textit{ROCK1} knockdown on K1 cell motility.

Results: Our results demonstrate that altering the miR-584 levels affects human thyroid cancer cell migration, but has no effect on cell proliferation. The relative \textit{ROCK-1} expression levels were 1 and 0.54 in the scrambled-sequence control group and the miR-584 group, respectively. K1 cells transfected with siRNA-ROCK-1 showed weaker cell migration than cells transfected with siRNA-NC (negative control); the cell motility ratios were 18\% and 27\%, respectively.

Conclusion: These results indicate that miR-584 could inhibit the expression of \textit{ROCK1}, and \textit{ROCK1} knockdown would further affect the migration ability of K1 cells.
Materials and Methods

Materials

The TC cell line K1 was purchased from KeyGene Biotechnology (Nanjing). Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). miR-584 mimics, miR-584 inhibitor and the negative control (NC) were purchased from Ambion (Austin, TX, USA). ROCK1 short interfering RNA (siRNA) and NC-siRNA were obtained from BGI Company (Shanghai, China). The transfection reagent Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Kits for reverse transcription-polymerase chain reaction (RT-PCR) and PCR and the total RNA extraction reagent were all obtained from TaKaRa Biotechnology (Dalian, China). Kits for protein concentration determination and enhanced chemiluminescence (ECL) measurements were from Thermo Scientific (Rockford, IL, USA). The antibodies, including anti-α-tubulin mouse monoclonal antibody, anti-ROCK-1 antibody, and horseradish peroxidase (HRP)-labeled goat anti-mouse antibody, were ordered from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell Culture and Transfection

K1 TC cells were cultured in DMEM with 10% FBS, then inoculated into 6-well plates at 2 × 10^5 cells/well in the exponential phase and cultured at 37 °C and 5% CO_2. Transfections were performed using Lipofectamine 2000 when the cells had been cultured to 90% confluence. After keeping the cells at room temperature for 20 min, 2.5 μl siRNA and 5 μl Lipofectamine 2000 were diluted in 250 μl DMEM and mixed; the mixed liquid was then put into the 6-well plates to obtain a final concentration of 20 nmol/l of cell culture. The cells were then transferred to fresh complete medium.

siRNA Sequence Design

siRNA for ROCK1 was designed using the siRNA Target Finder and Design tools (Ambion, Austin, TX, USA) following the siRNA design principles on the basis of the human ROCK1 gene sequence from GenBank. Candidate sequences were identified by sequence alignment by the Basic Local Alignment Search Tool (BLAST) to exclude homologous expressed sequence tag (EST) sequences and coding sequences of other genes. The negative control siRNA (NC-siRNA) was randomly selected from siRNA sequences. The ROCK1 siRNA and NC-siRNA were produced by BGI Company. The ROCK1 siRNA sequence is as follows: 5'-UGAUCUUGUAGCUCCCGCAUGUGUC-3'.

Western Blot Analysis

At 48 h after transfection, the cells were washed in phosphate-buffered saline (PBS) and then a protein lysis buffer was added. The total proteins were extracted by centrifugation at 14,000 rpm for 10 min. The total cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred (semi-dry method) to nitrocellulose membranes after 15 min of soaking the membranes in buffer, which were then incubated overnight with primary antibodies at 4 °C. On the second day, the membranes were kept at 37 °C for 30 min and then shaken 3 times 5 min in phosphate buffer, followed by incubation with the HRP-labeled secondary antibodies. Two hours later, specific bands were visualized by ECL after 4 times 3 min of shaking in double-distilled water at 37 °C for 3 min.

Fluorescent Quantitative PCR

Cells in each group were collected 48 h after transfection. The Trizol method was applied to extract the total RNA, followed by reverse transcription and target fragment amplification performed with the miScript PCR system with RNA; the U18 small nuclear RNA was used as the reference for mRNA expression. Fluorescent quantitative PCR (FQPCR) system (25 μl): ddH_2O 8.5 μl, 2 × SYBR Premix Ex Taq II 12.5 μl, cDNA 2 μl, forward and reverse primers (10 μmol/l) 1.0 μl each. Cycling was as follows: activation: 30 s, 95 °C; denaturation, 5 s, 95 °C; extension, 30 s, 60 °C for 40 cycles, then increasing the temperature to 95 °C with a 0.2 °C gradient; the melting curves of the genes were then analyzed. The samples in each group were assayed in three duplicates. Inter-group comparisons were made among the mean values. mRNA expression levels were determined using the 2^-ΔΔCt method.

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Fig. 1. miR-584 is up-regulated in the human papillary thyroid cancer cell line K1. Error bar indicates the mean ± SE.

Wound Healing

Cells transfected with the miR-584 mimic, the miR-584 inhibitor, the NC and the ROCK1 siRNA (siR-ROCK1) were seeded into 6-well plates at 1 × 10^5 cells/l, followed by cultivation in DMEM to maintain adherent growth of the cells for 6 h. Scratching (wounding) was performed using a 10-μl Eppendorf tip. The cells were cultured in 5% CO_2 at 37 °C for 24 h after washing in serum-free medium for 3 times. Pictures were taken to measure the distances between the scratches using Image-Pro Plus6.0 software. The mean values and standard deviations were calculated for intra-group comparisons. The experiment was repeated 3 times. The transfer ability was expressed as mobility ratio: mobility ratio = (prime scratch width - current scratch width)/prime scratch width.

MTT Assay

Cells transfected with the miR-584 mimic and the miR-584 inhibitor and normal cells as control were plated at a density of 2000 cells adjusted to 100 μl culture solution per well in 96-well plates, to be cultured at 37 °C. The absorbance of different laryngeal cancer cell subpopulations was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 7 days of continuous culture after inoculation. 3 duplicates were set to detect the absorbance at 490 nm wavelength using a microplate reader in order to draw a cell growth curve of mean absorbance values along the culture time.

Statistical Analysis

All statistical analyses were performed using SPSS 13.0 software (IBM Software, USA). Experimental data are presented as mean ± standard error (SE), and the two-tailed Student’s t-test was adopted for comparison between 2 groups. A p value of 0.05 was regarded as statistically significant.

Results

Expression Levels of miR-584 in TC

We compared the expression levels of miR-584 in 4 human normal tissues and the human PTC cell lines K1, TCP-1 and W3, using FQPCR. As shown in figure 1, stronger relative expression of the miRNA was observed in the human PTC cell lines compared to the normal tissues (p < 0.01). Our real-time RT-PCR results revealed that miR-584 was overexpressed in human PTC cells.
To investigate whether miR-584 could regulate human PTC cell migration, a miRNA mimic and an inhibitor of miR-584 were applied. Cell migration was measured using the wound healing assay. K1 cells were transfected with the miR-584 mimic and the inhibitor and seeded to grow to confluence in a monolayer. Wounding was performed with an Eppendorf tip. Cell migration was calculated as the percentage of the surface area covered by the cells after stimulation for 24 h. As shown in figure 2, compared to the control, the miR-584 mimic attenuated the migration of K1 cells while the miR-584 inhibitor enhanced wound closure. Nevertheless, our result revealed that cell proliferation showed no significant differences among the groups (data not shown). These results indicate the inhibitory effect of miR-584 on K1 cell migration, but no effect on cell proliferation.

**Cell Migration Ability and Cell Proliferation of K1 Cells Transfected with miR-584 Mimic, miR-584 Inhibitor and NC**

To determine the toxicity of transfection, the ROCK-1 expression level in K1 cells without the addition of reagent was considered as negative control. At 72 h after transfection, the total protein was extracted and the ROCK-1 protein expression levels were analyzed by Western blot. α-Tubulin was used as loading control. The band intensity was measured by Quantity One software and the given band intensity ratio is relative to that of α-tubulin. As shown in figure 3, the relative ROCK-1 expression levels were 0.94 and 0.42 in the scrambled sequence- and the miR-584 mimic-treated group, respectively, indicating that miR-584 can inhibit the expression of ROCK-1 in K1 cells. This means that miR-584 has a knockdown effect on the oncogene ROCK1.

**ROCK1 Deficiency Inhibits the Migration of K1 Cells**

To evaluate the effects of a ROCK1 knockdown on the K1 cell motility, another wound healing experiment was carried out to measure the degree of motility of the K1 cells. ROCK1 siRNA (siR-ROCK-1) or siRNA-NC was transfected into the K1 cells; cells without addition of reagent were considered as negative control group. At 48 h after transfection, wounding was performed with a tip, and the surface area of the scratch was measured 24 h after scratching. Our results clearly demonstrate that K1 cells trans-

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**Fig. 2.** miR-584 inhibits K1 cell migration. The surface area of the scratch covered by migrating cells was measured as the graph shows. The error bar indicates the mean ± SE; \( p < 0.05 \).

**Fig. 3.** miR-584 can inhibit the expression of ROCK-1 in K1 cells. The error bar indicates the mean ± SE; \( p < 0.05 \).
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miRNA analysis revealed dysregulated expression levels of numerous miRNAs in tumor tissues related to common cells, which even was reported as a potential diagnostic tool for malignant neoplasms [4]. Many kinds of miRNAs have been identified to play roles in papillary carcinoma progression, such as miR-146 [11–14], miR-221 [11, 12], miR-222 [11–13, 15], miR-181 [11], miR-222 [12], miR-196 [14], miR-499 [14], let-7a [15, 16], miR-146-5p [16, 17], miR-335 [17], miR-451 [18], miR-2861 [18] and miR-375 [19], with up- or down-regulated expression levels. In spite of this, little is known of the role of miRNAs in suppressing invasion and migration of TC, let alone their functions and mechanisms of action. Chip screening, tumor migration modeling, and gene expression profiling were carried out in our previous studies to find miRNAs that showed dysregulated expression in TC, and, finally, miR-584 was identified, which is related to tumor migration, but which was never reported in TC. Few studies have demonstrated the dysregulated expression level of miR-584 in tumor tissues relative to normal tissues. Guled et al. [10] reported that the expression level of miR-584 in malignant mesothelioma was higher compared with normal mesothelium, but a functional analysis was not performed in that report. Regulation of miR-584 and regulation of its novel target PHACTR1 were reported as the necessary steps for breast cancer cell migration [1]. And in 2011, a study revealed that miR-584 acts as tumor suppressor by directly targeting the oncogene Rock-1 and decreasing the invasion ability of human clear-cell renal cell carcinoma [6]; this was the first report to show the tumor suppressor functions of miR-584 and the related mechanism. In this study, we found the overexpression of miR-584 in thyroid cancer K1 cells (fig. 1), which is consistent with previous studies, indicating that miR-584 may also have an important role as a tumor suppressor in thyroid cancer. And the result of miR-584 overexpression inhibiting thyroid cancer cell motility further verified the tumor suppressor role of miR-584 in thyroid cancer.

ROCK-1, which is a serine/threonine kinase that belongs to the Rho family of GTPase proteins which facilitate the reorganization of the actin cytoskeleton, plays important roles in mediating the downstream signaling of RhoA and in cell migration, cell death, and survival [20, 21]. It has been reported that ROCK-1 is overexpressed in brain tumors [22] and related to metastasis in bladder, lung, and prostate cancer [23–25]. Chen et al. [24] provided strong evidence that ROCK-1 (designated Rock-1 in their paper) at least partly contributes to the increased motility in lung cancer, and further indicated that ROCK-1 has a potential therapeutic value in lung cancer. The relationship between ROCK-1 and miRNAs has been reported by numerous researchers for various cancer tissues. So far, miR-126, miR-335, miR-584, and miR-186 have been reported to suppress the proliferation and/or invasion of cancer cells by inhibiting ROCK-1 in colon cancer [26], osteosarcoma [27], human renal cell carcinoma [6], and non-small-cell lung cancer [28], with all of these results indicating that ROCK1 could function as an oncogene. To determine the mechanism of action of miR-584 in thyroid cancer, we examined the relationship between the tumor suppressor miR-584 and the ROCK1 target oncogene protein expression levels, and the effects of a ROCK1 knockdown on K1 cell motility. The results indicated that miR-584 could diminish the expression of ROCK-1, and ROCK1 deficiency in turn attenuated the migration ability of K1 cells. These results indicate that miR-584 could diminish the expression of ROCK1, which results in the inhibition of K1 cell migration.

It has been reported that miR-126 suppresses colon cancer cell proliferation and invasion via inhibiting the RhoA/ROCK signal-
ing pathway [26]. Kong et al. [29] revealed that miR-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Combining these experimental results, it can be postulated that miR-584 might inhibit the RhoA/ROCK signaling pathway by targeting the oncogene ROCK1, which results in the suppression of cell invasion in thyroid cancer; however, this still needs further exploration.

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Disclosure Statement

The authors declare that they have no conflicts of interest concerning this article.

References