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Association of functional polymorphisms in promoter regions of *IL5*, *IL6* and *IL13* genes with development and prognosis of autoimmune thyroid diseases

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Summary

To clarify the association of genetic producibility of interleukin (IL)-5, IL-6 and IL-13, which are secreted by T helper type 2 (Th2), with the development and prognosis of autoimmune thyroid disease (AITD), we genotyped *IL5* −746C/T, *IL6* −572C/G and *IL13* −1112C/T polymorphisms, which are functional polymorphisms in the promoter regions of the genes regulating these cytokines. Fifty-seven patients with intractable Graves' disease (GD), 52 with GD in remission, 52 with severe Hashimoto's disease (HD), 56 with mild HD and 91 healthy controls were examined in this study. The *IL13* −1112T allele, which correlates with higher producibility of IL-13, was more frequent in patients with GD in remission than in those with intractable GD [$P = 0{\cdot}009$, odds ratio (OR) = 3·52]. The *IL5* −746T allele, which may correlate with lower levels of IL-5, was more frequent in patients with GD in remission than controls ($P = 0{\cdot}029$, OR = 2·00). The *IL6* −572G allele carriers (CG and GG genotypes), which have higher producibility of IL-6, were more frequent in AITD patients ($P = 0{\cdot}033$, OR = 1·75), especially in GD in remission ($P = 0{\cdot}031$, OR = 2·16) and severe HD ($P = 0{\cdot}031$, OR = 2·16) than in controls. Interestingly, both allele and genotype frequencies of Th2 cytokine genes were similar between GD and HD patients. In conclusion, functional polymorphisms in the genes encoding Th2 cytokines are associated differently with the development and prognosis of AITD from each other.

Keywords: IL-13, IL-5, IL-6, intractability, severity, single nucleotide polymorphism

Introduction

Autoimmune thyroid disease (AITD), such as Hashimoto's disease (HD) and Graves' disease (GD), are archetypes of organ-specific autoimmune disease [1,2]. The severity of HD and the intractability of GD, which are very difficult to predict, vary among patients. Some patients develop hypothyroidism early in life, and some maintain a euthyroid state in old age despite the passage of time [3,4]. Some patients with GD achieve remission through medical treatment, while others do not. We have reported previously on an association between the genetic producibility of some cytokines and the prognosis of AITD [5,6]. We showed that the CC genotype of *IL4* −590C/T polymorphism, which correlates with lower levels of interleukin (IL)-4 produced by T helper type 2 (Th2) cells, and the T allele of +874A/T polymorphism in the interferon (IFN)-γ gene, which correlates with higher levels of IFN-γ produced by T helper type 1 (Th1) cells, were both associated with development of hypothyroidism in HD [5,6]. These findings suggest that HD patients with genetically higher levels of Th1 cytokine and/or lower levels of Th2 cytokine may develop hypothyroidism caused by autoimmune destruction of thyroid follicles. Therefore, we hypothesized that the functional polymorphisms in the genes encoding other Th2 cytokines, such as IL-13, IL-5 and IL-6, may be associated with the pathogenesis and prognosis of AITD.

Among these cytokines, IL-13 is a major cytokine involved in immunoglobulin (Ig)E synthesis and is associated with Th2-mediated disease [7,8]. The T allele of the −1112C/T polymorphism in the *IL13* gene (rs1800925) correlates more with higher promoter activity than the C allele [9]. One report showed that this polymorphism was associated with susceptibility to GD [10], while others showed no association [11,12]. Therefore, the association between the *IL13* −1112C/T polymorphism and susceptibility to GD remains unclear.

IL-5 plays an important role in the growth of eosinophils and the C allele of the −746C/T polymorphism in the *IL5* gene (rs2069812), and is associated with the pathogenesis of some allergic diseases [13–15]. Because IL-5 is also elevated in the peripheral blood of AITD patients and some allergies have been reported to aggravate GD [16], it is possible that the *IL5* −746C/T polymorphism may be associated with the prognosis of AITD, especially in GD.

IL-6 is an inflammatory cytokine produced by various cells such as lymphocytes and monocytes. In GD patients, serum levels of anti-thyrotrophin receptor antibody (TRAb) correlated with the numbers of peripheral T cells bound to IL-6 [17]. In Japanese patients with type 2 diabetes, the −572G allele of the *IL6* −572C/G polymorphism (rs1800796) was related to elevated production of IL-6 by peripheral blood mononuclear cell *in vitro* [18]. These findings suggest that the *IL6* −572C/G polymorphism is functional and may be related to the prognosis of AITD.

In this study, we examined the distribution of alleles and genotypes of these functional polymorphisms in Japanese patients with HD and GD to clarify their association with disease severity and intractability.

Materials and methods

Subjects

We screened for these polymorphisms in 52 patients (nine men and 43 women) with HD who developed moderate to severe hypothyroidism before 50 years of age, and were treated daily with thyroxine at least 1·5 μg per kg body weight (severe HD) and in 56 untreated euthyroid patients (two men and 54 women) with HD who were more than 50 years of age (mild HD). All patients with HD were positive for anti-thyroid microsomal antibody (McAb) or anti-thyroglobulin antibody (TgAb) and all patients with mild HD had palpable diffuse goitres.

We also examined 57 euthyroid patients (11 men and 46 women) with GD who had been treated with methimazole for at least 5 years and were still positive for anti-thyrotrophin receptor antibody (TRAb) (intractable GD), 52 patients (four men and 48 women) with GD in remission who had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication (GD in remission) and 91 healthy volunteers (control subjects; 10 men and 81 women) who were euthyroid and negative for thyroid autoantibodies. All patients and control subjects were Japanese and unrelated to each other. They were followed-up closely for more than 5 years as out-patients at our thyroid clinic. Written informed consent was obtained from all subjects, and the study protocol was approved by the Ethics Committee of Osaka University. Clinical characteristics of the examined subjects are given in Table 1. The titres of TgAb and McAb were significantly higher in patients with severe HD than in those with mild HD, as was the case in our previous report [19], and the goitre sizes were larger in patients with intractable GD than those with GD in remission, as in our previous report [20]. The goitre sizes were smaller in patients with severe HD than those with mild HD probably because of severe tissue destruction in patients with severe HD.

Isolation of genomic DNA

Genomic DNA was isolated from ethylenediamine tetraacetic acid (EDTA)-treated peripheral blood mononuclear cells with a commercially available kit (DrGenTLE™, Takara Bio Inc., Shiga, Japan).

Genotyping of −1112C/T polymorphism in the *IL13* gene

The target sequence of the *IL13* gene was amplified using polymerase chain reaction (PCR). The forward primer was 5′-GGA ATC CAG CAT GCC TTG TGA GG-3′; the reverse primer was 5′-GTC GCC TTT TCC TGC TCT TCC CGC-3′. The PCR protocol was as follows: 96°C for 3 min and 33 cycles of denaturing at 94°C for 60 s, annealing at 54°C for 60 s, extension at 72°C for 60 s and a single final extension at 72°C for 5 min. The PCR product [247 base pairs (bp)] was digested by the addition of BstUI and incubation at 60°C for 2 h. BstUI digests PCR fragments with the −1112C allele to produce 224 bp fragments.

Genotyping of −746C/T polymorphism in the *IL5* gene

The target sequence of the *IL5* gene was amplified using PCR. The forward primer was 5′-GCT CAT TGA ACA GAA TAC GTA-3′; the reverse primer was 5′-GAA GGT ATT GGC TCA TAG AAC-3′. The PCR protocol was as follows: 94°C for 2 min and 30 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s, and a single final extension at 72°C for 5 min. The PCR product (162 bp) was digested by addition of RsaI and incubation at 37°C for 2 h. RsaI digests PCR fragments with the −746C allele to produce 141 bp fragments.

Genotyping of −572 C/G polymorphism in the *IL6* gene

The target sequence of the *IL6* gene was amplified using PCR. The forward primer was 5′-GAG ACG CCT TGA AGT AAC TG-3′; the reverse primer was 5′-AAC CAA AGA TGT TCT GAA CTG-3′. The PCR protocol was as follows: 95°C for 10 min and 36 cycles of denaturing at 95°C for 45 s, annealing at 54°C for 50 s, extension at 72°C for 1 min, and a single final extension at 72°C for 5 min. The PCR product (182 bp) was digested by the addition of BsrBI and incubation at 37°C for 6 h. BsrBI digests PCR fragment with the −572G allele to produce 122 bp + 60 bp fragment. We then

Table 1. Clinical characteristics of the patients with autoimmune thyroid disease at the time of sampling.

		Graves' disease		Hashimoto's disease	
		Past clinical history of thyrotoxicosis with elevated TRAb		Diffuse goitre and positive TgAb and/or McAb	
	Controls	Intractable	In remission	Severe	Mild
n (female/male)	91 (81/10)	57 (46/11)	52 (48/4)	52 (43/9)	56 (54/2)
Age of onset (years)	44·1 ± 12·2‡	33·6 ± 13·7	32·9 ± 11·7	37·5 ± 11·0	56·5 ± 8·5‡
(range)	(21–58)	(11–66)	(16–66)	(10–49)	(50–76)
Goitre size (cm)	n.d.	5·11 ± 1·42*	4·03 ± 0·51	4·16 ± 1·01**	5·43 ± 1·34
Free T4 (ng/dl)	n.d.	1·43 ± 0·92	1·31 ± 0·42	1·35 ± 0·37	1·25 ± 0·24
Free T3 (pg/ml)	n.d.	3·33 ± 2·99	2·98 ± 1·34	2·52 ± 0·65	2·82 ± 0·35
TSH (μU/ml)	n.d.	1·31 ± 1·15	1·81 ± 1·13	1·77 ± 1·36	2·53 ± 2·17
TRAb (IU/l)	< 1·0	7·53 ± 13·1	< 1·0	< 1·0	< 1·0
(range)		(1·1–71·0)			
TgAb ($2^n \times 100$)	Negative	2·9 ± 0·7	1·7 ± 1·5	7·7 ± 3·1**	1·7 ± 2·7
McAb ($2^n \times 100$)	Negative	4·7 ± 2·7	5·6 ± 2·1	5·6 ± 2·9**	2·3 ± 2·4
Current treatment	None	Methimazole or PTU	None	L-thyroxine	None
Duration of treatment (years)	None	13·1 ± 7·7	3·36 ± 0·96§	11·6 ± 9·4	None
Current dose of anti-thyroid drug (mg/day)†	None	14·3 ± 13·7	None	None	None
(range)		(2·5–60)			
Current dose of L-thyroxine (μg/day)	None	None	None	102·2 ± 39·2	None
(range)				(50–250)	

Data are expressed as mean ± standard deviation. *$P < 0{\cdot}01$ (*versus* Graves' disease in remission); **$P < 0{\cdot}01$ (*versus* mild Hashimoto's disease). †Doses are expressed as the comparable dose of Methimazole (50 mg of PTU was converted to 5 mg of Methimazole). ‡Age at the time of sampling. §Duration of treatment with anti-thyroid drug before remission. n.d., not determined; T4, thyroxine; T3, triiodothyronine; TSH, thyrotrophin; TRA, anti-thyrotrophin receptor antibody; McAb, anti-thyroid microsomal antibody; TgAb, anti-thyroglobulin antibody; PTU, propylthiouracil.

categorized into CC (low producibility group) and CG + GG groups (high producibility group) according to the producibility of IL-6.

Thyroid function and autoantibodies

The serum concentration of free thyroxine (FT4) was measured with a commercial radio immunoassay 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). The normal range of serum FT3 is 2·4–4·6 pg/ml (3·8–7·2 pmol/l). Serum thyrotrophin (TSH) concentration was measured with radioimmunoassay kit (Daiichi Radioisotope Laboratories Ltd, Tokyo, Japan). The normal range of serum TSH is 0·6–5·4 μU/ml. TgAb and McAb were measured with a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titre of > 1:100 was considered positive. Serum TRAb was measured by radioreceptor assay with a commercial kit (Cosmic Corporation, Tokyo, Japan).

Statistical analysis

We used the χ^2 test to evaluate the significance of differences in the frequencies of genotypes and alleles among the groups. The Mann–Whitney *U*-test was used to analyse difference in serum titres of McAb, TgAb and TRAb levels. Data were analysed with JMP8 software (SAS Institute Inc., Tokyo, Japan). Probability values of < 0·05 were considered significant.

Results

IL13 –1112C/T polymorphism

The distribution of genotypes and alleles did not differ between control subjects and all AITD, GD and HD patients (Table 2). The distributions of genotype differed between in patients with intractable GD and those with GD in remission ($P = 0{\cdot}0386$; Table 3) and the T allele of this polymorphism, which correlates with higher producibility of IL-13, was significantly more frequent in patients with GD in remission than in those with intractable GD [$P = 0{\cdot}009$, odds ratio (OR) = 3·52; Table 3]. We found no difference in genotype and allele frequencies of this polymorphism between the patients with severe HD and those with mild HD (Table 3).

IL5 –746C/T polymorphism

The distributions of genotypes and alleles did not differ between control subjects and all AITD, GD and HD patients (Table 2). The T allele, which may correlate with lower

Table 2. Genotype and allele frequencies of *IL5* –746 C/T, *IL6* –572C/G and *IL13* –1112C/T polymorphisms in patients with autoimmune thyroid diseases and control subjects.

		Genetic producibility	Control	All patients with autoimmune thyroid disease		All patients with Graves' disease		All patients with Hashimoto's disease	
IL5	TT	(Low)	39 (42·9%)	99 (52·4%)		52 (54·2%)		47 (50·5%)	
–746	CT		42 (46·2%)	78 (41·3%)	n.s.†	36 (37·5%)	n.s.†	42 (45·2%)	n.s.†
	CC	(High)	10 (10·9%)	12 (6·3%)		8 (8·3%)		4 (4·3%)	
	T allele	(Low)	120 (65·9%)	276 (73·0%)	n.s.†	140 (72·9%)	n.s.†	136 (73·1%)	n.s.†
	C allele	(High)	62 (34·1%)	102 (27·0%)		52 (27·1%)		50 (26·9%)	
IL6	CC	Low	58 (66·7%)	114 (53·3%)		53 (50·0%)		61 (56·5%)	
–572	CG	High	25 (28·7%)	87 (40·6%)	n.s.†	47 (44·3%)	n.s.†	40 (37·0%)	n.s.†
	GG	High	4 (4·6%)	13 (6·1%)		6 (5·7%)		7 (6·5%)	
	CC	Low	58 (66·7%)	114 (53·3%)	$P = 0{\cdot}0333$†	53 (50·0%)	$P = 0{\cdot}0198$†	61 (56·5%)	n.s.†
	CG + GG	High	29 (33·3%)	100 (46·7%)	OR = 1·75 (1·04–2·95)	53 (50·0%)	OR = 2·00 (1·11–3·49)	47 (43·5%)	
	C allele	Low	141 (81·0%)	315 (73·6%)	n.s.†	153 (72·2%)	$P = 0{\cdot}0420$†	162 (75·0%)	n.s.†
	G allele	High	33 (19·0%)	113 (26·4%)	($P = 0{\cdot}0536$)	59 (27·8%)	OR = 1·65 (1·02–2·67)	54 (25·0%)	
IL13	CC	Low	53 (77·9%)	108 (74·5%)		60 (76·9%)		48 (71·6%)	
–1112	CT		14 (20·6%)	35 (24·1%)	n.s.†	16 (20·5%)	n.s.†	19 (28·4%)	n.s.†
	TT	High	1 (1·5%)	2 (1·4%)		2 (2·6%)		0 (0%)	
	C allele	Low	120 (88·2%)	251 (86·6%)	n.s.†	136 (87·2%)	n.s.†	115 (85·8%)	n.s.†
	T allele	High	16 (11·8%)	39 (13·4%)		20 (12·8%)		19 (14·2%)	

Analysed by χ^2 tests; OR, odds ratio (95% confidence intervals); n.s., not significant; †*versus* control.

producibility of IL-5, was significantly more frequent in patients with GD in remission than in controls ($P = 0{\cdot}0289$, OR = 2·00; Table 3). We found no difference in genotype and allele frequencies of this polymorphism between the patients with severe HD and those with mild HD (Table 2).

IL6 –572C/G polymorphism

The G allele carriers (CG and GG genotypes), which correlate with higher producibility of IL-6, were significantly more frequent in all examined AITD and GD patients

Table 3. Genotype and allele frequencies of *IL5* –746 C/T, *IL6* –572C/G and *IL13* –1112C/T polymorphisms in patients with Graves' disease, Hashimoto's disease and in control subjects.

		Genetic producibility	Control	Graves' disease			Hashimoto's disease		
				Intractable	In remission		Severe	Mild	
IL5	TT	(Low)	39 (42·9%)	27 (47·4%)	25 (64·1%)		20 (50·0%)	27 (50·9%)	
–746	CT		42 (46·2%)	24 (42·1%)	12 (30·8%)	n.s.†	20 (50·0%)	22 (41·5%)	n.s.‡
	CC	(High)	10 (10·9%)	6 (10·5%)	2 (5·1%)		0 (0%)	4 (7·6%)	
	T allele	(Low)	120 (65·9%)	78 (68·4%)	62 (79·5%)§	n.s.‡	60 (75·0%)	76 (71·7%)	n.s.‡
	C allele	(High)	62 (34·1%)	36 (31·6%)	16 (20·5%)		20 (25·0%)	30 (28·3%)	
IL6	CC	Low	58 (66·7%)	28 (51·9%)	25 (48·1%)		25 (48·1%)	36 (64·3%)	
–572	CG	High	25 (28·7%)	26 (48·1%)	21 (40·4%)	$P = 0{\cdot}0357$†	22 (42·3%)	18 (32·1%)	n.s.‡
	GG	High	4 (4·6%)	0 (0%)	6 (11·5%)		5 (9·6%)	2 (3·6%)	
	CC	Low	58 (66·7%)	28 (51·9%)	25 (48·1%)	n.s.†	25 (48·1%)	36 (64·3%)	n.s.‡
	CG + GG	High	29 (33·3%)	26 (48·1%)	27 (51·9%)¶		27 (51·9%)¶	20 (35·7%)	
	C allele	Low	141 (81·0%)	82 (75·9%)	71 (68·3%)	n.s.†	72 (69·2%)	90 (80·4%)	n.s.‡
	G allele	High	33 (19·0%)	26 (24·1%)	33 (31·7%)		32 (30·8%)	22 (19·6%)	
IL13	CC	Low	53 (77·9%)	41 (85·4%)	19 (63·3%)		25 (75·8%)	23 (67·6%)	
–1112	CT		14 (20·6%)	7 (14·6%)	9 (30·0%)	$P = 0{\cdot}0386$†	8 (24·2%)	11 (32·4%)	n.s.‡
	TT	High	1 (1·5%)	0 (0%)	2 (6·7%)		0 (0%)	0 (0%)	
	C allele	Low	120 (88·2%)	89 (92·7%)	47 (78·3%)	$P = 0{\cdot}009$†	58 (87·9%)	57 (83·8%)	n.s.‡
	T allele	High	16 (11·8%)	7 (7·3%)	13 (21·7%)	OR = 3·52 (1·31–9·41)	8 (12·1%)	11 (16·2%)	

Analysed by χ^2 tests; OR, odds ratio (95% confidence intervals); n.s., not significant. †Intractable Graves' disease (GD) *versus* GD in remission; ‡severe Hashimoto's disease (HD) *versus* mild HD; §$P = 0{\cdot}0289$, OR = 2·00 (1·06–3·70) (*versus* control); ¶$P = 0{\cdot}0306$, OR = 2·16 (1·07–4·36) (*versus* control).

than in controls ($P = 0{\cdot}0333$, OR = 1·75 and $P = 0{\cdot}0198$, OR = 2·00, respectively). The G allele of this polymorphism was also more frequent in GD patients than in controls ($P = 0{\cdot}0420$, OR = 1·65; Table 2). Significant differences in genotype frequencies between patients with intractable GD and those with GD in remission ($P = 0{\cdot}0357$) was observed, and the GG genotype was absent in patients with intractable GD (Table 3). The G allele carriers were significantly more frequent in patients with GD in remission or with severe HD than in controls ($P = 0{\cdot}0306$, OR = 2·16; Table 3).

Examined polymorphisms and clinical characteristics

We found no association between each allele of examined polymorphisms and TRAb levels, McAb titres, TgAb titres or goitre size with the genotypes (data not shown).

Discussion

In a previous report by Hiromatsu *et al.* [10], the *IL13* −1112T allele, which correlates with higher producibility of Il-13 [9], occurred less frequently in GD patients than in controls, whereas others reported no difference in the frequencies of the T allele between the groups [11,12], similar to our results (Table 2). Because many GD patients examined by Hiromatsu *et al.* [10] have ophthalmopathy, it is possible that this group of GD patients may have included many intractable GD patients, in which the −1112T allele is clearly found less frequently compared to GD patients in remission (Table 3). Furthermore, our finding that the *IL13* −1112T allele with higher producibility of IL-13 is more frequent in patients with GD in remission suggests that IL-13 may induce the remission of GD. We have reported previously that Th17 cells, which are induced by transforming growth factor (TGF)-β, IL-1β, IL-6 and IL-23 [21], increase in patients with intractable GD more than in those with GD in remission [22], and that higher genetic producibility of TGF-β and IL-1β were associated with the intractability of GD, probably through the induction of Th17 cells [23,24]. Interestingly, it has been reported that IL-13 inhibits the generation of Th17 cells [8,25]. These observations are consistent with our present data.

In the case of the *IL5* −746C/T polymorphism, the T allele, which may correlate with lower levels of IL-5, was significantly more frequent in patients with GD in remission than in controls (Table 3). This finding is consistent with previous reports, which showed that the T allele may be protective against the development of GD [26], that IL-5 is elevated in peripheral blood from GD patients [16] and promote the proliferation of $CD5^+$ B cells [27], which increase in various autoimmune diseases including GD [28], and that IL-5 also plays an important role in the growth of eosinophils and may be associated with the recurrence of GD after an attack of allergic rhinitis [29].

The *IL6* −572G carrier (CG and GG genotypes), which has a higher producibility of IL-6 [18], was significantly more frequent in patients with AITD, especially with GD in remission or with severe HD, than in controls (Table 3). In a Chinese population, this carrier was also found more frequently in HD patients than in controls [30]. These may be related to the findings that the IL-6 promote the production of inflammatory cytokines such as IFN-γ and TNF-α [31] and the differentiation of Th17 cells [21], which increased in GD and HD patients [24]. These results indicate that the higher genetic producibility of IL-6 may affect the development of AITD not only by promotion of inflammatory cytokines [31], but also by induction of Th17 cells [21]. Interestingly, conversely, we could not find the *IL6* −572GG genotype in patients with intractable GD (Table 3), in spite of the fact that the producibility of IL-6 is not different between CG and GG genotypes [18].

The most striking findings in this study may be the similarity of GD and HD in both allele and genotype frequencies of Th2 cytokine genes. It is often considered that GD is a Th2 disorder, whereas HD is a Th1 disorder, but the two diseases might be more of a spectrum rather than distinct diseases.

A limitation of this study may be the small sample numbers. Our data need to be confirmed with further investigation, such as a replication study on another sample, to increase confidence in our findings.

In conclusion, these functional polymorphisms in genes encoding Th2 cytokines are associated differently with the development and prognosis of AITDs from each other. The lower producibility of IL-13 may be associated with the intractability of GD; the higher producibility of IL-13 and the lower producibility of IL-5 may induce the remitting of GD, and the higher producibility of IL-6 may induce the development of Graves' hyperthyroidism and Hashimoto's hypothyroidism. Therefore, IL-5, IL-6 and IL-13 have completely different effects on GD, although they are all Th2 cytokines.

Disclosure

The authors have no financial conflicts of interest.

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