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Long noncoding RNA MIAT promotes thyroid cancer progression by regulating the Wnt/β-catenin signaling pathway

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Abstract: Background: Thyroid cancer is deemed as a prevalent endocrine cancer, which possesses relatively high occurrence and migration globally. MIAT has been reported to play an oncogenic role in a variety of cancers. Nevertheless, MIAT remains to be explored in thyroid cancer. This study is aimed to investigate the roles of MIAT in thyroid cancer. **Materials and methods:** The mRNA expression of genes was tested by microarray and RT-qPCR assay. CCK-8 and colonies formation assays were operated to evaluated cell proliferative capacity. Cell migration was examined by transwell assay. The interaction between miR-29b-3p and MIAT (or GPATCH2) was confirmed by luciferase reporter, RIP and RNA pull down assays. Western blot assay was performed to measure the protein expression of Wnt, β-catenin and GPATCH2. **Results:** We discovered that MIAT displayed high expression in thyroid cancer tissues and cells. Inhibition of MIAT suppressed proliferation and migration of thyroid cancer cells. Additionally, miR-29b-3p interacted with MIAT and miR-29b-3p amplification limited cell proliferation or migration in thyroid cancer. Moreover, miR-29b-3p negatively regulated the expression of GPATCH2. MIAT served as a ceRNA to sponge miR-29b-3p and target GPATCH2. At last, the inhibitive influence of MIAT deficiency on cell proliferation, migration and protein expression of Wnt, β-catenin and GPATCH2 was abrogated by GPATCH2 amplification. **Conclusions:** MIAT/miR-29b-3p/GPATCH2 promotes thyroid cancer progression via the Wnt/β-catenin signaling pathway, which may serve a novel therapeutic point for thyroid cancer patients.
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Key words: MIAT, miR-29b-3p, GPATCH2, thyroid cancer, Wnt/β-catenin signaling pathway

Running title: MIAT promotes thyroid cancer progression

Introduction

Thyroid carcinoma is considered as one of prevalent endocrine tumors around the world. With increasing morbidity by approximately 4% year by year (1, 2), thyroid cancer accounted for almost 0.5% of cancer - related deaths due to the occasional distant metastasis and slow differentiation (3, 4). Radiotherapy (5), chemotherapy (6) are usually used for cancer patients. Recently, more and more researchers paid attention to the underlying regulatory mechanism of thyroid cancer and the result showed that several signaling pathways associated with molecular derangement played an essential role in the cellar processes of the thyroid cancer (7). Such discoveries provided a novel insight for novel therapeutic targets, which contributed to the treatment of thyroid cancer.

Long noncoding RNAs (lncRNAs) with more than 200 nucleotides have been widely reported to frequently take part in several tumor cellar processes including cell proliferation, migration, invasion, as well as apoptosis (8-11). The aberrant expression of lncRNAs has been observed in multiple diseases and cancers. For instance, the overexpression of LINC01234 mediated by SP1 acted as a ceRNA to promotes non-small-cell lung cancer development by targeting OTUB1(12). PANDAR inhibition limits cell proliferation and invasion, and suppresses EMT process in breast cancer (13). Myocardial Infarction Associated Transcript (MIAT) has been reported to interfere with various heart conditions and be related to increasing risk of nervous system tumors (14).

Moreover, LncRNA MIAT modulates miR-128-3p/VEGFC axis to promotes osteosarcoma progression (15). Nevertheless, neither the biological role nor the molecular mechanism of MIAT has been investigated in thyroid cancer.

MicroRNA (miRNA) is another class of non-coding RNA with 22-24 nucleotides (16). Emerging explorations proposed that lncRNAs could bind to miRNAs to regulate target gene expression, acting as competing endogenous RNAs (ceRNA). For instance, sponged by SNHG8, miR-542-3p regulates the non-small-cell lung cancer progression by targeting CCND1/CDK6 (17). LncRNA SNHG14 regulates miR-206/YWHAZ axis to facilitate the development of cervical cancer (18). Therefore, we predicted that MIAT also functioned in this pattern and miR-29b-3p was selected to validate our hypothesis. Although miR-29b-3p has been reported to take part in the regulation of proliferation and apoptosis pancreatic cancer and epithelial-mesenchymal transition process in colorectal cancer (19, 20). The roles of miR-29b-3p in thyroid cancer remain to be explored. G-patch domain containing 2 (GPATCH2) was reported to be involved in several cellar process of breast cancer cells and testes cells (21, 22). GPATCH2 dysregulation has been observed in a variety of cancers (22, 23). However, the function of GPATCH2 in MIAT/miR-29b-3p/ GPATCH2 axis in thyroid cancer is unknown as well.

In this current research, we assessed the function of MIAT/miR-29b-3p/ GPATCH2 axis in thyroid cancer, especially the biological role and regulatory mechanism of MIAT. We discovered that MIAT promotes the proliferation and migration of cells through the Wnt/β-catenin signaling pathway, providing a novel therapeutic point for patients with thyroid cancer.

Materials and methods

Tissue samples and patients

Thyroid cancer specimens (n=3) and adjacent normal tissues (n=3) were obtained from patients with thyroid cancer at the First Affiliated Hospital of Zhengzhou University. All the tissue samples were immediately preserved at -80℃ in liquid nitrogen. All patients have not received any anticancer treatment before surgery. This study has been approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Cell culture and transfection

For cell culture, the human normal thyroid follicular cell Nthy-ori 3-1 and thyroid cancer cells including K1, SW579, TPC-1 and NPA were collected from Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultivated in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York, USA) in a humid atmosphere with 5% CO_2 at 37℃.

For cell transfection, shRNAs targeting MIAT (sh-MIAT#1, sh-MIAT#2 and sh-MIAT#3) were used to knockdown MIAT expression with sh-NC as scramble control. MiR-29b-3p mimics were adopted to amplify miR-29b-3p expression with NC mimics as internal control. GPATCH2 expression was upregulated by pcDNA3.1/GPATCH2 transfection normalized by the transfection of pcDNA3.1. Vectors used in this assay were purchased from GenePharma (Shanghai, China). According to the manufacturer's instruction, all transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Microarray analysis

Total RNA was extracted from tissues of thyroid cancer patients and purified by RNeasy mini kit (QIAGEN, Germany) following manufacturer's instructions. RNA quality was measured by Qubit Fluorometer (Thermo Fisher Scientific, USA). The mRNA expression of thyroid cancer tissues was shown in our study. Raw data was processed using the manufacturer's standard protocol.

Real-time quantitative PCR

Total RNA was isolated from thyroid tissues and cells with TRIzol reagent (Invitrogen, USA), and then reversely transcribed into cDNA using M-MLV Reverse Transcriptase Kit (Invitrogen, USA). Real-time PCR analyses were carried out in triplicate for each sample using SYBR Green PCR Master Mix (TOYOBO) on a LightCycler 480 system (Roche). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. For detecting miRNA expression level, cDNA was synthesized using a TaqMan® miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and U6 small nuclear RNA served as the endogenous control. All primers are listed below:

MIAT: forward, 5′-GGACGTTCACAACCACACTG-3' and reverse, 5′-TCCCACTTTGGCATTCTAGG-3′;

MiR-29b-3p: forward, 5′-TGCGGTAGCACCATTTGAAAT-3' and reverse, 5′- CCAGTGCAGGGTCCGAGGT-3′;

GPATCH2: forward, 5′-GCGCAGGCCGTCATCAAC-3' and reverse, 5′-TCCCACCAGGGCACAACTC-3′;

GAPDH: forward, 5′-GAA GGT GAA GGT CGG AGT C-3′ and reverse, 5′-GAA GAT GGT GAT GGG ATT TC-3′;

U6: forward, 5′-ATT GGA ACG ATA CAG AGA AGA TT-3′ and reverse, 5′-GGA ACG CTT CAC GAA TTT G-3′.

Cell Counting Kit 8（CCK-8）assays

The influence of MIAT suppression on cell proliferation was evaluated using Cell Counting Kit-8 assay kits (CCK-8, Dojindo, Japan) under manufacturer's instructions. The transfected cells was grown in a 96-well plate ($1x10^3$cells/well), followed by the cultivation for 96 hours. Subsequently, 10 ul CCK-8 solution was added in each well at 0, 24, 48, 72 and 96 hours. The cell proliferation was assessed by a wave of 450 nm in length in microplate reader (Bio-Tek, Winooski, VT).

Colony formation assay

Forty eight hours after transfection, the cells were trypsinized into single-cell status. Then, these cells were grown in a 6-well plate at a density of 800 cells per well and cultivated for 2 weeks at 37℃ to form a colony. After the incubation, the plate was gently rinsed with 10% PBS. The cells were immobilized with 1% methanol solution, and then dyed with 0.1% crystal violet. Colonies over 50 cells were observed and manually counted under an inverted microscope.

Transwell assay

Transwell chambers inserts (24-wells, 8 μm pore size, 6.5mm diameter; Corning, NY, USA) without matrigel were employed in migration assay. The transfected cells were cultivated in the medium without serum, and then placed in the upper chambers. At the same time, DMEM with 10% FBS were added in the lower chamber. After incubation for 48 h, migrated cells were fixed with 1% methanol for 15 min and stained with 0.5% crystal violet for 15 min. Then, the stained cells were captured by a microscope (Nikon).

Luciferase reporter assay

MIAT-WT (or GPATCH2-WT) and MIAT-Mut (or GPATCH2-Mut) were constructed into pGL3 plasmids (Promega, Madison, USA). The constructed plasmids were co-transfected with miR-29b-3p mimics or NC mimics into TPC-1 and K1 cells for 48 hours. Luciferase reporter assay system (Promega, Madison WI) was adopted to examine the relative luciferase activities.

RNA immunoprecipitation (RIP) assay

To confirm whether miR-29b-3p could bind with MIAT, magna RNA immunoprecipitation (RIP) kit (Millipore, Billerica, USA) was adopted. Magnetic beads containing Ago2 or IgG (negative control) antibodies were added into cell lysate which was preserved in RIP buffer before. The relative expression of miR-29b-3p and MIAT were detected by RT-qPCR.

RNA pull down assay

MiR-29b-3p-WT and miR-29b-3p-Mut were transcribed employing Transcript Aid T7 High Yield Transcription Kit (Thermo Fisher Scientific, USA). Biotinylated miR-29b-3p-WT, miR-29b-3p-Mut and negative control (Bio-NC) were co-cultivated with cell lysate (TPC-1 and K1) at 4℃ for 1 hour. After purifying, elution buffer was used to elute RNAs and RT-qPCR was performed to examine the expression of MIAT and GPATCH2.

Western blot assay

Cells were lysed in chilled RIPA buffer (Beyotime) supplemented with 1 mmol/L protease inhibitor mixture (Sigma-Aldrich). An equal amount of each protein sample was separated on a 10% SDS-PAGE gel and was transferred onto a PVDF membrane (Millipore Corporation, USA). The membranes were blocked with 5% nonfat dry milk at RT for 1 h and were incubated with specific primary antibodies overnight (S5-table), followed by an incubation with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Proteintech, USA). The peroxidase reaction was detected using enhanced chemiluminescence reagent (ECL, Thermo, Rockford, USA).

Statistical analysis

Statistics were analyzed utilizing the SPSS 16.0 software system (SPSS, Chicago, IL). The experiments mentioned were all conducted in triplicate and all data were expressed taking the form of the mean ± standard error (SD). Student's t-test or one-way analysis of variance (ANOVA) was employed to evaluate differences between two groups or more than two groups. $P < 0.05$ was regarded to be statistically significant.

Result

MIAT displays high expression to facilitate proliferation and migration of thyroid cancer cells

To figure out lncRNAs involved in thyroid cancer, total RNAs were measured by microarray (Arraystar, Rockville, MD) and differently expressed lncRNAs were depicted by heatmap. According to the result, 53 lncRNAs expression was upregulated and 109 lncRNAs was downregulated in thyroid tissues. The rest expressed no difference (Fig. 1A). To specify which lncRNA played the most important role in thyroid cancer, the top five upregulated lncRNAs (MALAT1, H19, PVT1, MIAT and UCA1) were chosen for following experiment. RT-qPCR assay uncovered that MIAT presented the highest expression among these five RNAs (Fig.1B). To explore the specific roles of MIAT in thyroid cancer, following experiments were performed. At first, the result of RT-qPCR assay demonstrated that thyroid cancer cells (TPC-1, K1, SW579, NPA) indicated higher expression than normal thyroid cell (Nthy-ori 3-1) (Fig. 1C). The transfection of sh-MIAT#1, sh-MIAT#2 or sh-MIAT#3 caused a decrease of MIAT expression in TPC-1 and K1 cells, and sh-MIAT#1 and sh-MIAT#2 displayed better knockdown efficiency of MIAT expression (Fig. 1D). Thereby, sh-MIAT#1 and sh-MIAT#2 were chosen for following assays. CCK-8

assay demonstrated that MIAT suppression triggered an apparent depletion of proliferative capacity of TPC-1 and K1 cells (Fig. 1E). Likewise, the result of colonies assay showed that the number of colonies was reduced by transfection of sh-MIAT#1 or sh-MIAT#2 into TPC-1 and K1 cells (Fig. 1F). Transwell assay using TPC-1 and K1 cells was carried out to evaluate the ability of cell migration. Compared with sh-NC group, sh-MIAT#1 or sh-MIAT#2 group possessed larger number of migrated cells (Fig. 1G). Collectively, MIAT displays high expression in thyroid cancer tissues and cells. Inhibition of MIAT suppresses cell proliferation and migration in thyroid cancer.

MiR-29b-3p interacts with MIAT and acts a tumor suppressor in thyroid cancer

Abundant reports proposed that lncRNA functions as a ceRNA to regulate tumor development by sponging miRNA and targeting mRNA. Therefore, we predicted that MIAT also work in this pattern. According to the prediction of starBase (http://starbase.sysu.edu.cn), four miRNAs (miR-29a-3p, miR-29b-3p, miR-29c-3p and miR-150-5p) had the potential binding sites for MIAT (Fig. 2A). Then, RT-qPCR assay elucidated that TPC-1 and K1 cells transfected with sh-MIAT presented much higher expression of miR-29b-3p and similar expression of miR-29a-3p, miR-29c-3p and miR-150-5p, compared with that transfected with sh-NC. (Fig. 2B). Thus, miR-29b-3p was selected for following assays. As depicted in Fig. 2C, miR-29b-3p expression was prominently amplified by the treatment of miR-29b-3p mimics. Then, the gain-of-function assays were implemented. At first, CCK-8 assay indicated that cell proliferation was limited by the transfection of miR-29b-3p mimics (Fig. 2D). Similarly, the evidently decreasing number of colonies was observed in TPC-1 and K1 cells by colony formation assay (Fig. 2E). Last, by transfecting miR-29b-3p mimics into TPC-1 and K1 cells, the ability of cell migration was obviously prevented (Fig. 2F). To conclude, MiR-29b-3p interacts with MIAT and acts a tumor suppressor in thyroid cancer.

MIAT acts as a ceRNA to sponge miR-29b-3p and target GPATCH2 in thyroid cancer

To determine the target gene of miR-29b-3p, the starBase website was adopted. Four potential target genes (GPATCH2, AGPAT5, GPATCH3 and AGPAT3) were selected based on the prediction of result (Fig. 3A). Only the expression of GPATCH2 in TPC-1 and K1 cells was reduced by the transfection of miR-29b-3p mimics (Fig. 3B). Moreover, GPATCH2 expression was downregulated in miR-29b-3p mimics-transfected TPC-1 and K1 cells (Fig. 3C). The putative binding bites between miR-29b-3p and MIAT (or GPATCH2) were analyzed by starBase and the binding possibility was validated by luciferase reporter assay. According to result of luciferase reporter assay using TPC-1 and K1 cells transfected with miR-29b-3p mimics, an evident reduction of luciferase activity in MIAT-WT (or GPATCH2-WT) vector was observed but no subtle alteration was noticed in MIAT-Mut (or GPATCH2-Mut) vector (Fig. 3D-E). RIP assay demonstrated that the expression of MIAT, miR-29b-3p and GPATCH2 was enriched in Ago2 antibody group rather than IgG antibody group, which meant that MIAT, miR-29b-3p and GPATCH2 could be co-immunoprecipitated by Ago2 antibody instead of IgG antibody (Fig. 3F). Based on RNA pull down assay, the expression of MIAT and GPATCH2 was abundant in miR-29b-3p biotin probe but not miR-29b-3p no biotin probe in TPC-1 and K1 cells (Fig. 3G). All the data above led to the conclusion that MIAT acts as a ceRNA to sponge miR-29b-3p and target GPATCH2 in thyroid cancer.

MIAT promotes thyroid cancer progression via the Wnt/β-catenin signaling pathway

To validate the roles of MCM3AP-AS1/miR-3139/SEMA7A axis in thyroid cancer progression, rescue assays were carried out. At the very beginning, RT-qPCR assay delineated that GPATCH2 overexpression partially rescued the inhibitory effect of MIAT suppression on the mRNA expression of GPATCH2 (Fig. 4A). Subsequently, CCK-8 and colonies formation assays verified that the depletion of cell proliferation caused by MIAT knockdown was abolished by the transfection of pcDNA3.1/GPATCH2 into TPC-1 cells (Fig. 4B-C). Additionally, transwell assay using TPC-1 cells validated that the inhibitive influence of MIAT deficiency on the capability of cell migration was abrogated by GPATCH2 amplification (Fig. 4D). Last but not least, to further explore how GPATCH2 mediated the oncogenic effect of MIAT, western blot assay was operated to assess the activity of Wnt/β-catenin signaling pathway, which is tightly related to proliferation and migration of thyroid cancer cells. The result testified that GPATCH2 overexpression countervailed the inhibited role of MIAT silence on the protein expression of Wnt, β-catenin and GPATCH2 (Fig. 4E). In a word, MIAT/miR-29b-3p/GPATCH2 promotes thyroid cancer progression via the Wnt/β-catenin signaling pathway.

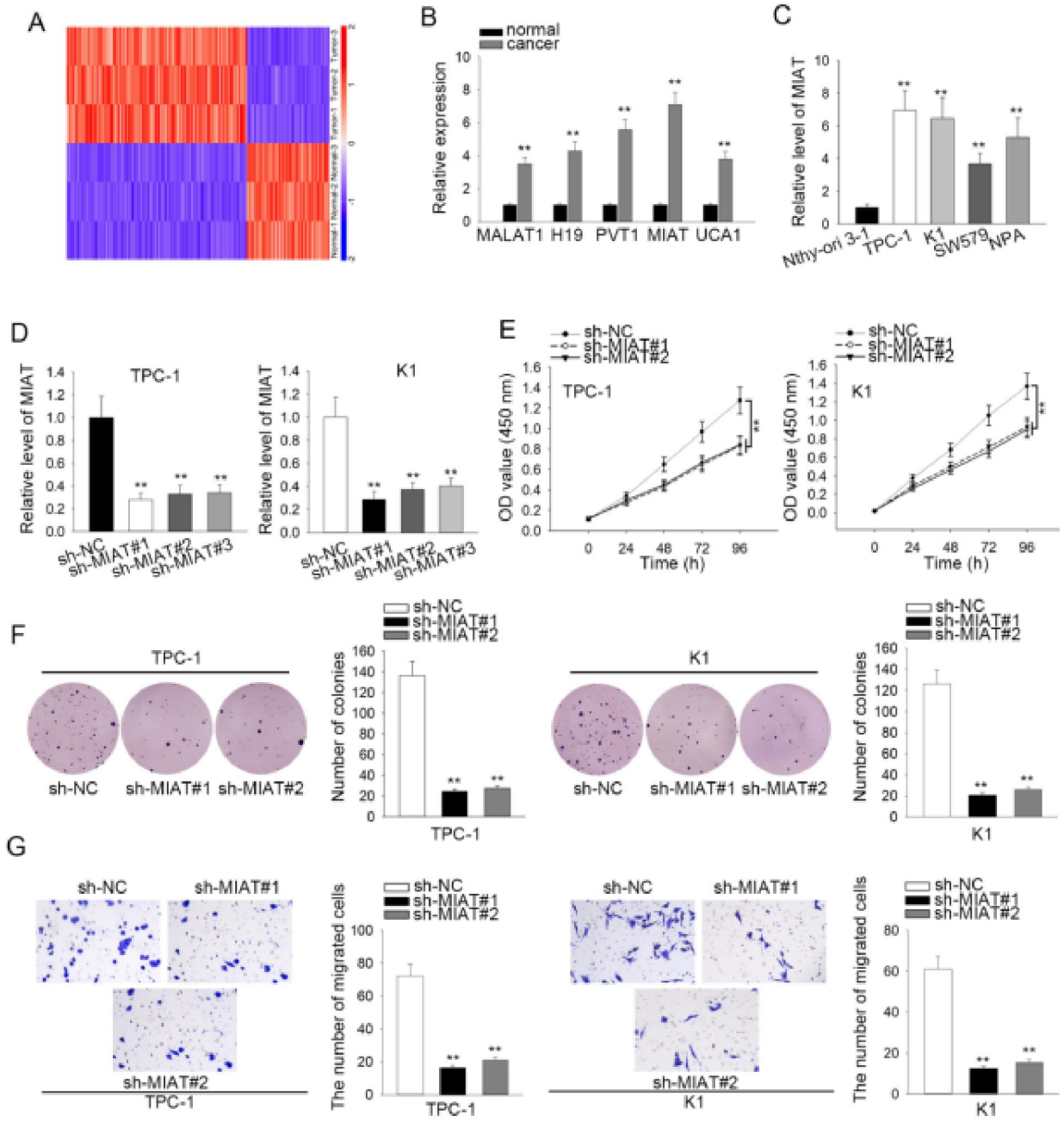

Figure 1 MIAT displays high expression to facilitate proliferation and migration of thyroid cancer cells.

(A) Microarray was performed to measure lncRNA expression in thyroid tissues. (B) RT-qPCR was implemented to examine the expression of MALAT1, H19, PVT1, MIAT and UCA1(C) The expression of MIAT was tested by RT-qPCR in TPC-1, K1, SW579, NPA and Nthy-ori 3-1 cells. (D) The knockdown efficiency of sh-MIAT#1/2/3 was evaluated by RT-qPCR assay. (E-F) The capacity of cell proliferation was assessed by CCK-8 and colony formation assays. (G) Transwell assay was operated to detect cell migration. $^{**}P< 0.01$.

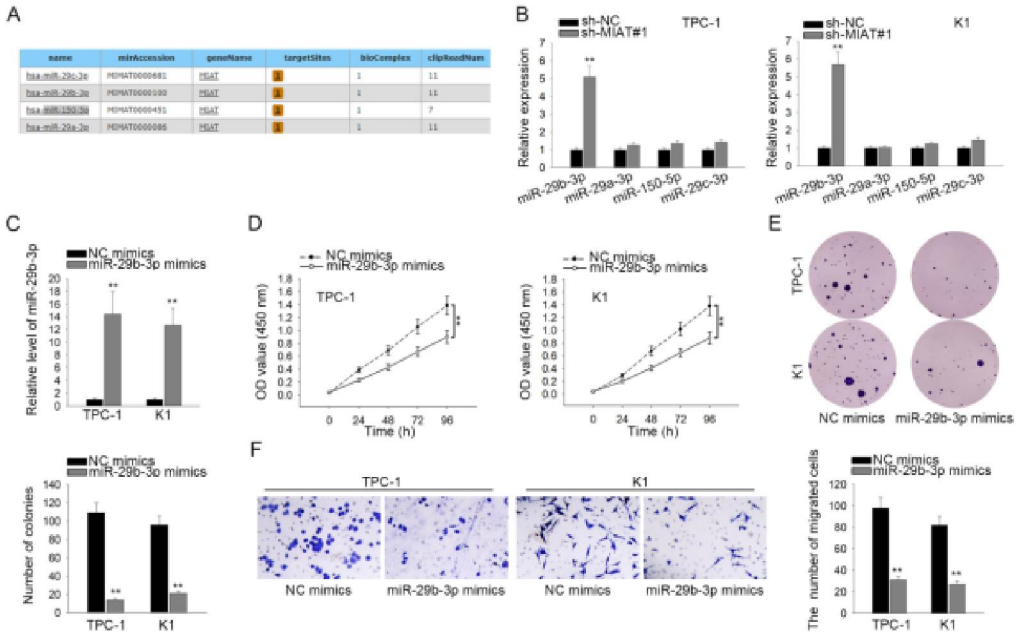

Figure 2 MiR-29b-3p interacts with MIAT and acts a tumor suppressor in thyroid cancer.

(A) The potential miRNAs were predicted by starBase. (B) The expression of potential miRNAs was tested by RT-qPCR in K1 and TPC1 cells transfected with sh-MIAT#1. (C) RT-qPCR assay was adopted to monitor the overexpression efficiency of miR-29b-3p mimics. (D-E) CCK-8 and colony formation assays were performed to measure cell proliferation in K1 and TPC1 cells transfected with miR-29b-3p mimics. (F) Cell migration of K1 and TPC1 cells transfected with miR-29b-3p mimics was assessed by transwell assay. $^{**}P< 0.01$.

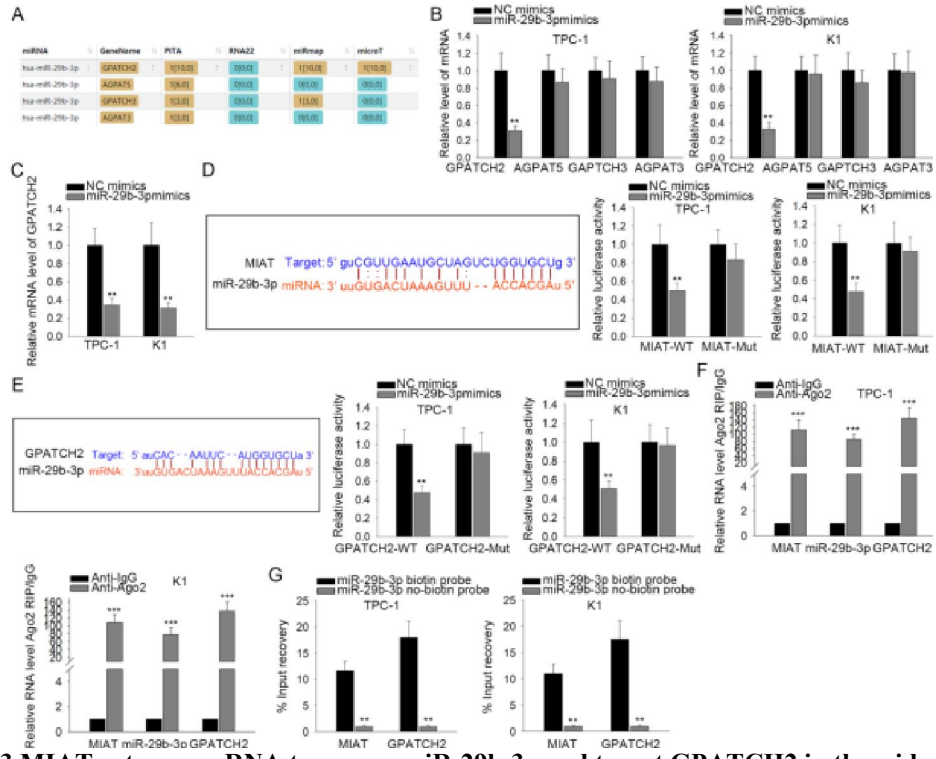

Figure 3 MIAT acts as a ceRNA to sponge miR-29b-3p and target GPATCH2 in thyroid cancer.

(A) The possible target genes were predicted by starBase. (B) The expression of potential mRNAs was detected by RT-qPCR in K1 and TPC1 cells transfected with miR-29b-3p mimics. (C) The expression of miR-29b-3p was tested by RT-qPCR assay. (D-E) The binding sites between miR-29b-3p and MIAT (or GPATCH2) predicted by starBase and proven by luciferase reporter assay. (F-G) The binding capacity between miR-29b-3p and MIAT (or GPATCH2) was evaluated by RIP and RNA pull down assays. $^{**}P< 0.01$, $^{***}P< 0.001$.

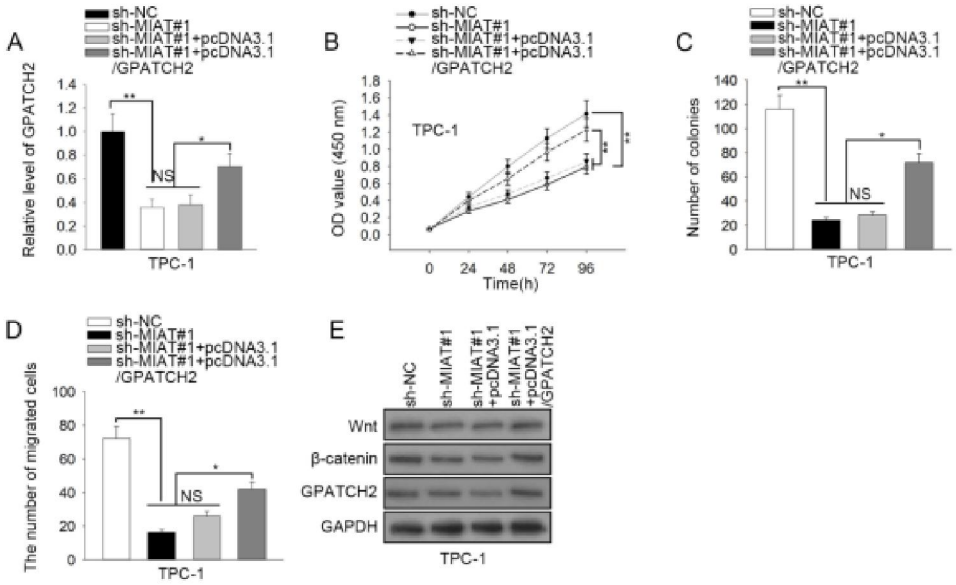

Figure 4 MIAT promotes thyroid cancer progression via the Wnt/β-catenin signaling pathway.

(A) The expression of GPATCH2 was detected by RT-qPCR assay. (B-C) CCK-8 and colony formation assays were implemented to test cell proliferation in TPC1 cells. (D) Cell migration of TPC1 cells was explored by transwell assay. (E) The protein expression of Wnt, β-catenin and GPATCH2 was studied by western blot assay. $^{*}P< 0.05$, $^{**}P< 0.01$.

Discussion

Thyroid cancer is known as an endocrine malignant cancer with the rising rate of morbidity worldwide. The standard treatment for the most patients was surgery and radioactive iodine. Unfortunately, the thyroid cancer still remains a poor prognosis due to the insufficient exploration concerning the tumorigenesis and development of thyroid cancer. Thus, it was significantly urgent to find out the underlying regulatory mechanism of thyroid cancer. In current research, MIAT was regarded as a regulatory factor that promotes the progression of thyroid cancer.

LncRNAs have been discovered to play diverse roles in various human cancers, and their abnormal expression is closely associated with the biological processes of tumor cells, such as cell apoptosis, metastasis and proliferation. Specifically, MIAT has been identified to drive cell proliferation and metastasis in non-small cell lung cell (24). Additionally, MIAT was reported to absorb miR-128-3p to facilitate osteosarcoma growth(15). Though upregulated expression of MIAT has been noticed in a variety of cancers, the expression status of MIAT is uncertain in Thyroid cancer. In present study, MIAT presented high expression in thyroid cancer tissues and cells. Moreover, inhibition of MIAT suppressed the proliferative and migratory capacity of thyroid cancer cells. To specify the regulatory of MIAT, starBase was employed to seek potential miRNA for MIAT. According to the result of prediction, miR-29b-3p was chosen for following experiments. MiR-29b-3p was reported to facilitate chondrocyte apoptosis and inhibit epithelial-mesenchymal transition process, however, there is no sufficient evidence to show that miR-29b-3p functions in the same pattern in thyroid cancer. In our investigation, miR-29b-3p interacted with MIAT and miR-29b-3p amplification limited cell proliferative and migratory ability in thyroid cancer. GPATCH2 served as a target gene of miR-29b-3p in thyroid cancer. Moreover, miR-29b-3p negatively regulated the expression of GPATCH2 and MIAT served as a ceRNA to sponge miR-29b-3p and target

GPATCH2. At last, the results of rescue assays uncovered that the inhibitive influence of MIAT deficiency on cell proliferation, migration was abrogated by GPATCH2 amplification.

Wnt/β-catenin pathway existed widely in kinds of cells, serving as a signal transduction pathway (25). Wnt and β-catenin proteins were the main proteins in Wnt/β-catenin pathway, which played a crucial regulatory role in biological processes including proliferation and migration (26). Additionally, cumulative evidences proved that many lncRNAs exert their oncogenic role via Wnt/β-catenin pathway (27-29). While the association between MIAT and Wnt/β-catenin pathway in thyroid cancer remains to be explored. In current exploration, GPATCH2 amplification restored the inhibitive influence of MIAT deficiency on the protein expression of Wnt and β-catenin.

All in all, our exploration elucidated that long noncoding RNA MIAT promotes thyroid cancer progression by regulating the Wnt/β-catenin signaling pathway, presenting a possible solution for treating patients with thyroid cancer.

Highlights:

1. MIAT displays high expression in thyroid cancer tissues and cells. Inhibition of MIAT suppresses cell proliferation and migration in thyroid cancer.
2. MiR-29b-3p interacts with MIAT and acts a tumor suppressor in thyroid cancer.
3. MIAT acts as a ceRNA to sponge miR-29b-3p and target GPATCH2 in thyroid cancer.
4. MIAT/miR-29b-3p/GPATCH2 promotes thyroid cancer progression via the Wnt/β-catenin signaling pathway.

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Conflicts of interest

The authors declare that there are no competing interests in this study.

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