Methylation levels of the *IL10* gene in peripheral blood are related to the intractability of Graves' disease

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Abstract

The prognosis of autoimmune thyroid diseases (AITDs), including Hashimoto's disease (HD) and Graves' disease (GD), is difficult to predict. DNA methylation regulates gene expression of immune mediating factors. Interleukin (IL)-10 is a Th2 cytokine that downregulates inflammatory cytokines produced by Th1 cells. To clarify the role of methylation of the *IL10* gene in the prognosis of AITD, we evaluated the methylation levels of two CpG sites in the *IL10* promoter using pyrosequencing. The methylation levels of the -185 CpG site of the *IL10* gene were related to age and GD intractability in GD patients. Furthermore, the C carrier of the *IL10*-592 A/C polymorphism was related to low methylation levels of the -185 CpG site. The methylation levels of the *IL10*-185 CpG site of the *IL10* gene were related to the intractability of GD and were lower in individuals with the C allele of the *IL10*-592 A/C polymorphism.

Keywords

IL-10, polymorphism, methylation, pyrosequencing, severity, intractability, autoimmune thyroid disease

1. Introduction

Autoimmune thyroid diseases (AITDs), including Hashimoto's disease (HD) and Graves' disease (GD), are archetypes of organ-specific autoimmune diseases [1]. GD is the common cause of hyperthyroidism, which is characterized by the presence of anti-thyrotropin receptor antibodies (TRAbs) and the infiltration of lymphocytes into the thyroid tissue. HD is characterized by the infiltration of lymphocytes into the thyroid tissue, the destruction of thyroid follicles, and subsequent hypothyroidism [2, 3]. The prognosis, which is the intractability of GD and the severity of HD, varies from patient to patient and is very difficult to predict at the time of diagnosis. Some patients with HD develop hypothyroidism earlier in life, whereas some maintain a euthyroid state even in old age. Some patients with GD achieve remission through medical treatment, while others do not. Therefore, we focused on gene polymorphisms as genetic factors to predict the development and prognosis of AITD and showed the significance of several gene polymorphisms [4-7]. However, autoimmune diseases, including AITDs, are believed to be caused by both genetic and environmental factors [8, 9]. In a twin study, it was recently reported that the heritability of AITD development is approximately 70-80% and that environmental factors contribute to the pathogenesis of AITD by approximately 20-30%

[9]. Therefore, it is important to examine environmental influences to predict the development and prognosis of AITDs more accurately.

DNA methylation is an epigenetic mechanism that regulates gene expression and can reflect environmental factors [10]. It mainly occurs at cytosine residues in cytosine-phosphate-guanosine (CpG) dinucleotides and forms 5-methylcytosine (5-mC) [11]. DNA methylation is established and maintained by enzymes, including DNA methyltransferases (DNMTs) and methionine synthase reductase (MTRR), and we have already reported that polymorphisms in the *DNMT1* and *MTRR* genes are involved in the prognosis of AITD [4]. DNA methylation plays a role in regulating gene expression by inhibiting the binding of transcription factors [12]. Several studies have reported an association of altered DNA methylation with some diseases, such as cancer and autoimmune diseases [13, 14]. We have also already shown the methylation levels of some cytokine genes are related to the development and prognosis of AITD [15-17].

Interleukin (IL)-10 was identified as a Th2 cytokine and it downregulates inflammatory cytokines produced by Th1 cells [18, 19]. Dysregulation of IL-10 expression is associated with various immune-related diseases, such as cancer, rheumatoid arthritis (RA), asthma, and infectious disorders [20]. We have already reported that the *IL10*-592A/C polymorphism, which affects IL-10 levels, is associated

with the severity of HD [21].

Methylation of the *IL10* promoter region is correlated with promoter activity [22, 23]. In this study, we focused on methylation of two CpG sites in IL10 promoter regions (-110, -185). *IL10*-110CpG is the binding site of transcription factors such as STAT3 [22], and *IL10*-185CpG is near the binding site of CCAAT/enhancer binding protein (C/EBP-β) [22]. High methylation levels of these two CpG sites play important roles in suppressing the activity of the *IL10* promoter [22]. It was also reported that hypomethylation of *IL10*-110CpG enhanced the mRNA expression of IL-10 in RA patients [24]. These results suggest that the methylation levels in the promoter region may be associated with IL-10 production and affect the pathogenesis and prognosis of AITD.

To clarify the epigenetic regulation of the *IL10* gene with the pathogenesis and prognosis of AITD, we evaluated the methylation levels of these two CpG sites in the *IL10* promoter using pyrosequencing. In addition, we also analyzed the methylation of these CpG sites in combination with genotypes of the *IL10*-592A/C polymorphism, which are genetic factors affecting IL-10 expression.

2.Materials and methods

2.1 Subjects for genotyping and methylation analysis

We examined 26 patients with HD who were positive for anti-thyroid microsomal antibody (McAb) and/or anti-thyroglobulin antibody (TgAb). Forty-three patients with GD had a clinical history of thyrotoxicosis with a positive test for anti-thyrotrophin receptor antibody (TRAb), and 13 healthy volunteers were negative for thyroid autoantibodies (control subject). Among the HD patients, 13 patients developed hypothyroidism before 50 years of age and were treated with thyroxine (severe HD), and 13 patients were euthyroid and untreated over 50 years of age (mild HD). Among GD patients, 22 patients had been treated with methimazole for at least 5 years and were still positive for TRAb (intractable GD), and 21 patients had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication (GD in remission).

All subjects were Japanese and unrelated. Written informed consent was obtained from all subjects, and the study protocol was approved by the Ethics Committee of Osaka University (564).

2.2 DNA extraction and measurement of methylation levels

Genomic DNA was isolated from whole blood with a commercially available kit (QIAamp® DNA Blood Mini Kit, QIAGEN, Tokyo, Japan). Isolated genomic DNA was treated with bisulfite using an Epitect® Plus DNA Bisulfite Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The primers for polymerase chain reaction (PCR) and sequencing of the PCR product were designed using Pyrosequensing™ Assay Design Software ver.2.0 SNP/SQA (QIAGEN, Tokyo, Japan). Detailed information on the primers and PCR conditions is shown in Table 1A. The PCR product was sequenced using a PyroMark Q24 pyrosequencer (QIAGEN, Tokyo, Japan) after pretreatment performed by a PyroMark Q24 Vacuum Workstation (QIAGEN, Tokyo, Japan). Epitect® PCR methylated and unmethylated control DNA (QIAGEN, Tokyo, Japan) were included in each pyrosequencing assay. Measurement of the methylation levels was performed in duplicate, and the methylation level was adopted when the difference between the two measured values was less than 5%.

2.3 Correction of measured methylation level

We evaluated methylation levels in five control DNA samples (0%, 25%, 50%, 75% and 100% methylated DNA) using pyrosequencing and prepared calibration curves. Methylation levels of isolated DNA samples measured by pyrosequencing were corrected

using this curve.

2.4 Genotyping

We used restriction fragment length polymorphism (RFLP) analysis for genotyping the *IL10*-592A/C polymorphism. The target sequence was amplified using PCR. The PCR product was digested by the addition of restriction enzyme. The sequences of the forward and reverse primers, PCR conditions, and restriction enzymes used are summarized in Table 1B.

2.5 Statistical analysis

We conducted Tukey Kramer's HSD test on subjects' age, free T3, free T4 and TSH levels, and the sex balance with Fisher's exact test. Dunnett's test was used to analyze the methylation levels of each group compared with control subjects. Comparisons between two disease conditions were conducted with Student's t-test. P values of less than 0.05 were considered significant. Multiple regression analysis was performed to minimize AIC. The data were analyzed with JMP13 software (SAS Institute Inc., Tokyo, Japan).

3. Results

3.1 Clinical Characteristics

The clinical characteristics of the subjects are shown in Table 2. Among subjects for -185CpG, the age at sampling was significantly higher in patients with mild HD than in patients with intractable GD (p=0.0364). The proportion of males in the control subjects was significantly higher than that in the other groups.

3.2 Correlation between age and methylation levels

The methylation levels of the -185 CpG site were positively correlated with the age at sampling for all measured subjects (r=0.26, p=0.0167) (Fig. 1). The methylation level of the -110CpG site was not correlated with any of the clinical characteristics.

3.3 Differences in the methylation levels among the patient groups

We could not find any significant differences between the two examined CpG sites among the groups (Table 3). The methylation levels of the -185CpG site in patients with intractable GD were significantly higher than in patients with GD in remission, but only in patients with a C carrier of -592 A/C (p=0.0331) (Table 4).

3.4 Multiple regression analysis

Because there was a positive correlation between age and the methylation level of the -185 CpG site (Fig. 1) and there was an association of the methylation level of the -185 CpG site with the intractability of GD in subsets of subjects with a C carrier of *IL10*-592 A/C (Table 4), we conducted multiple regression analysis using age, the -592 A/C genotype and clinical condition as explanatory variables. Age showed a significant positive effect on the methylation levels of the -185 CpG site in GD patients (Tables 5A and 5C). Patients with intractable GD showed a significant positive effect on the methylation levels of the -185 CpG site (Table 5C). The C carrier of *IL10*-592 A/C showed a negative effect on the methylation levels of the -185 CpG site (Tables 5B and 5C).

4. Discussion

We have previously reported that the CC genotype of the *IL10*-592A/C polymorphism, which is associated with higher expression of IL-10, is more frequent in patients with severe HD than in patients with mild HD [21]. Based on this study, we first hypothesized that the methylation level of the *IL10* promoter CpG was lower in severe HD patients than in patients with mild HD because IL-10 expression is higher in individuals showing lower methylation. However, we could not find any significant differences in the methylation level of the two examined CpG sites in the *IL10* gene promoter region between the control subjects and the patient groups or between the patient groups with different pathological conditions (Table 3). Although this will be a difficult matter, since there was a positive correlation between age and methylation level at the -185 CpG site (Fig. 1, Table 5), we suggest that aging may influence the methylation levels of the -185 CpG site.

In GD patients, although we could not find any differences in methylation levels at the -110 CpG and -185CpG sites by univariate analysis (Table 3) and multiple regression analysis (Table 5A), the methylation levels of the -185 CpG site were significantly higher in intractable GD patients than in GD patients in remission (Table

5C). Because the methylation levels in this promoter region of the *IL10* gene are negatively correlated with IL-10 production [22], we supposed that IL-10 production might be downregulated in intractable GD patients, who showed high methylation levels, and upregulated in patients with GD in remission. It has been reported that IL-10 suppresses the production of IL-6 [25], which promotes Th17 differentiation [26], and we have already reported that the proportion of Th17 cells in patients with intractable GD is higher than that in those with GD in remission [27]. Therefore, we suggest that in intractable GD patients, who show hypermethylation of the -185 CpG site and may show lower IL-10 expression, Th17 differentiation may be promoted, and those patients may become intractable. On the other hand, in patients with GD in remission, who show hypomethylation of the -185 CpG site, Th17 differentiation may be suppressed, and those patients may be achieve a prolonged remission.

In HD patients, we could not find any association even after multiple regression analysis. This indicates that methylation of these CpG sites might have minor roles in the development and prognosis of HD, although the patients with severe HD showed genetically high producibility of IL-10, as mentioned above [21].

Interestingly, we showed that the methylation levels of the -185 CpG site were significantly lower in the C carrier of -592 A/C than in those with the AA genotype (Table

5B). It has been reported that the *IL10*-592 C allele is associated with high IL-10 production in vitro [28, 29]. These results indicate that the *IL10*-592 C allele may influence IL-10 production via methylation of the -185 CpG site.

5. Conclusions

The methylation levels of the IL10-185 CpG site were related to the intractability of GD. Methylation levels were lower in individuals with the C allele of the IL10-592 A/C polymorphism. Our results suggest that methylation levels of *IL10* gene may be a useful new biomarker for the prognosis of autoimmune diseases.

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6. References

[1] A.A. Sinha, M.T. Lopez, H.O. McDevitt, Autoimmune diseases: the failure of self tolerance, Science (New York, N.Y.), 248 (1990) 1380-1388

[2] F. Menconi, Oppenheim, YL. , Tomer, Y., Graves' disease, in: Y. Shoenfeld, Cervera, R.,Gershwin, ME. (Ed.) Diagnostic Criteria in Autoimmune Diseases, Humana Press, Totowa, NJ, 2008, pp. 231-235.

[3] A.P. Weetman, Chlonic autoimmune thyroiditis, in: L.E. Braverman, R. Utiger (Eds.) The Thyroid: a Fundamental and Clinical Text., Lippincott Williams & Wilkins, Philadelphia, 2000, pp. 721-732.

[4] Y. Arakawa, M. Watanabe, N. Inoue, M. Sarumaru, Y. Hidaka, Y. Iwatani, Association of polymorphisms in DNMT1, DNMT3A, DNMT3B, MTHFR and MTRR genes with global DNA methylation levels and prognosis of autoimmune thyroid disease, Clin Exp Immunol, 170 (2012) 194-201.10.1111/j.1365-2249.2012.04646.x

[5] N. Inoue, M. Watanabe, M. Morita, K. Tatusmi, Y. Hidaka, T. Akamizu, Y. Iwatani, Association of functional polymorphisms in promoter regions of IL5, IL6 and IL13 genes with development and prognosis of autoimmune thyroid diseases, Clin Exp Immunol, 163 (2011) 318-323.10.1111/j.1365-2249.2010.04306.x

[6] F. Hayashi, M. Watanabe, T. Nanba, N. Inoue, T. Akamizu, Y. Iwatani, Association of the -31C/T functional polymorphism in the interleukin-1beta gene with the intractability of Graves' disease and the proportion of T helper type 17 cells, Clin Exp Immunol, 158 (2009) 281-286.10.1111/j.1365-2249.2009.04034.x

[7] T. Nanba, M. Watanabe, T. Akamizu, Y. Iwatani, The -590CC genotype in the IL4 gene as a strong predictive factor for the development of hypothyroidism in Hashimoto disease, Clin Chem, 54 (2008) 621-623

[8] F. Coppede, Epigenetics and Autoimmune Thyroid Diseases, Front Endocrinol (Lausanne), 8 (2017) 149.10.3389/fendo.2017.00149

[9] T.H. Brix, L. Hegedus, Twin studies as a model for exploring the aetiology of autoimmune thyroid disease, Clin Endocrinol (Oxf), 76 (2012) 457-464.10.1111/j.1365-2265.2011.04318.x

[10] R.L. Jirtle, M.K. Skinner, Environmental epigenomics and disease susceptibility, Nat Rev Genet, 8 (2007) 253-262.10.1038/nrg2045

[11] X. Zhang, J. Yazaki, A. Sundaresan, S. Cokus, S.W. Chan, H. Chen, I.R. Henderson, P. Shinn, M. Pellegrini, S.E. Jacobsen, J.R. Ecker, Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis, Cell, 126 (2006) 1189-1201.10.1016/j.cell.2006.08.003

[12] V. Muthusamy, M. Bosenberg, N. Wajapeyee, Redefining regulation of DNA methylation by RNA interference, Genomics, 96 (2010) 191-198

[13] A. Portela, M. Esteller, Epigenetic modifications and human disease, Nat Biotechnol, 28 (2010) 1057-1068.10.1038/nbt.1685

[14] K.S. Son, H.S. Kang, S.J. Kim, S.Y. Jung, S.Y. Min, S.Y. Lee, S.W. Kim, Y. Kwon, K.S. Lee, K.H. Shin, J. Ro, Hypomethylation of the interleukin-10 gene in breast cancer tissues, Breast, 19 (2010) 484-488.10.1016/j.breast.2010.05.011

[15] E. Morita, M. Watanabe, N. Inoue, H. Hashimoto, E. Haga, Y. Hidaka, Y. Iwatani, Methylation levels of the TNFA gene are different between Graves' and Hashimoto's diseases and influenced by the TNFA polymorphism, Autoimmunity, 51 (2018) 118-125.10.1080/08916934.2018.1448078

[16] N. Hirai, M. Watanabe, N. Inoue, R. Kinoshita, H. Ohtani, Y. Hidaka, Y. Iwatani, Association of IL6 gene methylation in peripheral blood cells with the development and prognosis of autoimmune thyroid diseases, Autoimmunity, 52 (2019) 251-255.10.1080/08916934.2019.1669568

[17] H. Hashimoto, M. Watanabe, N. Inoue, N. Hirai, E. Haga, R. Kinoshita, Y. Hidaka, Y. Iwatani, Association of IFNG gene methylation in peripheral blood cells with the development and prognosis of autoimmune thyroid diseases, Cytokine, 123 (2019)

154770.10.1016/j.cyto.2019.154770

[18] M.G. Roncarolo, R. Bacchetta, C. Bordignon, S. Narula, M.K. Levings, Type 1 T regulatory cells, Immunol Rev, 182 (2001) 68-79

[19] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu Rev Immunol, 19 (2001) 683-765

[20] K.N. Couper, D.G. Blount, E.M. Riley, IL-10: the master regulator of immunity to infection, J Immunol, 180 (2008) 5771-5777

[21] N. Inoue, M. Watanabe, M. Wada, M. Morita, Y. Hidaka, Y. Iwatani, IL-10 -592A/C polymorphism is associated with severity of Hashimoto's disease, Cytokine, 64 (2013) 370-374

[22] A. Szalmas, F. Banati, A. Koroknai, B. Laszlo, E. Feher, D. Salamon, L. Gergely, J. Minarovits, J. Konya, Lineage-specific silencing of human IL-10 gene expression by promoter methylation in cervical cancer cells, Eur J Cancer, 44 (2008) 1030-1038.10.1016/j.ejca.2008.02.046

[23] S. Alipour, M. Nouri, A. Khabbazi, N. Samadi, Z. Babaloo, S. Abolhasani, J. Farhadi, N. Roshanravan, G. Jadideslam, E. Sakhinia, Hypermethylation of IL-10 gene is responsible for its low mRNA expression in Behcet's disease, J Cell Biochem, 119 (2018) 6614-6622.10.1002/jcb.26809

[24] L.H. Fu, C.L. Ma, B. Cong, S.J. Li, H.Y. Chen, J.G. Zhang, Hypomethylation of proximal CpG motif of interleukin-10 promoter regulates its expression in human rheumatoid arthritis, Acta Pharmacol Sin, 32 (2011) 1373-1380.10.1038/aps.2011.98

[25] S.A. Robertson, A.S. Care, R.J. Skinner, Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice, Biology of reproduction, 76 (2007) 738-748.10.1095/biolreprod.106.056143

[26] E. Volpe, N. Servant, R. Zollinger, S.I. Bogiatzi, P. Hupe, E. Barillot, V. Soumelis, A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses, Nat Immunol, 9 (2008) 650-657.10.1038/ni.1613

[27] T. Nanba, M. Watanabe, N. Inoue, Y. Iwatani, Increases of the Th1/Th2 cell ratio in severe Hashimoto's disease and in the proportion of Th17 cells in intractable Graves' disease, Thyroid, 19 (2009) 495-501

[28] D.M. Turner, D.M. Williams, D. Sankaran, M. Lazarus, P.J. Sinnott, I.V. Hutchinson, An investigation of polymorphism in the interleukin-10 gene promoter, European journal of immunogenetics : official journal of the British Society for Histocompatibility and Immunogenetics, 24 (1997) 1-8

[29] C.J. Edwards-Smith, J.R. Jonsson, D.M. Purdie, A. Bansal, C. Shorthouse, E.E. Powell, Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa, Hepatology (Baltimore, Md.), 30 (1999) 526-530.10.1002/hep.510300207

7. Abbreviations

Autoimmune thyroid diseases: AITD

Hashimoto’s disease: HD

Graves’ disease: GD

5-methylcytosine: 5-mC

DNA methyltransferases: DNMTs

methionine synthase reductase: MTRR

methyl-binding domain proteins: MBDs

histone deacetylases: HDACs

Interleukin: IL

CCAAT/enhancer binding protein: C/EBP-β

Rheumatoid Arthritis: RA

anti-thyroid microsomal antibody: McAb

anti-thyroglobulin antibody: TgAb

anti-thyrotrophin receptor antibody: TRAb

polymerase chain reaction: PCR

Figure Captions

Fig. 1

Correlation between age at sampling and the methylation levels of the *IL10* -185 CpG site

Figure

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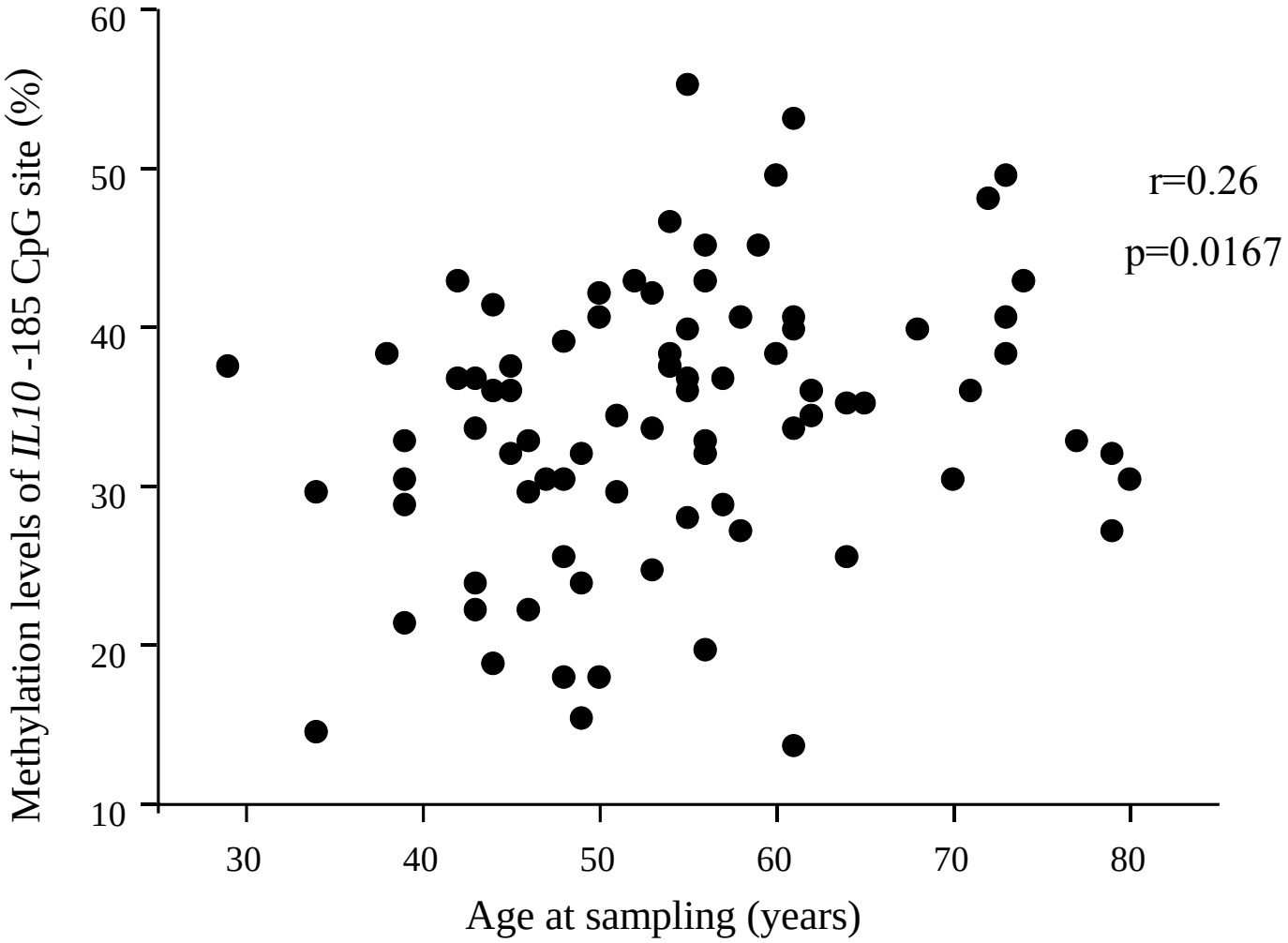

Table 1. Primers and PCR conditions used in pyrosequencing and RFLP analysis

(A) Pyrosequencing

CpG	Primers	PCR conditions
-185	F: 5'-biotin-AGGAAATTAATTTTTTTTAATTGAGAAC R: 5'-CCCCTAATATATAAACCTTCACCT-3' S: 5'-ATTCATTAAAAAACCACAATCA-3'	95°C for 15 min (94°C for 30 s, 53.8°C for 30 s, 72°C for 30 s) × 45 cycle 72°C for 10 min
-110	F: 5'-biotin-AGGAAATTAATTTTTTTTAATTGAGAAC R: 5'-CCCCACACAAAATTTTTTCTACTTAAAACT-3' S: 5'-CCTCTCTAATAAACTTAATTTTCA-3'	95°C for 15 min (94°C for 30 s, 53.8°C for 30 s, 72°C for 30 s) × 45 cycle 72°C for 10 min

F: forward primer, R: reverse primer, S: sequencing primer

(B) RFLP analysis

polymorphism	Primers	PCR conditions	Enzyme
-592 A/C	F: 5'-TCCAGCCACAGAAGCTTACAAC-3' R: 5'-AGGTCTCTGGGCCTTAGTTTCC-3'	94°C for 5 min (94°C for 30 s, 64°C for 45 s, 72°C for 60 s) × 35 cycle 72°C for 10 min	*RsaI*

F: forward primer, R: reverse primer

Table 2
Clinical characteristics of GD and HD patients and control subjects measured the methylation levels of each CpG sites of *IL10* gene

CpG		GD			HD			Control
		All	Intractable	In remission	All	Severe	Mild	
-110	n	29	15	14	26	13	13	13
	gender (male/female)	5 / 24	3 / 12	2 / 12	5 / 21	3 / 10	2 / 11	10 / 3
	age	53.6 ± 12.2	51.7 ± 11	55.6 ± 14	57.8 ± 12	53.8 ± 11	61.8 ± 11	54.1 ± 6
	FreeT4 (ng/dL)	1.27 ± 0.33	1.27 ± 0.4	1.28 ± 0.1	1.35 ± 0.4	1.46 ± 0.4	1.17 ± 0.3	ND
	FreeT3 (pg/mL)	2.76 ± 0.45	2.94 ± 0.5	2.49 ± 0.1	2.78 ± 0.2	2.71 ± 0.1	2.98 ± 0.3	ND
	TSH (μIU/mL)	2.09 ± 2.09	2.48 ± 2.5	1.43 ± 0.8	2.59 ± 1.7	2.56 ± 1.9	2.64 ± 1.3	ND
-185	n	43	22	21	26	13	13	13
	gender (male/female)	5 / 38	3 / 19	2 / 19	5 / 21	3 / 10	2 / 11	10 / 3
	age	51.9 ± 11.8	50.6 ± 10 *	53.3 ± 13	57.8 ± 12	53.8 ± 11	61.8 ± 11 *	54.1 ± 6
	FreeT4 (ng/dL)	1.28 ± 0.31	1.26 ± 0.4	1.30 ± 0.2	1.35 ± 0.4	1.46 ± 0.4	1.17 ± 0.3	ND
	FreeT3 (pg/mL)	2.75 ± 0.45	2.87 ± 0.5	2.51 ± 0.1	2.78 ± 0.2	2.71 ± 0.1	2.98 ± 0.3	ND
	TSH (μIU/mL)	1.95 ± 1.80	2.32 ± 2.2	1.39 ± 0.9	2.59 ± 1.7	2.56 ± 1.9	2.64 ± 1.3	ND

Values represent mean ± SD (%); Analyzed by Tukey Kramer's HSD-test; *: the age was significantly higher in patients with mild HD than in patients with intractable GD (p=0.0364); ND: not determined

Table 3 Methylation levels (%) of each CpG site of *IL10* gene in GD and HD patients and control subjects

CpG	GD	HD	GD		HD		Control
			Intractable	In remission	Severe	Mild	
-110	19.3 ± 6.2	16.6 ± 5.6	20.2 ± 6.4	18.4 ± 6.2	16.7 ± 6.2	16.6 ± 5.3	17.3 ± 5.3
-185	33.7 ± 9.9	33.7 ± 8.1	36.3 ± 8.7	31.1 ± 10.5	32.9 ± 9.1	34.5 ± 7.2	35.4 ± 5.6

Values represent mean ± SD (%); analyzed by Dunnett tests *versus* control; analyzed by Student's t-test about severe HD versus mild and intractable GD and GD in remission

Table 4 Methylation levels (%) of each CpG site of *IL10* gene in GD and HD patients and control subjects with the AC or CC genotype of *IL10* -592A/C polymorphism and in those with the AA genotype.

CpG	-592A/C	GD	HD	GD		HD		Control
				Intractable	In remission	Severe	Mild	
-110	AC + CC	18.7 ± 6.5	15.8 ± 5.6	21.1 ± 7.1	15.7 ± 4.5	15.9 ± 5.6	15.6 ± 5.9	16.8 ± 3.5
	AA	20.0 ± 6.3	18.3 ± 5.6	18.3 ± 6.1	21.2 ± 6.7	17.8 ± 7.6	18.8 ± 2.7	17.7 ± 6.8
-185	AC + CC	32.1 ± 11.5	32.1 ± 6.7	36.2 ± 10.7*	25.4 ± 10.0	32.1 ± 7.2	32.1 ± 6.6	33.8 ± 6.2
	AA	35.2 ± 8.2	36.8 ± 9.9	36.2 ± 5.6	34.6 ± 9.6	34.2 ± 12.3	40.1 ± 5.7	36.9 ± 5.1

Values represent mean ± SD (%); analyzed by Dunnett tests *versus* control; analyzed by Student's t-test about severe HD versus mild HD and intractable GD and GD in remission; *: significantly higher than GD in remission (p=0.0331)

Table 5 Multiple regression analysis in the effect of age, disease category, pathological conditions of GD or HD and genotype of *IL10* -592 A/C polymorphism on the methylation levels of each CpG site of *IL10* gene

(A)

CpG	age	GD	C carrier
-110	n.a.	n.a.	n.a.
-185	0.24 / 0.28	n.a.	n.a.

Values represent partial regression coefficient / β value; n.a.: not association; reference of disease conditions is control subjects; reference of genotype of *IL10* -592 A/C is AA genotype

(B)

CpG	age	HD	C carrier
-110	n.a.	n.a.	n.a.
-185	n.a.	n.a.	-4.3 / -0.30

Values represent partial regression coefficient / β value; n.a.: not association; reference of disease conditions is control subjects; reference of genotype of *IL10* -592 A/C is AA genotype

(C)

CpG	age	Intractable GD	C carrier
-110	n.a.	n.a.	n.a.
-185	0.27 / 0.33	6.93 / 0.35	-4.65 / -0.23

Values represent partial regression coefficient / β value; n.a.: not association; reference of disease prognosis is GD in remission; reference of genotype of *IL10* -592 A/C is AA genotype

(D)

CpG	age	Severe HD	C carrier
-110	n.a.	n.a.	n.a.
-185	n.a.	n.a.	n.a.

Values represent partial regression coefficient / β value; n.a.: not association; reference of disease prognosis is mild HD; reference of genotype of *IL10* -592 A/C is AA genotype