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LncRNA profile in Hashimoto's thyroiditis and potential function of NONHSAT079547.2

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Abstract

Hashimoto's thyroiditis (HT) is a common organ-specific autoimmune disease, which develops in 0.3–1.5/1000 subjects annually. The aims of this study were to determine the lncRNA profile in peripheral blood CD4⁺ T cells from HT patients and then to characterize the potential function of NONHSAT079547.2. A total of 37 HT patients and 50 sex- and age-matched healthy controls were enrolled for high-throughput sequencing. Another 43 HT patients and 50 sex- and age-matched controls were enrolled for validation via real-time PCR. Flow cytometry and CCK8 assays were used to measure cell apoptosis and growth levels. Western blotting was used for measuring the expression of growth- and apoptosis-associated proteins. IL-17 serum concentration and transcriptional level in CD4⁺ T cells of participants were detected by ELISA and real-time PCR, respectively. The mechanism of competitive endogenous RNA was determined using real-time PCR, ELISA, RNA immunoprecipitation, and dual-luciferase assays in Jurkat cells. A total of 7564 significantly differentially expressed lncRNAs were found, of which 3913 lncRNAs were upregulated and 3651 lncRNAs were downregulated in HT group when compared to control group. NONHSAT079547.2 was significantly upregulated in HT patients and was positively correlated with serum thyroid peroxidase antibody level. Further studies confirmed that NONHSAT079547.2 could promote cell growth and control IL-17 expression and secretion via the NONHSAT079547.2/miR-4716-5p/IL-17 axis. This is the first study to describe the lncRNA profile in CD4⁺ T cells of HT patients. The studies on the function of NONHSAT079547.2 might elucidate the underlying molecular mechanisms and represent potential biomarkers for HT.

Key Words

- ▶ Hashimoto's thyroiditis
- ▶ long non-coding RNAs
- ▶ NONHSAT079547.2
- ▶ miR-4716-5p
- ▶ IL-17

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Introduction

Hashimoto's thyroiditis (HT) is a common organ-specific autoimmune disease, which develops in 0.3–1.5/1000 individuals each year (Ragusa *et al.* 2019). The prevalence of HT among Caucasians is estimated at 5% and has shown a growing trend (Pyzik *et al.* 2015). It is characterized by a high level of the thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TgAb) in patients'

serum, and patients' goiters may have a large amount of lymphocyte infiltration or even form lymphoid follicles (Antonelli *et al.* 2015). The cells infiltrating the thyroid gland are mainly T cells, especially CD4⁺ T cells, which are directly related to inflammation and thyroid cell destruction (Luty *et al.* 2019). Pro-inflammatory cytokines and the chemokines produced by CD4⁺ T cells directly

lead to thyroid inflammation and recruit more T cells into the thyroid, aggravating the inflammatory response (Li *et al.* 2019b, Luty *et al.* 2019). The destruction of thyroid follicles may lead to hypothyroidism (Antonelli *et al.* 2015). Studies have shown that both environmental and genetic factors can contribute to HT (Guarneri & Benvenaga 2007); however, the detailed pathogenic mechanisms remain unclear.

Long ncRNAs (lncRNAs) are ncRNA molecules greater than 200 nucleotides in length. Most lncRNAs are produced by RNA polymerase II and have a 5'-end cap and a 3'-end poly-A tail (Derrien *et al.* 2012). In early studies, lncRNAs were described as transcriptional noise (Wright & Bruford 2011). However, increasing evidence has shown that lncRNAs can be involved in chromatin remodeling, transcriptional regulation, and post-transcriptional control (Man & Marsden 2019). They can act as 'sponges' for miRNA to control gene expression. LncRNA SNHG15 can protect the oncogene YAP1 from degradation by miR-200a-3p in papillary thyroid carcinoma (Wu *et al.* 2018). LncRNA ZFAS1 can also promote progression of papillary thyroid carcinoma by sponging miR-590-3p (Tong *et al.* 2019). Numerous studies have confirmed that lncRNAs are involved in autoimmune diseases (Wu *et al.* 2015, Aune *et al.* 2017); however, related studies pertaining to HT are rare.

Interleukin-17 (IL-17/IL-17A) is the first cytokine to be discovered from the IL-17 family and is a pro-inflammatory factor that can participate in adaptive and innate immunity (Isailovic *et al.* 2015). It is mainly secreted by CD4⁺ T cells; however, some other cells including macrophages, dendritic cells, natural killer T cells, CD8⁺ T cells, neutrophils, and eosinophils can also produce IL-17 (Li *et al.* 2019b). IL-17 binds to IL-17Rs on the surface of various cells (e.g. fibroblasts, epithelial cells, and endothelial cells) and activates the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) signal pathways to induce the production of various inflammatory factors and chemokines, thereby mobilizing, recruiting, and activating granulocytes and macrophages to reach the site of inflammation and promote T cell proliferation (Fouser *et al.* 2008). Numerous studies have shown that IL-17 is involved in autoimmune diseases such as rheumatoid arthritis, psoriasis, multiple sclerosis, inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE) (Catana *et al.* 2015, Kurte *et al.* 2018, Koga *et al.* 2019, Krueger *et al.* 2019, Mohammadi *et al.* 2019).

The aim of the current study was to detect lncRNA profile in peripheral blood CD4⁺ T cells of HT patients

and healthy controls. In addition, after verifying the differentially expressed lncRNA NONHSAT079547.2, we tried to determine its biological function and possible mechanism that may affect IL-17 expression and participate in the pathological process of HT.

Materials and methods

Subjects

A total of 80 female HT patients were enrolled in the First Hospital of China Medical University from 2017 to 2019. Among them, 37 patients were recruited for high-throughput sequencing and the remaining 43 patients were recruited for real-time PCR verification. The concentrations of free T3 (FT3), free T4 (FT4), thyrotropin (TSH), TPOAb, and TgAb in participants' serum were measured on a Cobas 6000 analyzer (Roche Diagnostic) according to the manufacturer's instructions. All patients had a serum TPOAb concentration greater than 200 IU/mL or were positive for both TPOAb and TgAb (TPOAb >34 IU/mL and TgAb >115 IU/mL). One hundred age-matched female participants without abnormal autoantibodies (TPOAb and TgAb) and a history of chronic inflammatory diseases, thyroid diseases, tumors, and other autoimmune diseases were included in the healthy control group. Peripheral blood samples were obtained from all participants. The clinical information of the participants for sequencing and verification is summarized in Tables 1 and 2, respectively. All participants signed an informed consent form, and all research procedures were approved by the Medical Research Ethics Committee of the First Hospital of China Medical University.

Isolation of CD4⁺ T cells

Peripheral blood mononuclear cells were collected by density-gradient centrifugation using Lymphoprep™ lymphocyte separation solution (Axis-Shield, Norway). Then, a positive-selection magnetic separation system (Miltenyi Biotec, Germany) was used to isolate CD4⁺ T cells. Flow cytometry was used to detect the purity of the isolated CD4⁺ T cells. CD4⁺ T cells with purity greater than 90% were used for subsequent studies.

RNA extraction and high-throughput sequencing

Total RNA of CD4⁺ T cells from the participants was extracted using Trizol reagent (Thermo Fisher) according to the manufacturer's instructions. Nanodrop 2000C

Table 1 Clinical characteristics of Hashimoto's thyroiditis patients and healthy controls for high-throughput sequencing.

	HT (n = 37)	Con (n = 50)	P value	Reference range
Age (years) ^a	46.89 ± 10.36	44.52 ± 11.64	0.33	-
FT3 (pmol/L) ^a	4.68 ± 0.60	4.98 ± 0.52	0.01	3.1–6.8
FT4 (pmol/L) ^a	16.05 ± 1.89	16.27 ± 1.45	0.53	12–22
TSH (mIU/L) ^a	2.40 ± 0.99	1.93 ± 0.92	0.03	0.27–4.2
TgAb (IU/mL) ^b	313.80 (97.13–544.15)	16.67 (12.84–20.83)	<0.01	0–115
TPOAb (IU/mL) ^a	319.96 ± 195.65	12.83 ± 3.98	<0.01	0–34

^aVariables are shown as the mean ± s.d. Comparisons between groups were performed using Student's *t*-test. ^bVariables are shown as median (IQR). Comparisons between groups were performed using Non-parametric Mann–Whitney test.

FT3, free T3; FT4, free T4; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TSH, thyrotropin.

(Nano Drop Technologies, USA) was used to determine RNA concentration. RNA samples with OD260/OD280 values ranging from 1.8 to 2.0 were eligible for experiments. High-throughput sequencing was performed by BGI Genomics with the Illumina Hiseq X-Ten platform. Raw reads were obtained and compared to those in the ribosomal database to filter and remove ribosomal reads by using the short reads alignment tool SOAP1. Up to five mismatches were allowed. Then, clean reads were obtained after filtering out reads containing adapter dimers or reads with more than 50% low-quality bases or more than 10% N bases. Thereafter, clean data were aligned to the Hg19 reference genome by using HISAT software and assembled by StringTie3 software. Only the transcripts with fragments per kilobase of transcript per million reads mapped (FPKM) > 0.5, coverage value > 1 and length > 200 bp were allowed for further quantitative analysis. Differentially expressed transcripts were filtered using DEGseq. A fold change (FC) of > 2 and a *P* value of < 0.05 were considered to indicate a significant difference.

Quantitative real-time PCR

PrimeScript™ RT Master Mix kit (Takara) was used for extracted RNA reverse-transcription and TB Green® Premix Ex Taq™ II kit (Takara) was used for cDNA amplification

on a LightCycler 480 machine (Roche). Specific primers of miRNAs were provided by Ribobio, and the specific primer sequences of other transcripts were as follows: NONHSAT079547.2, 5'-GTGACCACTCTGTCGCCATT-3' (forward); 5'-AGAATGAAGACCAACCGCCC-3' (reverse). IL-17, 5'-GAGATATCCCTCTGTGATCTGG-3' (forward); 5'-GACAGAGTTCATGTGGTAGTCC-3' (reverse). β-actin, 5'-GTCCACCTTCCAGCAGATGTG-3' (forward); 5'-GCATTTGCGGTGGACGAT-3' (reverse). β-Actin served as a reference gene for lncRNA and mRNA detection, U6 was used as a reference gene for miRNA detection. Primer specificity was verified by obtaining melting curves, and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Cell culture and transfection

Jurkat, Clone E6-1 cells (Stem Cell Bank/Stem Cell Core Facility, SCSP-513), were grown in RPMI-1640 medium (Hyclone, USA) with 10% fetal bovine serum (FBS; Biological Industries, Israel). Human embryonic kidney 293T cells (ATCC, CRL-3216) used for the luciferase reporter assay were grown in DMEM-high glucose medium (Hyclone, USA) with 10% FBS. We constructed three shRNA silencing plasmids (Sh1, Sh2, and Sh3; GenePharma, China) to knockdown the lncRNA NONHSAT079547.2 level in the cells. The shRNA

Table 2 Clinical characteristics of Hashimoto's thyroiditis patients and healthy controls for validation.

	HT (n = 43)	Con (n = 50)	P value	Reference range
Age (years) ^a	42.77 ± 12.10	44.54 ± 12.46	0.49	-
FT3 (pmol/L) ^a	4.43 ± 0.57	4.60 ± 0.58	0.15	3.1–6.8
FT4 (pmol/L) ^a	16.02 ± 2.09	16.85 ± 2.18	0.07	12–22
TSH (mIU/L) ^a	2.20 ± 0.90	1.87 ± 0.75	0.06	0.27–4.2
TgAb (IU/mL) ^b	248.80 (136.20–538.80)	17.36 (11.67–22.26)	<0.01	0–115
TPOAb (IU/mL) ^a	265.97 ± 165.24	12.98 ± 4.57	<0.01	0–34

^aVariables are shown as the mean ± s.d. Comparisons between groups were performed using Student's *t*-test. ^bVariables are shown as median (IQR). Comparisons between groups were performed using non-parametric Mann–Whitney test.

FT3, free T3; FT4, free T4; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TSH, thyrotropin.

sequences were as follows: Sh1, 5'-GCCCTCGTTGCT TGTAG AATATTCAAG AGATATTCTACAAGCAACGA GGGCTT-3'. Sh2: 5'-GCACAGGTTTCATGCTCCTTCAT TCAAGAGATGAAGGAGCATGAACCTGTGCTT-3'. Sh3: 5'-GGAATTTCTCATCTTTGACTCTTCAAGAGAGAGTCAA AGATGAGAAATTCCTT-3'. ShRNA negative control: 5'-GTTCTCCGAACGTGTCACGTCAAGAGATTACG TGACACGTTCCGAGAATT-3'. Lipofectamine™ 3000 reagent (Thermo Fisher) was used for shRNA plasmid transfection. To upregulate the level of miRNAs in the cells, miRNA mimics (20 µM, GenePharma, China) was transfected into Jurkat cells with Lipofectamine™ RNAiMAX reagent (Thermo Fisher). The sequences of the miRNA mimics were as follows: miR-30a-3p mimic, sense, 5'-CUUUCAGUCGGAUGUUUGCAGC-3'; antisense, 5'-UGCAAACAUCCGACUGAAAGUU-3'. MiR-4716-5p mimic: sense, 5'-UCCAUGUUUCCUCCCCCUUCU-3'; antisense, 5'-AAGGGGGAAGGAAACAUGGAUU-3'. Furthermore, to downregulate the level of miR-4716-5p in cells, miR-4716-5p inhibitors (20 µM, GenePharma, China) mixed with Lipofectamine™ RNAiMAX reagent were transfected into Jurkat cells. The sequence of the miR-4716-5p inhibitor was 5'-AGAAGGGGGAAGGAAACAUGGA-3'. The sequences of the negative controls were as follows: mimic negative control, sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Inhibitor negative control: 5'-CAGUACUUUUGUGUA GUACAA-3'.

Cell counting kit-8

A cell counting kit-8 (CCK-8; MCE, USA) assay was applied to assess cell growth. Jurkat cells were seeded into 96-well plates at a density of 10000 cells/well and 100 µL of RPMI-1640 medium containing 10% FBS was added to each well. ShRNA-NONHSAT079547.2 and shRNA negative controls were transfected into the wells, respectively. CCK-8 reagent was added to each well at 24, 48, 72, and 96 h after transfection. After incubation at 37°C for 2 h, the absorbance levels at 450 nm were measured.

Flow cytometry

Annexin V FITC Apop Dtec Kit (BD Pharmingen, USA) was used for apoptosis cell detection. Cells were mixed with 100 µL of cell suspension after washing three times with PBS. Thereafter, the cells were incubated with Annexin V-fluorescein isothiocyanate and propidium iodide for 15 min at room temperature in darkness, followed by

addition of 400 µL of Annexin V binding buffer. All the samples were analyzed on a BD LSRFortessa™ instrument (BD Biosciences, USA).

Western blotting

Total proteins in Jurkat cells were isolated by RIPA lysis buffer (Beyotime, China) with 1% phenylmethanesulfonyl fluoride (Beyotime, China) and 2% protease inhibitor (Beyotime, China). The BCA Protein Concentration Assay Kit (Beyotime, China) was used to measure the protein concentration. The protein samples were boiled at 100°C for 5–10 min and then subjected to the electrophoresis in 12% polyacrylamide gel. The protein samples were then transferred to polyvinylidene difluoride membranes (Millipore) electrophoretically. The membranes were blocked with 1.5% BSA for 2 h at room temperature. Then the primary antibodies against human Bcl-xl (1:1000 dilution; Abcam), proliferating cell nuclear antigen (PCNA; 1:1000 dilution; CST, USA), and β-actin (1:100 dilution; Boster, China) were conjugated onto the corresponding epitopes after incubation overnight at 4°C. The primary antibodies were marked using rabbit secondary antibodies (1:5000 dilution; Boster, China) for 2 h. The protein bands were visualized by Pierce™ ECL Western Blotting Substrate (Thermo Fisher) and analyzed using Image J software.

Cytokine detection by enzyme-linked immunosorbent assay (ELISA)

Concentration of IL-17 in the culture supernatant of Jurkat cells and the human serum was determined using the IL-17 ELISA kit (Cloud-Clone Corp, China). The OD values at 450 nm were measured. Standard curves were plotted using absorbance values against the gradient concentrations of the standards. A new standard curve was generated for each experiment.

Subcellular fractionation assay

Nuclear and cytosolic fractions were separated using the Cytoplasmic and Nuclear RNA Purification Kit (Norgen Biotec, Canada) according to the protocol. Approximately 1×10^7 cells were lysed in 200 µL Lysis Buffer J. The cytoplasmic RNA contained in the supernatant and the nuclear RNA contained in the precipitate were separated after maximum-speed centrifugation. The cytoplasmic

RNA and nuclear RNA were bound to spin columns with Buffer SK and 100% ethanol and purified after washing with Washing Solution A, separately. Real-time PCR was performed to detect the cytoplasmic and nuclear RNA levels.

Dual-luciferase reporter assay

The dual-luciferase reporter assay was used to detect the direct binding of NONHSAT079547.2/miR-4716-5p and miR-4716-5p/IL-17 3' UTR. The reporter vector pmiRGLO-NONHSAT079547.2-wild-type, pmiRGLO-NONHSAT079547.2-mutant-type, pmiRGLO-IL-17-wild-type, or pmiRGLO-IL-17-mutant-type containing the putative binding site and mutant type of miR-4716-5p were purchased from GenePharma and named as lnc-547.2-Wt, lnc-547.2-Mt, IL-17-Wt, and IL-17-Mt, respectively. One of the reporter vectors and miR-4716-5p mimics or miR-4716-5p mimic negative controls were co-transfected into HEK 293T cells, which were seeded into a 96-well plate the day before transfection using Lipofectamine™ 3000. Luciferase activity was determined using the Dual-Luciferase® Reporter Assay System (Promega). The renilla luciferase activity was used as the internal reference to normalize the firefly luciferase activities among different groups.

Anti-Ago2 RNA immunoprecipitation

The Magna RIP™ kit (Millipore) was used for RNA immunoprecipitation (RIP) assay according to the protocol. Cells (2×10^7) were lysed using 100 μ L of RIP Lysis Buffer after washing with cold PBS three times. Then the cell lysate was incubated with magnetic beads conjugated with human anti-Ago2 antibody (Abcam) or anti-IgG antibody (in the kit), respectively, rotating overnight at 4°C. After washing six times, the immunoprecipitated RNAs were separated from the magnetic beads by proteinase K digestion. The isolated RNA samples were analyzed by real-time PCR.

Statistical analysis

Normal distribution was verified using the Kolmogorov–Smirnov test. Mean values between two independent variables were compared using the Student's *t*-test. Median values between two independent variables were compared using non-parametric Mann–Whitney tests. Comparisons of mean values among multiple groups were performed

using one-way ANOVA tests. Bivariate correlations between clinical parameters and IL-17 mRNA expression with NONHSAT079547.2 expression were determined by the Pearson rank correlation method. All experiments were performed three times, and $P < 0.05$ was considered to indicate statistical significance. The analysis was based on SPSS 20.0 platform (IBM).

Results

LncRNA expression profile in peripheral blood CD4⁺ T cells in HT

We randomly selected peripheral blood CD4⁺ T cell samples from 37 female HT patients and 50 age-matched female healthy controls for high-throughput sequencing. In comparison with the control group, 75,117 lncRNAs, including known and novel lncRNAs, were differentially expressed in the CD4⁺ T cells of HT patients. After filtering and analyses, 7564 significantly differentially expressed lncRNAs were found, of which 3913 lncRNAs were upregulated and 3651 lncRNAs were downregulated in HT group when compared to the control group. The significantly differentially expressed lncRNAs that were expressed in 80% of the sequencing samples are shown in the heat map according to FPKM levels (Fig. 1). The number of sequencing reads and the percentage of reads aligned to the reference genome are summarized in Supplementary Table 1 (see section on

Figure 1

Heat map representation of selected differentially expressed lncRNAs in CD4⁺ T cells from Hashimoto's thyroiditis patients and healthy controls ($n = 37:50$). Candidate lncRNAs, with a fold change > 2 , $P < 0.05$, which were expressed in more than 80% of the participants.

supplementary materials given at the end of this article). The sequencing data have been submitted to NCBI Sequence Read Archive under the accession number SRP218685.

NONHSAT079547.2 was upregulated in peripheral blood CD4⁺ T cells in HT and was positively correlated with serum TPOAb concentration

We recruited another group of 43 female HT patients and 50 age-matched female healthy controls for validation of the differentially expressed lncRNAs by using real-time PCR. NONHSAT079547.2 showed significantly higher expression in CD4⁺ T cells from HT patients in comparison with the control group ($P < 0.01$; Fig. 2A). In addition, we analyzed the correlations between NONHSAT079547.2 expression and the clinical characteristics of HT patients. Among them, NONHSAT079547.2 expression was positively correlated with serum TPOAb concentration ($P < 0.01$; Fig. 2B), but no associations were noted between NONHSAT079547.2 expression and TgAb, FT3, FT4 or TSH levels ($P > 0.05$ for each; Fig. 2C, D, E and F). The correlations between NONHSAT079547.2 expression and the clinical characteristics of the control group are shown in Supplementary Fig. 1.

Knockdown of NONHSAT079547.2 inhibited cell growth but had no effect on apoptosis

To explore the function of NONHSAT079547.2, three shRNA-NONHSAT079547.2 plasmids (Sh1, Sh2, and Sh3) were designed and transfected into Jurkat cells. The

knockdown efficiency of Sh1, Sh2, and Sh3 in Jurkat cells is shown in Fig. 3A ($P < 0.01$ for each), and Sh1 was selected for further studies. The role of NONHSAT079547.2 in cell growth was detected by the CCK-8 assay. The results showed that knockdown of NONHSAT079547.2 significantly inhibited the growth of Jurkat cells ($P < 0.01$ for 24, 48, 72, and 96 h; Fig. 3B). Then, we performed a flow cytometry assay to detect cell apoptosis level, but there was no difference between the NONHSAT079547.2 knockdown group and the shRNA negative control group (Fig. 3C). In addition, NONHSAT079547.2 knockdown also inhibited the expression of the growth-associated protein PCNA, but no differences were observed in apoptosis-associated protein Bcl-xl between the knockdown group and the negative control group (Fig. 3D).

Knockdown of NONHSAT079547.2 downregulated IL-17 expression and secretion in HT

IL-17 transcriptional level in peripheral CD4⁺ T cells and the serum IL-17 level in HT patients and healthy controls were quantified by real-time PCR and ELISA, respectively. As shown in Fig. 4A and B, IL-17 mRNA expression and IL-17 concentration were both higher in the HT group ($P < 0.01$ for IL-17 mRNA expression and $P < 0.05$ for IL-17 serum concentration). Moreover, the NONHSAT079547.2 expression level was positively correlated with IL-17 mRNA expression ($P < 0.05$; Fig. 4C). Knockdown of NONHSAT079547.2 in Jurkat cells inhibited IL-17 mRNA expression in Jurkat cells ($P < 0.05$, Fig. 4D and Supplementary Fig. 2A) and IL-17 concentration in the culture supernatant ($P < 0.05$, Fig. 4E).

Figure 2

Validation of NONHSAT079547.2 expression by real-time PCR and correlation between NONHSAT079547.2 expression and clinical characteristics of HT patients. (A) Relative expression of NONHSAT079547.2 in peripheral blood CD4⁺ T cells of Hashimoto's thyroiditis patients and healthy controls ($n = 43:50$). β -actin was used as the control housekeeping gene. Data are presented as mean $\pm$ s.d. (B, C, D, E, and F) Correlations between NONHSAT079547.2 expression and serum thyroid peroxidase antibody (TPOAb), thyroglobulin antibody (TgAb), free T3 (FT3), free T4 (FT4), and thyrotropin (TSH) concentrations ($n = 43$). Student's *t*-test was used for comparisons between the HT and control groups. Bivariate correlations between clinical parameters and NONHSAT079547.2 expression were determined by the Pearson rank correlation method. R represents the Pearson correlation efficiency. ** $P < 0.01$.

Figure 3

Knockdown of NONHSAT079547.2 inhibited cell growth but not apoptosis. (A) NONHSAT079547.2 knockdown efficiency after Sh1, Sh2, Sh3, or shRNA negative control transfection in Jurkat cells. The statistical differences were analyzed using one-way ANOVA tests followed by Dunnett's *t*-test. (B) The CCK8 assay was applied to evaluate cell growth after transfection with shRNA-NONHSAT079547.2 or shRNA negative controls in Jurkat cells at 24, 48, 72, and 96 h. (C) Flow cytometry was used to evaluate cell apoptosis after transfection with shRNA-NONHSAT079547.2 or shRNA negative controls in Jurkat cells. (D) Western blotting was used to evaluate the growth- and apoptosis-associated protein expression after transfection with shRNA-NONHSAT079547.2 or shRNA negative controls in Jurkat cells. β-Actin was used as the control housekeeping gene for both mRNA and protein level evaluations. Data are presented as mean ± s.d. The statistical differences of B–D were analyzed using Student's *t*-test. Data were obtained from three independent experiments. Sh1, Sh2, and Sh3 represent the three NONHSAT079547.2 knockdown plasmids. *P < 0.05, **P < 0.01.

Knockdown of NONHSAT079547.2 inhibited IL-17 expression and secretion by upregulating miR-4716-5p

To further explain why knockdown of NONHSAT079547.2 could inhibit IL-17 expression and secretion, we focused on the mechanism of competitive endogenous RNA (ceRNA). Eight miRNAs that could not only target NONHSAT079547.2 but also had binding sites for IL-17 3' UTR were predicted by bioinformatics analysis and target prediction websites (LNCedting and TargetScan)

(Fig. 5A). As for lncRNA subcellular distribution, NONHSAT079547.2 expression was detected by real-time PCR in the nucleus and cytoplasm separately. As a result, NONHSAT079547.2 was found to be distributed in both the nucleus and cytoplasm, and more NONHSAT079547.2 was found in the cytoplasm (Fig. 5B). A real-time PCR assay was applied to confirm whether NONHSAT079547.2 knockdown could upregulate the predicted miRNAs. We found that miR-30a-3p (*P* < 0.05, Fig. 5C) and miR-4716-5p (*P* < 0.05, Fig. 5D and Supplementary Fig. 2B) were upregulated after NONHSAT079547.2 knockdown.

Figure 4

Knockdown of NONHSAT079547.2 inhibited IL-17 expression and secretion. (A) IL-17 mRNA expression in peripheral CD4⁺ T cells of Hashimoto thyroiditis patients and healthy controls (*n* = 43:50) by real-time PCR. (B) Assessment of IL-17 serum concentration in Hashimoto's thyroiditis patients and healthy controls by enzyme-linked immunosorbent assay. (C) Correlation between NONHSAT079547.2 expression and IL-17 mRNA expression (*n* = 43), determined by the Pearson rank correlation method. IL-17 mRNA expression in Jurkat cells (D) and IL-17 concentration in the cell culture supernatant (E) after transfection with shRNA-NONHSAT079547.2 or shRNA negative controls. β-Actin was used as the control housekeeping gene for IL-17 mRNA expression. Data are presented as mean ± s.d., analyzed by Student's *t*-test. Data were obtained from three independent experiments. *P < 0.05, **P < 0.01.

Figure 5

Knockdown of NONHSAT079547.2 upregulated miR-30a-3p and miR-4716-5p. (A) miRNA that could target both NONHSAT079547.2 and IL-17 3' UTR predicted by LNCediting and TargetScan. (B) NONHSAT079547.2 subcellular location in Jurkat cells by real-time PCR. β -Actin and U6 acted as cytoplasm and nucleus markers, respectively. (C) MiR-30a-3p expression after transfection with shRNA-NONHSAT079547.2 or shRNA negative controls in Jurkat cells. (D) MiR-4716-5p expression after transfection of shRNA-NONHSAT079547.2 or shRNA negative controls in Jurkat cells. U6 was used as the internal reference for miRNA expression. Data are presented as mean $\pm$ s.d., analyzed by Student's *t*-test. Data were obtained from three independent experiments. * $P < 0.05$.

Next, miR-30a-3p mimics and miR-4716-5p mimics were transfected into Jurkat cells to upregulate their expression. Eventually, only overexpression of miR-4716-5p could downregulate the expression of IL-17 ($P < 0.01$; Fig. 6D), while overexpression of miR-30a-3p could not reduce the IL-17 mRNA expression ($P > 0.05$; Fig. 6B), which meant that miR-4716-5p could not only target NONHSAT079547.2 but also had binding sites for IL-17. Moreover, miR-

4716-5p downregulation by the miR-4716-5p inhibitors led to higher expression of IL-17 compared to the negative control group ($P < 0.05$; Fig. 6F). In addition, overexpression and knockdown of miR-4716-5p could also downregulate and upregulate the IL-17 concentration in the culture supernatant ($P < 0.01$ for both; Fig. 6G and H). Then, the NONHSAT079547.2 knockdown plasmids, miR-4716-5p inhibitors, and the corresponding negative

Figure 6

Knockdown of NONHSAT079547.2 inhibited IL-17 expression and secretion by targeting miR-4716-5p. MiR-30a-3p overexpression efficiency (A) and IL-17 expression (B) after transfection with miR-30a-3p mimics or mimic negative controls in Jurkat cells. MiR-4716-5p overexpression efficiency (C) and IL-17 expression (D) after transfection with miR-4716-5p mimics or mimic negative controls in Jurkat cells. MiR-4716-5p interference efficiency (E) and IL-17 expression (F) after transfection with miR-4716-5p inhibitors or inhibitor negative controls in Jurkat cells. (G) IL-17 concentration in the Jurkat cell culture supernatant after transfection with miR-4716-5p mimics or mimic negative controls. (H) IL-17 concentration in Jurkat cell culture supernatant after transfection with miR-4716-5p inhibitors or inhibitor negative controls. IL-17 mRNA expression (I) and IL-17 concentration in the culture supernatant (J) after co-transfection with shRNA-NONHSAT079547.2, miR-4716-5p inhibitors, or the corresponding negative controls in Jurkat cells. β -Actin was used as the internal reference for IL-17 mRNA expression. U6 was used as the internal reference for miRNA expression. Data are presented as mean $\pm$ s.d. The statistical differences were analyzed by Student's *t*-test (A, B, C, D, E, F, G, and H) or Dunnett's *t*-test (I and J). Data were obtained from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

controls were co-transfected into Jurkat cells. The IL-17 mRNA expression and the IL-17 concentration in culture supernatant are shown in Fig. 6I and J. Although IL-17 expression and secretion level were upregulated by miR-4716-5p inhibitors, shRNA-NONHSAT079547.2 vectors could restore this effect ($P < 0.01$; $P < 0.05$). In addition, dual-luciferase reporter assays were applied to detect the direct binding of NONHSAT079547.2/miR-4716-5p and miR-4716-5p/IL-17 3' UTR. For the binding association between NONHSAT079547.2 and miR-4716-5p, we found that only lnc-547.2-Wt and miR-4716-5p mimics co-transfecting into Jurkat cells could downregulate luciferase activity, while the luciferase activity of the lnc-547.2-Mt and miR-4716-5p mimics co-transfecting group was the same as that of the negative control ($P < 0.01$ and $P > 0.05$; Fig. 7A). Similarly, overexpression of miR-4716-5p could reduce the luciferase activity of wild-type IL-17 3' UTR, but failed to reduce the luciferase activity of mutant-type IL-17 3' UTR ($P < 0.01$ and $P > 0.05$; Fig. 7B). After confirming that miR-4716-5p could directly target both NONHSAT079547.2 and IL-17 3' UTR, an RNA immunoprecipitation assay was conducted to determine whether NONHSAT079547.2 and miR-4716-5p interacted in an Ago2-dependent manner. The results revealed that

NONHSAT079547.2 and miR-4716-5p were preferentially enriched to Ago2 in comparison with IgG ($P < 0.01$ for both; Fig. 7C and D) and that overexpression of miR-4716-5p could increase the enrichment of NONHSAT079547.2 and miR-4716-5p to Ago2 ($P < 0.01$ for both; Fig. 7E and F).

Discussion

Our research on the prevalence of thyroid disorders in ten Chinese cities showed that the TPOAb- and TgAb-positive rates were 11.5% and 12.6, respectively (Shan *et al.* 2016). Therefore, it is important to identify the pathogenic mechanism underlying HT. In addition, HT is estimated to occur 4–10 times more often in women than men (Ragusa *et al.* 2019). Therefore, we recruited female participants in this study, hoping to find more prominent markers of HT. Less than 3% of the genes in the human genome are known to be protein-coding genes, while over 85% of the genome is transcribed (Hangauer *et al.* 2013). Previous studies have shown that a number of lncRNAs are widely involved in the immune system regulation, including the NF- κ B signal pathway, antiviral responses, immune cell differentiation, and inflammatory responses (Covarrubias *et al.* 2017, Sun *et al.* 2017, Zhang *et al.* 2019,

Figure 7

miR-4716-5p directly bound to NONHSAT079547.2 and 3' UTR of IL-17. (A) The predicted miR-4716-5p binding site of NONHSAT079547.2 and the designed mutant sequence are presented. Dual-luciferase assays were applied in HEK 293T cells transfected with lnc-547.2-Wt, lnc-547.2-Mt, miR-4716-5p mimics, or mimic negative controls. (B) The predicted miR-4716-5p binding site of IL-17 3' UTR and the designed mutant sequence are presented. Dual-luciferase assays were applied in HEK 293T cells transfected with IL-17-Wt, IL-17-Mt, miR-4716-5p mimics, or mimic negative controls. (C) The enrichment level of NONHSAT079547.2 by the Ago2 protein in comparison with IgG via RNA immunoprecipitation assay. (D) The enrichment level of miR-4716-5p by Ago2 protein in comparison with IgG via RNA immunoprecipitation assay. (E) The enrichment level of NONHSAT079547.2 by Ago2 and IgG after transfection with miR-4716-5p mimics or mimic negative controls via RNA immunoprecipitation assay. (F) The enrichment level of miR-4716-5p by Ago2 and IgG after transfection with miR-4716-5p mimics or mimic negative controls via RNA immunoprecipitation assay. Data are presented as mean $\pm$ s.d., analyzed by Student's *t*-test. Data were obtained from three independent experiments. ** $P < 0.01$.

Zhou *et al.* 2019). In addition, emerging evidence has shown that dysregulated lncRNAs play a vital role in the pathogenic processes underlying autoimmune diseases (Sigdel *et al.* 2015, Hur *et al.* 2019). Although lncRNA IFNG-AS1 has been reported to be involved in Th1 cell-associated pathogenic progress in HT (Peng *et al.* 2015), we still know little about the function and mechanism of most lncRNAs in HT. In this regard, we screened the lncRNA expression profile in the peripheral blood CD4⁺ T cells of patients with HT and healthy controls and obtained ~90,000 lncRNAs, of which 7564 lncRNAs were significantly differentially expressed between HT patients and healthy controls. The findings confirmed that in comparison with the control group, lncRNA NONHSAT079547.2 was upregulated in peripheral blood CD4⁺ T cells of patients with HT. We then found that the NONHSAT079547.2 expression was positively correlated with the concentration of TPOAb in serum, but not of TSH, FT3 or FT4. This suggests that an increase in the concentration of autoantibodies may result in an increase in the expression level of NONHSAT079547.2, rather than a hypothyroidism tendency in HT patients.

With the development of high-throughput technology, an increasing number of lncRNAs are being detected (Messemaker *et al.* 2018, Li *et al.* 2019a), but only a small number of lncRNAs have been characterized to date. To determine the function of NONHSAT079547.2, we selected the Jurkat cell line to verify the function of cell growth and apoptosis. We found that knockdown of NONHSAT079547.2 inhibited cell growth. Further studies had shown that apoptosis was not a factor contributing to cell growth.

Previous studies had confirmed a significant increase in IL-17 expression and secretion in patients

with HT (Kotkowska *et al.* 2015, Konca Degertekin *et al.* 2016), which was consistent with our result. Moreover, correlation analyses showed that the expression levels of NONHSAT079547.2 and IL-17 mRNA were positively correlated, which indicated that IL-17 might be a potential target of NONHSAT079547.2. Furthermore, we found that NONHSAT079547.2 can achieve control of IL-17 expression and secretion via the NONHSAT079547.2-miR-4716-5p-IL-17 axis.

The ceRNA mechanism was first reported in 2011 by Pier Paolo Pandolfi *et al.* who described it as regulation of the expression of target genes by competing for miRNAs through base complementation (Salmena *et al.* 2011). Subsequent studies have shown that lncRNAs can function as ceRNAs in the regulation of autoimmune diseases. lncRNA ANRIL was highly expressed in ulcerative colitis and protected TLR4 expression by targeting miR-323b-5p to activate the NF- κ B pathway (Qiao *et al.* 2019). Another lncRNA THRIL could sponge miR-34a to promote MCP-1 expression following activation of the JNK/Wnt pathway in SLE (Deng *et al.* 2018). Using bioinformatics analyses and target gene prediction websites, we predicted eight miRNAs that could not only target NONHSAT079547.2 but also bind to IL-17 3' UTR. The predicted miRNAs were further validated in NONHSAT079547.2-silenced cells. The results showed that miR-30a-3p and miR-4716-5p were upregulated after knockdown of NONHSAT079547.2. However, miR-4716-5p, but not miR-30a-3p mimics transfected into Jurkat cells, could inhibit IL-17 expression and secretion. At the same time, IL-17 expression and secretion were upregulated by miR-4716-5p inhibitors. For further studies, we co-transfected the shRNA-NONHSAT079547.2 plasmids, miR-4716-5p inhibitors, and the corresponding negative controls in Jurkat cells. We found that miR-4716-5p inhibitors promoted IL-17 expression and secretion and that knockdown of NONHSAT079547.2 could resume this effect. This indicated that the decrease in IL-17 expression and secretion after NONHSAT079547.2 silencing was achieved by upregulating miR-4716-5p. The subsequent dual-luciferase assay further demonstrated that miR-4716-5p directly targeted both NONHSAT079547.2 and 3' UTR of IL-17. Furthermore, miR-4716-5p targeted NONHSAT079547.2 in an Ago2-dependent manner.

In summary, lncRNA NONHSAT079547.2, which was significantly upregulated in peripheral blood CD4⁺ T cells in HT, might promote cell growth and serve as a ceRNA to promote IL-17 expression and secretion by sponging miR-4716-5p (Fig. 8). Our study describes the lncRNA profile in CD4⁺ T cells from HT for the first time,

Figure 8

Schematic of the molecular mechanism of NONHSAT079547.2 in HT. NONHSAT079547.2 functions as a competing endogenous RNA to regulate IL-17 expression by sponging miR-4716-5p.

which may prove useful for further studies. The function of NONHSAT079547.2 may provide the molecular mechanisms and represent the potential therapeutic targets and biomarkers of HT.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0239>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Weiping Teng, Tingting Liu, and Jingyi Luo conceived and designed the research. Jingyi Luo was responsible for performing the experiments and writing the manuscript.

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