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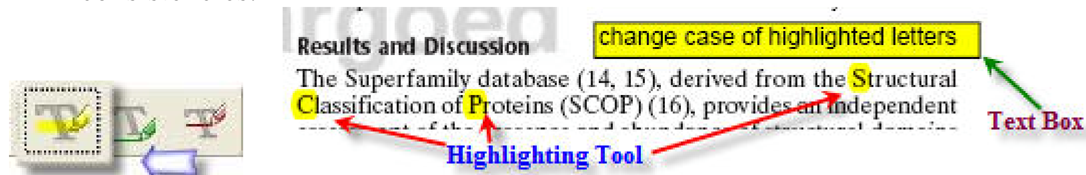

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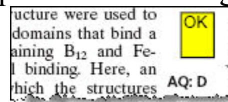

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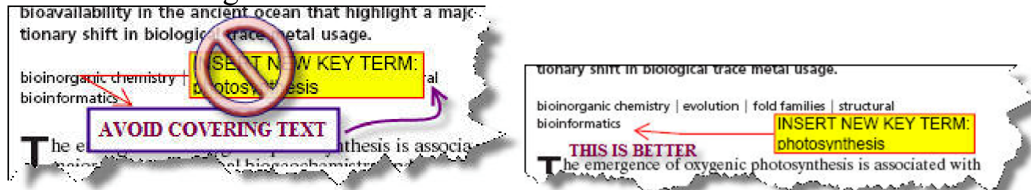

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Association of Interleukin-1β (*IL1B*) Polymorphisms with Graves' Ophthalmopathy in Taiwan Chinese Patients

Yu-Huei Liu,[1,2] *Rong-Hsing Chen,*[3] *Hsin-Hung Wu,*[4] *Wen-Ling Liao,*[1,2] *Wen-Chi Chen,*[5]
AQ: 1 *Yuhsin Tsai,*[2] *Chang-Hai Tsai,*[6,7] *Lei Wan,*[*,1,2,8] *and Fuu-Jen Tsai*[*,1,2,3,6,9,10]
7,8 9 7,8

Purpose. To evaluate whether variations in the *IL1B* gene could be associated with Graves' ophthalmopathy (GO) in patients with Graves' disease (GD).

Method. This case–control study included 471 Taiwan Chinese patients with GD (200 with GO and 271 without GO) and 160 healthy volunteers. Eight single-nucleotide polymorphisms (SNPs) in *IL1B* were genotyped with an allele-specific extension and ligation assay.

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Results. In the *IL1B* SNPs examined, the C allele of rs1143634 was associated with GD, whereas the T/T genotype of the SNPs rs1143634 and rs16944 were less associated with the disease. The A/A genotype of the SNPs rs3917368 and rs1143643, which had the strongest interaction, may increase the risk of GO (P = 0.024 and P = 0.017, respectively). Several GD susceptibility and insusceptibility *IL1B* haplotypes have been identified, and the Ht4-GCGCCTCC haplotype, composed of eight SNPs and associated with low circulating IL1β levels, may be protective against the development of GO (P = 0.025). Moreover, that the GO-susceptible genotype was associated with lower plasma IL1β concentrations implies that the origin of GO may go beyond the *IL1B* polymorphism-associated elevation of circulating IL1β.

Conclusions. The data for *IL1B* polymorphisms and the association of GD and GO with plasma IL1β levels show that *IL1B* polymorphisms may be associated with the development of GD and GO. (*Invest Ophthalmol Vis Sci.* 2010;51:000–000) DOI:10.1167/iovs.09-4965

Graves' disease (GD), with or without Graves' ophthalmopathy (GO), is an autoimmune disease characterized by hyperthyroidism, diffuse goiter, thyroid-specific autoantibodies, and dermopathy due to circulating autoantibodies.[1] GO is the most common extrathyroid manifestation of GD and affects 25% to 50% of GD patients.[2–5] Approximately 28% of patients with GO present as severe cases, with restricted mobility, diplopia, keratopathy, and optic neuropathy.[6,7] Several genes have been reported that promote the development of GO, including human leukocyte antigen (HLA) class I and class II molecules.[8] For example, the +49G allele of cytotoxic T-lymphocyte-associated antigen-4 (*CTLA4*) confers genetic susceptibility to GO, although meta-analyses did not support this finding. CT60A/G of *CTLA4* is one of the GD-associated polymorphisms that await further studies that examine their association with GO. In addition, polymorphisms in several immunomodulatory genes, such as the intron 1 (CA) repeat in interferon-γ (*IFNG*); G238A, C863A, and T1031C in tumor necrosis factor-α (*TNFA*); and A1405G in intracellular adhesion molecule-1 (*ICAM1*), have been reported to increase susceptibility to GO.[8] The combination of specific alleles among these genes would make the patient susceptible to GO.

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Interleukin-1 beta (*IL1B*), a proinflammatory cytokine expressed by activated macrophages and several other types of cells, is thought to play a crucial role in the pathogenesis of autoimmune diseases.[9,10] IL1β was initially known as one of the lymphocyte activating factors (LAFs), owing to its role in the induction of T-cell proliferation and maturation.[9,10] Recent studies have demonstrated that IL1β released by macrophages and fibroblasts can induce adipogenesis and accumulation of glycosaminoglycans (GAGs) and prostaglandin E2 (PGE2), which may result in the development of GO.[8,11,12] One study revealed that the single-nucleotide polymorphism (SNP) −511C of *IL1B* is associated with GO, whereas others did not.[13–15] Although the connection between *IL1B* polymorphism and GO remains controversial, several studies have demonstrated that polymorphisms of *IL1B* may correlate with IL1β expression in other diseases.[16–18] In addition, IL1β promotes the accumulation of GAGs through the upregulation of hyaluronan synthesis and accumulation of PGE2, stimulates adipogenesis, and hyperinduces the expression of interleukin (IL)-6 and -8 and macrophage chemoattractant protein (MCP)-1 in orbital fibroblasts derived from healthy individuals and patients with GO.[8,19–23] Moreover, the polymorphisms of the IL1 family are involved in several autoimmune diseases such as systemic lupus erythematosus (SLE),[24–26] rheumatoid arthritis,[27,28] autoimmune hemolytic anemia,[29] and GD.[30] These reports support that *IL1B* is a potential candidate gene in the development of GO.

Although previous reports have suggested that *IL1B* polymorphisms and expression lead to autoimmune diseases,[24–30] the genetic role of *IL1B* in GO remains to be elucidated. In the present study, we investigated SNPs in *IL1B* that may be protective against or causative of GO in Taiwan Chinese patients with GD.

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METHODS

Patients and Healthy Individuals

A group of 484 patients with a confirmed diagnosis of GD and a control group of 160 healthy volunteers at China Medical University Hospital in Taiwan were enrolled and actively observed. All individuals in this study provided informed consent, as approved by the ethics committee of China Medical University Hospital and in accordance with the guidelines in the Declaration of Helsinki.

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Patients. Diagnosis of GD was based on the typical clinical features of hyperthyroidism: diffuse enlargement of the thyroid gland, increased free thyroxine or triiodothyronine levels, suppressed thyroid-stimulating hormone levels, positive thyrotrophin-receptor autoantibodies, and the presence (or absence) of anti-thyroid peroxidase (anti-TPO) antibodies or antithyroglobulin antibodies. Information regarding sex, age at onset of GD, treatment of hyperthyroidism, personal history of cigarette smoking, history of systemic diseases, and family history of autoimmune thyroid disease was obtained. The inclusion criteria were (1) meeting the diagnostic criteria of GD at the time of examination; (2) being willing to participate and capable of giving informed consent; and (3) being a self-reported nonaboriginal Taiwanese with no parent or grandparent having an aboriginal background. The exclusion criteria were (1) being unable to understand or give informed consent or (2) being pregnant or having given birth within 1 year, to exclude the possibility of including subjects with postpartum thyroiditis. Patients with GO (GD/GO) were identified according to the following criteria: Normal upper eyelid position was 1.5 mm below the superior limbus, and normal lower eyelid position was at the level of the inferior limbus in primary gaze. Proptosis was measured by a Hertel exophthalmometer and was defined as the anteroposterior protrusion of the globe >19 mm from the lateral orbital rim in either eye or any discrepancy in the degree of protrusion of the two eyes by >1 mm. All individuals classified as affected were interviewed and examined by experienced clinicians. A full medical record review was conducted to obtain demographics (age and sex); history of tobacco use; recurrence of GD (patients with GD who have accepted medical treatment); and progression (patients with ongoing GD), treatment, and clinical features of the condition.

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Healthy Individuals. The healthy group was matched for sex according to the female predominance of GD, including 32 men (20.0%) and 128 women (80.0%). Age was significantly different between the groups of healthy volunteers (27.4 ± 6.4 years) and patients with GD (39.9 ± 12.2 years; $P = 1.028 \times 10^{-34}$).

All blood samples were collected from consenting individuals by venipuncture for subsequent genomic DNA isolation.

SNP Selection

IL1B SNP genotype information was downloaded in December 2008 from the HapMap CHB+JPT population. HapMap genotypes were analyzed in Haploview (Haploview software (http://www.broad.mit.edu/mpg/haploview/ provided in the public domain by The Broad

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TABLE 1. Background and Demographic Characteristics of Healthy Individuals and Graves' Patients with or without GO

Patients' Characteristics	Healthy (*n* = 160)	GD/GO (*n* = 200)	GD/non-GO (*n* = 271)	*P* GD vs. Healthy	*P* GD/GO vs. GD/nonGO
Age at diagnosis (mean ± SD)	27.4 ± 6.4	37.5 ± 10.8	41.7 ± 12.8	1.028×10^{-34}*	2.978×10^{-4}*
Sex					
Male	32 (20.0)	51 (25.5)	48 (17.7)	0.784†	**0.040**†
Female	128 (80.0)	149 (74.5)	223 (82.3)		
Smoking status‡					
Smoking		57 (28.5)	54 (19.9)		**0.030**†
Nonsmoking		146 (71.5)	217 (80.1)		
Recurrence					
Yes		99 (49.5)	128 (47.2)		NS†
No		101 (50.5)	143 (52.8)		
Treatment					
Radioiodine					
Yes		15 (7.5)	6 (2.2)		**0.006**†
No		185 (92.5)	265 (97.8)		
Thyroid gland surgery					
Yes		24 (12.0)	23 (8.5)		NS†
No		176 (88.0)	248 (91.5)		
Clinical features					
Goiter					
Grade 1		14 (7.0)	17 (6.3)		NS§
Grade 2		5 (2.5)	21 (7.7)		
Grade 3		22 (11.0)	32 (11.8)		
Grade 4		129 (64.5)	169 (62.4)		
Grade 5		30 (15.0)	32 (11.8)		
Nodular hyperplasia					
Yes		22 (11.0)	25 (9.2)		NS†
No		178 (89.0)	246 (90.8)		
Myxedema					
Yes		5 (2.5)	1 (0.4)		**0.042***
No		195 (97.5)	270 (99.6)		
Vitiligo					
Yes		2 (1.0)	2 (0.7)		NS†
No		198 (99.0)	269 (99.3)		

Data are *n* (%), unless otherwise indicated. Bold probabilities are statistically significant.

* Age and myxedema were determined by Mann-Whitney U test. †All characteristics except age, goiter, and myxedema were determined by χ^2 test using 2 × 2 contingency tables.

‡ Smoking category includes former, current, and ever smoker categories.

§ Goiter was determined by chi-square test using 2 × 5 contingency tables. $P < 0.05$ was statistically significant.

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Institute, Massachusetts Institute of Technology, Cambridge, MA), and Tag SNPs were selected by using the Tagger function and applying the following additional criteria: (1) a threshold minor allele frequency (MAF) in the HapMap CHB+JPT population of 0.10 for tag SNPs and (2) a genotyping score (Illumina, Inc., San Diego, CA) more than or equal to 0.6, as recommended by the manufacturer, to ensure a high genotyping success rate. Eight polymorphisms in the *IL1B* gene met the criteria and were selected, including the SNPs rs3917368 (A/G at 3′ UTR), rs2853550 (C/T at 3′ UTR), rs1143643 (A/G at intron 6), rs1143634 (C/T at exon 5, known as +3954A/G and +3962A/G), rs1143630 (A/C at intron 3), rs1143627 (C/T at 5′-UTR, known as −31C/T), rs16944 (C/T at 5′-UTR, known as −511C/T), and rs12621220 (C/T at 5′-UTR).

Genomic DNA Extraction and Genotyping

All blood samples from individuals were collected by venipuncture for genomic DNA isolation. The genomic DNA was extracted from peripheral blood leukocytes (Genomic DNA kit; Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. DNA concentration was quantified in all samples before genotyping. Thirteen of the 484 samples were excluded in this study because the amount of DNA was not enough to perform the assay. All eight single-nucleotide polymorphisms (SNPs) in *IL1B* were genotyped with an allele-specific extension and ligation assay according to the manufacturer's instructions (Illumina).

IL1β Quantitative Measurement

The plasma IL1β level was measured by using a quantitative enzyme-linked immunosorbent assay according to the manufacturer's instructions (eBioscience, San Diego, CA).

Statistical Analysis

Associations between each SNP and disease were assessed by χ^2 test. Allele and genotype frequencies in cases and controls were compared and odds rations (ORs) per SNP were estimated by applying unconditional logistic regression. Alleles and genotype frequencies of alleles, genotypes, haplotypes, and diplotypes were expressed as a percentage of the total number of alleles, genotypes, haplotypes, and diplotypes. Results reaching $P < 0.05$ were statistically significant. The OR, with the 95% confidence interval (CI), was calculated from the genotype and allelic frequencies. Associations between each SNP and IL1β plasma levels were assessed by Student's *t*-test (for two-category variable) or ANOVA (for a three or more category variable; SPSS for Windows, ver., 14.0; Chicago, IL). Haplotypes were inferred by using Phase 2.1, a computational tool based on Bayesian methods.[31] Linkage disequilibrium (LD) was performed with Haploview 4.1.[31] The multifactor dimensionality reduction (MDR) 1.1.0 of the open-source MDR software package (Dartmouth Medical School, Hanover, NH) was used to detect the best locus–locus interaction models with an estimated testing accuracy of >50% consistency. The interaction dendrogram was established according to a hierarchical clustering algorithm.[32–35]

Results

Basic Characteristics of Patients with GD and Correlation between the Factors

The demographics and clinical information of the participants are summarized in Table 1. We also examined the association of GO with age, sex, smoking status, recurrence, treatment, and clinical features. The χ^2 test and Mann-Whitney U test revealed that sex, smoking status, and radioiodine therapy were significantly associated with GO among patients with GD.

T1

Allele and Genotype Frequencies of the *IL1B* Polymorphisms

To identify the SNPs associated with GO, we genotyped eight SNPs in *IL1B*. As comparing with healthy individuals, the

Table 2. Allele Frequencies of *IL1B* Single-Nucleotide Polymorphism in Healthy Individuals and Patients with or without GO

Alleles	Healthy (n = 320) n (%)	GD/non-GO (n = 542) n (%)	GD/GO (n = 400) n (%)	P* (OR, 95% CI)† GD vs. Healthy	GD/GO vs. Healthy	GD/GO vs. GD/non-GO
rs3917368						
A allele	171 (53.4)	297 (54.8)	234 (58.5)	0.362	0.174	0.257
G allele	149 (46.6)	245 (45.2)	166 (41.5)			
rs2853550						
C allele	300 (93.8)	505 (93.2)	367 (91.8)	0.478	0.307	0.410
T allele	20 (6.3)	37 (6.8)	33 (8.3)			
rs1143643						
A allele	170 (53.1)	297 (54.8)	235 (58.8)	0.297	0.131	0.226
G allele	150 (46.9)	245 (45.2)	165 (41.3)			
rs1143634						
T allele	168 (52.5)	8 (1.5)	5 (1.3)	1.655×10^{-112}	1.414×10^{-57}	0.769
C allele	152 (47.5)	534 (98.5)	395 (98.8)	(78.984, 43.795–142.446)	(87.316, 35.184–216.689)	
rs1143630						
C allele	266 (83.1)	453 (83.6)	332 (83.0)	0.931	0.965	0.814
A allele	54 (16.9)	89 (16.4)	68 (17.0)			
rs1143627						
T allele	182 (56.9)	309 (57.0)	228 (57.0)	0.967	0.973	0.997
C allele	138 (43.1)	233 (43.0)	172 (43.0)			
Rs16944						
C allele	166 (51.9)	309 (57.0)	229 (57.3)	0.090	0.150	0.968
T allele	154 (48.1)	233 (43.0)	171 (42.8)			
rs12621220						
C allele	201 (62.8)	334 (61.6)	256 (64.0)	0.954	0.742	0.456
T allele	119 (37.2)	208 (38.4)	144 (36.0)			

* Allele frequencies were determined by χ^2 test using 2 × 2 contingency tables.

† OR and 95% CI per allele were estimated by applying unconditional logistic regression. The major allele was used as the reference. $P < 0.05$ is statistically significant.

Table 3. Genotype Frequencies of *IL1B* SNPs in Healthy Individuals and Patients with or without GO

Genotypes	Healthy (n = 160) n (%)	GD/non-GO (n = 271) n (%)	GD/GO (n = 200) n (%)	P* GD vs. Healthy	P* GO vs. Healthy	P* GD/GO vs. GD/non-GO	OR (95% CI)† GD vs. Healthy	OR (95% CI)† GO vs. Healthy	OR (95% CI)† GD/GO vs. GD/non-GO
rs3917368									
A/A	44 (27.5)	70 (25.8)	71 (35.5)	0.594	0.27	**0.030**			1
A/G	83 (51.9)	157 (57.9)	92 (46.0)						**0.578 (0.380–0.878)**
G/G	33 (20.6)	44 (16.2)	37 (18.5)						0.829 (0.479–1.434)
A/G+G/G				0.559	0.106	**0.024**			**0.633 (0.425–0.941)**
rs2853550									
C/C	141 (88.1)	234 (86.3)	167 (83.5)	0.123	0.202	0.391			
C/T	18 (11.3)	37 (13.7)	33 (16.5)						
T/T	1 (0.6)	0 (0)	0 (0)						
C/T+T/T				0.086	0.263	—			
rs1143643									
A/A	44 (27.5)	70 (25.8)	72 (36.0)	0.495	0.229	**0.022**			1
A/G	82 (51.3)	157 (57.9)	91 (45.5)						**0.564 (0.371–0.856)**
G/G	34 (21.3)	44 (16.2)	37 (18.5)						0.818 (0.473–1.413)
A/G+G/G				*0.526*	0.086	**0.017**			**0.619 (0.416–0.921)**
rs1143634									
T/T	53 (33.1)	0 (0)	0 (0)				1	1	
C/T	31 (19.4)	263 (97.0)	195 (97.5)	**1.065×10^{-91}**	**2.993×10^{-51}**	0.767	2.387×10^{10} (0.0–0.0)	10.162×10^{9} (0.0–0.0)	
C/C	76 (47.5)	8 (3.0)	5 (2.5)				2.763×10^{8} (0.0–0.0)	1.063×10^{8} (0.0–0.0)	
C/T+C/C				**6.283×10^{-39}**	**1.207×10^{-18}**	0.767	7.111×10^{9} (0.0–0.0)	3.020×10^{9} (0.0–0.0)	
rs1143630									
C/C	112 (70.0)	186 (68.6)	138 (69.0)	0.447	0.877	0.496			
A/C	42 (26.3)	81 (29.9)	56 (28.0)						
A/A	6 (3.8)	4 (1.5)	6 (3.0)						
A/C+A/A				0.775	0.838	0.933			
rs1143627									
T/T	46 (28.8)	83 (30.6)	65 (32.5)	0.528	0.379	0.710			
C/T	90 (56.3)	143 (52.8)	98 (49.0)						
C/C	24 (15.0)	45 (16.6)	37 (18.5)						
C/T+C/C				0.481	0.379	0.592			
rs16944									
C/C	48 (29.9)	84 (31.0)	66 (33.0)	0.041	0.21	0.638	1		
C/T	70 (43.8)	143 (52.8)	97 (48.5)				0.911 (0.599–1.387)		
T/T	42 (26.3)	44 (16.2)	37 (18.5)				0.563 (0.356–0.889)		
C/T+T/T				0.664	0.543	0.645			
rs12621220									
C/C	58 (36.3)	101 (37.3)	83 (41.5)	0.358	0.296	0.642			
C/T	85 (53.1)	132 (48.7)	90 (45.0)						
T/T	17 (10.6)	38 (14.0)	27 (13.5)						
C/T+T/T				0.527	0.311	0.352			

Bold data indicate statistical significance.
* Genotype frequencies were determined by χ^2 test using 2 × 3 or 2 × 2 contingency tables.
† OR and 95% CI per genotype were estimated by applying unconditional logistic regression. $P < 0.05$ was statistically significant.

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presence of the C allele of SNP rs1143643 may increase the risk of GD ($P = 1.655 \times 10^{-112}$) and GO ($P = 1.414 \times 10^{-57}$), although all eight allele distributions of the *IL1B* polymorphisms did not differ significantly between GD patients with or without GO (Table 2). Table 3 summarizes the genotype distributions of the *IL1B* polymorphisms in all individuals. The T/T genotype of SNP rs1143634 was present only in healthy individuals, and the T/T genotype of SNP rs16944 was less frequent in patients with GD ($P = 0.041$). In addition, the A/A genotype of SNPs rs3917368 and rs1143643 may increase the risk of GO among patients with GD ($P = 0.024$ and $P = 0.017$, respectively). The interaction dendrogram of the eight SNPs in the *IL1B* gene were constructed with the MDR software, and the results revealed a strong interaction of the rs3917368 and rs1143643 loci in the *IL1B* gene in modulating the risk of GO (Fig. 1). These results suggest that patients with the T/T genotype at SNPs rs1143634 and rs16944 have a lower risk of developing GD. In addition, patients with the A/A genotype of SNPs rs3917368 and rs1143643 may have a higher risk of developing GO.

Haplotype Frequencies of the *IL1B* Polymorphisms

Combination of the eight selected SNPs in *IL1B* by tagging SNPs in the HapMap CHB+JPT population may represent different *IL1B* haplotypes. In addition, MDR analysis indicated that the best interaction model for predicting the development of GO is the eight-locus model composed by all eight SNPs analyzed in the present study (testing accuracy, 63.9%; cross-validation consistency, 100/100; $P < 0.0001$). Therefore, we determined the haplotypes in the eight SNPs that had frequencies of >5% and identified the 13 haplotypes shown in Table 4). Ht3-GCGCACTT, Ht5-ACACACTT, and Ht6-GTGCCCTC were found only in patients with GD, whereas Ht9-ACATCTTC, Ht10-ACATCTCC, Ht11-GCGCCCCT, Ht12-ACACCTTC, and HT13-GCGTCCCT were found only in the healthy individuals. Haplotype-specific analysis showed that the Ht1-ACACCTCC and Ht2-GCGCCCTT haplotypes may increase the risk of GD ($P = 1.241 \times 10^{-44}$ and 7.388×10^{-8}; OR, 21.599 and 3.917; 95% CI, 12.221–38.175 and 2.304–6.658, respectively) compared with the risk in healthy individuals. Ht4-GCGCCTCC may reduce the risk of GO among the patients with GD ($P = 0.025$; OR, 0.502; 95% CI, 0.273–0.925). Table 5 showed that 141 patients with GD bore the diplotype A-A/A-A, and it appeared more frequently in patients with GO than did A-A/G-G or G-G/G-G ($P = 0.008$; OR, 1.650; 95% CI, 1.141–2.384). In 419 patients with GD, the non-Ht4/non-Ht4 diplotype was less frequently found in the patients who also had GO compared with at least one Ht4 haplotype (diplotypes Ht4/Ht4 and Ht4/non-Ht4, $P = 0.007$; OR, 0.414; 95% CI, 0.214–0.797). These findings confirm that the results from genotype and haplotype analysis. In addition, the LD plots of *IL1B* in the healthy individuals and GD patients with or without GO showed an apparent variation in these polymorphisms (Fig. 2). These observations suggest that Ht1-ACACCTCC and Ht2-GCGCCCTT haplotypes put the individual at risk for the development of GD, whereas Ht4-GCGCCTCC may play a protective role against the development of GO.

GO-Associated *IL1B* Polymorphisms Related to Circulating IL1β Levels among Patients with GD

We performed enzyme-linked immunosorbent assays on plasma proteins from 432 of 471 GD patients and examined their association with *IL1B* genotypes and diplotypes (Table 6). The mean plasma IL1β concentration in patients with GO was 181.5 ± 580.1 pg/mL, which was significantly higher than in those without GO (88.8 ± 190.9 pg/mL, $P = 0.038$). Stratified analysis showed that patients with Ht4-GCGCCTCC had much lower concentrations of IL1β ($P = 0.042$). Although patients with the A-A/G-G and G-G/G-G diplotypes of the SNPs rs3917368 and rs1143643 are less susceptible to GO (GO versus nonGO, 45.9% vs. 57.8%, respectively), they had unexpectedly higher IL1β concentrations than did those with the A-A/A-A diplotype ($P = 0.029$). Genotype analysis is consistent with the observation. Our results demonstrated that higher circulating IL1β concentrations may correlate with GO development. The GO-protective haplotype Ht4-GCGCCTCC may be used to predict IL1β-induced GO in patients with GD. However, patients with A-A/G-G and G-G/G-G, although less susceptible to GO, may have higher plasma IL1β concentrations than do those with A-A/A-A. These findings imply that the involvement of IL1β in GO development may be more than the *IL1B* polymorphism-associated circulating IL1β elevation in patients with GD.

Discussion

We found that the SNPs rs3917368 and rs1143643 in the 3′ UTR and intron regions of *IL1B*, respectively, may be risk genotypes for development of GO. Persons with the genotypes containing both rs3917368 A/A and rs1143643 A/A may bear a

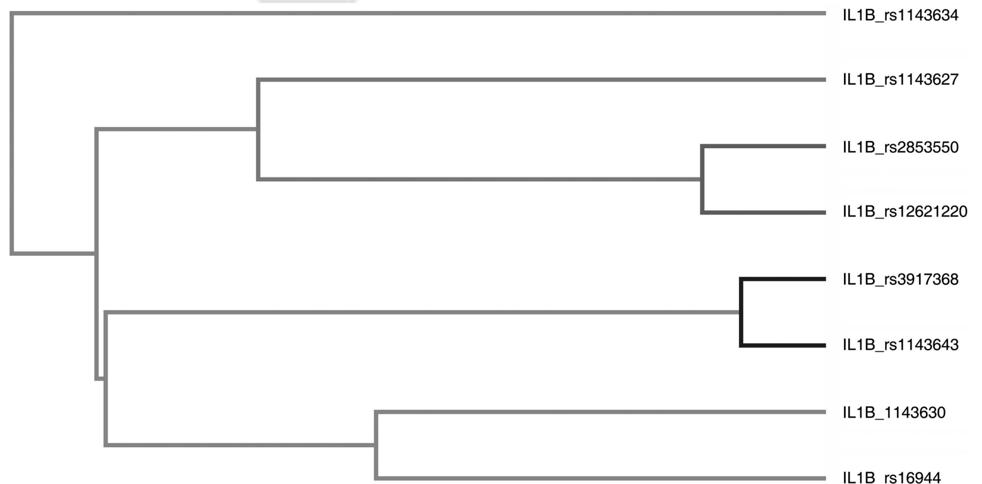

Figure 1. Interaction dendrogram. The location of the longitudinal connecting bars indicates the strength of the dependence: *left* is weaker and *right* is stronger. The hierarchical cluster analysis placed *IL1B* rs3917368 and rs1143643 on the same branch, demonstrating the strong interaction between these two SNPs. There were interactions between *IL1B* rs3917368-rs1143643 and other SNPs as shown in the dendrogram. *IL1B*, interleukin-1β gene; SNP, single nucleotide polymorphism.

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TABLE 4. Haplotypes from SNPs of *IL1B* in Healthy Individuals and Patients with or without GO

			GD		*P*, Global†			*P*, Individual‡ OR (95% CI)§		
Haplotypes*		Healthy *n* (%)	Non-GO *n* (%)	GO *n* (%)	GD vs. Healthy	GD/GO vs. Healthy	GD/GO vs. GD/nonGO	GD vs. Healthy	GD/GO vs. Healthy	GD/GO vs. GD/nonGO
Ht1	ACACCTCC	13 (4.1)	251 (46.3)	199 (49.8)				**1.241×10^{-44}** **21.599 (12.221–38.175)**	**9.684×10^{-41}** **23.380 (12.979–42.118)**	0.296
Ht2	GCGCCCTT	16 (5.0)	97 (17.9)	64 (16.0)				**7.388×10^{-8}** **3.917 (2.304–6.658)**	**3.058×10^{-6}** **3.619 (2.048–6.395)**	0.445
Ht3	GCGCACTT	0 (0.0)	53 (9.8)	37 (9.3)				9.598×10^{-9} 6.068×10^{8} (0.0–0.0)	2.232×10^{-8} 1.424×10^{9} (0.0–0.0)	0.785
Ht4	GCGCCTCC	13 (4.1)	39 (7.2)	15 (3.8)				0.250	0.829	**0.025** **0.502 (0.273–0.925)**
Ht5	ACACACTT	0 (0.0)	36 (6.6)	31 (7.8)				9.454×10^{-7} 5.908×10^{8} (0.0–0.0)	3.566×10^{-7} 1.401×10^{9} (0.0–0.0)	0.513
Ht6	GTGCCCTC	0 (0.0)	14 (2.6)	15 (3.8)				1.496×10^{-3} 5.662×10^{8} (0.0–0.0)	4.639×10^{-4} 1.343×10^{9} (0.0–0.0)	0.305
Ht7	GTGCCCTT	1 (0.3)	9 (1.7)	9 (2.3)	1.643×10^{-173}	1.793×10^{-100}	0.348	0.043 6.214 (0.826–46.737)	0.273	0.514
Ht8	GCGCCCTC	5 (1.6)	12 (2.2)	12 (3.0)				0.310	0.207	0.449
Ht9	ACATCTTC	39 (12.2)	0 (0.0)	0 (0.0)				1.370×10^{-27} 0.000 (0.0–0.0)	7.009×10^{-13} 0.000 (0.0–0.0)	—
Ht10	ACATCTCC	35 (10.9)	0 (0.0)	0 (0.0)				7.485×10^{-25} 0.000 (0.0–0.0)	1.191×10^{-11} 0.000 (0.0–0.0)	—
Ht11	GCGCCCCT	29 (9.1)	0 (0.0)	0 (0.0)				8.971×10^{-21} 0.000 (0.0–0.0)	7.954×10^{-10} 0.000 (0.0–0.0)	—
Ht12	ACACCTTC	23 (7.2)	0 (0.0)	0 (0.0)				1.003×10^{-16} 0.000 (0.0–0.0)	5.047×10^{-8} 0.000 (0.0–0.0)	—
Ht13	GCGTCCCT	23 (7.2)	0 (0.0)	0 (0.0)				1.003×10^{-16} 0.000 (0.0–0.0)	5.047×10^{-8} 0.000 (0.0–0.0)	—
Remaining		123 (38.4)	31 (5.7)	18 (4.5)				—	—	—
Total		320	542	400						

* Order of SNPs comprising the *IL1B* haplotypes: rs3917368, rs2853550, rs1143643, rs1143634, rs1143630, rs1143627, rs16944, and rs12621220. The haplotypes were identified by the Bayesian statistical method available in the program Phase 2.1.

† Global test for haplotype frequency in relation to GD or GO development was determined by χ^2 test using $2 \times n$ contingency tables (where n is 13 +1 (haplotype >5% plus remaining haplotypes). $P < 0.05$ is statistically significant.

‡ Individual haplotype frequency in relation to GD or GO development was determined by χ^2 test using 2×2 contingency tables. $P < 0.05$ is statistically significant.

§ OR and 95% CI for genotypes were estimated by applying unconditional logistic regression.

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Table 5. Distribution of *IL1B* Diplotypes and Their Associations with GO

Diplotypes*	Without GO (n = 271) n (%)	With GO (n = 200) n (%)	Cross Validation Consistency	P	OR (95% CI)
A–A/A–A	70 (25.8)	71 (35.5)			1
A–A/G–G	157 (57.9)	92 (44.0)		0.030§	**0.578 (0.380–0.878)**‖
G–G/G–G	44 (14.2)	37 (18.5)			0.830 (0.480–1.434)‖
A–A/G–G + G–G/G–G	201 (74.2)	129 (64.5)	~~100/100~~	0.024§	**0.633 (0.425–0.941)**‖
			100/100	0.008†	**1.650 (1.141–2.384)‡**
Non-Ht4/non-Ht4	232 (85.6)	187 (93.5)			1
Ht4/non-Ht4	39 (14.4)	11 (5.5)		0.002§	**0.350 (0.174–0.702)**‖
Ht4/Ht4	0 (0.0)	2 (1.0)			2.004×10^9 (0.0–0.0)‖
Ht4/Ht4 + Ht4/non-Ht4	39 (75.0)	13 (25.0)		0.007§	**0.414 (0.214–0.797)**‖

* The order of the SNPs comprising the *IL1B* haplotype was rs3917368 and rs2853550. The haplotypes were identified by the Bayesian statistical method available in the program Phase 2.1.

† Significance of diplotype in relation to GO development was determined by applying multifactor dimensionality reduction (MDR) models. $P < 0.05$ was statistically significant.

‡ ORs and 95% CIs for A–A/A–A and non A–A/A–A was estimated by applying multifactor dimensionality reduction (MDR) models.

§ Genotype frequencies were determined by χ^2 test using 2×2 or 2×3 contingency tables. $P < 0.05$ was statistically significant.

‖ ORs and 95% CIs for non-Ht4/non-Ht4 (reference) and Ht4/Ht4 and Ht4/non-Ht4 was estimated by applying unconditional logistic regression.

higher risk of developing GO. Clinical association studies showed that the presence of the A-A/A-A diplotype was significantly associated with a higher risk of GO, but the presence of the diplotype along with the Ht4-GCGCCTCC haplotype may be protective against GO. In addition, the circulating IL1β concentrations have been analyzed in GD patients and the association with GO as well as genotypes have been made. Our results showed that *IL1B* polymorphisms may be associated with the level of circulating IL1β as well as GO development in patients with GD.

Several reports have predicted the genetic association of *IL1B* with the development of GD and GO. Hayashi et al.[36] found a significant association between GD and the −31T (rs1143627) allele in the promoter region in their study of the Japanese population. Liu et al.[13] found an association between −511C/T (rs16944) and GD with GO in China, whereas Lacka et al.[14] and Khalilzadeh et al.[15] did not find an association between the *IL1B* polymorphisms -511C/T (rs16944) and +3954 C/T (rs1143634) and GO in their studies in Iran and Poland, respectively. We found that the C allele of +3954 (rs1143634) is related to the development of GD and GO. In addition, the T/T genotype of −511 (rs16944) is related to less susceptibility to GD. However, in the present study, the genotypes −31C/T (rs1143627) and -511C/T (rs16944) were not associated with the development of GO. The effect of population differences in the determination of such associations should not be underestimated. In the present study, for the first time, we found the association between A/A genotypes of rs3917368 and rs1143643 and the risk for GO, although the significance was weak. Although we identified haplotypes with the Phase program, we found much more diverse haplotype distribution in healthy individuals than in the patients with GD. In addition, patients with GO had more similar haplotype distribution than did those without GO. This observation is

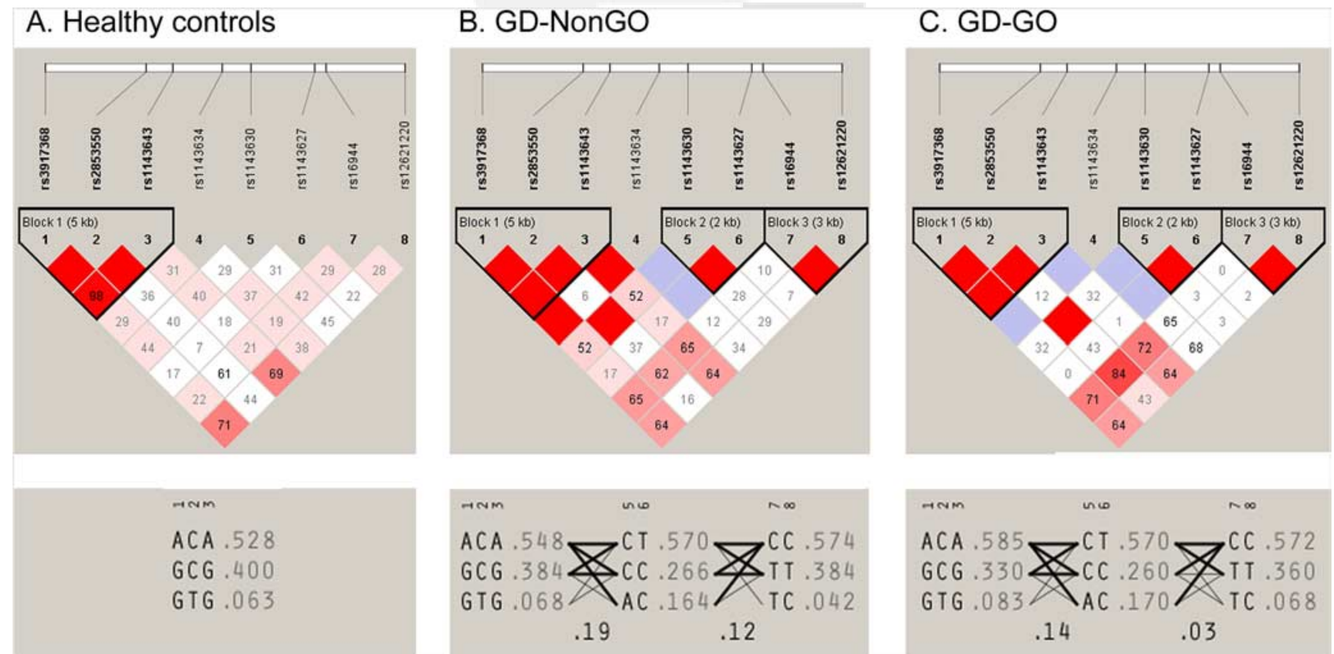

Figure 2. Pairwise LD measures of D′ (*top*) and r^2 (*bottom*) for the SNPs of the *IL1B* locus. The scale *above* the figures indicates the site of each SNP around the *IL1B* gene region in healthy individuals (**A**), patients with Graves' disease without ophthalmopathy (**B**), and patients with Graves' disease with ophthalmopathy (**C**).

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Table 6. Stratified Analysis of Plasma IL1β Levels on GO Risk by *IL1B* Genotypes

Variables	GD/non-GO	GD/GO	P*	P†
—	88.8 ± 190.9 (249)	181.5 ± 580.1 (183)	**0.038**	
rs3917368				
A/A	62.5 ± 80.9 (63)	65.7 ± 124.8 (65)		0.868
A/G	102.1 ± 224.8 (144)	302.8 ± 825.9 (84)	**0.030**	**0.032**
G/G	81.0 ± 180.1 (42)	103.7 ± 200.3 (34)		0.606
	P = 0.377‡‡	**P = 0.031‡**		
A/G+G/G	97.3 ± 215.2 (186)	245.4 ± 709.5 (118)	**0.029**	
rs1143643				
A/A	62.5 ± 80.9 (63)	65.8 ± 123.9 (66)		0.859
A/G	102.1 ± 224.8 (144)	305.5 ± 830.6 (83)	**0.029**	**0.031**
G/G	81.0 ± 180.1 (42)	103.7 ± 200.3 (34)		0.606
	P = 0.377‡	**P = 0.029‡**		
A/G+G/G	97.3 ± 215.2 (186)	246.8 ± 712.4 (117)	**0.029**	
A–A/A–A	62.5 ± 80.9 (63)	65.6 ± 124.8 (65)		0.868
A–A/G–G	102.1 ± 224.8 (144)	302.8 ± 825.9 (84)	**0.030**	**0.032**
G–G/G–G	81.0 ± 180.1 (42)	103.7 ± 200.3 (34)		0.606
	P = 0.377‡	**P = 0.031‡**		
A–A/G–G + G–G/G–G	97.3 ± 215.2 (186)	245.4 ± 709.5 (118)	**0.029**	
Non-Ht4/non-Ht4	91.1 ± 202.0 (213)	189.0 ± 598.1 (171)		**0.042**
Ht4/non-Ht4	73.1 ± 103.7 (36)	86.6 ± 178.5 (10)	**0.604**	**0.042**
Ht4/Ht4	—	14.2 ± 10.9 (2)		0.758
	P = 0.402‡	P = 0.547‡		
Ht4/non-Ht4 + Ht4/Ht4	73.1 ± 103.7 (36)	74.6 ± 163.9 (12)	**0.042**	

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Data are expressed as mean ± SD plasma IL1β levels (in picograms/milliliter), with the number of subjects in parentheses. $P < 0.05$ is statistically significant (data in bold).

* Significance of genotypes and haplotypes toward plasma IL1β levels or plasma IL1β levels in relation to GO development in all patients with GD were determined by Student's *t*-test or ANOVA, as suitable.

† Significance of each genotype or haplotype in relation to plasma IL1β levels in all patients with GD were determined by Student's *t*-test.

‡ Significance of genotypes and haplotypes in relation to plasma IL1β levels in patients with or without GO were determined by ANOVA.

consistent with our results from LD analysis. These results may provide new information for prediction of development of GD and GO by different *IL1B* haplotypes.

Recent studies have shown that a positive feedback cycle composed of mechanical, immunologic, and cellular processes is involved in the development of GO.[8,21,22] Interstitial accumulation of GAGs, PGE2, and adipogenesis in the orbital tissue contributes to GO in GD patients and these observations and a series of thyroid-related factors, such as the thyrotropin receptor antigen, are thought to be a consequence of the release of certain cytokines, including IL1β, from T cells, monocytes, and activated fibroblasts.[8,21–23,37] Polymorphisms may cause alternations in the expression and function of *IL1B*,[16–18] which may affect the downregulation of T-cell activation and the subsequent inflammatory diseases, autoimmune diseases, cancer, and GAG accumulation. Given the important role of T cells in the pathogenesis of GO, *IL1B* may be a candidate gene for the induction of these autoimmune reactions. Our results demonstrated that GO patients have higher plasma IL1β levels than those without GO, which supports this hypothesis.

Although our hypothesis was that polymorphisms of *IL1B* relate to the development of GO in patients with GD, the −31C and −511T of *IL1B*, the polymorphism associated with decreased and increased transcriptional activity,[38,39] were associated with neither IL1β levels nor GO development in our study. We found that the Ht4-GCGCCTCC is related to lower circulating IL1β concentration and less susceptibility toward GO, indicating that Ht4-GCGCCTCC may be used to predict the IL1β-induced GO in patients with GD. However, the A-A/G-G and G-G/G-G diplotypes (less susceptible for GO were related to high circulating levels of IL1β. It is notable that the IL1β concentration examined in our patients (0–3.843 ng/mL) was lower than the IL1β dosage most used in the in vitro experiments (10 ng/mL),[19,20,40,41] and the outliers (IL1β >1 ng/mL) were only the patients with the A-A/G-G genotype. Although plasma IL1β in healthy individuals is yet to be determined, it would be interesting to know whether there are critical dose-dependent levels of IL1β in protection against or promotion of the development of GO. The linkage among the *IL1B* polymorphisms, IL1β level, and GO development should be further confirmed in studies with larger samples.

In the present study, our results suggest that *IL1B* genotypes are associated with the development of GO, the most common orbital disease in GD. This report provides evidence from examination of patient outcomes that polymorphisms of the *IL1B* gene may predict the development of GO.

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