MiR-630 inhibits papillary thyroid carcinoma cell growth, metastasis, and epithelial-mesenchymal transition by suppressing JAK2/STAT3 signaling pathway


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Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence is on the increase. Papillary thyroid carcinoma (PTC) is the most frequent type of thyroid tumors, accounting for about 80% of all thyroid cancer cases. Risk factors that increase the incidence of TC can be divided into two main types: exogenous and endogenous. Currently, the main therapies for PTC treatment include surgery, chemotherapy and radiotherapy and most PTC cases have excellent prognosis and therapeutic response after system treatments. However, the rate of disease recurrence of this type of cancer is high due to increasing incurability rates. In addition, there are approximately 30% cases with lymph node metastases with poor prognosis. Therefore, exploring the molecular basis of PTC is necessary for designing new therapeutic agents that will improve long-term survival rate. MicroRNAs (miRNAs) are a class of small, non-coding RNA that are ~20 nucleotides in length that can bind the 3'-untranslated region (3'-UTR) of specific genes to inhibit the translation of corresponding mRNA targets. Dysregulation of miRNAs has been confirmed to be implicated in a variety of cellular processes, including cell differentiation, proliferation, migration and apoptosis. Interestingly, growing evidence demonstrates that miRNAs play an important role in tumor progression including cellular migration, proliferation, and angiogenesis, and miRNAs are able to act either as oncogenes or as tumor suppressors by modulating the expression of their target genes. However, the potential mechanism of miRNAs involved invasion and metastasis during the progression of PTC. A better understanding of the...
role of miRNAs in PTC development may help in creating a novel therapeutic strategy for PTC treatment. Previously, miR-630 was identified as a tumor-suppressive miRNA in multiple types of cancers, including ovarian cancer, breast cancer, gastric cancer and glioma14-17. Frequent down-regulation of miR-630 was observed in these tumors. However, the expression of miR-630 and its role in PTC have not been investigated. In this study, we showed that miR-630 expression was down-regulated in both PTC tissues and cell lines, and its overexpression could suppress PTC cell proliferation and metastasis by inhibiting JAK2/STAT3 signaling pathway.

Patients and Methods

Clinical Specimens
This research was approved by the Ethics Committee of Gansu Provincial Hospital, and informed consent was obtained from 47 thyroid carcinoma patients who did not receive any local or systemic anti-cancer treatment before the surgery. 47 paired of PTC tissues and matched normal non-tumor tissues, which were immediately frozen using liquid nitrogen after surgery, were collected from April 2014 to May 2016.

Cell Lines and Cell Transfection
The cell lines used in this study (thyroid follicular epithelial cells: Nthy-ori3-1; thyroid carcinoma cells: SW1736, 8505C and TPC-1) were all purchased from Shanghai YuBo Biotechnology Co., Ltd. (Minhang, Shanghai, China), and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (containing 10% fetal bovine serum (FBS) and 1% antibiotics solution). The cell transfection was conducted by the use of an X-tremeGENE transfection reagent (Saixin Biotech, Changchun, Jilin, China). The miR-630 mimics and negative control (NC) mimics were synthesized by ZoonBio Biotechnology Co., Ltd. (Nanjing, Jiangsu, China).

RNA Isolation and Quantitative Real-Time PCR
Total RNA from thyroid carcinoma tissue samples and cell lines was extracted using the TRIzol reagent (Yaji Mall, Minhang, Shanghai, China). Subsequently, the expression of miR-630 was detected by Invtrogen miRNA qPCR analysis kit (Huijia Biotech, Xiamen, Fujian, China). The qRT-PCR assays for miR-630 detection were conducted on a SLAN-96P Real-time PCR instrument (Sansure Biotech, Changsha, Hunan, China). The primers for miR-630 and U6 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PCR primers used were as follows: miR-630 forward: 5’-TCAGCGCATCTTGTAC-3’ and reverse 5’-GGATCTTGCACAGCACA-3’, U6 forward: 5’-CTCGCTTCGGCAGCACA-3’ and reverse 5’-AACGCTTCACAGAATTTCGT-3’. We used U6 snRNA as internal control and the 2$^{-ΔΔCt}$ method to calculate the relative expression.

Western Blot Analysis
RIPA lysis buffer (CWBio Biotech, Changping, Beijing, China) was applied to extract total protein from TPC-1 and SW1736 cells after treatment. Afterwards, 25 μg proteins were mixed with 2 × SDS loading buffer, and separated by 8-12% polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, and the membranes were blocked by 5% skim milk, following by being probed with primary antibodies at 4°C overnight. After rinsing with TBST for three times, the membranes were incubated with matched secondary antibodies, and the protein bands were examined by a Promega ECL Western Blotting substrate kit (TaizeXingye Biotech, Wuqing, Tianjin, China). The primary antibodies against caspase3, p-JAK2, p-STAT3, JAK2 and vimentin were purchased from Abcam Co., Ltd. (Cambridge, MA, USA). The primary antibodies against caspase 9, STAT3, N-cadherin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from CUSABio Biotechnology Co., Ltd. (Wuhan, Hubei, China).

Cell Proliferation Assays
Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) kit (PuheBio, Wuxi, Jiangsu, China). Briefly, two thousands of miR-630 mimics or NC mimics-transfected TPC-1 or SW1736 cells were placed into 96-well plates, and subsequently 10 μl CCK-8 solution was added into each well. Next, the cells were cultured for 1-2 h, and an HBS-1096C Pro Microplate Photometer (Detie, Nanjing, Jiangsu, China) was utilized to examine the absorbance at 450 nm.

Cell Colony Formation Assays
The miR-630 mimics or NC mimics-transfected TPC-1 or SW1736 cells were digested using trypsin into a single-cell suspension. Subsequently, the cells (500 cells per well) were placed in
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6-well plates and allowed to grow for about 2 weeks. Prior to stain with crystal violet (0.1%; PsaiTong, Haidian, Beijing, China), the colonies were fixed with methanol, and imaged by a Motic AE30/31 microscope (Xiamen, Fujian, China).

**Cell Apoptosis Analysis**

Flow cytometry was employed to determine cell apoptotic rates via a Sigma Annexin V-FITC/PI apoptosis assay kit (Sigma-Aldrich, St. Louis, MO, USA). In short, the cells were rinsed with phosphate-buffered saline (PBS), harvested using trypsin digestion, resuspended in binding buffer, and stained with Annexin V-FITC/PI for 20 min in the dark. Next, the cell apoptosis was assessed by a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA).

**Wound Healing Assays**

In brief, the miR-630 mimics or NC mimics-transfected TPC-1 or SW1736 cells (2 × 10^5 cells) were seeded into 12-well culture plates, and maintained to 90% confluence. Thereafter, these confluent TPC-1 or SW1736 cell monolayers were 'scratch'-wounded by a sterilized 200 μl pipette tip. Finally, the wounded areas were imaged under a Motic AE30/31 microscope (Xiamen, Fujian, China) after scratching at 0 h and 48 h.

**Transwell Invasion Assays**

Briefly, TPC-1 or SW1736 cells (NC mimics or miR-630 mimics-transfected) resuspended in serum-free medium were plated on the top chambers of Costar 8 μl pore size transwell inserts pre-coated with Matrigel, while the bottom wells were added with complete medium (containing 15% FBS). Cells were allowed to invade through the insert membrane, and the cells attached to the lower surface of the insert membrane were fixed and stained using crystal violet (0.1%; PsaiTong, Haidian, Beijing, China). Finally, the invaded cells were photographed using a Motic AE30/31 microscope (Xiamen, Fujian, China).

**Statistical Analysis**

SPSS 19.0 (SPSS Inc., Armonk, NY, USA) was employed for statistical analysis. A two-tailed Student’s *t*-test was applied for comparisons of two independent groups, and One-way ANOVA and Tukey post-hoc test were performed to analyze the difference among three or above groups. Differences were considered statistically significant if *p* < 0.05.

**Results**

**miR-630 Expression was Downregulated in PTC Tissues and Cell Lines**

To explore dysregulated miRNAs in PTC progression, we performed miRNAs analysis of PTC and normal tissues from a public database GSE97070 and identified 16 down-regulated miRNAs in PTC samples (Figure 1A). Among the 16 down-regulated miRNAs, miR-630 was one of the most down-regulated miRNAs (Figure 1B). Then, we validated miR-630 expression levels in 47 PTC tissues and adjacent non-tumor tissues using qRT-PCR analysis (Figure 1C). In addition, we examined the expression status of miR-630 in PTC cell lines including SW1736, 8505C and TPC-1, and normal cell line Nthy-ori3-1, finding that miR-630 expressions were significantly reduced in SW1736, 8505C and TPC-1 cells compared with that in Nthy-ori3-1cells (Figure 1D). These results suggested that miR-630 might play an important role in PTC progression. TPC-1 cells exhibited the lowest level of miR-630, followed by SW1736 cells. Thus, TPC-1 and SW1736 cells were chosen for further analysis.

**Ectopic Expression of miR-630 Suppressed Proliferation and Promoted Apoptosis in TPC-1 and SW1736 Cells**

In order to explore whether miR-630 influenced PTC cell proliferation and apoptosis, we transfected TPC-1 and SW1736 cells with miR-630 mimics, and employed qRT-PCR assays to evaluate the miR-630 expression. The results suggested that miR-630 mimics transfection significantly increased the expression of miR-630 in TPC-1 and SW1736 cells (Figure 2A). Additionally, CCK-8 assays demonstrated that, compared with the negative control (NC) mimics-transfected TPC-1 and SW1736 cells, the miR-630 mimics-transfected cells grew remarkably slower (Figure 2B and C). Moreover, cell colony formation assays were carried out, and the data confirmed that enhancing expression of miR-630 in TPC-1 and SW1736 cells notably reduced the colony formation efficiency (Figure 2D and E). We next wondered whether the cell apoptosis was affected by miR-630. Hence, flow cytometry analysis was performed and the data indicated that increased miR-630 expression markedly accelerated the apoptotic rates of TPC-1 and SW1736 cells (Figure 2F). Since miR-630 modulated the cell apoptosis, we next conducted Western blot assays to assess whether the expression
of caspase 3 and caspase 9, two apoptosis related molecules, were changed in TPC-1 and SW1736 cells after transfecting miR-630 mimics. The data certified that treatment with miR-630 mimics resulted in a marked reduction of caspase 3 and caspase 9 expression (Figure 2G). Taken together, our data suggested that miR-630 played essential roles in regulating the cellular growth and apoptosis of PTC cells.

**Enforced Expression of miR-630 Repressed the Migration and Invasion of TPC-1 and SW1736 Cells**

To study the influence of miR-630 on cellular migration and invasion, we next transfected miR-630 mimics into TPC-1 and SW1736 cells, and utilized wound healing and Matrigel-coated transwell invasion assays. In contrast with the NC mimics-transfected cells, the wounded areas of TPC-1 or SW1736 cells transfected with miR-630 mimics were remarkably wider, indicating that increased miR-630 expression inhibited the migratory capacity of PTC cells (Figure 3A). Furthermore, to assess the influence of miR-630 on cellular invasion, we forced expression of miR-630 in TPC-1 and SW1736 cells, and found that the number of invaded cells was notably lower when they were transfected with miR-630 mimics (Figure 3B). To examine whether miR-630 affected epithelial-mesenchymal transition markers expression, we next compared the level of N-cadherin and vimentin in TPC-1 and SW1736 cells.
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Enhancing Expression of miR-630 Blocked the Activity of JAK/STAT3 Signaling in TPC-1 and SW1736 Cells

JAK/STAT3 signaling was a well-studied pathway orchestrating diverse aspects of multiple cancer types. Therefore, we wondered whether miR-630 could modulate this signaling since our above findings proved that miR-630 was involved in regulation of PTC growth and metastasis. The results from Western blot assays indicated that, aside from markedly decreased p-JAK2 levels, we also found that miR-630 overexpression in TPC-1 cells resulted in a significant decline of p-STAT3 expressing levels (Figure 4A). However, the changes of JAK2 and STAT3 expressing levels were not observed in TPC-1 cells after treatment with miR-630 mimics. Similar results were also found in miR-630 mimics-transfected with SW1736 cells (Figure 4B). In summary, these data suggested that overexpression of miR-630 abrogated the activity of JAK/STAT3 signaling in TPC-1 and SW1736 cells.
Discussion

In this study, we downloaded microarray data to screen dysregulated miRNAs and found that miR-630 was significantly down-regulated in PTC tissues. Subsequently, we evaluated miR-630 expression in 47 pairs of fresh PTC and matched adjacent normal tissue specimens, finding that miR-630 expression was reduced in PTC tissues. Furthermore, we also observed that miR-630 expression was significantly down-regulated in five PTC cell lines. Thus, our data revealed miR-630 as a down-regulated miRNA in PTC. Then, functional investigation showed that forced miR-
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MiR-630 expression could suppress PTC cells proliferation, migration and invasion, and promote apoptosis. In addition, mechanistic investigation suggested that up-regulation of miR-630 directly inhibited the phosphorylation of JAK2 and the downstream STAT3 phosphorylation. Overall, our findings revealed that miR-630 served as a tumor suppressor in PTC by suppressing JAK2/STAT3 signaling pathway.

Recently, numerous pieces of evidence showed that down-regulation of miR-630 is proved to contribute in tumor progression in several tumors and can be used to develop as biomarkers and therapy target. For instance, overexpression of miR-630 inhibited lung cancer cells proliferation by targeting CDC7 kinase. Forced expression of miR-630 inhibited invasion and metastasis in esophageal squamous cell carcinoma. Up-regulation of miR-630 suppressed the proliferation and metastasis by targeting metadherin. In addition, the important prognostic value of miR-630 in gastric cancer and colorectal cancer was also reported. However, it was uncertain whether miR-630 was associated with the progression of PTC. In this study, we provided first evidence by performing gain-of-function assays that overexpression of miR-630 inhibited the proliferation and promoted apoptosis. In addition, the results of Western blot showed that forced miR-630 expression could suppress the expression levels of caspase-3 and caspase-6, which were the central regulators of apoptosis. Moreover, we also showed that up-regulation of miR-630 inhibited the migration and invasion, suggesting that miR-630 may act as a negative regulator in metastasis progression of PTC. EMT, an essential step in invasion and metastasis of human cancers, happens when epithelial cells transform into mesenchymal phenotype during various physiological as well as pathological processes. We further explored whether miR-630 was involved in the regulation of EMT progress and the results of Western blot suggested that up-regulation of miR-630 suppressed the expression of vimentin and N-cadherin, suggesting that miR-630 displayed its tumor-suppressive roles in metastasis progression by suppressing EMT progress.

Janus kinase 2 (JAK2) is a receptor-associated protein tyrosine kinase involved in signaling via the JAK/signal transducer and activator transcription (JAK-STAT) pathway, which has been confirmed to mediate tumor cell proliferation, survival, motility and angiogenesis. It has been demonstrated that the JAK2/STAT3 pathway is required to sustain EMT-associated phenotypes in various tumors, including PTC. However, the upstream regulator involved in JAK2/STAT3 pathway remains largely unclear. In this study, in order to explore the potential mechanism by which miR-630 inhibited PTC cells proliferation, metastasis and EMT, TPC-1 and SW1736 cells were transduced with miR-630 mimics and we found the protein level of p-JAK2 and p-STAT3 remarkably reduced, suggesting that JAK2/STAT3 signaling pathway was inversely regulated by miR-630 in PTC cells. Overall, our results revealed that miR-630 suppressed the proliferation and metastasis of PTC cells by activating the JAK2/STAT3 pathway.

Conclusions

We observed an upregulation of miR-630 in PTC tissues and cell lines. Overexpression of miR-630 can suppress the proliferation, metastasis and EMT, and promote the apoptosis of PTC cells, potentially through modulating JAK2/STAT3 pathway, highlighting a novel therapeutic approach for the treatment of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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