MicroRNA-1236-3p/translationally controlled tumor protein (TPT1) axis participates in congenital hypothyroidism progression by regulating neuronal apoptosis

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Abstract. Congenital hypothyroidism (CH) is an endocrine disease caused by congenital thyroid hormone (TH) deficiency. MicroRNAs (miRNAs or miRs) have been reported to inhibit the progression of congenital hypothyroidism. However, the expression and role of miR-1236-3p in CH remains unclear. To address this, 12 day old Sprague-Dawley rats were divided into five groups: Control; Congenital hypothyroidism (CH), miR-1236-3p inhibitor control (inhibitor control); miR-1236-3p inhibitor (inhibitor); and miR-1236-3p inhibitor + translationally-controlled tumor protein 1 (TPT1)-small interfering (si)RNA (inhibitor + siRNA). Propylthiouracil (50 mg/day) was injected intraperitoneally into pregnant rats to generate pups with CH. The levels of miR-1236-3p and TPT1 were detected via reverse transcription-quantitative PCR and western blot analysis. Bioinformatics analysis was performed to predict the targets of miR-1236-3p, which was confirmed using dual luciferase reporter assay. Flow cytometry and MTT assay were used to measure neuronal cell apoptosis and cell viability, whereas western blotting was applied to detect the expression of Pim-3, p-Bad (Ser112), Bad and Bcl-xL, proteins associated with apoptosis. The results revealed that miR-1236-3p expression was significantly upregulated, whilst TPT1 expression was significantly downregulated in the hippocampus tissues of CH rats compared with the control group. TPT1 was confirmed as a target of miR-1236-3p expression in hippocampal tissues of CH rats compared with the control group. TPT1 inhibitor prevented hippocampal neuron apoptosis induced by CH induction, which was reversed by TPT1-siRNA transfection. In addition, following miR-1236-3p inhibitor transfection, neuronal cell apoptosis significantly reduced compared with the control group, which was accompanied by significantly increased expressions of Pim-3, p-Bad (Ser112) and Bcl-xL expression. These effects were reversed by TPT1-siRNA co-transfection. These results indicated that inhibition of miR-1236-3p expression inhibited neuron apoptosis in vivo and in vitro by targeting TPT1, serving a protective role in CH.

Introduction

Congenital hypothyroidism (CH) is a form of general endocrine disease caused by congenital thyroid hormone (TH) deficiency (1). The incidence of CH in newborn infants ranges between 1/3,000 and 1/4,000 (2). This condition is treatable if diagnosed early, but later diagnoses can lead to physical and mental abnormalities (3). Therefore, the accurate timing of thyroid hormone replacement therapy in humans is critical for optimizing neurocognitive recovery. CH results in cognitive deficiency (4), motor deficits (5) and behaviors associated with anxiety (6). Timely and effective screening of newborns would enable early diagnosis and treatment, with the aim of improving the prognosis of children. In addition, quality care can improve the completion rate of CH screening in newborns more effectively. Therefore, early detection, diagnosis, scientific and effective nursing and treatment are crucial for improving the prognosis of children with congenital hypothyroidism and improving the quality of the population.

TH is essential for the proper development of the mammalian brain. TH deficiency results in serious structural and functional damage to the central nervous system (7). In particular, CH leads to neutrophil damage, abnormal cerebellar growth and differentiation (8-10). The hippocampus is an important center responsible for cognitive activities in humans. Indeed, children with CH who experience neonatal thyroid hormone deficiency exhibit reduced hippocampal volumes compared with healthy controls (11). A previous study has reported that perinatal hypothyroidism may induce apoptosis in hippocampal neurons in rats (12). Following early diagnosis of CH, neurodevelopmental dysplasia could still occur if treatment is not optimized within the first 2-3 years of birth (13). Therefore, patients with CH must undergo early treatment followed by close follow-up.

MicroRNAs (miRNAs) are non-coding single stranded small RNAs consisting of 18-25 nucleotides that do not encode...
proteins (14). Mature miRNA directly bind to mRNAs to regulate their expression (15). Recent research has suggested that miRNAs participate widely in diseases involving the nervous system, including congenital hypothyroidism (16). For example, a previous study has demonstrated that miR-124 inhibits the progression of congenital hypothyroidism (16). Previous studies have revealed that miR-1236 can serve different roles in a tissue- or physiological-dependent manners. miR-1236 attenuates human lymphatic endothelial cell migration and tube formation (in vitro angiogenesis detection) in addition to angiogenesis in the lymphatic system in vivo (17). In contrast, miR-1236-3p has been found to repress ovarian cancer metastasis (18). However, the role of miR-1236-3p in congenital hypothyroidism remains unclear.

Translationally-controlled tumor protein 1 (TPT1) is a highly conserved protein that has been reported to be strongly expressed in a variety of malignant tumors, where it regulates cell proliferation, invasion, cell cycle and apoptosis (19-21). Indeed, TPT1 downregulation has been demonstrated to inhibit cell proliferation and induce cell cycle arrest and apoptosis in pancreatic cancer (22). Furthermore, miR-489-3p has been revealed to inhibit glioblastoma progression by acting through the downregulation of TPT1 (23).

The present study aimed to clarify the role of miR-1236-3p in CH by investigating the function of this miRNA in hippocampal neuron apoptosis in vivo and in vitro using a rat model.

Materials and methods

Reagents. Propylthiouracil was obtained from Beyotime Institute of Biotechnology. This protocol followed and dosage of Propylthiouracil used was performed/selected according to a previous study (24). The miR-1236-3p inhibitor and its corresponding negative control (inhibitor control), TPT1-siRNA (cat no. XWCRR9262; Zhejiang Huijia Biotechnology Co., Ltd.) and control-siRNA (cat no. 9500C-1; Zhejiang Huijia Biotechnology Co., Ltd.) were purchased from Shanghai GenePharma Co., Ltd.

Experimental animals. A total of 50 female pregnant Sprague-Dawley rats (weight, 200±10 g; age, 6 weeks) obtained from Vital River Laboratories Co., Ltd. were used. All rats were maintained at room temperature with a humidity of 55% and ad libitum access to standard pellet feed and water under a 12-h light/dark cycle. Propylthiouracil (50 mg/day) was injected intraperitoneally into pregnant rats on day 15 of gestation and then carried out every day thereafter until parturition to generate pups with congenital hypothyroidism (24). For the treatment of CH pups, animals were anesthetized with pentobarbital (40 mg/kg) by intraperitoneal injection and sacrificed by cervical dislocation (death defined as the lack of heartbeat and breathing). The brain hippocampus tissue was subsequently harvested following euthanasia. The mother rats (24 h after the pups were born) from which the pups were obtained were also anesthetized with pentobarbital (40 mg/kg) and then sacrificed by cervical dislocation, with death defined as the lack of heartbeat and breathing. The experimental procedure of the present study regarding the establishment of the CH rat model and treatment process is presented in Fig. S1. All animal care and experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and approved by the Animal Ethics Committee of the First Hospital of Jilin University.

Cell culture and transfection. Hippocampal neurons were prepared from the hippocampus of postnatal day 0 rat pups from the control group, as previously described (26). Neurons were cultured using Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in poly-D lysine-coated plates at a density of 1x10⁶ cells/ml. After culturing for 6 h at 37°C and 5% CO₂, the cell culture medium was replaced with neurobasal medium (Gibco; Thermo Fisher Scientific, Inc.) containing 2% B27 (cat. no. 17504-044; Life Technologies; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 g/ml streptomycin and 0.5 mM glutamine (Gibco; Thermo Fisher Scientific, Inc.) and then incubated at 37°C in a humidified atmosphere under 5% CO₂. The culture medium was replaced once every 3 days.

Neurons were seeded into 6-well plates (5x10⁴ cells/well) and transfected with miR-1236-3p inhibitor, miR-1236-3p inhibitor control (inhibitor control), TPT1-siRNA, control-siRNA, miR-1236-3p inhibitor + control-siRNA or miR-1236-3p inhibitor + TPT1-siRNA using Lipofectamine®2000 (Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Neurons were collected for subsequent experiments after 48 h of transfection.
**MTT assay.** Cell viability of neurons was measured via an MTT assay. Cells were first cultured in 96-well plates (1x10^3 cells/well) at 37°C for 24 h and then transfected with the miR-1236-3p inhibitor, inhibitor control, miR-1236-3p inhibitor + control-siRNA or miR-1236-3p inhibitor + TPT1-siRNA for 48 h, as aforementioned. Following incubation, 10 µl MTT solution was added into each well and incubated for a further 4 h at 37°C under 5% CO₂. A total of 150 µl dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals. Optical density at 490 nm was measured in each well using a microplate reader.

**Flow cytometry.** A BD FACSCalibur™ Flow cytometer (BD Biosciences) was used to analyze neuronal cell apoptosis. Neurons (1x10^3 cells/well) were first digested using 0.25% trypsin, washed with PBS and fixed in 70% ice-cold ethanol overnight at 4°C. The neurons were subsequently added with 5 µl fluorescein isothiocyanate-labeled Annexin V and 5 µl propidium iodide (PI; cat. no. 6592; Cell Signaling Technology, Inc.). Then, the neurons were incubated at 4°C for 30 min in the dark. Cell apoptosis was analyzed and calculated (right quadrants) using FlowJo software (version 7.6.1; Tree Star Inc.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the hippocampus tissues and hippocampus neurons using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Biototechnology Co., Ltd.) according to manufacturer's protocol. qPCR was performed using Maxima™ SYBR Green qPCR Master Mix (2x) (Fermentas; Thermo Fisher Scientific, Inc.) according to manufacturers' protocols. The primer sequences used for qPCR were as follows: TPT1 forward, 5'-ATGATTATCTACCGGACCTC-3' and reverse, 5'-TACATTTTTTCATTTCAACCATCC-3'; GAPDH forward, 5'-CTTTTGATATCCTGGAGAAGACT-3' and reverse, 5'-GTAGAGCCAGGGATGATGTTCTT-3'; U6 forward, 5'-CTGCTCAGGCAGACATATACT-3' and reverse, 5'-ACGCTTTCAAGATTTTCGTTGC-3'; miR-1236-3p forward, 5'-CCAATCAGCTCCTTCCCTTCT-3' and reverse, 5'-TATGGTTATCACGACTCTTAC-3'. The thermocycling conditions were as follows: 5 min at 95°C and 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. U6 for (miRNA) and GAPDH (for mRNA) were used as the internal control. Quantification was performed using the 2^(-ΔΔCq) method (27).

**Western blot analysis.** After cell transfection, the neuronal cells were homogenized in cell lysis buffer (10x; Cell Signaling Technology, Inc.) containing protease inhibitors. Proteins were quantified using Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Inc.) and subsequently separated (30 µg/lane) via 12% SDS-PAGE prior to transferal onto PVDF membranes. The membranes were blocked with 5% skim milk in TBS containing 0.1% Tween at room temperature for 2 h, and then incubated with primary antibodies against TPT1 (cat. no. 5128; 1:1,000; Cell Signaling Technology, Inc.), Serine/Threonine Kinase Pim-3 (Pim-3; cat. no. 4165; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (p)-Bad (Ser112) (cat. no. 5284; 1:1,000; Cell Signaling Technology, Inc.), Bcl-xL (cat. no. 2764; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. 4970; 1:1,000; Cell Signaling Technology, Inc.) at 4°C overnight. The membranes were incubated at 4°C for 30 min in the dark. Cell apoptosis was analyzed and calculated (right quadrants) using FlowJo software (version 7.6.1; Tree Star Inc.).

**Dual luciferase reporter assay.** Bioinformatics software 7.1 (http://www.targetscan.org/vert_71/) was used to predict potential targets of miR-1236-3p. The binding sites between the 3'-untranslated region (3'-UTR) of TPT1 and miR-1236-3p were observed. A dual luciferase reporter assay was used to investigate whether miR-1236-3p directly targets the 3'-UTR of TPT1. The wild-type (WT) 3'UTR of TPT1 or the mutant (MUT) 3'UTR TPT1 were cloned into the dual-luciferase reporter vector pmir-RB-REPORT (Guangzhou RiboBio Co., Ltd.) following the manufacturer's instructions. Hippocampal neurons were subsequently prepared from the hippocampus of postnatal day 0 rat pups in the control group (5x10⁴ cells per well) were first seeded into 24-well plates and co-transfected with either TPT1-3'-UTR-wild type (WT) or TPT1-3'-UTR-mutant (MUT) 3'UTR TPT1 were cloned into the dual-luciferase reporter vector pmir-RB-REPORT (Guangzhou RiboBio Co., Ltd.) following the manufacturer's instructions. Hippocampal neurons were subsequently prepared from the hippocampus of postnatal day 0 rat pups in the control group.
Statistical analysis. All experiments were repeated three times. Data were presented as the mean ± standard deviation. The significance of differences between groups was evaluated using Student's t-test or analyzed via one-way ANOVA followed by a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

MiR-1236-3p is upregulated in rats with CH. Changes in miR-1236-3p expression in the hippocampus tissue of newborn pups after the establishment of CH were first measured using RT-qPCR. Compared with the newborn pups of the control group, the expression of miR-1236-3p was significantly higher in the hippocampus tissue of newborn pups with CH (Fig. 1).

TPT1 is a target of miR-1236-3p. TargetScan revealed a potential miR-1236-3p binding site on the 3'-UTR of TPT1 (Fig. 2A). Dual-luciferase reporter assay results demonstrated that transfection with miR-1236-3p mimic significantly reduced luciferase activity in neurons that were also co-transfected with TPT1-WT group but not in those co-transfected with TPT1-MUT (Fig. 2B). This observation indicated that TPT1 is a direct target of miR-1236-3p. To support this, levels of TPT1 expression in the hippocampus tissue of newborn pups with or without CH was determined using RT-qPCR and western blotting. Compared with the control group, TPT1 protein (Fig. 2C) expression markedly reduced in the hippocampus tissues of rats with CH. Compared with the control group, TPT1 mRNA (Fig. 2D) expression was significantly reduced in the hippocampus tissues of rats with CH.

Inhibition of miR-1236-3p mitigates CH newborn pups hippocampal neuronal cell apoptosis in vivo. The effects of miR-1236-3p on neuronal cell apoptosis in rats with CH was assessed via flow cytometry. Compared with the control group, neuronal cells isolated from rats in the CH group exhibited significantly increased cell apoptosis (Fig. 3A and B). Injection with miR-1236-3p inhibitor significantly reduced CH-induced neuronal cell apoptosis, an effect that was reversed by co-injection with TPT1-siRNA (Fig. 3A and B). Overall, these results indicated that inhibition of miR-1236-3p prevented neuronal cell apoptosis in rats with CH.

Inhibition of miR-1236-3p protects cultured neurons from apoptosis in vitro. Neuronal cells were isolated from the hippocampus of postnatal day 0 rat pups from the control group and subsequently transfected with either miR-1236-3p inhibitor, inhibitor control or miR-1236-3p inhibitor + TPT1-siRNA. Transfection efficiency was first assessed via RT-qPCR. Transfection with the miR-1236-3p inhibitor significantly reduced the levels of miR-1236-3p in neurons (Fig. 4A), whilst TPT1-siRNA transfection significantly reduced TPT1 mRNA level in cultured neurons (Fig. 4B). TPT1-siRNA transfection also markedly reduced TPT1 protein level in cultured neurons (Fig. 4C). Compared with the inhibitor control group, transfection with the miR-1236-3p inhibitor significantly increased TPT1 mRNA expression, which was reversed by
Figure 3. Effect of miR-1236-3p inhibition and/or TPT1 knockdown on CH newborn pups hippocampal neuronal cell apoptosis in vivo, using Annexin V FITC/PI assay. Flow cytometry was used to measure apoptosis of neurons in newborn rats from their respective treatment groups. (A) representative dot plots of flow cytometry; (B) quantified data of cell apoptosis. *P<0.01 vs. Control; **P<0.01 vs. inhibitor control; ***P<0.01 vs. Inhibitor. miR, miRNA; TPT1, translationally-controlled tumor protein 1; CH, congenital hypothyroidism; PI, propidium iodide.

Figure 4. miR-1236-3p negatively regulates TPT1 expression in cultured neurons. (A) The levels of miR-1236-3p expression in cultured neurons transfected with either miR-1236-3p inhibitor or inhibitor control. (B) TPT1 mRNA and (C) protein expression in cultured neurons transfected with either TPT1-siRNA or control-siRNA. (D) TPT1 mRNA and (E) protein expression in cultured neurons co-transfected with either miR-1236-3p inhibitor or inhibitor control and control-siRNA or TPT1-siRNA. *P<0.01 vs. inhibitor control; **P<0.01 vs. Inhibitor + control-siRNA. miR, microRNA; TPT1, translationally-controlled tumor protein 1; siRNA, small interfering RNA.
co-transfection with TPT1-siRNA (Fig. 4D). Similar results were observed from western blot assay (Fig. 4E).

Next, the effects of miR-1236-3p on cultured neuronal cell apoptosis in vitro was assessed. Compared with the inhibitor control group, transfection with the miR-1236-3p inhibitor significantly increased the neuronal cell viability after 48 h (Fig. 5A) whilst inhibiting neuronal cell apoptosis (Fig. 5B and C), effects that were significantly reversed by co-transfection with TPT1-siRNA.

**Discussion**

In the present study, it was demonstrated that miR-1236-3p was upregulated in the hippocampus of newborn rats with CH. TPT1 was a direct target of miR-1236-3p, which was revealed to be downregulated in the hippocampus of newborn rats with CH. Inhibition of miR-1236-3p expression protected hippocampal neurons from apoptosis induced by CH in vivo. In addition, the present study also revealed that miR-1236-3p inhibition prevented neurons from apoptosis and upregulated Pim-3, p-Bad (Ser112) and Bcl-xL protein expression in vitro, all of which were reversed by TPT1 knockdown.

Inhibition of miR-1236-3p upregulates Pim-3, p-Bad and Bcl-xL expression. To explore the molecular mechanism by which miR-1236-3p inhibition reduced neuronal apoptosis, the expressions of Pim-3, p-Bad, Bad and Bcl-xL, proteins associated with apoptosis, were analyzed via western blotting. Compared with the inhibitor control group, transfection with miR-1236-3p inhibitor significantly increased protein levels of Pim-3, p-Bad (Ser112) and Bcl-xL in neuronal cells (Fig. 6), which were in turn reversed by co-transfection with TPT1-siRNA (Fig. 6).

Figure 5. Effect of miR-1236-3p inhibitor and/or TPT1 knockdown on cell viability and apoptosis of cultured neurons in vitro. (A) Cultured neuronal cell viability was determined after 48 h using MTT assay. (B and C) Flow cytometry was used to analyze cultured neuronal cell apoptosis. (B) Representative dot plots in cultured neuronal cells co-transfected with either miR-1236-3p inhibitor or inhibitor control and TPT1-siRNA or control siRNA. (C) Quantified results of (B). *P<0.01 vs. inhibitor control; **P<0.01 vs. Inhibitor + control-siRNA. miR, microRNA; TPT1, translationally-controlled tumor protein 1; siRNA, small interfering RNA; PI, propidium iodide; FITC, fluorescein isothiocyanate.
CH leads to decreased developmental quotient, mental retardation and increased depression/anxiety scores in patients (6,28‑31). Clinical phenomenon (including short stature and mental retardation) suggested that thyroid hormone synthesis and secretion reduction leads to developmental delay and neurological deficits (32). In addition, TH deficiency can lead to hippocampal neuronal cell injury and cell apoptosis (33). miRNAs have been revealed to serve a critical role in diseases associated with the nervous system, including autoimmune neuroinflammation (34), Alzheimer's disease (35), Parkinson's disease (36) and CH (16). A previous study has reported that miR -124 can protect neurons from apoptosis in rat models of hypothyroidism (16). In the present study, it was determined that miR -1236-3p was significantly upregulated in the hippocampal tissue of newborn rats with CH, where TPT1 was identified as one of its direct targets. Supporting this notion, it was also identified that TPT1 was downregulated in the hippocampus tissues of newborn rats with CH. TPT1 is a translationally controlled oncogene, where it was found to be upregulated in a variety of malignancies including glioma (20), pancreatic (21,22), breast (38) and colorectal cancer (39). It was also revealed to enhance tumor cell proliferation whilst inhibiting cell apoptosis. Hippocampal neuronal cell apoptosis is one of the main characteristics of CH (11,12,16). Therefore, the results from the present study suggested that miR-1236-3p may affect CH progression by regulating neuronal cell apoptosis by targeting TPT1. Subsequently, the effect of miR-1236-3p on hippocampal neuronal cell apoptosis in rats with CH was investigated. The results of the present study revealed that CH induction increased apoptosis of neurons in the hippocampus of rats, which was alleviated by the inhibition of miR-1236-3p expression in vivo. The in vitro results also indicated that miR-1236-3p inhibition enhanced neuronal cell viability and prevented apoptosis.

Pim-3 is a member of the proto-oncogene Pim family and is reported to regulate cell survival, proliferation and cell cycle in during tumor development (40). A previous study demonstrated that Pim-3 may inhibit cell apoptosis by upregulating the phosphorylation of Bad and the expression of the anti-apoptotic molecule Bcl-xL (41). In particular, TPT1 can interact with Pim-3 to enhance Pim-3 protein stability (22). A previous study has reported that miR-216b-5p regulates pancreatic cancer progression by regulating TPT1/Pim-3 signaling (42). miR-216b-5p upregulation suppressed Pim-3, p-Bad and Bcl-xL protein expression in pancreatic cancer cells, which was partly reversed by TPT1 upregulation (42). In the present study, the results revealed that the inhibition of miR-1236-3p significantly increased TPT1, Pim-3, p-Bad (Ser112) and Bcl-xL protein expression in hippocampal neurons. In addition, it was demonstrated that TPT1-siRNA transfection reversed the upregulation of Pim-3, p-Bad (Ser112) and Bcl-xL protein expression caused by miR-1236-3p inhibition. Therefore, miR-1236-3p could regulate neuronal cell apoptosis in the hippocampus in a CH rat model by regulating TPT1/Pim-3 signaling.

In conclusion, the present study indicated that miR-1236-3p was upregulated in new born rats with CH. Inhibition of miR-1236-3p protected neurons from apoptosis by upregulating the TPT1/Pim-3 axis. These results indicated that miR-1236-3p may be a new potential therapeutic target for the treatment of CH.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
TM and SS contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. CL and XL contributed to data collection and statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All animal care and experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and approved by the Animal Ethics Committee of the First Hospital of Jilin University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
References


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