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Association of *IFNG* gene methylation in peripheral blood cells with the development and prognosis of autoimmune thyroid diseases

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ABSTRACT

The intractability of Graves' disease (GD) and the severity of Hashimoto's disease (HD) vary among patients. Both genetic and environmental factors may be associated with their prognoses. To clarify the role of methylation of the *IFNG* gene in the pathogenesis and prognosis of (AITDs), we examined interferon gamma (IFNG) methylation levels at various CpG sites and genotyped *IFNG* +874 A/T and +2109 C/T polymorphisms. We analyzed methylation 59 patients with HD, 57 patients with GD and 26 healthy volunteers by pyrosequencing. We genotyped *IFNG* gene polymorphisms from 207 patients with GD, 208 patients with HD, and 102 healthy controls. The methylation levels of *IFNG* −54 CpG were higher in patients with intractable GD than in those with GD in remission, but there was no difference between patients with severe and mild HD. In carriers of *IFNG* +2109 T (CT + TT) (85.5% in controls), the −54 CpG methylation levels were significantly higher in patients with intractable GD than in those with GD in remission. On the other hand, in carriers of *IFNG* +2109 CC, the −4293 CpG methylation levels were higher in intractable GD patients. The methylation levels of *IFNG* −54 CpG and −4293 CpG were negatively correlated with the age in HD, especially severe HD, patients and GD patients, respectively. There was no circadian variation but considerable daily variation in the methylation levels of *IFNG* −54 CpG. In conclusion, both the methylation levels of CpG sites and the functional polymorphisms in the *IFNG* gene were associated with the pathogenesis and prognosis of AITD, especially with GD intractability.

1. Introduction

Autoimmune thyroid diseases (AITDs), such as Graves' disease (GD) and Hashimoto's disease (HD), are typical autoimmune diseases [1,2]. The intractability of GD and the severity of HD varies among patients. However, the intractability of GD and the severity of HD are very difficult to predict at the first diagnosis. Therefore, we have focused on gene polymorphisms as genetic factors to predict the development and prognosis of AITD and have previously shown the significance of several gene polymorphisms [3–7]. However, autoimmune diseases, including AITD, are believed to develop through both genetic and environmental factors [8]. Therefore, it is also important to clarify environmental influences to predict the development and prognosis of AITD more strictly, it is also important to clarify the environmental influences. DNA methylation is an epigenetic mechanism for the regulation of gene expression, which can reflect the effects of environmental factors [9–12]. These findings suggest that alterations in DNA methylation may affect the development and prognosis of AITD.

We previously reported that both thyroid autoantibodies and cytotoxic T cells are independently involved in the disease severity of HD [13]. Interferon-gamma (IFN-γ) activates cytotoxic T cells, increases cell-mediated cytotoxicity [14,15]. We also previously reported that the proportion of T-helper (Th)1 cells were higher in severe HD than in mild HD [16]. Therefore, IFN-γ may be one of major factors influencing the severity of HD. We focused this study on the *IFNG* +874 A/T and +2109 C/T gene polymorphisms, which are associated with the

Abbreviations: AITDs, Autoimmune thyroid diseases; GD, Graves' disease; HD, Hashimoto's disease; CpG, cytosine-phosphate-guanosine; DNMT, DNA methyltransferase; MTRR, methionine synthase reductase; IFN-γ, Interferon-gamma; Th, T-helper; CNS1, conserved noncoding sequence 1; PBMC, peripheral blood mononuclear cells; McAb, anti-thyroid microsomal antibody; TgAb, anti-thyroglobulin antibody; TRAb, anti-thyrotropin receptor antibody; EDTA, ethylenediaminetetraacetic acid; RFLP, restriction fragment length polymorphism; P-values, probability values; CV, coefficient of variance

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production of IFN-γ [17–19]. We have previously reported that the *IFNG* +874 T allele is associated with the severity of HD [20]. Furthermore, methylation of the *IFNG* promoter and the conserved non-coding sequence 1 (CNS1) region are correlated with *IFNG* gene expression [21]. Therefore, we also focused this study on the methylation of cytosine-phosphate-guanosine (CpG)s in the promoter (−54) and CNS1 region (−4399, −4377, −4360, −4325, −4293, −4278 and −4229). Methylation of the *IFNG* −54 CpG inhibits promoter activity by 60–80% in peripheral blood mononuclear cells (PBMC) [22,23]. The CNS1 region possessed functional enhancer activity [24]. These data suggest that the methylation levels in these regions may affect the pathogenesis and prognosis of AITD.

To clarify the role of methylation of the *IFNG* gene in the pathogenesis and prognosis of AITD, we evaluated the methylation levels of eight CpG sites in the *IFNG* promoter and CNS1 regions. Moreover, we analyzed the circadian and daily variations in *IFNG* −54 CpG methylation levels to clarify physiological changes.

2. Material and methods

2.1. Subjects for methylation analysis

We examined 59 patients with HD who were positive for anti-thyroid microsomal antibody (McAb) and/or anti-thyroglobulin antibody (TgAb), 57 patients with GD who had a clinical history of thyrotoxicosis with a positive test for anti-thyrotropin receptor antibody (TRAb), and 26 healthy volunteers who were euthyroid and negative for thyroid autoantibodies (control subjects).

Among the HD patients, 23 developed hypothyroidism before age 50 and were treated with thyroxine (severe HD), and 20 were euthyroid and untreated over 50 years of age (mild HD) and 16 patients who could not be categorized to severe or mild HD groups at the time of sampling. GD patients were also categorized three groups. 24 GD patients had been treated with methimazole for at least 5 years and were still positive for TRAb (intractable GD) and their thyroid function can be controlled to normal as long as they receive medical treatment by anti-thyroid drug. 15 GD patients had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication (GD in remission). 18 GD patients who could not be categorized to intractable GD or GD in remission groups at the time of sampling.

All patients and control subjects were Japanese and were unrelated to each other. Genomic DNA was isolated from ethylenediaminetetraacetic acid (EDTA)-treated whole blood cells with a commercially available kit (QIAamp® DNA Blood Mini Kit; QIAGEN, Tokyo, Japan). Written informed consent was obtained from all patients and controls and the study protocol was approved by the Ethics Committee of Osaka University.

2.2. Evaluation of methylation levels

We evaluated the methylation levels of eight CpG sites in the *IFNG* promoter and CNS1 regions. Genomic DNA was bisulfite treated using the Epitect® Plus DNA Bisulfite Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The primers for polymerase chain reaction (PCR) and the PCR product sequences were designed using Pyrosequencing™ Assay Design Software ver. 2.0 SNP/SQA (QIAGEN, Tokyo, Japan). According to previous studies [25], we carried out a touchdown PCR for the measurement of the −54 CpG methylation level. Detailed information on the primers and PCR conditions are shown in Table 1. After a pretreatment was performed using the PyroMark Q24 Vacuum Workstation (QIAGEN, Tokyo, Japan), the PCR product was sequenced using a PyroMark Q24 pyrosequencer (QIAGEN, Tokyo, Japan). Epitect® PCR methylated and unmethylated control DNA (QIAGEN, Tokyo, Japan) was included in each pyrosequencing assay.

2.3. Correction of measured methylation level

According to previous studies [26], we assessed methylation levels in control DNA (0%, 25%, 50%, 75%, 100% methylated DNA) using pyrosequencing and applied the data to a cubic equation in order to create a calibration curve. Methylation levels measured by pyrosequencing were corrected using this curve.

2.4. Subjects for genotyping

We genotyped *IFNG* gene polymorphisms from 207 patients with GD, 208 patients with HD, and 102 healthy controls. Among them, there were 71 patients with intractable GD, 45 patients with GD in remission, 64 patients with severe HD, and 48 patients with mild HD.

2.5. Genotyping

We used restriction fragment length polymorphism (RFLP) analysis for genotyping the *IFNG* +2109C/T polymorphism. The target sequence was amplified using PCR and the PCR product was digested using restriction enzymes. The forward and reverse primer sequences, the PCR conditions, and the restriction enzymes used are summarized in Table 2. The TaqMan® SNP Genotyping Assay (Applied Biosystems, Tokyo, Japan) was used to genotype the *IFNG* +874 A/T polymorphism.

2.6. Subjects for analyzing circadian and daily variations

We analyzed diurnal and daily variations in the *IFNG* −54 CpG methylation level in 13 healthy controls, including 6 males (23.2 ± 2.5 years old) and 7 females (23.3 ± 1.4 years old).

2.7. Circadian and daily variations in IFNG −54 CpG methylation level

To evaluate the daily variation in *IFNG* −54 CpG methylation, we collected blood samples between 8:30 and 9:00 am, before meals, every day over the course of 10 weeks. On 3 days within the 10 weeks, blood was also collected three times a day, before meals (at 8:30–9:00, 12:30–13:00, and 16:30–17:00), to evaluate the circadian rhythm of *IFNG* −54 CpG methylation. Methods used in these analyses were mentioned above, however, the methylation levels of samples from the same person were measured on the same plate to assess intraindividual variation.

2.8. Statistical analysis

We used Tukey-Kramer's HSD test to analyze the differences in methylation level among the subject groups. The χ^2 test was used to evaluate the significance of differences between the genotype and allele frequencies among the respective groups. We used paired t tests to analyze the differences in circadian rhythm and the daily variations in −54 CpG methylation. Bonferroni correction was applied to the probability values (P-values) of the circadian rhythm and daily variations for multiple comparisons. Data were analyzed with JMP Pro13 software (SAS Institute Inc., Tokyo, Japan). P-values of less than 0.05 were considered significant.

3. Results

3.1. Age at sampling of the subjects for methylation analysis

The mean age ± SD of each subject group was as follows: GD patients, 52.1 ± 14.3 years old (intractable GD, 52.4 ± 12.7 years old and GD in remission, 54.2 ± 13.8 years old); HD patients, 57.8 ± 15.0 years old (severe HD 54.2 ± 13.7 years old and mild HD 68.2 ± 11.1 years old); control subjects, 49.0 ± 10.5 years old. The

Table 1
Primers and PCR conditions used in pyrosequencing.

	Primers	PCR conditions
−54	F: 5′-GAATGGTATAGGTGGGTATAATGG-3′ R: 5′-biotin-TCAAAACAATATACTACACCTCCTCTA-3′ S: 5′-ATTATTTTATTTTAAAAAATTTGTG-3′	94 °C for 15 min {94 °C for 30 s, 64 °C for 30 s(−0.5 °C/cycle), 72 °C for 30 s} × 20 cycles {94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s} × 25 cycles 72 °C for 10 min
−4399, −4377, −4360, −4325	F: 5′-GTATAAAAGAAAAGGGGGGATTTAGA-3′ R: 5′-biotin-CCTAACACTCACAACCAAATTATCT-3′ S: 5′-ATTTAGAAAAATTTTTTTTTAAT-3′	94 °C for 15 min {94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s} × 45 cycles 72 °C for 10 min
−4293, −4278, −4229	F: 5′-GTATAAAAGAAAAGGGGGGATTTAGA-3′ R: 5′-biotin-CCTAACACTCACAACCAAATTATCT-3′ S: 5′-TGTATGATGTTAGGAGTTT-3′	94 °C for 15 min {94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s} × 45 cycles 72 °C for 10 min

age at sampling was significantly different between HD patients and control subjects ($p = 0.0292$) and was significantly higher in patients with mild HD than in those with intractable GD, GD in remission, severe HD and control subjects ($p = 0.0037$, $p = 0.0478$, $p = 0.0159$, and $p = 0.0002$, respectively).

3.2. Methylation of IFNG −54 CpG

There were no significant differences in the −54 CpG methylation levels between patients with GD or HD and control subjects (Supplemental Table 1). However, the methylation levels were significantly higher in patients with intractable GD (90.5 ± 8.7%) than in those patients with GD in remission (80.2 ± 9.0%) ($p = 0.0203$) (Fig. 1A and Supplemental Table 2). Furthermore, the methylation levels were significantly lower in patients with mild HD (79.4 ± 8.4%) than in control subjects (88.6 ± 12.0%) ($p = 0.0209$) (Fig. 1A and Supplemental Table 2). However, the methylation level was not different between patients with severe HD (54.2 ± 13.7 years old) (86.8 ± 10.6%) and those with mild HD (68.2 ± 11.1 years old) (79.4 ± 8.4%) (Fig. 1A and Supplemental Table 2), or between patients with severe HD (63.7 ± 9.0 years old) (81.7 ± 9.6%) and mild HD (68.2 ± 11.1 years old) (79.4 ± 8.4%) (Fig. 1B) when we excluded patients with severe HD that were less than 50 years old to match the ages between those with severe and mild HD.

The −54 CpG methylation levels were negatively correlated with age at sampling in HD patients, especially in patients with severe HD (HD: $r = -0.36$, $p = 0.0067$; severe HD: $r = -0.45$, $p = 0.0306$) (Fig. 2A and B). However, there was no correlation between methylation levels and methimazole dose (Fig. 3).

3.3. Methylation of the IFNG CNS1 region

The methylation levels of individual CpGs within the CNS1 region (−4399, −4377, −4360, −4325, −4293, −4278, and −4229 CpG) were not significantly associated with the development and prognosis of AITD (Supplemental Tables 1 and 2).

Correlations between the methylation levels of CpGs within the CNS1 region and the age at sampling are shown in Supplemental Tables 3 and 4 and Fig. 4. Methylation levels of −4399, −4293, −4278, and −4229 CpG were negatively correlated with the age at sampling (Fig. 4). Methylation levels of −4377, −4360 and −4325 CpG were not correlated with the age at sampling (Supplemental Tables 3 and 4). There was no correlation between methylation levels of individual CpGs within the CNS1 region and methimazole dose (Fig. 3).

3.4. IFNG polymorphisms

The frequency of the *IFNG* +874 TT genotype, which is related to higher IFN-γ secretion, was significantly higher in GD patients than in healthy controls ($p = 0.0326$). The frequency of the *IFNG* +2109 CC genotype, which is related to lower IFN-γ secretion, was significantly higher in GD patients than in healthy controls ($p = 0.0237$). However, we could not find any significant differences in the distribution of this polymorphism between the controls and HD patients. There were no significant differences in the genotype or allele frequencies of this polymorphism between patients with intractable GD and GD in remission, or between those with severe HD and mild HD (Supplemental Tables 5 and 6).

3.5. Association of IFNG methylation levels with IFNG +874 A/T polymorphism

In both subjects with the *IFNG* +874 T carrier (AT + TT) and AA genotype, methylation levels of the *IFNG* gene were not involved in the development and prognosis of AITD (Supplemental Tables 7 and 8).

3.6. Association of IFNG methylation levels with the IFNG +2109 C/T polymorphism

In subjects with the *IFNG* +2109 TT genotype, the methylation level of −4360 CpG was significantly lower in GD patients (82.8 ± 3.8%) than in controls (88.3 ± 3.8%) ($p = 0.0261$) (Supplemental Table 9). However, there were no significant changes in the methylation levels of the other CpG sites in GD and HD patients with the *IFNG* +2109C carrier or TT genotype (Supplemental Table 9). Furthermore, there were no significant difference in the methylation levels of all CpG sites examined between patients with intractable GD and GD in remission, and between patients with severe and mild HD in both subjects with the *IFNG* +2109C carrier and with the TT genotype (Supplemental Table 10). Also, there were no significant changes in the methylation levels of all CpG sites examined in GD and HD patients with the *IFNG* +2109 T carrier or +2109 CC genotype (Supplemental

Table 2
Primers, restriction enzymes, and PCR conditions used in genotyping.

	Primers	PCR conditions	Enzyme
+2109 C/T	F: 5′-TTCATCACAGTTCCTTGGTG-3′ R: 5′-CCAGTAAGAGAATCGCTGAAG-3′	94 °C for 5 min (94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s) × 35 cycles 72 °C for 7 min	*AciI*
+874 A/T	TaqMan® SNP Genotyping Assays (Applied Biosystems, Tokyo, Japan)		

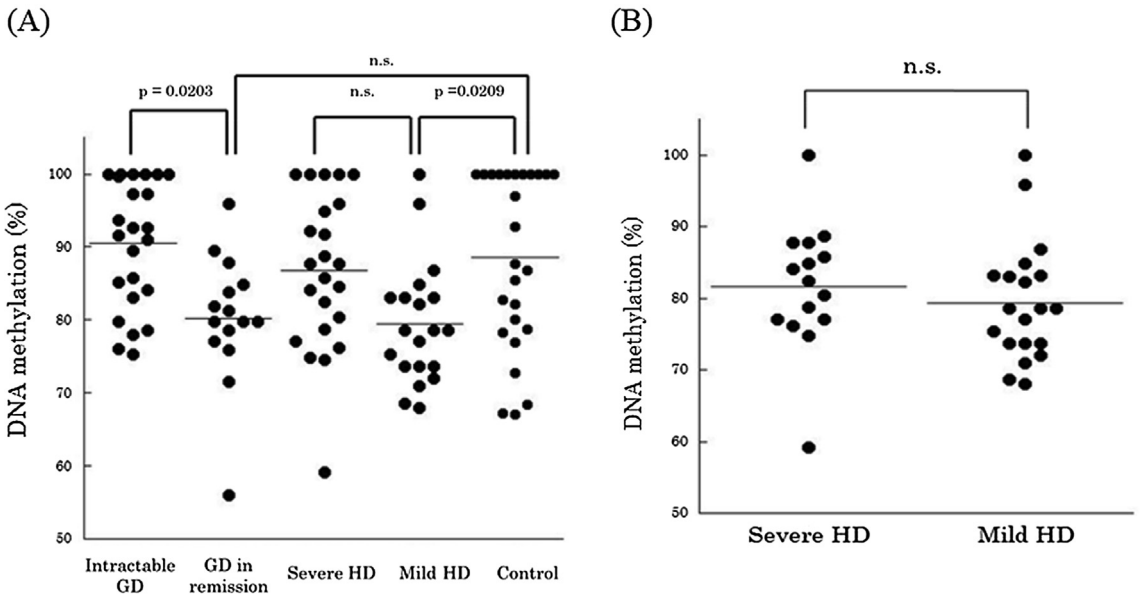

Fig. 1. Methylation levels of *IFNG* −54 CpG in GD patients with varied intractability, HD patients with different severities and control subjects (A), and in HD patients with different severities with age-matched samples (B).

Table 11). Moreover, in subjects carrying the *IFNG* +2109 T carrier (CT + TT) (85.5% in control subjects), the methylation level of −54 CpG was significantly higher in patients with intractable GD (90.0 ± 9.0%) than in those with GD in remission (78.2 ± 8.5%) (p = 0.0182) (Supplemental Table 12). In the subjects with the *IFNG* +2109 CC genotype, the methylation level of −4293 CpG was significantly higher in patients with intractable GD (95.5 ± 4.5%) than in those with GD in remission (82.1 ± 16.0%) (p = 0.0376) (Supplemental Table 12).

3.7. Circadian variation in IFNG −54 CpG methylation levels

There were no significant differences in the −54 CpG methylation levels when we compared between samples taken at 9:00 and 13:00 (p = 0.659), between samples taken at 13:00 and 17:00 (p = 0.088), or between samples taken at 9:00 and 17:00 (p = 0.178) (Fig. 5).

3.8. Daily variation in IFNG −54 CpG methylation levels

The daily variations in *IFNG* −54 CpG methylation levels are shown in Fig. 6. We excluded samples taken on days in which the subjects showed clinical evidence of inflammation. The mean coefficient of variance (CV) in the daily variation of methylation was 2.2% (range of 0.7–4.0%), and the mean daily intraindividual variation in the methylation level during the entire 10 weeks was 6.2% (range of 1.8–12.0%).

4. Discussion

Although we could not find any significant difference in the circadian variation of −54 CpG methylation levels (Fig. 5), we observed an average value of 6.2% daily variance in the methylation levels of the same subjects (Fig. 6). In previous studies, the methylation level of −54 CpG was also altered by approximately 10% compared to samples from the same subjects over 4–7 days [27]. These data indicate that it is

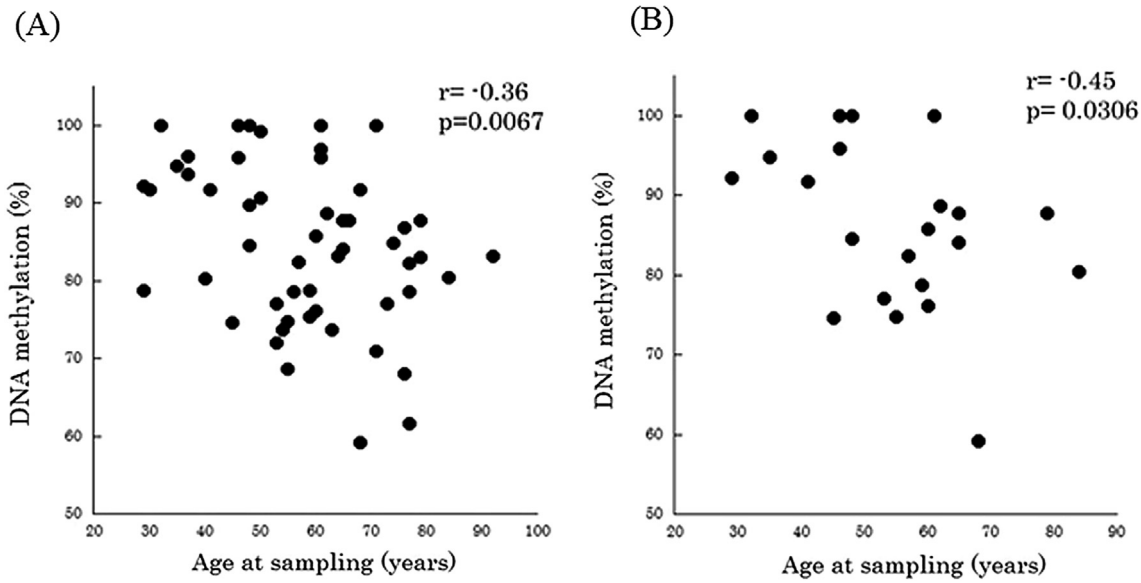

Fig. 2. Correlation between the methylation level of *IFNG* −54 CpG and the age at sampling in (A) all HD patients and (B) patients with severe HD.

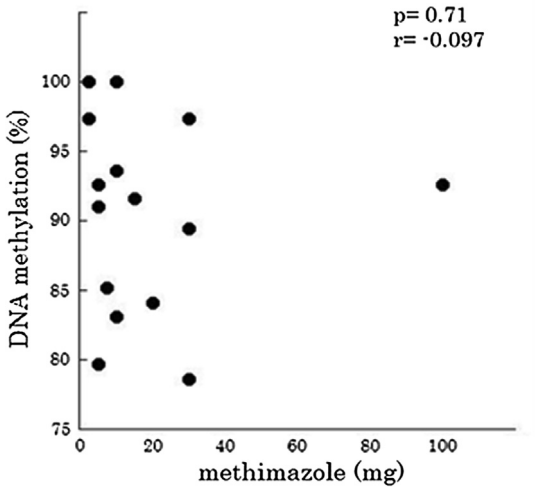

Fig. 3. Correlation between the methylation level of *IFNG* −54 CpG and the dose of methimazole in patients with intractable GD.

important to consider the daily intraindividual variances in methylation levels when we analyze the significance of *IFNG* methylation.

We expected that *IFNG* methylation in patients with severe HD, which is related to a genetically higher production of IFN-γ (23), would be lower than in those patients with mild HD because high methylation of the *IFNG* gene suppresses gene expression. Contrary to our expectation, however, the methylation level of −54 CpG was not lower in patients with severe HD compared to those with mild HD. The methylation level was not significantly different between patients with severe and mild HD (Fig. 1A and Supplemental Table 2). In particular, when we age-matched the patient groups at the time of sampling (severe HD: 63.7 ± 9.0 years old, mild HD: 68.2 ± 11.1 years old) the methylation levels of −54 CpG were almost the same between severe and mild HD patients (Fig. 1B). Therefore, we suggest that epigenetic effects on IFN-γ production may be minor in determining the severity of HD.

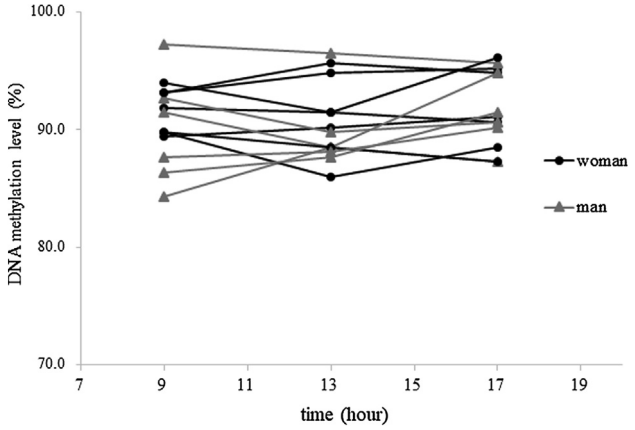

Fig. 5. Circadian variation in *IFNG* −54 CpG methylation level in control subjects.

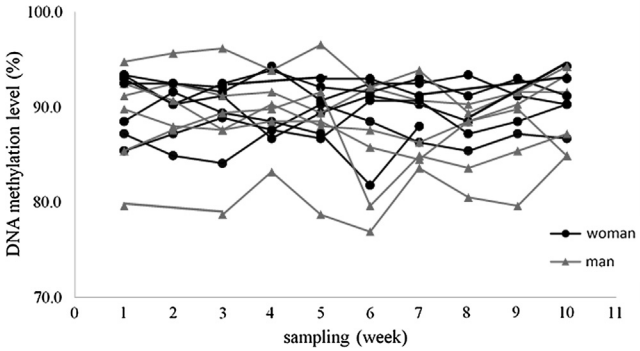

Fig. 6. Daily variations in *IFNG* −54 CpG methylation level in control subjects over 10 weeks.

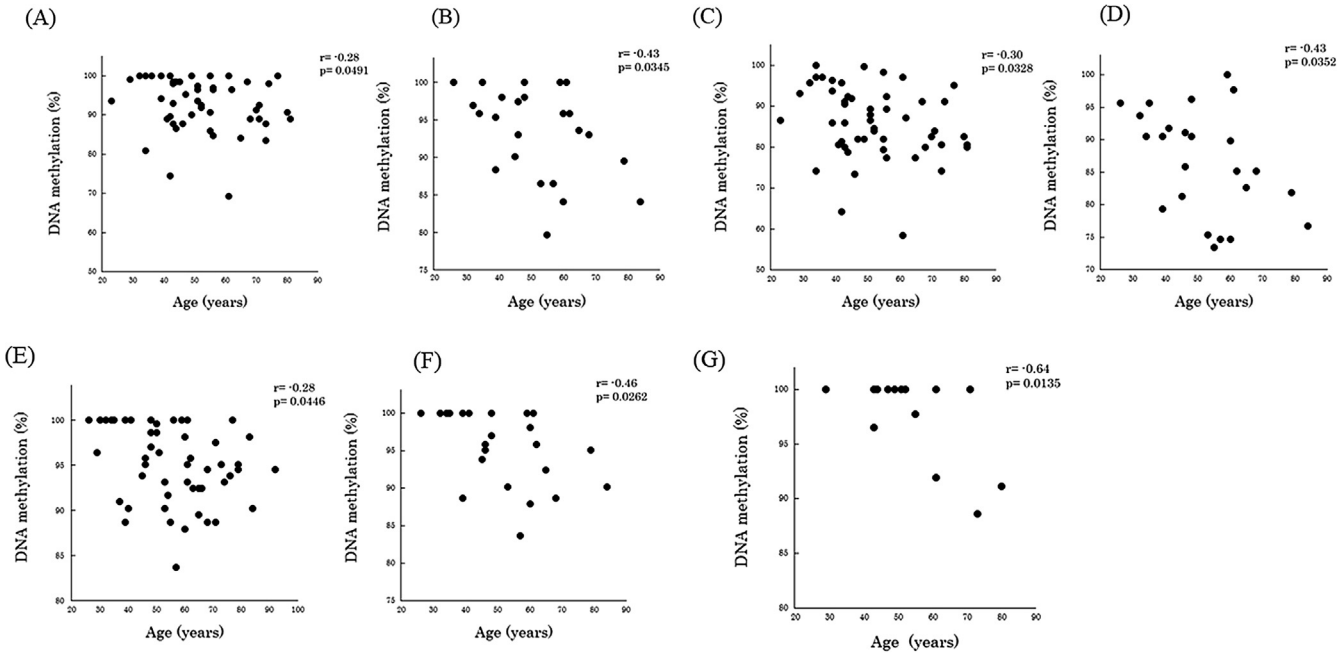

Fig. 4. Correlation between methylation rates of the *IFNG* CNS1 region (−4293, −4278, −4229 CpG) and the age at sampling. Correlation between methylation of −4293 CpG and the age at sampling in (A) all GD patients and (B) patients with severe HD. Correlation between methylation of −4278 CpG and the age at sampling in (C) all GD patients and (D) patients with severe HD. Correlation between methylation of −4229 CpG and the age at sampling in (E) all HD patients and (F) patients with severe HD. Correlation between methylation of −4399 CpG and the age at sampling in (G) patients with GD in remission.

We also found that, in HD patients, there was a negative correlation between the methylation level of this site and the age at sampling, especially in patients with severe HD (Fig. 2A and Supplemental Table 3, Fig. 2B and Supplemental Table 4). In this regard, it has been shown that *IFNG* methylation is decreased in adults compared to neonates [28,29] and that the methylation of −54 CpG is lower in adults with allergic asthma compared to children [30]. Therefore, IFN-γ production, which is related to the cytotoxicity of T cells, may increase with the age of HD patients. This is compatible with the high incidence of hypothyroidism in middle-aged and elderly patients with HD (2).

On the other hand, the −54 CpG methylation level was significantly higher in patients with intractable GD compared to those with GD in remission (Fig. 1A and Supplemental Table 2), and this difference was 10.3%, which was over the average daily variance (6.2%). These data suggest that higher methylation levels in patients with intractable GD may be important. We could not find any correlations between methylation levels and methimazole dosage (Fig. 3), suggesting that methimazole may not have any effect on the methylation of −54 CpG. These data indicate that high methylation of this site may be associated with the intractability of GD. We have previously shown that a high proportion of Th17 cells is related to the intractability of GD [16] and that Th17 differentiation is suppressed by IFN-γ [31]. We suppose, therefore, that higher methylation levels of −54 CpG may suppress the expression of IFN-γ that is followed by an enhancement of Th17 differentiation, and thereby, GD patients may become intractable. These data suggest that the methylation of −54 CpG may be an important epigenetic factor in the intractability of GD.

The methylation levels of each CpG site in the CNS1 region were not significantly associated with the development and prognosis of AITD (Supplemental Tables 1 and 2). Although the demethylation of CNS1 is associated with Th1 differentiation and IFN-γ expression (24), our data suggested that, compared to the −54 CpG site, CNS1 may not be essential in the regulation of IFN-γ expression. Similar to *IFNG* −54 CpG methylation, the methylation levels of −4293, −4278 and −4229 CpG were negatively correlated with the age at sampling in AITD patients (Fig. 4A, C, E and Supplemental Table 3), especially in patients with severe HD (Fig. 4B, D, F and Supplemental Table 4). The methylation level of −4399 CpG was also negatively correlated with the age at sampling in patients with GD in remission (Fig. 4G and Supplemental Table 4), but this may not reliable because of the small number of samples. Thus, we suggest that the methylation of −4293, −4278 and −4229 CpG may also be affected by age.

In this study, we also found that the *IFNG* +2109 CC genotype, which is related to lower IFN-γ secretion, was associated with the development of GD (Supplemental Table 5). This suggests that reduced IFN-γ production may promote Th17 differentiation and thereby may influence GD development because Th17 cells are increased in AITD, especially in GD [16]. In addition, it has been shown that the T allele of the *IFNG* +874 A/T polymorphism is associated with higher IFN-γ production (21). Therefore, we expected that the methylation levels of the *IFNG* gene may be higher in intractable GD patients with the T allele of this polymorphism (which correlates with higher IFN-γ production) because these GD patients become intractable despite their genetically high production of IFN-γ, which in turn suppress Th17 differentiation (35). However, in subjects carrying the *IFNG* +2109 TT genotype, which have a high production of IFN-γ, the methylation level of −4360 CpG in GD patients was significantly lower than in control subjects (Supplemental Table 9). These findings suggest that methylation of −4360 CpG may have an unknown role in the development of GD, especially in individuals with a genetically higher production of IFN-γ. Because the differences in the methylation of −4360 CpG were 4.1% in +874 T carriers and 5.5% in those with the +2109 TT genotype and both were less than the mean daily variation in *IFNG* methylation (6.2%), we considered that these differences may not always be significant or a major factor in the development of GD. Moreover, in the subjects with carrying the *IFNG* +2109 T carrier (CT + TT), which correlates with a high production of IFN-γ, the methylation level of −54 CpG in patients with intractable GD was significantly higher than in those with GD in remission (Supplemental Table 12). In the subjects with the *IFNG* +2109 CC genotype, which is associated with low IFN-γ production, the methylation level of −4293 CpG was significantly higher in patients with intractable GD than in those with GD in remission (Supplemental Table 12). These −54 CpG and −4293 CpG methylation results may be reliable because they were higher than the mean daily variation (6.2%), although the −4293 CpG results may be less reliable because of the small number of samples. We considered that the methylation of −54 CpG may be important for the prognosis of GD under specific genetic backgrounds and suggest that the higher methylation levels of −54 CpG in the subjects with a high production of IFN-γ may suppress IFN-γ expression and promote Th17 differentiation, thereby causing GD patients to become intractable despite their genetically high production of IFN-γ.

There were limitations in this study. The sample numbers of intractable GD, GD in remission, severe HD and mild HD may not large enough in this study. These were weakness of this study. However, this is because we categorized patients very strictly and excluded many obscure cases. This policy of categorize is the strength of this study. In this study, 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. We could not subdivide patients further for clinical manifestations to ascertain IFN-γ mediated effect because of insufficient sample numbers for reliable analysis. As shown in Supplemental Tables 3 and 4, and Figs. 2 and 4, the methylation levels of *IFNG* CpG sites were negatively correlated with age at sampling. Therefore, we supposed that younger patients tended to be higher methylation patterns.

5. Conclusions

Both the methylation levels of CpG sites and the functional polymorphisms in the *IFNG* gene were associated with the pathogenesis and prognosis of AITD, especially with GD intractability. Additionally, there was no circadian variation but considerable daily variation in *IFNG* methylation levels (6.2 ± 2.8%).

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154770.

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