Increases of microRNA let-7e in peripheral blood mononuclear cells in Hashimoto’s disease

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Abstract. MicroRNA (miRNA) is a family of non-coding RNAs that have important roles in various vital functions. It has been reported that let-7e, a miRNA, may be involved in the regulation of interleukin (IL)-10 production. The purpose of this study was to evaluate the role of let-7e as a regulator of IL-10 production in the pathological processes of autoimmune thyroid diseases (AITDs). We evaluated the association between let-7e expression and intracellular expression of IL-10 in the peripheral blood mononuclear cells (PBMCs) collected from 11 healthy volunteers. Then we investigated the expression levels of let-7e in the PBMCs of 50 patients with Graves’ disease (GD), 42 patients with Hashimoto’s disease (HD) and 28 healthy controls. We found negative correlations between the expression level of let-7e and IL-10 messengerRNA (mRNA) and between the expression level of let-7e and proportion of IL-10+ cells in stimulated PBMCs from healthy volunteers (r = -0.44, p = 0.0267 and r = -0.49, p = 0.0166, respectively). The expression levels of let-7e were significantly increased in HD patients compared with those in GD patients and healthy volunteers (p = 0.0003 and p = 0.0011, respectively). let-7e may be associated with the pathogenesis of HD through the regulation of intracellular IL-10 expression.

Key words: MicroRNA, let-7e, Interleukin-10, Autoimmune thyroid disease, Hashimoto’s disease

AUTOIMMUNE THYROID DISEASES (AITDs) are typically organ-specific autoimmune diseases [1-3]. The pathological processes of AITDs vary among patients. To develop a diagnostic method for predicting the development and prognosis of AITDs, their associations with various immunoregulatory factors, such as cytokines and costimulatory molecules involved in antigen presentation, have been clarified [4-9].

MicroRNA (miRNA) has recently gained attention as another class of molecules that may act as immunoregulatory factors. miRNAs are approximately 22 nucleotides in length and are non-coding RNAs that mediate posttranscriptional silencing of target genes [10]. miRNAs play some roles in maintaining the balance of gene regulatory networks and in determining the fate of cells [11]. A bioinformatic analysis showed that miRNAs can control the expression of one-third of the human proteome [12]. Recent studies have shown that miRNAs act as micromanagers of various stages of immune regulation and are involved in various states of autoimmune diseases, including AITDs, such as Hashimoto’s disease (HD) and Graves’ disease (GD) [13-17].

The miRNA let-7 family was found as miRNAs that regulate cell proliferation and differentiation during development in different species [18]. Recently, let-7e was shown to directly target the 3’ untranslated region of interleukin (IL)-10 genes, leading to the repression of those genes [19-21]. Generally, IL-10 is classified as a Th2 cytokine, and it has anti-inflammatory functions and suppresses Th1 cytokine production [22, 23]. The expression level of let-7e was shown to be increased in infiltrated mononuclear cells in the brain and spinal cord of experimental autoimmune encephalomyelitis (EAE) mice [21]. It was also thought that let-7e modulates the balance of Th1/Th2 cytokines and the development of EAE by targeting IL-10 genes [21]. Therefore, let-7e may be a principal regulator that controls the expression of IL-10 in various physiological and pathological processes, including autoimmune dis-
accordance with the manufacturer’s protocol. cDNAs of IL-10 were generated using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Tokyo, Japan). A StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used to detect and quantify let-7e and IL-10 mRNA. The let-7e expression levels were normalized to U6. The expression levels of IL-10 mRNA were normalized to GAPDH. All reactions were performed in triplicate. The relative expression level of each miRNA was calculated by the 2-ΔΔCt method. We analysed the proportion of IL-10+ cells in the PBMCs, which were incubated with anti-human IL-10 antibody, with a FACS Accuri flow cytometer (Becton Dickinson, San Jose, CA).

**Materials and Methods**

**Samples to evaluate the association between let-7e and IL-10**

To evaluate the association between the expression level of let-7e and intracellular expression of IL-10, we collected peripheral blood samples from 11 healthy volunteers. Six volunteers were 22 years old and 5 volunteers were 23 years old. Written informed consent was obtained from all volunteers. PBMCs were isolated from heparin-treated peripheral blood samples and were isolated by density gradient centrifugation with Lymphoprep® (Axis-Shield PoC AS, Oslo, Norway) and washed in phosphate-buffered saline (PBS). Separated PBMCs were resuspended at 5 × 10^5 to 1 × 10^6 cells/mL in roswell park memorial institute (RPMI) containing 10% fetal bovine serum (FBS). PBMCs were cultured in RPMI containing 10% FBS, 25 ng/mL of phorbol myristate acetate and 1 μg/mL of ionomycin for 4 hours at 37 °C in a humidified atmosphere containing 5% CO₂. After the incubation, total RNA from one portion of the cultured PBMCs was isolated with the mirVana™ PARIS™ Kit (Ambion, Austin, TX, USA) for quantification of let-7e and IL-10 mRNA. Additionally, aliquots of cultured PBMCs were incubated with anti-human IL-10 antibody (eBioscience, San Diego, CA) for 20 minutes following the manufacturer’s protocol to stain the intracellular IL-10. The PBMCs were resuspended in 400 μL PBS.

**let-7e, IL-10 mRNA and IL-10+ cells in the cultured PBMCs**

Reverse transcription of let-7e with total RNA from the cultured PBMCs was performed using a TaqMan® MicroRNA RT Kit and target-specific stem loop primers provided in TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed using the TaqMan® MicroRNA Assays (Applied Biosystems) in accordance with the manufacturer’s protocol. cDNAs of IL-10 were generated using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Tokyo, Japan). A StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used to detect and quantify let-7e and IL-10 mRNA. The let-7e expression levels were normalized to U6. The expression levels of IL-10 mRNA were normalized to GAPDH. All reactions were performed in triplicate. The relative expression level of each miRNA was calculated by the 2-ΔΔCt method. We analysed the proportion of IL-10+ cells in the PBMCs, which were incubated with anti-human IL-10 antibody, with a FACS Accuri flow cytometer (Becton Dickinson, San Jose, CA).

**Samples to evaluate let-7e in AITDs patients**

To evaluate the expression levels of let-7e in PBMCs from AITD patients, we examined the expression levels of let-7e in PBMCs from 50 patients with GD who had a clinical history of thyrotoxicosis with elevated thyroid stimulating hormone receptor antibodies (TRAb), 42 patients with HD who were positive for anti-thyroid microsomal antibody (McAb) and/or thyroglobulin antibodies (TgAb), and 28 healthy volunteers. Among the GD patients, 28 had been treated with methimazole for at least five years and were still positive for TRAb (intractable GD) and 22 had maintained a euthyroid state and had been negative for TRAb for more than two years without medication (GD in remission). Among the HD patients, 26 had developed moderate to severe hypothyroidism before 50 years of age and had been treated with thyroxine (severe HD) and 16 had been untreated and maintained a euthyroid state to over 50 years of age (mild HD). All patients with mild HD had a palpable diffuse goitre. All healthy volunteers were euthyroid and negative for thyroid-specific autoantibodies. All patients and control subjects were Japanese and were unrelated to each other. Written informed consent was obtained from all patients and controls, and the study protocol was approved by the Ethics Committee of Osaka University. The clinical characteristics of the subjects are shown in Table 1. The serum concentration of free thyroxine (FT4) was measured with a commercial radioimmunoassay kit® (Eiken chemical Co., Ltd., Tokyo, Japan). The normal range of serum FT4 is 1.0–1.6 ng/dL (12.9–20.6 pmol/L). The serum concentration of free triiodothyronine (FT3) was measured with a radioimmunoassay kit® (Japan Kodak diagnostic Co., Ltd., Tokyo, Japan).
**Statistical analysis**

The pearson correlation coefficient was used to analyse the associations of the expression levels of let-7e with the age, and with the proportions of intracellular IL-10 expression. We used Student’s *t*-test to analyse the significance of differences in the expression levels of let-7e between the two groups. The data were analysed using JMP9 software (SAS Institute, Inc., Tokyo, Japan). Probability values of less than 0.05 were considered statistically significant.

**Table 1 Clinical characteristics of subjects**

|                      | Graves’ disease | Hashimoto’s disease | Controls |
|----------------------|-----------------|---------------------|----------|
|                      | Intractable     | In remission        | Severe   | Mild   | Controls |
| n (female/male)      | 28 (24/4)       | 22 (19/3)           | 26 (19/7) | 16 (15/1) | 28 (19/9) |
| Age of sampling (years) | 54.5 ± 11.7 (38 - 83) | 53.3 ± 14.7 (26 - 87) | 53.6 ± 15.4 (26 - 83) | 60.3 ± 8.6 (50 - 74) | 51.1 ± 7.6 (35 - 66) |
| Goiter size (cm)     | 5.0 ± 2.5       | 4.4 ± 0.5           | 4.5 ± 1.3 | 4.7 ± 1.3 | n.d.     |
| Free T4 (ng/dL)      | 1.2 ± 0.5       | 1.3 ± 0.2           | 1.3 ± 0.3 | 1.2 ± 0.3 | 1.2 ± 0.2 |
| Free T3 (pg/dL)      | 3.6 ± 2.3       | 2.9 ± 0.3           | 2.7 ± 0.3 | 2.8 ± 0.3 | 2.4 ± 0.2 |
| TSH (μU/mL)          | 1.7 ± 1.4       | 2.4 ± 1.7           | 4.5 ± 5.3 | 2.9 ± 1.9 | 2.2 ± 1.5 |
| TRAb (IU/L)          | 17.2 ± 32.3 (2 - 117) | <1.0               | <1.0  | <1.0  | <1.0  |
| TgAb (2n × 100)      | 3.2 ± 4.1       | 2.6 ± 3.5           | 5.3 ± 3.9 | 3.5 ± 3.3 | Negative |
| McAb (2n × 100)      | 4.0 ± 2.9       | 4.5 ± 3.5           | 4.3 ± 3.4 | 3.2 ± 3.1 | Negative |
| Current treatment    | Methimazole or PTU | L-thyroxine      | L-thyroxine | L-thyroxine | L-thyroxine |
| Current dose of anti-thyroid drug (mg/day) (range) | 13.0 ± 9.3 (2.5 - 30)* | None | None | None | None |
| Current dose of L-thyroxine drug (μg/day) (range) | None | None | 77.3 ± 33.5 (25 - 150) | None | None |

Data are means ± standard deviations. * Dose were expressed as comparable dose of methimazole (50 mg of PTU was converted to 5 mg of methimazole). T4, thyroxine; T3, triiodothyronine; TSH, thyrotropin; TRAb, anti-thyrotropin receptor antibody; TgAb, anti-thyroglobulin antibody; McAb, anti-thyroid microsomal antibody; n.d., not determined; PTU, propylthiouracil.

**Expression level of let-7e inAITDs patients**

For every AITD patient and healthy volunteer, we collected blood samples in EDTA-treated tubes and isolated PBMCs with Lymphoprep® (Axis-Shield PoC AS, Oslo, Norway). PBMCs were washed once in sterile PBS and preserved in RNAlater® solution (Ambion, Austin, TX, USA) at -80°C until use. Total RNA was isolated from preserved PBMCs using the mirVana™ PARIS™ Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer’s protocol. The expression levels of let-7e were detected and quantified by the previous described method.

**Clinical characteristics of the AITDs patients**

The clinical characteristics of the subjects are shown in Table 1. All patients showed normal serum concentrations of FT4, FT3, and TSH. The mean age in each GD group and each HD group did not differ significantly from that of the control subjects. No statistical difference was detected between the mean ages of the two GD groups and those of the two HD groups.

**Association between the expression level of let-7e and intracellular expression of IL-10**

We evaluated the association between let-7e expression and intracellular expression of IL-10 in the PBMCs collected from 11 healthy volunteers. They had no history of thyroid disease and were negative for...
TRAb, thyroid peroxidase antibodies and TgAb. All volunteers were female and were apparently free of any signs or symptoms of infection. As shown in Fig. 1, there was a negative correlation between the expression level of let-7e and IL-10 mRNA (Fig. 1A) and between the expression level of let-7e and the proportion of peripheral IL-10+ cells in PBMCs (Fig. 1B) ($r = -0.44$, $p = 0.0267$ and $r = -0.49$, $p = 0.0166$, respectively).

**Expression level of let-7e in PBMCs of AITD patients**

We investigated the expression levels of let-7e in the PBMCs of 50 patients with GD, 42 patients with HD and 28 healthy controls. The expression level of let-7e was not correlated with age ($r = 0.016$, $p = 0.474$). As shown in Fig. 2, the expression levels of let-7e were significantly higher in HD patients than in GD patients or control subjects ($p = 0.0003$ and $p = 0.0011$, respectively). We found no significant differences in the expression levels of let-7e between GD patients and control subjects. Additionally, as shown in Fig. 3, the expression levels of let-7e were significantly lower in severe HD patients than in mild HD patients ($p = 0.0246$). We found no significant differences in the expression levels of let-7e between the patients of the two GD groups.

![Fig. 1](image1.png) **Fig. 1** Correlation between the expression level of let-7e and the IL-10 mRNA (A) and between the expression level of let-7e and the proportion of IL-10+ cells (B). The Pearson correlation coefficient was used to analyse these correlations.

![Fig. 2](image2.png) **Fig. 2** PBMC let-7e levels in patients with AITDs and control subjects. Student’s $t$-test was used to analyse the significance of differences in the expression levels of let-7e between AITDs and control.

![Fig. 3](image3.png) **Fig. 3** PBMC let-7e levels in patients with AITDs with different prognoses. Student’s $t$-test was used to analyse the significance of differences in the expression levels of let-7e between intractable GD and GD in remission (A) and between severe and mild HD (B).
Discussion

In this study, we revealed that the expression level of IL-10 mRNA and the proportion of peripheral IL-10+ cells in PBMCs was negatively correlated with the expression level of let-7e (Fig. 1). We used quantitative RT-PCR-based approaches to assess miRNA let-7e expression in PBMCs from patients with AITDs. For the first time, we found that the expression levels of let-7e were significantly increased in patients with HD compared in those with GD and healthy volunteers (Fig. 2).

IL-10 is produced by various types of immune cells, such as monocytes/macrophages, neutrophils, mast cells, basophils, natural killer cells and effector T-cell subsets, including Th1, Th2, Th9, Th17 and Treg cells [24]. IL-10 suppresses inflammation by reducing the synthesis of pro-inflammatory Th1 cytokines, such as tumor necrosis factor-α, IL-1, and IL-6, by suppressing the expression of cytokine receptors and by inhibiting the activation of cytokine receptors [22, 23]. In patients with HD, it has been reported that IL-10 synthesis in PBMCs is suppressed and that serum levels of Th1 cytokines are elevated [25, 26]. It would be needed to clarify whether the decreased IL-10 expression and the elevated let-7e expression could induce the production of Th1 cytokines in patients with HD increased or not.

We expected higher expression levels of let-7e in patients with severe HD than in those with mild HD because Th1 cytokine levels may be higher in patients with more severe HD. Contrary to our expectation, the expression levels of let-7e were lower in patients with severe HD than in those with mild HD (Fig. 3). However, this result was consistent with our previous report that the frequency of the CC genotype of the -592 A/C polymorphism, which is associated with higher production of IL-10, was higher in patients with severe HD than in those with mild HD [4] Interestingly, it has been indicated that IL-10, in combination with IL-2, enhances the cytotoxic activity of CD8+ T cells in mice [27, 28]. In addition, IL-10 promotes inflammation in systemic lupus erythematosus (SLE) [29] and accelerates the onset of SLE [30]. Moreover, local production of IL-10 accelerates the onset and increases the prevalence of T cell-dependent autoimmune diabetes [31]. Therefore, IL-10 has been confirmed to play both immunoprotective and immunodestructive roles in diabetes [32]. Because the target tissues of HD and autoimmune diabetes have been shown to be destroyed by cytotoxic T cells [1-3], we hypothesized that local production of IL-10 may accelerate the cytotoxic activity of T cells in the thyroid glands of HD patients and that once thyroid-specific autoimmune destruction occurs, lower expression of let-7e may cause higher production of IL-10 and induce greater destruction of the thyroid glands in patients with severe HD.

Although statistic significances were found in this study, a limitation may be the small sample numbers. As described above, all patients were categorized very strictly with their pathological condition from all registered patients and excluded many obscure cases. In this study, however, we could find statistically significant differences between patients’ groups despite the moderate numbers of samples, and so we think that such differences would be major.

In conclusion, let-7e may negatively regulate the expression of IL-10 and play an important role in regulating the immune response in HD.

Disclosure

The authors declare that no competing financial interests to disclose.

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