Silencing long non-coding RNAs nicotinamide nucleotide transhydrogenase antisense RNA 1 inhibited papillary thyroid cancer cell proliferation, migration and invasion and promoted apoptosis via targeting miR-199a-5p

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Abstract. The increasing incidence of papillary thyroid cancer (PTC) has attracted many researchers to investigate the mechanism underlying PTC progression. This study explored the growth and apoptosis of PTC cells based on an lncRNA regulatory mechanism. The expression of nicotinamide nucleotide transhydrogenase antisense RNA 1 (NNT-AS1) in PTC cell lines and PTC tissues was analyzed by qRT-PCR. The mutual binding site between NNT-AS1 and miR-199a-5p was predicted by starBase and confirmed by dual-luciferase reporter assay. The correlation between NNT-AS1 and miR-199a-5p was shown by Pearson correlation test. The viability, clone formation, migration, invasion and apoptosis of TPC-1 and IHH-4 cells were examined by CCK-8, colony formation, wound-healing, transwell, and flow cytometry assays, respectively. The expressions of Bax, cleaved Caspase-3, Bcl-2, E-Cadherin, N-Cadherin and SNAIL in TPC-1 and IHH-4 cells were determined by Western blot or qRT-PCR. NNT-AS1 expression was upregulated in PTC cells and tissues. In TPC-1 cells, silencing NNT-AS1 inhibited viability, clone formation, migration, and invasion as well as the expressions of N-Cadherin, SNAIL and Bcl-2, but promoted the expressions of E-Cadherin, Bax, and cleaved caspase-3. The effects of NNT-AS1 overexpression on IHH-4 cells were opposite to those of silencing NNT-AS1. In PTC tissues, miR-199a-5p was low-expressed and targeted by NNT-AS1, and it was negatively correlated with NNT-AS1. MiR-199a-5p inhibitor promoted TPC-1 cell progression, but miR-199a-5p mimic inhibited IHH-4 cell progression. NNT-AS1 and miR-199a-5p exerted opposite effects on PTC cells. Silencing NNT-AS1 inhibited PTC cell proliferation, migration and invasion, but promoted apoptosis via upregulation of miR-199a-5p.

Key words: Nicotinamide nucleotide transhydrogenase-antisense RNA1, Papillary thyroid cancer, MiR-199a-5, Apoptosis, Migration

THYROID CANCER has become the most common endocrine malignant cancer worldwide with an increasing incidence [1]. Thyroid cancer is classified into four subtypes according to histology, with the major subtype being Papillary thyroid cancer (PTC), which accounts for almost 90% of total thyroid cancer cases [2, 3]. PTC is also a type of differentiated thyroid carcinoma (DTC) originating from thyroid follicular cells [4]. DTC tends to result in a favorable prognosis and a low mortality rate [5]. However, among DTC patients, around 5% still develop distant metastasis and 10–15% suffer from cancer recurrence [6]. Moreover, early lymph node metastasis is often detected in PTC patients [7]. Extranodal spread of PTC to surrounding soft tissues caused by enlarged lymph nodes usually results in a worse prognosis [8, 9]. Therefore, exploring the biological properties of PTC and developing new therapeutic approaches may contribute to a more satisfactory prognosis of PTC.

Long non-coding RNAs (lncRNAs) have a protein-encoding function and consist of more than 200 nucleotides [10], and they regulate a wide range of physiological and pathological processes through diverse molecular mechanisms [11]. Study has discovered that lncRNA-Nicotinamide Nucleotide Transhydrogenase-antisense RNA1 (NNT-AS1) promotes the growth of malignant tumors, such as cholangiocarcinoma [12], osteosarcoma [13], and gastric cancer [14]. Located on 5p12 with three exons, NNT-AS1 can be reverse-transcribed to NNT and
does not overlap with NNT [15]. It has been previously demonstrated that upregulated NNT-AS1 in osteosarcoma signifies a poor prognosis and serves as an independent risk factor for predicting the survival of osteosarcoma patients [13]. Silencing NNT-AS1 inhibits the proliferation, migration and invasion as well as suppresses the epithelial-mesenchymal transition (EMT) of colorectal cancer cell lines [16]. Nonetheless, the specific role of NNT-AS1 in PTC has not been fully elucidated.

In cancers, lncRNAs could act as competing endogenous RNAs to bind to microRNAs (miRNAs), thereby blocking miRNAs from interacting with their targets [17]. MiR-199a-5p has been found low-expressed in colorectal cancer cell lines [16]. Nonetheless, the specific role of NNT-AS1 in PTC has not been fully elucidated.

This study focused on investigating the roles of NNT-AS1 and miR-199a-5p in the progression of PTC and revealing the relation between NNT-AS1 and miR-199a-5p, with a hope to discover a novel molecular mechanism in PTC.

Materials and Methods

Ethical statement

The approval of this study was granted by the Ethics Committee of Shanxi Provincial People’s Hospital (approval number: DE20191234), and written informed consents were obtained from all the participants in any experimental work involving humans.

Clinical sample

Paired PTC tissues and peritumoral normal tissues (n = 30) were collected from 30 PTC patients aged 39–73 years during surgical treatment at Shanxi Provincial People’s Hospital in 2019. All the patients had not received preoperative chemotherapy or radiotherapy, and were informed of the use of their samples for clinical research. The peritumoral normal tissues were taken at least 2 cm away from the margin of PTC tumor. All the samples were snap-frozen by liquid nitrogen and stored at –80°C.

Cell culture

Human thyroid epithelial (Nthy-ori3-1) cells and PTC cell lines (TPC-1 and IHH-4 cells) were ordered from COBIOER (CBP61205, CPB60257, CPB61201, Nanjing, China, http://www.cobioer.com/). PTC cell line (MDA-T68 cells) was obtained from ATCC (CRL-3353, Manassas, VA, USA). Nthy-ori3-1 cells were cultured in RPMI-1640 medium (22400105, ThermoFisher, Walthma, MA, USA) which was supplemented with 10% fetal bovine serum (FBS, F8318, Sigma-Aldrich, St. Louis, MO, USA). TPC-1 cells were cultured in RPMI-1640 containing 10% FBS and 100 U/mL streptomycin/penicillin (V900929, Sigma-Aldrich, USA). IHH-4 cells were cultured in a mixed medium comprising DMEM (A4192101, ThermoFisher, USA) and RPMI-1640 at a ratio of 1:1 and supplemented with 10% FBS. MDA-T68 cells were cultured in RPMI-1640 containing 10% FBS and 2 mM L-glutamine (G7513, Sigma-Aldrich, USA). All cells were cultured at 37°C with 5% CO2.

Transfection

TPC-1 and IHH-4 cells were seeded into each well of 96-well plates (60180-K100, ThermoFisher, USA) at 1 × 104 cells to 80% confluence. Next, TPC-1 cells were transfected with siRNA-NNT-AS1 (siNNT-AS1, 5’-UAA CAUAUGCUCUAAUCAUCU-3’, RIBOBIO, Guangzhou, China) alone or in combination. IHH-4 cells were transfected with NNT-AS1 overexpression plasmid (produced by pBABE-puro, Addgene, Watertown, MA, USA) or miR-199a-5p mimic (5’-GGGUCACAAGUG CUGAUGGACAAG-3’, miR20000231-1-5, RIBOBIO, Guangzhou, China) alone or in combination. The cell transfection was performed using Lipofectamine 3000 reagent (L3000015, ThermoFisher, USA). RNA negative control and siRNA-negative control were purchased from Qiagen (1022076, Hilden, Germany). Five μL of the Lipofectamine 3000 reagent, 0.2 μg of NNT-AS1 overexpression plasmid and 0.2 μg of siNNT-AS1 were separately diluted in Opti-MEM media (22600134, ThermoFisher, USA) by thorough mixing. P3000 reagent was added into the diluted Lipofectamine 3000 reagent solution and NNT-AS1 overexpression plasmid solution. Further, the diluted siNNT-AS1 or NNT-AS1 overexpression solution was added with the diluted Lipofectamine 3000 reagent solution at a ratio of 1:1, and incubated at room temperature for 10 min. Afterwards, 10 μL of RNA-lipid complex and the incubated mixed solution were added into TPC-1 or IHH-4 cells for 48 h of incubation at 37°C. At 24 h after the cell transfection, the culture media were refreshed, and the transfection rate of RNA and the silencing effects of siRNA were analyzed via quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Cell counting kit (CCK-8) assay

One hundred μL of cell solution was seeded into each well of 96-well plates. Ten μL of CCK-8 reagent (HY-K0301-100T, MedChemExpress, Monmouth Junction,
colonies were incubated with culture medium at 37°C for two weeks, and the medium was renewed every three days during cell cloning. Two weeks later, the TPC-1 and IHH-4 cells were washed by PBS, fixed in methanol (34860, Sigma-Aldrich, USA) for 15 min, and then dyed by 1% crystal violet (332488, Sigma-Aldrich, USA). The cell clone formation was quantified by counting the stained TPC-1 and IHH-4 cells.

**Dual-luciferase reporter assay**

Mutual binding sites between NNT-AS1 and miR-199a-5p were predicted by starBase and confirmed by using a Dual-Luciferase Reporter Assay System (E1910, Promega, Fitchburg, WI, USA). 2 × 10^4 TPC-1 or IHH-4 cells were first seeded into 24-well plates (12684049, ThermoFisher, USA) for 24 h to 70% confluence. The sequences of NNT-AS1-Mutant Type (5'-TTTAGGATTTATAAATGGAAGCTC-3') and NNT-AS1-Wild Type (5'-TTTAGGATTTATAAATGGAAGCTG-3') were separately cloned into 50 ng of pMirGLO luciferase vectors (E1330, Promega, USA). Next, the TPC-1 and IHH-4 cells were co-transfected with the reconstructed pMirGLO and miR-199a-5p mimic using Lipofectamine 3000 transfection reagent for 48 h. After the co-transfection, the TPC-1 and IHH-4 cells were lysed by Lysis buffer, dyed using Giemsa (32884, Sigma-Aldrich, USA), incubated for 15 min at room temperature, and finally observed with an inverted microscope (IXplore Standard, Olympus, Japan).

**Annexin-V and Propidium Iodide (PI) staining**

TPC-1 or IHH-4 cell apoptosis was measured in a flow cytometer (FACSCalibur, BD bioscience, Franklin Lakes, NJ, USA) using an Annexin V-APC/PI apoptosis detection kit (E-CK-A217, Elabsscience, Wuhan, China). Annexin V Binding Buffer was diluted (1:10) by deionized water. 2 × 10^5 TPC-1 cells transfected with siNNT-AS1 and IHH-4 cells transfected with NNT-AS1 overexpression plasmid were separately digested using Trypsin, washed by Annexin V Binding Buffer, and centrifuged at 2,000 × g for 5 min. Then, TPC-1 or IHH-4 cells were resuspended in 500 μL of Annexin V binding buffer and added with 5 μL of Annexin V-APC solution. After thorough mixing, 5 μL of PI solution was added into TPC-1 or IHH-4 cells by vortex, followed by incubation for 5 min at room temperature in the dark. Finally, TPC-1 and IHH-4 cell suspensions were prepared for analysis in a flow cytometer (FACSCalibur, BD bioscience, Franklin Lakes, NJ, USA).

**qRT-PCR**

Total mRNA and miRNA in PTC tissues, TPC-1 cells and IHH-4 cells were isolated by TRIzol LS Reagent (10296010, ThermoFisher, USA) and a RNAiso for Small RNA kit (9753Q, TaKaRa, Liaoning, China), respectively. The extracted mRNA and miRNA were reverse-transcribed with SuperScript IV reverse transcriptase (18090010, ThermoFisher, USA) and a TaqMan miRNA Reverse Transcription Kit (4366597, ThermoFisher, USA) was then added into the TPC-1 or IHH-4 cell solution in each well, and the cells were incubated at 37°C for 4 h. Optical absorbance was determined at 450 nm using a microplate reader (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany).
ThermoFisher, USA) respectively to synthesize single-stranded cDNA. cDNA was amplified using PowerTrack SYBR Green Master Mix (A46012, ThermoFisher, USA) in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were set as follows: at 50°C for 2 min and at 95°C for 2 min, followed by 40 cycles of at 95°C for 15 s and at 60°C for 1 min. The relative expressions of NNT-AS1, Bax and Bcl-2 were normalized to that of GAPDH, whereas miR-199a-5p expression was normalized to U6 expression. The primers used for amplifying cDNA were listed in Table 1. The relative changes in the RNA expressions were calculated by the 2−ΔΔCt method and expressed as fold change [20].

**Western blot**

Total protein was extracted by lysing the transfected TPC-1 and IHH-4 cells with RIPA Buffer (89900, ThermoFisher, USA) containing protease inhibitors and it was quantitated using a BCA protein assay kit (A53227, ThermoFisher, USA). Then, the protein (30 μg/lane) was loaded onto 12% SDS-PAGE gel (P0053A, Beyotime, Shanghai, China) and electrophoresed, and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (P2438, Sigma-Aldrich, USA). The protein-attached PVDF membranes were blocked in 5% non-fat milk for 1 h at room temperature and diluted with Tris Buffered Saline and 1% Tween 20 (TBST) (TA-125-TT, ThermoFisher, USA). After that, the membranes were probed and incubated overnight at 4°C with the following primary antibodies: Bax (ab32503, 21 KDa, 1:1,000, abcam, Cambridge, MA, USA), Cleaved caspase-3 (ab2302, 17 KDa, 1:1,000, abcam, Cambridge, USA), Bcl-2 (ab59348, 26 KDa, 1:1,000, abcam, Cambridge, USA), E-Cadherin (#14472, 135 kDa, 1:1,000, Cell Signaling, Boston, MA, USA), N-Cadherin (#14215, 140 kDa, 1:1,000, Cell Signaling, USA), SNAIL (ab53519, 68 kDa, 1:1,000, abcam, Cambridge, USA), and GAPDH (ab8245, 36 kDa, 1:1,000, abcam, Cambridge, USA). After being washed in TBST, the membranes were further incubated for 2 h at room temperature with the following secondary antibodies: Biotinylated Anti-mouse IgG (H+L) (14709S, 1:2,000, Cell Signaling, USA), Goat Anti-Rabbit IgG H&L (HRP) (ab205718, 1:2,000, abcam, USA) and Donkey Anti-Goat IgG H&L (HRP) (ab6885, 1:1,000, abcam, USA). The protein bands were visualized with an enhanced chemiluminescence reagent kit (WP20005, ThermoFisher, USA) and their images were captured on an imaging System (iBright CL1500, A44240, ThermoFisher, USA). The relative density of the protein bands was analyzed by ImageJ software (1.52s version, National Institutes of Health, Bethesda, MA, USA).

**Statistical analysis**

Each experiment was conducted in triplicate. Statistical analyses were performed using the SPSS19.0 software (version 19, IBM, Chicago, IL, USA) and mapped by GraphPad Prism 6 Software (GraphPad, San Diego, CA, USA). The relationship between NNT-AS1 expression and clinical characteristics of PTC patients was analyzed by Chi-square test. Pearson correlation coefficient analysis was performed to determine the correlations. Comparisons between groups were analyzed by one-way ANOVA followed by Bonferroni post-hoc test. p < 0.05 was considered as statistically significant.

**Results**

**NNT-AS1 was high-expressed in PTC tissues and cells, and it regulated the viability, cell clone formation and migration of PTC cell lines**

To investigate the role of NNT-AS1 in PTC, NNT-AS1 expression in PTC tissue and cells was detected. NNT-AS1 expression was increased in PTC tissues compared with that in peritumoral normal tissues (p < 0.001, Fig. 1A). In addition, according to the median expression level of NNT-AS1, the patients were divided into NNT-AS1 high expression group and NNT-AS1 low expression group to investigate the relationship between NNT-AS1 expression and clinical characteristics of PTC.

| Table 1 Primers used in qPCR for the target genes |
|---|
| **Gene** | **Species** | **Forward** | **Reverse** |
| NNT-AS1 | human | 5'-AGTTCCACCAAGTTTCTTCA-3' | 5'-AGGTTTTGCCAGCATAGAC-3' |
| miR-199a-5p | human | 5'-GTTGTCGTATCCAGTGCAA-3' | 5'-CGTATCCAGTGCTGTCGTG-3' |
| Bax | human | 5’-CCCAGAGAGGTCTTTTCCCGAG-3’ | 5’-CCAGGCCATGATGTTCTGATG-3’ |
| Bcl-2 | human | 5’-GGTGGGGTGCTATGTTGTTGG-3’ | 5’-CGTTACGATCTACGATCAC-3’ |
| GAPDH | human | 5’-GGAGCGAGATCCCTCCAAAT-3’ | 5’-GGGTGGTTGCTATCTCCTCATG-3’ |
| U6 | human | 5’-CTGCTTTCGGACGCA-3’ | 5’-AAGCTTCAGAATTGCGT-3’ |
patients was analyzed, and the result showed that high
expression of NNT-AS1 was associated with tumor size,
lymph node metastasis and TNM stage (Table 2). Similar
to the aforementioned results, an increase in NNT-AS1
expression was observed in TPC-1, IHH-4 and MDA-
T68 cells as compared with Nthy-ori3-1 cells (p < 0.001,
Fig. 1B). After TPC-1 and IHH-4 cells were transfected
with siNNT-AS1 and NNT-AS1 overexpression plasmid
respectively, it was found that the expression of NNT-
AS1 was decreased in the siNNT-AS1 group compared
with the NC group (p < 0.001, Fig. 2A), while the
expression of NNT-AS1 was increased in the NNT-AS1
group compared with the NC group (p < 0.001, Fig. 2B).
We found that the viability of TPC-1 cells transfected
with siNNT-AS1 was decreased at 24 h, 48 h and 72 h
versus those without transfection, while the viability of
IHH-4 cells transfected with NNT-AS1 overexpression
plasmid was increased at 48 h and 72 h as compared with
those without transfection (Fig. 2C, D, p < 0.05). Fur‐
thermore, the transfection of siNNT-AS1 inhibited the
clonal formation and migration of TPC-1 cells (p < 0.001,
< 0.001), while the transfection of NNT-AS1 overex‐
pression plasmid promoted the clone formation and
migration force of IHH-4 cells, as compared to the non-
transfected cells (Fig. 2E–J, p < 0.001, p < 0.01). The
results suggested that NNT-AS1 overexpression pro‐
moted the clone formation and migratory ability of PTC
cell lines.

NNT-AS1 regulated the invasiveness, apoptosis and
EMT of PTC cell lines

The transfection of siNNT-AS1 inhibited invasiveness
and decreased the protein expressions of SNAIL and N-
Cadherin, but promoted apoptosis and increased E-
Cadherin protein expression in TPC-1 cells, as compared
with the non-transfected cells (Fig. 3A–C, E and Fig. 4A,
B, p < 0.001, p < 0.01). However, the transfection of
NNT-AS1 overexpression plasmid promoted invasiveness
and increased the protein expressions of SNAIL and
N-Cadherin, but inhibited apoptosis and decreased E-
Cadherin protein expression in IHH-4 cells, as compared
with the non-transfected cells (Fig. 3A, B, D, F and
Fig. 4C, D, p < 0.001). The results indicated that NNT-
AS1 overexpression promoted invasiveness and EMT
but inhibited apoptosis in PTC cell lines.

Table 2 The relationship between NNT-AS1 expression
and clinical characteristics

| Variable         | n | NNT-AS1 expression | p value |
|------------------|---|--------------------|---------|
|                  |   | Low | High |       |
| Total            | 30| 15  | 15   |        |
| Gender           |   |     |      |        |
| Male             | 8 | 5   | 3    | 0.409  |
| Female           | 22| 10  | 12   |        |
| Age              |   |     |      |        |
| ≤45              | 16| 9   | 7    | 0.464  |
| >45              | 14| 6   | 8    |        |
| Tumor size (cm)  |   |     |      |        |
| ≤2               | 18| 12  | 6    | 0.025  |
| >2               | 12| 3   | 9    |        |
| TNM stage        |   |     |      |        |
| I-II             | 19| 14  | 5    | 0.001  |
| III-IV           | 11| 1   | 10   |        |
| Lymph node metastasis | |     |      |        |
| No               | 17| 13  | 4    | 0.001  |
| Yes              | 13| 2   | 11   |        |
| Multifocality    |   |     |      |        |
| No               | 14| 9   | 5    | 0.143  |
| Yes              | 16| 6   | 10   |        |
Low-expressed miR-199a-5p in PTC tissues targeted NNT-AS1 and was negatively correlated with NNT-AS1

Previous study showed that miR-199a-5p has an anti-tumor effect on PTC occurrence and progression [18], so we examined miR-199a-5p expression and its relation with NNT-AS1 in PTC in this research. Online database starBase predicted a mutual binding site between miR-199a-5p and NNT-AS1 wild-type (WT) (Fig. 5A).
Dual-luciferase reporter assay demonstrated that miR-199a-5p mimic reduced the luciferase activity of NNT-AS1 WT in TPC-1 cells ($p < 0.01$) and IHH-4 cells ($p < 0.001$), yet it showed no effect on the luciferase activity of NNT-AS1 mutant-type, which confirmed the targeted relation between miR-199a-5p and NNT-AS1 (Fig. 5B, C). A decrease in the expression of miR-199a-5p was detected in PTC tissues when compared with peritumoral normal tissues ($p < 0.001$). Meanwhile, correlation analysis showed that constantly upregulated NNT-AS1 expression displayed a downward linear relation to miR-199a-5p in PTC (Fig. 5D).

**MiR-199a-5p reversed the effect of NNT-AS1 on the viability and clone formation of PTC cell lines**

Experiments were conducted to unveil the effect of miR-199a-5p on the viability and clone formation of PTC cell lines with high-expressed NNT-AS1 or low-expressed NNT-AS1. Our data revealed that silencing NNT-AS1 increased miR-199a-5p RNA expression in TPC-1 cells as compared with siNC-transfected TPC-1 cells, while overexpressing NNT-AS1 decreased the RNA expression of miR-199a-5p in IHH-4 cells relative to non-transfected IHH-4 cells (Fig. 6A, B, $p < 0.01$, $p < 0.001$). MiR-199a-5p inhibitor reversed the promoting effect of siNNT-AS1 on the RNA expression of miR-199a-5p in TPC-1 cells, while miR-199a-5p mimic reversed the inhibitory effect of NNT-AS1 overexpression on miR-199a-5p RNA expression in IHH-4 cells (Fig. 6A, B, $p < 0.01$, $p < 0.001$). However, we observed that siNNT-AS1 reversed the effect of miR-199a-5p inhibitor on miR-199a-5p RNA expression in TPC-1 cells ($p < 0.01$), and that NNT-AS1 overexpression reversed the effect of miR-199a-5p mimic on miR-199a-5p RNA expression in IHH-4 cells (Fig. 6A, B, $p < 0.01$, $p < 0.01$).

Moreover, the viability and clone formation of TPC-1 cells were promoted by miR-199a-5p inhibitor, but miR-199a-5p mimic inhibited the viability and cell clone formation of IHH-4 cells, as compared with the non-transfected cells (Fig. 6C–G, $p < 0.05$, $p < 0.001$).
Notably, miR-199a-5p inhibitor reversed the inhibitory effect of siNNT-AS1 on inhibiting the viability and clone formation of TPC-1 cells, and miR-199a-5p mimic reversed the effect of NNT-AS1 overexpression on promoting the viability and clone formation of IHH-4 cells (Fig. 6C–G, \( p < 0.05, p < 0.001 \)). Moreover, siNNT-AS1 reversed the promoting effect of miR-199a-5p inhibitor on the viability and clone formation of TPC-1 cells, and NNT-AS1 overexpression reversed the inhibitory effect of miR-199a-5p mimic on the viability and clone formation of IHH-4 cells (Fig. 6C–G, \( p < 0.05, p < 0.001 \)). These results indicated a mutual inhibition between the effects of miR-199a-5p and NNT-AS1 on the viability and clone formation of PTC cell lines.

**MiR-199a-5p reversed the effect of NNT-AS1 on migration and invasiveness in PTC cell lines**

The effect of miR-199a-5p on the migration and invasiveness of PTC cell lines with high-expressed NNT-AS1 or low-expressed NNT-AS1 was explored. The results demonstrated that miR-199a-5p inhibitor promoted the migration and invasiveness of TPC-1 cells, and miR-199a-5p mimic inhibited the migration and invasiveness of IHH-4 cells, as compared with the non-transfected cells (Fig. 7A–F, \( p < 0.01, p < 0.001, p < 0.01 \)). As expected, miR-199a-5p inhibitor reversed the inhibitory effect of siNNT-AS1 on the migration and invasiveness of TPC-1 cells, and miR-199a-5p mimic reversed the effect of NNT-AS1 overexpression on enhancing the migration and invasiveness of IHH-4 cells (Fig. 7A–F, \( p < 0.01, p < 0.001 \)). In contrast, siNNT-AS1 reversed the promoting effect of miR-199a-5p inhibitor on the migration and invasiveness of TPC-1 cells, and NNT-AS1 overexpression reversed the effect of miR-199a-5p mimic on inhibiting the migration and invasiveness of IHH-4 cells (Fig. 7A–F, \( p < 0.01, p < 0.001 \)). These data also indicated a mutual inhibition between the effects of miR-199a-5p and NNT-AS1 on the migration and invasiveness of PTC cell lines.

**MiR-199a-5p reversed the effect of NNT-AS1 on the expressions of apoptosis-associated and EMT-associated factors in PTC cell lines**

To further explore the effect of miR-199a-5p on the apoptosis and EMT of PTC cell lines with high-expressed NNT-AS1 or low-expressed NNT-AS1, the
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protein and mRNA expressions of apoptosis- and EMT-associated factors were determined. Silencing NNT-AS1 was found to increase the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin as well as decrease the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL in TPC-1 cells (Fig. 8A, C, D, G, H, \(p < 0.001\)). In contrast, NNT-AS1 overexpression decreased the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin, but increased the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL in IHH-4 cells (Fig. 8B, E, F, I, J, \(p < 0.001\)). Moreover, the transfection of miR-199a-5p mimic increased the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin, and decreased the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL in IHH-4 cells (Fig. 8B, E, F, I, J, \(p < 0.001\)), showing a similar effect to that of NNT-AS1 silencing on
MiR-199a-5p reversed the effect of NNT-AS1 on the viability and clone formation of PTC cell lines. (A). MiR-199a-5p RNA expression in TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both was analyzed by qRT-PCR, with U6 serving as a reference gene. (B). MiR-199a-5p RNA expression in IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both was analyzed by qRT-PCR, with U6 serving as a reference gene. (C). The viability of TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both was analyzed by CCK-8 assay. (D). The viability of IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both was analyzed by CCK-8 assay. (E). Representative pictures of the clone formation of TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both and IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both (F). The colony number of TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both was analyzed by colony formation assay. (G). The colony number of IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both was analyzed by colony formation assay. *p < 0.05; **p < 0.01; ***p < 0.001; vs. siNC; ^p vs. siNNT-AS1; #p vs. siNC+Inhibitor; &p vs. NC; +p vs. NNT-AS1; ^p vs. NC+Mimic (siNNT-AS1, siRNA-NNT-AS1; NC, negative control; siNC, siRNA negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction)
TPC-1 cells. We also found that the transfection of miR-199a-5p inhibitor decreased the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin, but increased the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL in TPC-1 cells (Fig. 8A, C, D, G, H, p < 0.001), demonstrating a similar effect to that of NNT-AS1 overexpression on IHH-4 cells. Furthermore, miR-199a-5p inhibitor reversed the effect of siNNT-AS1 on inhibiting the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL, and reversed the effect of siNNT-AS1 on promoting the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin in TPC-1 cells (p < 0.001). However, miR-199a-5p mimic reversed the promoting effect of NNT-AS1 overexpression on the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL, and also reversed the inhibitory effect of NNT-AS1 overexpression on the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin in IHH-4 cells (Fig. 8A–J, p < 0.001). In addition, siNNT-AS1 reversed the effect of miR-199a-5p inhibitor on promoting the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL, and also reversed the effect of miR-199a-5p inhibitor on inhibiting the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin in TPC-1 cells (p < 0.001, p < 0.01), whereas NNT-AS1 overexpression reversed the inhibitory effect of miR-199a-5p mimic on the protein and mRNA expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL, and also reversed the inhibitory effect of miR-199a-5p mimic on inhibiting the protein and mRNA expressions of Bax and the protein expressions of cleaved Caspase-3 and E-Cadherin in IHH-4 cells (Fig. 8A–J, p < 0.001).
expressions of Bcl-2 and the protein expressions of N-Cadherin and SNAIL, and also reversed the promoting effect of miR-199a-5p mimic on the protein and mRNA expressions of cleaved Caspase-3 and E-Cadherin in IHH-4 cells (Fig. 8A-J, p < 0.001). These results indicated a mutual inhibition between the effects of miR-199a-5p and NNT-AS1 on the apoptosis and EMT of PTC cell lines.

Discussion

LncRNAs play crucial roles in various cancers [11], and their expressions or mutations are closely related to tumorigenesis and metastasis [11]. LncRNAs function as either a tumor suppressor or an oncogene in thyroid cancer [21]. Specifically, XIST expression is remarkably increased in thyroid cancer tissues and cells, whereas its knockdown inhibits cell proliferation and tumor volume, pointing to the oncogenic function of XIST in thyroid cancer [22]. Moreover, some other lncRNAs (BANCR, MALAT1, HOTAIR, H19) have also been recognized as cancer-promoting factors in thyroid cancer, and are employed as biomarkers in the diagnosis and therapy of the cancer [23]. However, certain lncRNAs, such as MEG3, LINCO00271, NAMA, and PTCSCL1/2/3, are downregulated and function as tumor suppressors in thyroid cancer [24]. Here, we discovered that the RNA expression of NNT-AS1 was pronouncedly increased in PTC tissues and differentially upregulated in PTC cell lines (TPC-1, IHH-4 and MDA-T68). Therefore, we speculated that NNT-AS1 might be able to promote PTC development as well.

Aberrantly expressed lncRNAs will affect cancer cell proliferation, tumor growth and metastasis [25]. Lei’s study showed that lncRNA-TUG1 is overexpressed in PTC like NNT-AS1, and increased expression of TUG1 promotes tumor cell proliferation, migration and invasion, while knocking down TUG1 produces the opposite effects [26]. Wang observed that the clone formation of BCPAP cells was enhanced by upregulated expression of lncRNA-BANCR, which has been previously found to be overexpressed in thyroid tumor tissues yet low-expressed in BCPAP cells [27]. In our study, silencing NNT-AS1 exerts an anticancer effect through hindering the proliferation, clone formation, migration and invasion of TPC-1 cells, while NNT-AS1 overexpression leads to oncogenesis in IHH-4 cells.

Existing studies reported that the inhibition of thyroid cancer cell growth or thyroid cancer metastasis caused by regulation of lncRNA expressions is relevant to increased apoptosis [23, 28]. Liu’s study highlighted the role of LINCO00704 as a thyroid cancer-associated lncRNA and the effect of its downregulation on inhibiting thyroid cancer cell growth and metastasis and increasing apoptosis [27]. Consistent with Liu’s findings, our results showed that silencing NNT-AS1 promoted TPC-1 cell apoptosis, while NNT-AS1 overexpression inhibited IHH-4 cell apoptosis.

EMT is an essential cell biological process involved in various pathological processes [29], and it is considered as a vital mechanism for the development of carcinoma metastasis [30]. EMT is featured by the silence of the epithelial marker E-cadherin and the upregulation of mesenchymal markers such as N-cadherin [30]. Moreover, transcription factors like SNAIL also exert functions in transcription, translation and post-translation during EMT [31]. Previously recognized as a survival promotor and cell movement inducer, SNAIL is able to initiate the EMT program in metastatic cancer [32]. Studies discovered that SNAIL inhibits E-cadherin expression in human glioblastoma cells [33], but its inhibition leads to E-cadherin activation during breast carcinoma metastasis [34]. Similar to their findings, in the current study, TPC-1 cells with silenced NNT-AS1 exhibited increased E-Cadherin protein expression and decreased protein expression of N-cadherin and SNAIL, while the opposite results were obtained in IHH-4 cells with overexpressed NNT-AS1.

MiR-199a-5p, a tumor suppressor in PTC [18], is normally low-expressed in PTC tissues and cells; however, it can suppress PTC cell progression via inhibition of cell migration, invasion and EMT when upregulated [18]. Similarly, our study reaffirmed the low expression of miR-199a-5p in PTC tissues, and revealed that miR-199a-5p was negatively associated to PTC cell viability, migration, and invasion as well as the expressions of N-cadherin and SNAIL, but was positively associated to E-cadherin expression. Zhu’s study found that miR-199a-5p could induce colorectal cancer cell apoptosis [35]. A previous study detected a decreased expression of Bcl-2 and increased expressions of Bax and cleaved caspase-3 in apoptotic prostate cancer cells after anticancer treatment [36]. In our study, these apoptosis-associated factors showed similar expression patterns in PTC cells after miR-199a-5p expression was greatly upregulated, but inhibiting miR-199a-5p expression caused the opposite trends of their expressions, pointing to a pro-apoptotic effect of miR-199a-5p. Also, we found that contrary to miR-199a-5p, NNT-AS1 exerted an anti-apoptotic effect. LncRNAs are known for their regulatory mechanism that they can sponge miRNAs to modulate cancer progression [17]. Accordingly, we hypothesized that miR-199a-5p might be a regulatory target of NNT-AS1. As expected, bioinformatics analysis showed a potential relation between miR-199a-5p and NNT-AS1. Dual-luciferase reporter assay verified this
Fig. 8 MiR-199a-5p reversed the effect of NNT-AS1 on apoptosis-associated and EMT-associated factors in PTC cell lines. (A). The mRNA expressions of Bax and Bcl-2 in TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both were analyzed by qRT-PCR, with GAPDH serving as a reference gene. (B). The mRNA expressions of Bax and Bcl-2 in IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both were analyzed by qRT-PCR, with GAPDH serving as a reference gene. (C). Photos of the protein bands of Bax, C Caspase-3 and Bcl-2 in TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both were analyzed by Western blot, with GAPDH serving as a reference gene. (D). Photos of the protein bands of Bax, C Caspase-3 and Bcl-2 in IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both. (E). The protein expressions of Bax, C Caspase-3 and Bcl-2 in TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both were analyzed by Western blot, with GAPDH serving as a reference gene. (F). The protein expressions of Bax, C Caspase-3 and Bcl-2 in IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both were analyzed by Western blot, with GAPDH serving as a reference gene. (G). Photos of the protein bands of E-Cadherin, N-Cadherin and snail in TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both. (H). The protein expressions of E-Cadherin, N-Cadherin and snail in TPC-1 cells transfected with siNNT-AS1, miR-199a-5p inhibitor or both were analyzed by Western blot, with GAPDH serving as a reference gene. (I). Photos of the protein bands of E-Cadherin, N-Cadherin and snail in IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both. (J). The protein expressions of E-Cadherin, N-Cadherin and snail in IHH-4 cells transfected with NNT-AS1 overexpression plasmid, miR-199a-5p mimic or both were analyzed by Western blot, with GAPDH serving as a reference gene. ^p < 0.01; **p < 0.001; ***p < 0.0001; vs. NC; "vs. siNC; ^vs. siNNT-AS1; ¥ vs. siNNT-AS1+Inhibitor; & vs. NC; " vs. NNT-AS1; + vs. NC+Mimic (siNNT-AS1, siRNA-NNT-AS1; NC, negative control; siNC, siRNA negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction)
relation, and subsequent expression examination verified an inverse correlation between the two in PTC tissues. Subsequently, the interaction of miR-199a-5p and NNT-AS1 in PTC cell progression was further confirmed. It turned out that there was a mutual inhibition between the effects of miR-199a-5p and NNT-AS1 on PTC cell proliferation, clone formation, migration, and invasion as well as the expressions of apoptosis-associated factors and EMT-associated factors in PTC cells. In addition, it has been reported that NNT-AS1 acts as a major mediator of cisplatin chemoresistance through MAPK/Slug pathway in non-small cell lung cancer [37]. Nonetheless, the relationship between NNT-AS1 and drug assistance in PTC remains to be further understood, and the effect of NNT-AS1 in PTC needs further confirmation through in vivo experiments.

In conclusion, NNT-AS1 is overexpressed yet miR-199a-5p is low-expressed in PTC. Silencing of NNT-AS1 inhibits proliferation, migration, invasion and EMT, but promotes apoptosis in PTC cell lines through upregulating miR-199a-5p expression. NNT-AS1 may be a useful diagnostic biomarker and potential therapeutic target for PTC.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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