Interleukin-12B Gene Polymorphism does not Confer Susceptibility to Graves' Ophthalmopathy in Japanese Population

YUJI HIROMATSU, TOMOKA FUKUTANI, MICHIKO ICHIMURA, TOKUNORI MUKAI, HIROO KAKU, IKUYO MIYAKE AND KENTARO YAMADA

Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan

Abstract. Graves' disease (GD) is an autoimmune disorder with genetic predisposition and frequently associated with Graves' ophthalmopathy (GO). Interleukin 12 (IL-12) is an important mediator of inflammatory immune responses and is expressed in the thyroid and orbit. IL-12B gene, which encodes the p40 subunit of IL-12, is located at chromosome 5q31–33. The aim of the present study was to investigate whether IL-12B gene polymorphism is associated with the development of GD or GO. IL-12B gene polymorphism was studied in Japanese GD patients (n = 329) and healthy control subjects without anti-thyroid autoantibodies or a family history of autoimmune disorders (n = 226). The A/C polymorphism at position 1188 of the 3' untranslated region (3'UTR) of the IL-12B gene was analyzed using the polymerase chain reaction – restriction fragment length polymorphism method. There was no difference in allele or genotype frequency of the IL-12B gene polymorphism (1188A/C) between GD patients and control subjects. There was no association of the IL-12B gene polymorphism with ophthalmopathy, severity of hyperthyroidism or serum IgE levels. There was no association of the IL-12B gene polymorphism with serum IL-12 levels, which were significantly elevated in hyperthyroid phase of GD. In conclusion, IL-12B gene 1188A/C polymorphism is not associated with GD or GO susceptibility in Japanese.

Key words: Polymorphism, IL-12, Graves' disease, Graves' ophthalmopathy

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GRAVES' disease (GD) is an autoimmune disorder frequently associated with ophthalmopathy [1]. Although the thyroid-stimulating hormone (TSH) receptor has been proposed as an autoantigen in GD patients, the nature of autoimmune reactions in the thyroid and orbit, and the mechanisms linking GD and Graves' ophthalmopathy (GO) have not been fully elucidated [2]. Several lines of research support the involvement of environmental factors, such as smoking, and genetic factors in both GD and GO [2, 3]. The genetic sus-

ceptibility of these diseases is thought to be polygenic. It has been reported that major histocompatibility complex (MHC) gene [3, 4], cytotoxic T lymphocyte antigen-4 (CTLA-4) gene [4–6], thyrotropin receptor (TSHR) gene [7], PTPN22 gene [8], CD40 gene [9, 10], interferon- γ (IFN- γ) gene [11], tumor necrosis factor- α (TNF- α) gene [12, 13] and interleukin-13 (IL-13) gene [14] polymorphisms are associated with GD and their interactions may influence disease phenotype and severity [3]. However, their contribution to susceptibility to GO awaits confirmation.

Recent genome-wide researches have provided evidence for the linkage of GD to loci on multiple chromosomes, including loci on chromosome 5 in regions 5q31–q33 [15, 16], which have been linked to autoimmune thyroid disorders including GD in Japanese and Chinese populations, but not in Caucasian popu-

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Correspondence to: Dr. Yuji HIROMATSU, Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan

lations. This might suggest that these loci are specific to East Asian populations. The loci in regions 5q31–q33 have also been identified as being susceptible to IgE synthesis [17, 18]. This region encodes a cluster of cytokine genes, including IL-3, IL-4, IL-5, IL-9, IL-12B and IL-13, which are involved in inflammation in both thyroid and orbit, and IgE synthesis [19].

IL-12 is an important immunoregulatory protein produced primarily by macrophages, B cells and other antigen-presenting cells [20]. It drives the differentiation of CD4⁺ T cells into T helper 1 (Th1) cells. IL-12 induces IL-2, IFN- γ and TNF- β from Th1 cells and IFN- γ from NK (natural killer) cells. Increased serum levels of IL-12 have been reported in GD [21–23]. Furthermore, IL-12B mRNA expression has been demonstrated in thyroid tissues from patients with GD [24] and in orbital fat tissues from patients with active GO [25], but not in extraocular muscle tissues (EOM), or EOM-derived T cell lines from patients with GO undergoing corrective strabismus surgery [26]. Numerous single nucleotide polymorphisms (SNP) have recently been identified in the IL-12B gene [27] and the 1188A/C polymorphism in the 3' untranslated region (3'UTR) of the IL-12B gene is associated with IL-12B mRNA expression levels [28] and IL-12 secretion levels from lipopolysaccharide (LPS) and purified protein derivative (PPD) stimulated peripheral blood mononuclear lymphocytes (PBML) [29]. Further, IL-12B gene has been proposed as a susceptibility gene in type 1 diabetes mellitus [28, 30, 31] with controversy [32–34], asthma [35] and hepatitis C virus infection [36]. Thus, IL-12B gene might be a potential candidate gene contributing to the development of GD or influencing its clinical course. The aim of the present study was to investigate whether IL-12B gene polymorphism is associated with the development of GD and GO.

Subjects and Methods

Subjects

In total, 329 GD patients (74 males, 255 females; aged 11–83 years, mean age 41.9 ± 15.6 years) being treated at Kurume University Hospital were enrolled in this study. GD diagnosis was determined by the presence of hyperthyroidism and serum anti-thyrotropin receptor antibodies and/or an increased ^{123}I uptake ratio

with diffuse uptake. Ophthalmopathy was classified according to the system recommended by the American Thyroid Association (ATA) Committee [37]. One hundred and three of the GD patients, 24 males and 79 females, showed ophthalmopathy defined as ATA class III or greater and were classified as GO. Two hundred and twenty-six patients showed no ophthalmopathy (ATA class 0), signs of ophthalmopathy without symptoms (ATA class I), or only soft tissue involvement (ATA class II). Two hundred and twenty-six healthy unrelated Japanese volunteers (101 males and 125 females; aged 20–71 years, mean age 34.0 ± 8.4 years) with no family history of autoimmune diseases and no detectable anti-thyroid autoantibodies were enrolled as control subjects. The study plan was reviewed and approved by the institutional review committee, and informed consent was obtained from all patients and control subjects.

IL-12B gene polymorphism

Genomic DNA extracted from peripheral blood was subjected to polymerase chain reaction (PCR) to amplify the polymorphic regions. The A/C polymorphism in the 3'UTR (1188A/C) of the IL-12B gene was analyzed using a PCR-RFLP method previously described by Huang *et al.* [27]. PCR was performed using 50 ng of genomic DNA, 1.25 units of Taq DNA polymerase (AmpliTaq, Applied Biosystems, Foster City, CA), 0.5 μM of each primer (forward, 5'-TTTGGAGGAA AAGTGAAGA-3'; reverse, 5'-AACATTCCATAC ATCCT-3'), 1.5 mM MgCl_2 and 200 μM of each deoxynucleotide triphosphate under the following conditions: 35 cycles of PCR consisting of 30 sec at 95°C, annealing for 30 sec at 50°C, extension for 1 min at 72°C and a final extension for 5 min at 72°C in a thermocycler (Gene Amp PCR system 9600, Perkin Elmer Applied Biosystems, Foster City, CA). The PCR products were digested using TaqI (Toyobo, Osaka, Japan), and the digested products were separated on a 3% agarose gel and identified by ethidium bromide staining. Some of the PCR products were directly sequenced using an ABI sequencer (ABI PrismTM 3100 Genetic Analyzer, Perkin Elmer Applied Biosystems) to determine the A/C polymorphism in the 3'UTR (1188A/C) of the IL-12B gene.

Measurement of serum IL-12 levels

Serum IL-12 levels were measured by a sandwich ELISA using a human IL-12 ELISA Kit (BioSource International, Camarillo, CA), according to the manufacturer's instructions.

Laboratory tests

Serum concentrations of free T3, free T4 and TSH were determined by enzyme immunoassays (EIAs). TSH receptor antibodies were measured using commercial kits [TRAb, Diasorin Inc., Stillwater, MN; thyroid stimulating antibody (TSAb), Yamasa Co., Tokyo, Japan]. Anti-thyroglobulin (TgAb) and anti-thyroid peroxidase antibodies (TPOAb) were measured by radioimmunoassay using commercial kits (RSR Limited, Cardiff, UK). The cut-off values for TRAb, TSAb, TgAb and TPOAb were 10%, 180%, 0.3 kU/L and 0.3 kU/L, respectively. Serum IgE was measured by nephelometric assay (Dade Behring Marburg GmbH, Marburg, Germany).

Statistical analysis

The clinical data were expressed as the means $\pm$ standard deviation (SD). Differences in the clinical data between groups were evaluated using a Student's *t* test or Welch's *t* test. The statistical significance of any differences in frequency between each polymorphic allele and genotype of the patient and control groups was evaluated using the χ^2 test or Fisher's exact probability test. Linear correlation analysis was used to examine the correlation between IL-12 levels and other parameters. In this study, a *P*-value of <0.05 was considered statistically significant.

Results

Association between IL-12B gene polymorphisms and Graves' disease

The distributions of alleles for both groups (control and GD) were in good agreement with the Hardy-Weinberg equilibrium. There was no difference in the genotype frequency or the allele frequency between GD patients and the control subjects (Table 1).

Association between the IL-12B gene polymorphisms and ophthalmopathy

There was no significant difference in genotype or allele frequencies of IL-12B gene polymorphisms between the patients with evident ophthalmopathy (ATA class III or more; GO) and those without or with mild ophthalmopathy (ATA class 0~II; Table 2).

Association between the IL-12B gene polymorphisms and the severity of Graves' hyperthyroidism

There were no significant differences in the levels of serum FT₄, FT₃, TRAb or TSAb among the genotypes of 1188A/C polymorphism (Table 3). There were no differences in the serum IgE levels, or positive ratios (>170 kIU/l) among the genotypes of 1188A/C polymorphism (Table 4).

Serum IL-12 levels in Graves' disease

The mean serum IL-12 level in 109 patients with GD was significantly higher than that in 57 control subjects (173 ± 100 pg/ml vs. 96 ± 38 pg/ml, respectively, $P < 0.0001$, Fig. 1). The serum levels of IL-12 were significantly correlated with the serum FT3 ($r = 0.422$, $P < 0.0001$), FT4 ($r = 0.304$, $P = 0.0013$) and TRAb levels ($r = 0.265$, $P = 0.0063$). The mean serum IL-12 level in hyperthyroid phase was significantly higher than that in euthyroid phase (203 ± 99 pg/ml vs. 115 ± 54 pg/ml, respectively, $P < 0.0001$, Fig. 1). There was no significant association of the IL-12B gene polymorphism with the serum IL-12 levels in hyperthyroid phase of GD or in control subjects (Fig. 1). There was no association of the IL-12 levels in hyperthyroid phase with ophthalmopathy (195 ± 88 pg/ml in ophthalmopathy group vs. 207 ± 105 pg/ml in group without ophthalmopathy).

Discussion

Graves' disease is an organ-specific autoimmune disorder characterized by a diffuse goiter and thyroid hormone oversecretion as a result of thyrotropin receptor antibody stimulation. Although the etiology of GD remains unclear, it is believed to be caused by a complex interaction between genetic and environmental factors. Increased serum levels of IL-12 have been re-

Table 1. IL-12B gene polymorphisms in patients with Graves' disease and healthy control subjects

IL-12B gene polymorphism		Graves' disease n = 329	Control subjects n = 226	χ^2 test <i>P</i>
A1188C	Genotype frequencies			
	AA	77 (24)	39 (17)	$\chi^2 = 3.063$
	AC	162 (49)	120 (53)	<i>P</i> = 0.2162
	CC	90 (27)	67 (30)	
	Allele frequencies			
	A	316 (48)	198 (44)	$\chi^2 = 1.918$
	C	342 (52)	254 (56)	<i>P</i> = 0.1661

Values in parentheses are percentages of the group. *P* values were calculated with χ^2 test comparing patients with Graves' disease and healthy control subjects.

Table 2. IL-12B gene polymorphisms in patients with Graves' ophthalmopathy and in patients with Graves' hyperthyroidism without clinically evident ophthalmopathy

IL-12B gene polymorphism		Ophthalmopathy		χ^2 test <i>P</i>
		ATA class III~VI n = 103	ATA class 0~II n = 226	
A1188C	Genotype frequencies			
	AA	22 (21)	55 (24)	$\chi^2 = 0.442$
	AC	51 (50)	111 (49)	<i>P</i> = 0.8017
	CC	30 (29)	60 (27)	
	Allele frequencies			
	A	95 (46)	221 (49)	$\chi^2 = 0.437$
	C	111 (54)	231 (51)	<i>P</i> = 0.5084

ATA class: classification by the American Thyroid Association

Values in parentheses are percentages of the group. *P* values were calculated with χ^2 test comparing patients with Graves' disease with ophthalmopathy and without evident ophthalmopathy.

Table 3. Association of IL-12B gene polymorphisms with laboratory features of untreated patients with Graves' disease

IL-12B gene polymorphism	n	FT3 (pg/ml)	FT4 (ng/dl)	TRAb (%)	TSAb (%)
A1188C genotype					
AA	49	14.2 ± 6.9	5.6 ± 3.2	38.0 ± 25.3	389 ± 341
AC	102	14.3 ± 6.6	5.3 ± 2.9	33.3 ± 24.0	483 ± 750
CC	59	13.1 ± 6.9	5.1 ± 3.0	33.1 ± 25.2	495 ± 341

n, number of patients; FT3, free T3; FT4, free T4; TRAb, anti-thyrotropin receptor antibody; TSAb, thyroid stimulating antibody.

Table 4. Association of IL-12B gene polymorphisms with serum total IgE in patients with Graves' disease and control subjects

IL-12B gene polymorphism	Graves' disease (n = 297)			Control subjects (n = 224)		
	IgE > 170 kIU/l	Mean ± SD (kIU/l)	Median (kIU/l)	IgE > 170 kIU/l	Mean ± SD (kIU/l)	Median (kIU/l)
A1188C genotype						
AA	21/68 (31)	326.0 ± 696.2	97.6	9/39 (23)	205.3 ± 376.8	76
AC	55/146 (38)	311.3 ± 513.8	119.5	36/116 (31)	249.4 ± 330.8	84
CC	28/83 (34)	334.3 ± 693.9	73.7	19/66 (29)	183.0 ± 228.8	89
total	104/297 (35)	321.1 ± 742.0	105	64/221 (29)	222.1 ± 443.2	84

Values in parentheses are percentages of the group.

There were no differences in the serum IgE levels, or positive ratios (>170 kIU/l) among the genotypes of 1188A/C polymorphism.

Fig. 1. Serum IL-12 levels in patients with Graves' disease and control subjects.

Hyper: hyperthyroid phase, Eu: euthyroid phase, Cont: control subjects, GH: Graves' disease without ophthalmopathy, GO: Graves' ophthalmopathy

ported in patients with GD [21–23]. The increased expression of IL-12 mRNA in thyroid and orbital fat tissues has been reported in GD and in active GO, respectively [24, 25]. Furthermore, IL-12 is involved in the development of animal model of autoimmune thyroid disorders [38]. Therefore, IL-12 may play a role in the pathogenesis of GD and GO. Recent genome-wide researches have provided evidence for the linkage of GD to loci on chromosome 5 in regions 5q31–q33 in Japanese [15] and Chinese [16] populations. This region encodes a cluster of cytokine genes, including IL-3, IL-4, IL-5, IL-9, IL-12B and IL-13, which are involved in inflammation and IgE synthesis [19]. Therefore, we hypothesized that IL-12B gene might be a potential candidate gene contributing to the development of GD or influencing its clinical severity and course, especially GO.

Recently, Ikeda *et al.* [39] performed a genetic association study using 90 GD patients and 123 control subjects in Japanese. They could not find any association between the IL-12B gene polymorphisms (A/T

polymorphism in the intron 4 or 1188A/C in the 3'UTR) and GD. Dahlman *et al.* [33] also failed to show any association between the IL-12B gene 1188A/C polymorphism and GD in white European patients. However, they did not investigate the association of the polymorphism with GO. In the present study, we confirmed the lack of association of IL-12B gene polymorphism with GD. Furthermore, our subanalysis showed no association of this polymorphism with GO, severity of hyperthyroidism, or serum IgE levels.

In the present study, we confirmed the increased levels of IL-12 in GD, especially in hyperthyroid phase. The IL-12 levels correlated with the serum FT3 levels, but not the presence of ophthalmopathy. Although there are conflicting reports on the relationship between the SNP and functional IL-12 gene expression on cell lines [28, 33], Yilmaz *et al.* [29] reported that LPS and PPD stimulation of PBML have induced higher levels of IL-12 in CC homozygotes *in vitro*. Bergholdt *et al.* [34] also reported the higher secretion of IL-12 by stimulation of PBML with IFN γ and LPS. The present study, however, showed no association of the elevated levels of serum IL-12 in hyperthyroid phase of GD with the genotypes of the IL-12B gene polymorphism.

In conclusion, the 1188A/C polymorphism in the 3'UTR of the IL-12B gene does not confer genetic susceptibility to GD or does not contribute to the development of ophthalmopathy in Japanese patients with GD. This SNP does not contribute to the elevated levels of serum IL-12 in hyperthyroid phase of GD. Further investigations of the other polymorphisms of the IL12B gene or haplotype analysis would be helpful to clarify the role of IL-12 in the etiology of GD and GO.

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