Chinese Journal of Physiology 57(2): 69-75, 2014
DOI: 10.4077/CJP.2014.BAB150

Association of Interleukin-16 Polymorphisms with Graves' Disease in a Taiwanese Population

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Abstract

Graves' disease (GD) is a complex, organ-specific autoimmune disease wherein the thyroid gland becomes enlarged and overactive. During GD progression, T cells secrete interleukin-16 (IL-16) to promote inflammation, act as chemoattractants that recruit more inflammatory cells, and activate target cells to enhance the development of GD. To investigate the role of IL-16 in GD, we genotyped 474 patients with GD at 8 single-nucleotide polymorphisms (SNPs) in the *IL-16* gene. The *IL-16* SNP rs8028364 was found to be associated with GD when compared with the control subjects ($P = 2.93 \times 10^{-17}$; CG genotype: odds ratio [OR] = 0.2 [0.07, 0.59]; CC genotype: OR = 0.03 [0.01, 0.09]). The rs1131445 polymorphism was found to be associated with GD under the allelic model ($P = 0.01$; G allele: OR = 1.97 [1.17, 3.32]). Sliding-window haplotype analysis by the PLINK program showed that the most significant haplotype was provided by the 6-SNP haplotype window, consisting of rs7182786, rs8028364, rs12907134, rs4128767, rs4072111 and rs8031107 ($P = 2.31 \times 10^{-51}$). We found 2 protective haplotypes: GCAAGG ($P = 8.69 \times 10^{-7}$; OR = 0.22 [0.12, 0.41]) and AGAAGG ($P = 0.0012$; OR = 0.26 [0.12, 0.6]). In addition, GGGGAA ($P = 0.39$; OR = 2.32 [1.08, 4.99]) and GGGAGA ($P = 1.18 \times 10^{-5}$; OR = 5.54 [2.50, 12.31]) were found to be the two high-risk haplotypes. These results suggest that polymorphisms in *IL-16* may be used as genetic markers for the diagnosis and prognosis of GD.

Key Words: Graves' disease, IL-16, polymorphisms

Introduction

Graves' disease (GD) is a complex, organ-specific autoimmune disease wherein the thyroid gland becomes enlarged and overactive (21). GD is characterized by a variety of clinical features such as hyperthyroidism, diffuse goiter, presence of autoantibodies against thyroid-specific antigens and dermopathy. Results of genetic studies from homozygous twins have revealed the importance of genetic factors in the development of GD and suggest that approximately 80% of GD development is determined by genetic factors (2). The infiltration of T cells into the thyroid gland is an important feature of GD. The accumulation of T

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Received: September 14, 2012; Revised (Final Version): March 15, 2013; Accepted: June 11, 2013.
 ISSN : 0304-4920. http://www.cps.org.tw

cells in the thyroid gland is considered an important factor in promoting inflammatory responses and tissue remodeling, which worsen the progression of GD (7, 18). T cells secrete a variety of cytokines, including IL-16, that promote inflammation, act as chemoattractants to recruit more inflammatory cells, and activate target cells to enhance the progression of GD (5, 11, 13, 14).

IL-16, a known T-cell chemoattractant, promotes the migration of T cells to target sites. Several cell types express IL-16, including T cells, eosinophils, dendritic cells, fibroblasts, epithelial cells and neuronal cells (5). The serum levels of IL-6, IL-1β and tumor necrosis factor (TNF)-α are higher in GD patients than in control subjects (15). Gianoukakis *et al*. showed that treating thyrocytes with IL-1β increases chemotactic activities by upregulating the expression levels of IL-16 (8). In addition, fibroblasts treated with IgG isolated from GD patients also increase the expression level of IL-16 (17). These studies suggest the importance of IL-16 in the pathogenesis of GD. IL-16 is a CD4-specific ligand required for the initiation of the bioactivities. By binding to the CD4 molecule, IL-16 activates T cells, monocytes, macrophages and dendritic cells, and increases the production of TNF-α, IL-1β and IL-6 leading to inflammatory responses (3, 4, 12). In GD, T cells infiltrate the thyroid gland and collaborate with thyrocytes and fibroblasts to form a paracrine loop between IL-16 and IL-1β, which maintains the inflammatory responses. This vicious cycle continuously induces inflammatory responses in the thyroid gland and subsequently induces GD.

To determine the genetic role of IL-16 in GD pathogenesis, we investigated single-nucleotide polymorphisms (SNPs) in the *IL-16* gene that may be associated with the protection against or risk factors for GD in Taiwan Chinese patients.

Materials and Methods

Patients

A total of 474 patients with GD were enrolled in this study. The diagnosis of GD was performed by endocrinologists and was assessed by ophthalmologists. The diagnosis of GD was made on the basis of clinical symptoms and biochemical affirmation of hyperthyroidism, multinodular goiter and a positive result for at least one of the following biochemical tests: thyroid-stimulating hormone receptor antibody, diffusely increased iodine-131 uptake in the thyroid gland and exophthalmos. Patients with GD were classified in accordance with the NOSPECS system recommended by the American Thyroid Association. Blood samples were collected for genomic DNA isolation and serological tests. This study was approved by the ethical committee and institutional review board of the China Medical University Hospital. Informed consent was obtained from all patients or their guardians.

SNP Genotyping

IL16 SNP genotype information was retrieved from the HapMap database for the HCB + JPT populations. The gene boundary of IL-16 was determined by SNPper program (http://snpper.chip.org) which are -10 kb from the transcription start site and +10 kb from the stop codon. Tagging SNPs were selected through the Tagger function in the Haploview software program (version 4.2). The selection criteria were: [1] the minor allele frequency should be greater than 10%; [2] SNPs that potentially affect transcription efficiency, translation efficacy or protein functions were selected first; and [3] the availability of probes or primers that passed the manufacturer's qualification requirements (Applied Biosystems Inc., Foster City, CA, USA). We used aggressive tagging method which use 2- and 3-marker haplotypes and set a maximum of 8 tags. We forced to include rs4072111 which may influence the splicing efficiency. The rest of the SNPs were determined by the Haploview software. Eight polymorphisms were selected according to these criteria: rs7182786 (A/G), rs8028364 (C/G), rs12907134 (A/G), rs4128767 (A/G), rs4072111 (A/G), rs8031107 (A/G), rs4072680 (A/G) and rs1131445 (A/G).

Statistical Analysis

PLINK (v. 1.07; http://pngu.mgh.harvard.edu/~purcell/plink/) was used for the analysis of the genotype frequency and allelic frequency distributions of the polymorphisms in controls and patients with GD. Hardy-Weinberg equilibrium (HWE) was performed separately for controls and GD patients. Haplotype analysis with sliding windows were analyzed with PLINK. Haplotype blocks were created with Haploview (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). A *P*-value less than 0.05 was considered statistically significant. The odds ratio (OR) was calculated from genotype frequencies and allelic frequencies with a 95% confidence interval (CI).

Results

Eight SNPs were examined; these were designated S1 to S8 in sequential order from the 5′-end of the *IL-16* gene for easy reference (Table 1). All SNPs were in Hardy-Weinberg equilibrium. The genotype

Table 1. Genotype count, Hardy-Weinberg equilibrium testing, minor allele frequency and association of IL-16 SNPs

	Allele		Genotype counts (11/12/22)		HWE (*P*-value)		Minor allele freq		Association (best result)	
SNP	1	2	Cases	Controls	Cases	Controls	Cases	Controls	*P*-value	Model
rs7182786 (S1)	A	G	39/194/241	3/19/23	1.0000	1.0000	0.2869	0.2778	0.85	Add
rs8028364 (S2)	C	G	40/203/231	23/18/4	0.6622	1.0000	0.2985	0.2889	2.93×10^{-17}	Geno
rs12907134 (S3)	A	G	104/227/143	10/24/11	0.4594	0.7701	0.4589	0.4889	0.42	Dom
rs4128767 (S4)	G	A	16/156/302	1/21/23	0.5628	0.2392	0.1983	0.2556	0.17	Geno
rs4072111 (S5)	A	G	10/148/316	1/20/24	0.1555	0.2518	0.1772	0.2444	0.10	Add
rs8031107 (S6)	G	A	102/225/147	11/25/9	0.3553	0.5574	0.4525	0.4778	0.12	Dom
rs4072680 (S7)	G	A	113/230/131	11/25/9	0.5808	0.5574	0.4810	0.4778	0.27	Dom
rs1131445 (S8)	G	A	63/201/210	2/15/28	0.1866	1.0000	0.3449	0.2111	0.01	Allelic

The SNPs are designated as S1 to S8 for easy reference and discussion. The two alleles of each SNPs are assigned as "1" or "2" and the genotypes counts are the exact numbers of genotypes 11, 12 and 22 in either the GD patients or control individuals. HWE stands for Hardy-Weinberg equilibrium. The association analysis was performed using five different models for each SNP: genotypic (Geno), additive (Add), dominant (Dom), recessive (Rec) and allelic models. The most significant results are shown in this table.

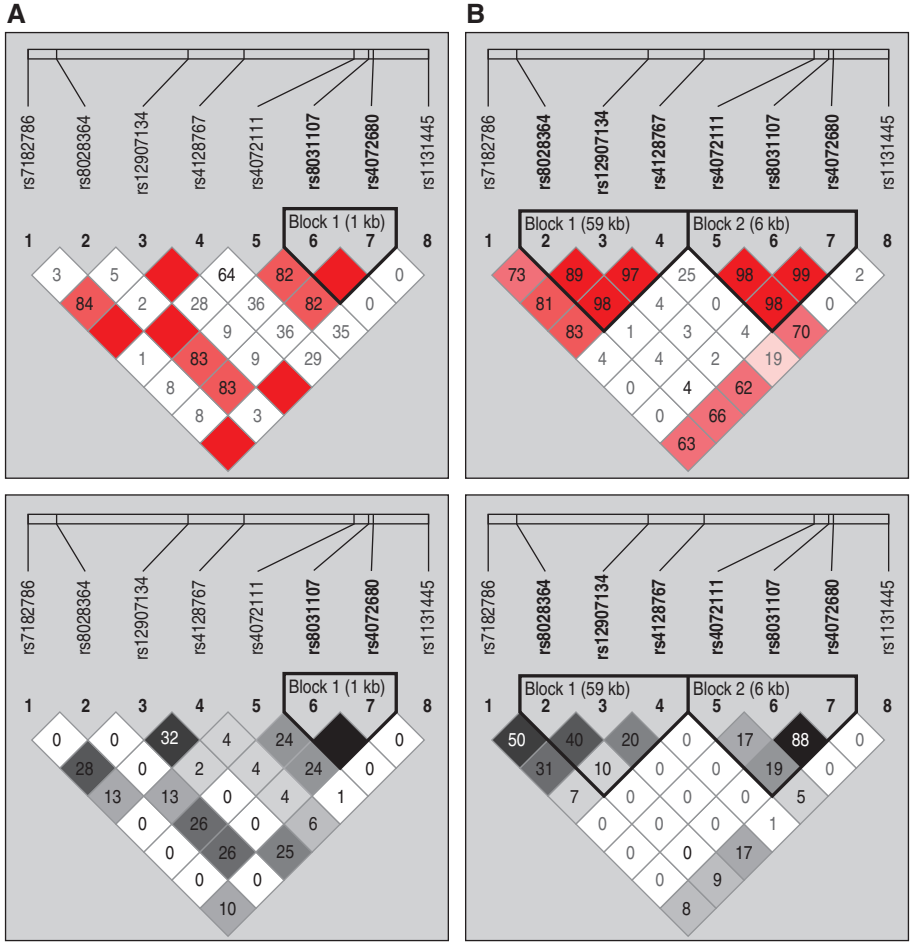

Fig. 1. Linkage disequilibrium (LD) plots of the SNPs in *IL-16* identified from 45 control subjects and 474 GD patients. The gene orientation is from 5′ to 3′ (left to right) relative to the positive strand of *IL-16* in control subjects (A) and GD patients (B). The plots were constructed using Haploview ver. 4.2. The D′ (×100) (red), and r2 (×100) (grey) values are shown as diamond-shaped data points. Strong LD is indicated by dark gray/red, whereas light gray/pink and white indicate uninformative and low confidence values, respectively.

Table 2. Genotype distribution among different ethnic populations

SNP	1	2	HCB	CEU	JPT	YRI	P^{a}_{CEU}	P^{b}_{JPT}	P^{c}_{YRI}
rs7182786 (S1)	A	G	3/19/23	71/17/2	4/22/19	18/38/34	1.16×10^{-16}	0.69	0.1
rs8028364 (S2)	C	G	23/18/4	5/31/54	21/17/6	57/25/8	2.9×10^{-11}	0.78	0.34
rs12907134 (S3)	A	G	10/24/11	19/51/20	9/23/13	8/32/49	0.93	0.89	0.002
rs4128767 (S4)	G	A	1/21/23	11/48/31	1/23/21	4/34/52	0.06	0.91	0.54
rs4072111 (S5)	A	G	1/20/24	1/27/62	0/24/21	0/11/79	0.2	0.46	4.0×10^{-5}
rs8031107 (S6)	G	A	11/25/9	18/49/20	13/20/11	6/25/57	0.85	0.63	3.5×10^{-6}
rs4072680 (S7)	G	A	11/25/9	19/50/20	13/20/11	9/35/46	0.9	0.63	0.001
rs1131445 (S8)	G	A	2/15/28	7/52/31	5/17/23	7/45/38	0.009	0.39	0.09

Genotype frequencies were determined by *Chi*-square test using 2 × 3 contingency tables. HCB was compared with CEU[a] or JPT[b] or YRI[c]. *P*-values less than 0.05 were considered significant. HCB: Han Chinese in Beijing, China; CEU: Utah residents with ancestry from northern and western Europe; JPT: Japanese in Tokyo, Japan; YRI: Yoruba in Ibadan, Nigeria.

and allele distributions of the 8 polymorphisms are shown in Table 1. Five different genetic models (genotypic, additive, dominant, recessive and allelic) were used to determine the difference between the healthy population of Han Chinese in Beijing (HCB) (obtained from the PubMed SNP database) and GD patients. Comparison of the genotypes and allele frequencies between the control subjects and GD patients showed no significant differences in 6 of the 8 polymorphisms, including S1, S3, S4, S5, S6 and S7 (Table 1); however, significant differences were found for S2 and S8. The lowest *P*-values among the 5 different genetic models were observed in the genotypic model for S2 ($P = 2.93 \times 10^{-17}$) and the allelic model for S8 ($P = 0.01$) (Table 1). The 8 SNPs were then subjected to a linkage disequilibrium (LD) plot by using the Haploview program. The D′ and r^2 values are presented as diamond-shaped points on the graph (Fig. 1). The LD map showed distinct differences between the 2 groups; an apparent variation in the S2 polymorphism was detected, indicating that this SNP may play some role in developing GD (Fig. 1).

The selected 8 SNPs (S1 to S8) are regarded as evolutionarily stable because no significant differences were found between HCB and Japanese in Tokyo, Japan (JPT). For S2 and S8, we did not detect significant differences between HCB and a more distant population of Yoruba in Ibadan, Nigeria (YRI) ($P = 0.34$ and 0.09, respectively), indicating they are stable between different ethnic groups (Table 2).

The CG and CC genotypes at S2 had a 0.2- and 0.03-fold lower risk of developing GD, respectively. The C allele in S2 showed a protective role in GD development and was 0.17-fold lower than the G allele. The G allele at S8 showed a 1.97-fold increase in risk for the development of GD compared with the A allele (Table 3). Taken together, these results showed a significant difference between the control groups and GD patients with regard to genotype or allele distribution for the S2 polymorphism. Furthermore, the frequency of the C allele was significantly lower in GD patients.

Haplotype frequencies were estimated among the 8 polymorphisms. The haplotypes were determined using a sliding-window approach and were examined in haplotypes of all possible sizes (Table 4). We used 36 sliding windows in all, and 14 of these were significantly associated with GD (omnibus test $P < 0.05$). For sliding windows with a size of up to 3 SNPs per window, the omnibus test result was significant when S2 was included in the window. The importance of S2 was more obvious when the sliding windows at 6 SNPs per window showed the significant results. The relative importance of SNPs within a sliding window was less apparent when the SNPs increased beyond 6 SNPs per window (Table 4). The details of haplotype analysis for the 6-SNP window are shown in Table 5. We found 2 protective haplotypes: GCAAGG ($P = 8.69 \times 10^{-7}$; OR = 0.22 [0.12, 0.41]) and AGAAGG ($P = 0.001$; OR = 0.26 [0.12, 0.6]). The two high-risk haplotypes were GGGGAA ($P = 0.39$; OR = 2.32 [1.08, 4.99]) and GGGAGA ($P = 1.18 \times 10^{-5}$; OR = 5.54 [2.50, 12.31]).

Discussion

In this study, we investigated the role of IL-16 polymorphisms in the risk of GD pathogenesis. Among the eight tagSNPs tested, rs8028364 and rs1131445 showed significant association with GD. Moreover, haplotype analysis showed that GCAAGG and AGAAGG played a protective role, whereas GGGGAA and GGGAGA were risk factors for GD.

In the study of the association between IL-16 polymorphisms with GD, Gu *et al*. found that the IL-16 polymorphisms were associated with GD as well as

Table 3. Odds ratios of SNPs

SNP	Genotype	Odds ratio	Allele	Odds ratio
rs7182786 (S1)	AA	1.24 (0.36-4.33)	A	1.05 (0.65-1.69)
	AG	0.97 (0.52-1.84)	G	1
	GG	1		
rs8028364 (S2)	CC	0.03 (0.01-0.09)	C	0.17 (0.11-0.28)
	CG	0.2 (0.07-0.59)	G	1
	GG	1		
rs12907134 (S3)	AA	0.8 (0.33-1.95)	A	0.89 (0.58-1.37)
	AG	0.73 (0.35-1.53)	G	1
	GG	1		
rs4128767 (S4)	GG	1.22 (0.15-9.6)	G	0.72 (0.44-1.19)
	GA	0.57 (0.3-1.05)	A	1
	AA	1		
rs4072111 (S5)	AA	0.76 (0.09-6.18)	A	0.67 (0.4-1.11)
	AG	0.56 (0.3-1.05)	G	1
	GG	1		
rs8031107 (S6)	GG	0.57 (0.23-1.42)	G	0.76 (0.49-1.17)
	GA	0.55 (0.25-1.21)	A	1
	AA	1		
rs4072680 (S7)	GG	0.71 (0.28-1.76)	G	0.85 (0.55-1.31)
	GA	0.63 (0.29-1.39)	A	1
	AA	1		
rs1131445 (S8)	GG	4.2 (0.97-18.12)	G	1.97 (1.17-3.32)
	GA	1.79 (0.93-3.44)	A	1
	AA	1		

Table 4. Sliding window (SW) haplotype analysis based on omnibus tests for all windows of all possible sizes across the eight SNPs

Sliding window (SW)		SW with omnibus test $P < 0.05$			Significant result	
SNPs/SW	No. of SW	No. of SW	First SW	Last SW	SW	*P*-value
1	8	0	–	–	–	–
2	7	3	S1-S2	S7-S8	S1-S2	3.11×10^{-37}
					S2-S3	5.10×10^{-41}
					S7-S8	0.05
3	6	2	S1-S3	S2-S4	S1-S3	1.38×10^{-46}
					S2-S4	3.08×10^{-42}
4	5	2	S1-S4	S2-S5	S1-S4	6.74×10^{-47}
					S2-S5	3.11×10^{-39}
5	4	2	S1-S5	S2-S6	S1-S5	1.55×10^{-43}
					S2-S6	1.29×10^{-42}
6	3	2	S1-S6	S2-S7	S1-S6	$\mathbf{2.31 \times 10^{-51}}$
					S2-S7	2.25×10^{-41}
7	2	2	S1-S7	S2-S8	S1-S7	3.92×10^{-36}
					S2-S8	4.16×10^{-19}
8	1	1	S1-S8	S1-S8	S1-S8	8.92×10^{-11}

The lowest *P*-values of overall haplotype distribution (omnibus) is shown in boldface.

Table 5. Haplotype analysis for 6-SNP windows showing the lowest *P*-value among the all possible sliding windows

Haplotypes	Cases	Controls	*P*-value	OR (95% CI)
rs7182786 – rs8028364 – rs12907134 – rs4128767 – rs4072111 – rs8031107				
OMNIBUS			2.31×10^{-51}	
ACAAGG	0.2251	0.1823	0.38	1.3 (0.75, 2.27)
GCAAGG	0.04888	0.1865	8.69×10^{-7}	**0.22 (0.12, 0.41)**
AGAAGG	0.02622	0.09279	0.0012	**0.26 (0.12, 0.6)**
GGAAGG	0.1213	0.05436	0.076	2.4 (0.95, 6.10)
GCGAGG	0.01448	0.03991	0.090	0.35 (0.11, 1.15)
GGGGAA	0.174	0.08334	0.039	**2.32 (1.08, 4.99)**
GGGAGA	0.3093	0.07476	1.18×10^{-5}	**5.54 (2.50, 12.31)**

The odds ratios (OR) are shown in boldface when their corresponding haplotypes are significantly associated with GD ($P < 0.05$). Only haplotypes with a frequency of 0.02 or above in either cases or controls are shown. PLINK calculates OR for a particular haplotype with reference to all the other haplotypes, and hence the reference haplotypes are different for different individual haplotypes under study.

Graves' ophthalmopathy (GO) (9). However, we did not find any association between IL-16 polymorphisms with GO. There are two possible explanations for the discrepancies: [1] we did not include in our study rs4778889 and rs4778641 which are the two significant SNPs in the study of Gu *et al*.; [2] it is not possible to get two exactly the same clinical samples with the same genetic background often resulting in different association datasets.

The potential biological functions of the SNPs tested in this study were predicted through the website: http://snpinfo.niehs.nih.gov/snpfunc.htm. The website predicts the following potential biological functions: transcription factor binding site, non-synonymous coding SNPs, stop codon, damaging SNPs, splicing regulation and microRNA (miRNA) binding site. No potential influences on the biological functions of S1, S2, S3, S4 and S7 were found. For S5 (rs4072111) and S6 (rs8031107), the results show S5 and S6 to be exonic splicing enhancers (ESEs) that influence mRNA transcription.

Exons in mammalian cells generally comprise only a fraction of the length of a pre-mRNA transcript. Accurate splicing requires the identification of shorter exonic sequences from lengthy intronic sequences. This identification is achieved by the binding of spliceosomal components to intronic and exonic splicing sequence elements. These short sequences can either enhance (ESEs) or reduce (exonic splicing silencers or ESSs) splicing at a nearby splice site (10, 20). The presence of a higher density of ESEs in authentic exons than in pseudo-exons may contribute to the recognition of the correct exons, whereas the presence of ESSs in pseudo-exons may suppress their splicing (6, 16). Thus, both classes of elements may contribute significantly to the specificity of pre-mRNA splicing.

According to the results of the PolyPhen-2 analysis, the non-synonymous amino change in S5 (Pro434Ser) (with a score of 0.958) may influence the structure and function of IL-16 (1). S8 (rs1131445), located in the 3′-untranslated region (3′-UTR), is predicted to be a potential miRNA binding site for the miRNAs *hsa-miR-1184*, *hsa-miR-1301*, *hsa-miR-135*, *hsa-miR-18* and *hsa-miR-624*. The binding of miRNA would post-trancriptionally down-regulate the IL-16 expression level.

An exhaustive haplotype analysis of all potential window sizes (sliding-window strategy) was performed to identify the most appropriate and significant associations between GD patients and the control subjects. The results show that there is at least 1 haplotype significantly associated with GD (Table 4). The 6-SNP window, consisting of rs7182786 (S1), rs8028364 (S2), rs12907134 (S3), rs4128767 (S4), rs4072111 (S5) and rs8031107 (S6) shows the most significant association with GD ($P = 2.3 \times 10^{-51}$). Among the SNPs, S3 and S6 seem to play important roles in determining whether the haplotypes are a risk or protective haplotype. GC[A]AG[G] and AG[A]AG[G] are protective haplotypes, whereas GG[G]GAA and GG[G]AG[A] are risk haplotypes. The alleles A in S3 and G in S5 are protective alleles, whereas the alleles G in S3 and A in S5 are risk alleles in combination with the effects of S1, S2, S4, and S6.

There are some drawbacks in our studies. First, the sample size of the control groups was small. Second, although we did re-genotype all the patient samples on rs8028364 and confirmed the genotype and allele frequencies, one may still speculate the possibility of mis-typing the genotype CC to GG or GG to CC. Moreover, we did not determine the IL-16 concentrations in the GD sera. An extended study is currently being conducted to remedy the above drawbacks.

In conclusion, the results of the present study suggest that the *IL-16* genotypes and haplotypes may be associated with GD. This report provides evidence that polymorphisms of the *IL-16* gene provide insight into the prediction of GD.

Acknowledgments

We thank Yu-Hui Liang for cataloging the samples and preparing the clinical data for the controls and GD patients. The authors also show special thanks to Professor Cheng-Wen Lin for his critical reading of this manuscript. This study was supported by a grant from the National Science Council (101-2320-B-039-038-), Taipei, Taiwan, and grants from the China Medical University (CMU99-asia-17), China Medical University Hospital (DMR-102-084), Taichung, Taiwan and Chi-Mei Hospital, Liouying, Tainan, Taiwan (CLFHR10212).

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